

**LOW-DOSE CARBON MONOXIDE EXPOSURE IN PREGNANCY;  
A POTENTIAL THERAPEUTIC FOR PRE-ECLAMPSIA**

by

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## Abstract

Preeclampsia (PE) is a maternal disorder of pregnancy, characterized by late-onset hypertension and proteinuria. It affects roughly 5-7% of all pregnancies worldwide and is a leading cause of maternal and fetal/neonatal morbidity and mortality. Cigarette smoking in pregnancy is associated with a 33% reduction in the incidence of PE, and this is dose dependent. It is hypothesized that carbon monoxide (CO), a combustion product in cigarettes, may confer cytoprotective and regulatory properties leading to the decreased incidence of PE. CO is produced endogenously by the enzyme heme oxygenase (HO), and it is thought that the manipulation of the HO/CO system in pregnancy can ameliorate or reduce the pathophysiologic signs of PE.

The exposure of pregnant mice to 250 ppm CO led to an increase in each of the maternal uterine blood flow, vascularity of the placenta and vessel diameter, with a shift towards angiogenesis in the placenta tissue proteins. Exposure of human placental villous explants to 250ppm CO led to a decreased production and release of the soluble vascular endothelial growth factor (VEGF) receptor -1 (sFlt-1). This molecule is increased in maternal plasma and placenta tissue of women with PE and it binds with molecules of angiogenesis, limiting their ability to interact with the endothelium. Using an AdsFlt-1 PE-like mouse model, the exposure of mice to 250ppm chronic CO prevented the hypertension, proteinuria and glomerular alterations, supporting the use of CO as a future therapeutic for women with PE.

We completed a pilot study to evaluate the exposure of healthy volunteers to two, one hour inhalations of 250ppm CO. We determined the half-life of CO and we provide baseline kinetics data for males and females following CO inhalation. These data are important for future therapeutic studies in order to better establish proper dosing, concentration of CO and method of delivery.

The results of this thesis contribute to the understanding of the pathophysiology of PE and provide evidence to support the use of CO as a therapeutic for this disorder.

## Co-Authorship

All basic research presented in this thesis was performed by Carolina Cynthia Venditti (CCV) with the help of several co-authors as listed below. Analysis of data, interpretation of results and manuscript preparations were completed by CCV with input from authors, as listed below. Dr. Graeme N. Smith (GNS) was involved in obtaining funding, designing experiments, analyzing data and discussion of results for all experiments in this thesis.

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This chapter was written by CCV and edited by GNS.

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**Chapter 3:** co- authored by Richard Casselman and Graeme N. Smith

RC aided with experimental design, proper delivery of CO into incubator chambers and technical assistance throughout the experiment. Data interpretation and discussion of results were completed with assistance from RC and GNS.

**Chapter 4:** co-authored by Richard Casselman, Iain Young (IY), S.Ananth Karumanchi (SAK) and Graeme N. Smith

Technical assistance, including adenovirus amplification and plaque forming unit number was provided by RC. Observation of blinded renal histology and discussion of pathology alterations was provided by IY. Adenovirus was provided by SAK. Analysis of data, and interpretation of results were assisted by GNS.

**Chapter 5:** co-authored by Richard Casselman, Kanji Nakatsu (KN) and Graeme N. Smith

KN and GNS assisted in experimental design. Technical assistance provided by RC, including ordering of proper medical gas and exposure of volunteer to accurate CO flow rates. KN and GNS assisted in analysis of results and interpretation of data.

**Chapter 6:** co-authored by Richard Casselman, Hendrik Vreman (HV) and Graeme N. Smith

Technical assistance was provided by RC and HV. Analysis of results and interpretation of data was assisted by HV. Discussion of results was completed with GNS.

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For my mother, who believed in me every step of the way

For my husband, who stood beside me on this journey

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## List of Abbreviations

%COHb	Percent carboxyhemoglobin
% spO <sub>2</sub>	Oxygen saturation
Ad	Adenovirus
BP	Blood pressure
BVR	Biliverdin reductase
CO	Carbon monoxide
CO <sub>2</sub>	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CoPP	Cobalt protoporphyrin
CORM	CO- Releasing Molecule
CSE	Cigarette smoke extract
CTB	Cytotrophoblast
DMEM	Dulbecco's modified eagle medium
DNA	deoxynucleic acid
EDV	End- diastolic velocity
ELISA	Enzyme-linked immunosorbent assay
EtCO	End- tidal breath CO
GC	Gas chromatography
GD	Gestation day
Hb	Hemoglobin
H&E	Hematoxylin and eosin
HgO	Mercuric oxide
HO	Heme oxygenase
HTN	Hypertension
HTR8-svNeo	Immortalized trophoblast cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Micro CT	Micro- computed tomography
mm	millimeter
mRNA	messenger ribonucleic acid
MV	Mean velocity over a cardiac cycle
O <sub>2</sub>	Oxygen
ODQ	1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PE	Preeclampsia
PFA	Paraformaldehyde
PFU	Plaque forming units
PGF	Placental growth factor

PI	Pulsatility index
ppm	parts per million
PSV	Peak systolic velocity
RI	Resistance index
ROS	Reactive oxygen species
RUPP	Reduced uterine perfusion pressure
SD	Standard deviation
SEM	Standard error of the mean
sEng	Soluble endoglin
sFlt-1	Soluble <i>fms</i> -like tyrosine kinase -1
sGC	Soluble guanylyl cyclase
siRNA	Silencing ribonucleic acid
SnMP	Tin mesoporphyrin
SnPP	Tin protoporphyrin
SSA	Sulfosalicylic acid
ST	Smokeless tobacco
TGF- $\beta$ 1	Transforming growth factor $\beta$ -1
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
T-reg	T-regulatory cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UMB A	Umbilical artery
UMB V	Umbilical vein
UtA	Uterine artery
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular adhesion molecule-1
YC-1	3-(5-hydroxymethyl-2-furyl)-1-benzylindazole
ZnPP	Zinc protoporphyrin

## Chapter 1

### **Introduction**

## **1.1 Preeclampsia (PE)**

### **1.1.1 The epidemiology of PE**

PE is a life threatening pregnancy disorder diagnosed by new-onset hypertension (HTN) ( $>140/90\text{mmHg}$ ) and proteinuria ( $>300\text{mg}/24\text{hr}$ )<sup>1-3</sup>. The syndrome affects 5-7% of all pregnancies worldwide<sup>4</sup>. PE continues to be one of the leading causes of preterm birth and significant maternal morbidity<sup>5</sup>. It is widely accepted to be a disorder of placental origin which ultimately has an adverse impact on the maternal vascular endothelium, leading to the clinical signs of HTN and proteinuria. We now know that several pre-existing maternal factors place a woman at increased risk of developing PE, including: underlying HTN, pre-gestational diabetes, young/advanced maternal age, nulliparity, high body mass index, a history of PE and multi-fetal gestation, as explained in a review by Young *et al.*<sup>6</sup>.

While the exact etiology of PE is not fully understood, it is widely considered to be a disorder of placental origin. In the traditional two-stage theory of PE pathophysiology, first described by Redman CW *et al.*<sup>7</sup> in 1991, an inefficient utero-placental circulation is thought to initiate hypoxic stress and damage within the developing placenta (Stage 1). Placental debris and placenta-derived factors are released into the maternal circulation where they result in inflammation and widespread endothelial dysfunction leading to the clinical condition of PE (Stage 2). In addition, it is thought that predisposing maternal factors, rendering the mother's endothelium more sensitive to the circulating placental products<sup>4</sup>, are also necessary to develop PE.

### **1.1.2 Stage 1: The impaired development of the placental vasculature in PE**

The first stage of PE is thought to be initiated by the compromised establishment of the utero-placental circulation at the maternal-fetal interface. In a healthy pregnancy, the extravillous

invasive cytotrophoblast (CTB) placental cells, invade the decidualized maternal endometrium, infiltrating the uterine spiral arteries and replacing the endothelial layers of these vessels. In doing so, they transform these vessels into high flow, low resistance conduits, insensitive to vasoactive factors and capable of transporting the expanded blood volume necessary for fetal growth late in gestation<sup>8</sup>. In a pregnancy complicated by PE, there is often shallow extravillous CTB invasion and minimal remodeling of the uterine spiral arteries; a finding in many placental bed biopsies of PE patients<sup>9</sup>. These poorly modified arteries continue to respond to vasoactive stimuli in the maternal circulation, creating a pulsatile blood flow to the placenta<sup>9</sup>, and maintaining a low flow, high resistance structure, significantly reducing utero-placental perfusion<sup>4:10</sup>. The consequences of decreased blood flow to the placenta are localized areas of placental oxidative stress and hypoxic injury. The pulsatile blood flow causes a re-introduction of oxygen (O<sub>2</sub>) on an inconsistent basis, leading to a production of reactive oxygen species (ROS), resulting in ischemic- reperfusion injury within the placenta. The PE placenta demonstrates evidence of increased apoptosis and necrosis<sup>11</sup>, increased shedding of placental debris into the maternal circulation, and leading to an inflammatory response<sup>7</sup>. The trophoblast invasion and utero-placental circulation are established by 20-22 weeks of gestation<sup>12</sup>, therefore, although PE is usually diagnosed later in pregnancy, it appears that development of PE begins in the first half of pregnancy.

### **1.1.3 Stage 2: Maternal widespread endothelial dysfunction in PE**

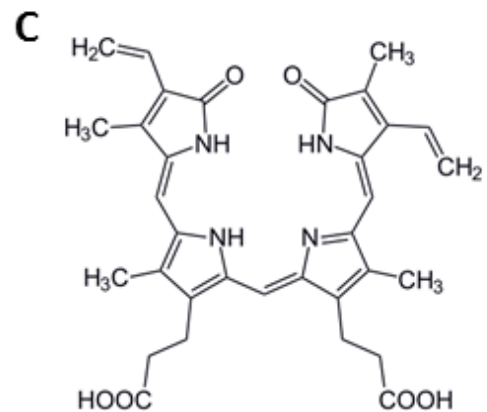
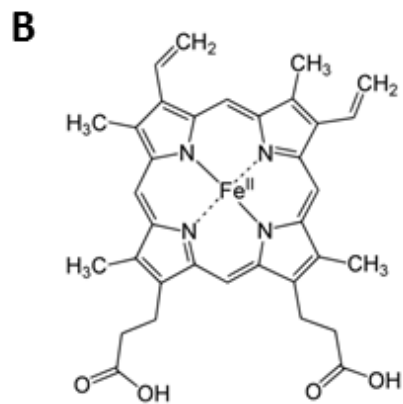
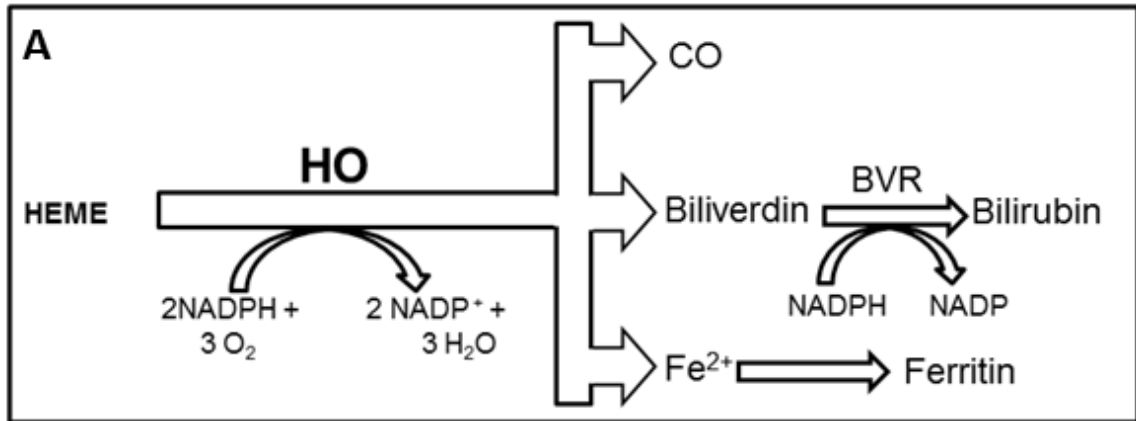
While the impaired placental development is of central importance in the establishment of PE, we now know that maternal pre-existing or contributing factors are of equal importance in the progression of this disorder<sup>4:13-15</sup>. Vascular impairment in PE patients is evident through increased reactivity to vasopressors<sup>16</sup> and by impairment of endothelium- derived vasorelaxation<sup>17</sup>. Levine and Karumanchi<sup>18</sup> suggest that the HTN, proteinuria, coagulopathy and liver dysfunction of PE can all be explained by systemic endothelial dysfunction. In fact, one of

the best characterized abnormalities of PE is glomerular endotheliosis, found in up to 80% of women with the disease<sup>19</sup>, and is characterized by glomerular endothelial damage<sup>19</sup>. This renal abnormality is not seen in any other form of HTN, and disappears following delivery, suggesting that it is not a secondary occurrence to HTN or hypoperfusion<sup>14</sup>. Following delivery, the clinical signs of PE largely dissipate, however, maternal impaired vascular function can remain for years<sup>20</sup>. We have shown that women with PE demonstrate an alteration in number and function of endothelial progenitor cells (markers of endothelial reparative capacity) at 2 months postpartum and endothelial colony forming units at 2 and 6 months postpartum<sup>21</sup>. Research has shown that several risk factors for the development of PE are akin to those of cardiovascular disorders: obesity<sup>22</sup>, HTN<sup>22;23</sup>, diabetes mellitus<sup>24</sup>, familial cardiovascular disease<sup>25</sup>; and that a diagnosis of PE identifies women with underlying cardiovascular risk factors<sup>26</sup>. Taken together, these results support the hypothesis that underlying maternal vascular dysfunction may have a role in the pathology of PE specifically sensitizing the mother's vasculature to respond to the increased placental factors abnormally.

## **1.2 The Heme Oxygenase (HO)/ Carbon Monoxide (CO) system and pregnancy**

### **1.2.1 The HO/CO system in the body**

While considered a toxic gas at high concentrations, CO is produced endogenously at low levels by almost every nucleated cell in our body<sup>27</sup>. Using the substrate heme, the enzyme HO creates three products in equimolar ratios: CO, biliverdin, which is converted to bilirubin, and iron (Figure 1.1), each possessing biological activity. Heme is found abundantly in hemoglobin<sup>28</sup>, and broken down by HO most abundantly in the reticuloendothelial system, the liver, kidney and mainly the spleen<sup>29</sup>. The main conversion of heme is to biliverdin,



**Figure 1.1: The pro-oxidant molecule heme is broken down by heme oxygenase (HO) to produce biliverdin, free iron and carbon monoxide (CO)**

(A). Biliverdin is further broken down to bilirubin by biliverdin reductase (BVR) and free iron is sequestered by the protein ferritin. The structure of heme is shown in (B) and that of biliverdin is shown in (C).

which is subsequently converted to bilirubin<sup>28</sup>. Both biliverdin/bilirubin (Figure 1.1) have potent antioxidant effects<sup>30</sup>, while elemental iron leads to the production of ferritin, an iron sequestering protein, reducing free iron levels and thereby oxidative stress<sup>31</sup>. CO has been implicated in maintaining vascular tone, increasing angiogenesis, and reducing inflammation and apoptosis<sup>32</sup>. HO exists in three isoforms; HO-1, the inducible form, HO-2, the constitutive isozyme and HO-3, a constitutive isoform, but with little information currently known. While HO-2 controls HO activity during steady state conditions<sup>33</sup>, HO-1, a heat shock protein, is induced by diverse stress-related conditions, and is capable of cytoprotective and antiapoptotic actions, protection against inflammation and oxidative stress<sup>34</sup>. This thesis will explain HO-1 and HO-2 in pregnancy, but will focus on the HO-1 isoenzyme.

### **1.2.2 The HO/CO system in pregnancy**

In pregnancy, a woman's total HO activity level is increased compared to that of a non-pregnant woman<sup>35</sup>. In the myometrium, both HO-1 and HO-2 have been identified at a 15- fold increase in pregnant women versus non- pregnant<sup>36</sup>. Several groups have reported finding each isoform in the placenta in humans<sup>37-39</sup>, rats<sup>40;41</sup> and mice<sup>42</sup>, however, study results are inconsistent between groups. There is no doubt that both messenger ribonucleic acid (mRNA)<sup>37;37-39</sup> and protein levels<sup>38;38;39;43</sup> of HO-1 and HO-2 have been identified in the placenta. The literature is inconsistent when discussing relative amounts, location and temporal changes in each isoenzyme, and this has been reported in recent reviews<sup>44-46</sup>. Using the rat and mouse as placental models, researchers have shown that HO-1 levels are largely elevated at the time of placental vascular development<sup>42</sup> and localized to the feto- maternal interface<sup>40;42</sup> decreasing at term<sup>42</sup>. Additionally, at this time- point, transcript levels of HO-1 are still highest in the placenta compared to various organs, functional and actively producing CO<sup>47</sup>; performing at 90% of the levels measured in the spleen<sup>42</sup>, the organ known to have the highest HO activity<sup>48</sup>. Though discrepancies exist in the

literature, HO-1 and HO-2 are spatially and temporally localized throughout the placenta and gestation, indicating a potential role in placental function, placenta development and maintenance of a healthy pregnancy.

### **1.2.3 The HO/ CO system and complications of pregnancy**

If the HO/CO system is involved in the progression of a healthy pregnancy, then it would be hypothesized that impairment of this system could result in pregnancy complications. Indeed this has been observed in cases of early pregnancy loss<sup>49</sup> and PE<sup>39;50;51</sup>. Studies have shown lower protein levels of both HO-1 at the feto- maternal interface and HO-2 in invasive trophoblasts of placentas with spontaneous abortion, hydatiform mole, and PE<sup>49</sup>. A significant reduction in HO-1 protein using western blot analysis was shown in the placentas of women with PE at term<sup>39</sup>, and a decrease in both isoforms of HO in damaged infarcts of placenta compared to normal areas, using immunohistochemistry<sup>51</sup>. In addition, HO-1 mRNA is decreased in the blood of women with PE<sup>52</sup>. These data suggest a relation between complications of pregnancy and an alteration in the HO system, but whether it is a cause or consequence of the disorder is not clearly shown by these results.

While it is evident that changes in the HO system occur in abnormalities of pregnancy, the localization of both isoenzymes to the chorionic villi, the underlying CTBs and the vascular endothelium<sup>51</sup>, in addition to strong expression of HO-1 at the distal ends of CTB cell columns<sup>53</sup> suggest a role in trophoblast invasion and regulation of placental function. Further, HO protein expression is decreased in the placentas of PE patients<sup>39;53;54</sup>, suggesting a role for reduced HO expression in the pathogenesis of PE. Importantly, Farina and colleagues<sup>50</sup> measured HO-1 levels in chorionic villi of women at 11 weeks of gestation and noted that those women who went on to develop PE had decreased levels of HO-1 in these localized areas of the placenta.

Rodent models of pregnancy have been used to elucidate the role of HO in maintaining a healthy pregnancy. Researchers have been able to partially (HO-1<sup>+/-</sup>)<sup>55</sup> and fully (HO-1<sup>-/-</sup>)<sup>36;56;57</sup> knockout HO-1 in mice, and in both cases, have reported adverse effects in pregnancy. Both HO-1<sup>-/-</sup> and HO-1<sup>+/-</sup> mice exhibit a prolonged time for blastocyst attachment versus normal mice<sup>57</sup>. Maintenance of pregnancy is poor in HO-1<sup>-/-</sup> mice, as extremely low birth rates<sup>56-58</sup> and poor survival are reported<sup>56</sup>. Further, HO-1<sup>+/-</sup> mice demonstrate impaired spiral artery remodeling, evident through vascular corrosion casting of the uteroplacental unit<sup>55</sup>. Studies *in vitro*, have shown that human trophoblast invasion is prevented by the HO inhibitor zinc protoporphyrin-9 (ZnPP), or by HO-2 antibodies<sup>59</sup>, further supporting the role of HO in facilitating trophoblast invasion of the spiral arteries. HO-1<sup>-/-</sup> mice also exhibit macromolecular oxidative damage, tissue injury, and chronic inflammation<sup>60</sup> in addition to anemia, and accumulation of hepatic and renal iron<sup>60</sup>. This is similar to a clinical case reported in 1999, a rare double deletion of the maternal and paternal allele for HO-1, leading to no endogenous HO-1 production<sup>61</sup>. In this case, a young boy was identified with severe growth restriction, subsequent developmental delay, erythrocyte fragmentation and persistent intravascular hemolysis, and an abnormal coagulation/fibrinolysis system<sup>61</sup>. The close comparison of HO-1 knockout mice and the clinical case of HO deletion support the use of these mouse models for comparison of the effects of HO in human pregnancy.

## **1.3 Cigarette smoking and PE**

### **1.3.1 The incidence of PE in women who smoke in pregnancy**

Ironically, the only thing shown to decrease the chance of developing PE is cigarette smoke, reducing the risk by 33%<sup>62;63</sup>, and in a dose dependent manner<sup>64;65</sup>. While this seems paradoxical, the finding has been reported in multiple countries and over many years of published reports<sup>62;66-68</sup>. It is well known that smoking cigarettes during pregnancy is associated with a

multitude of adverse fetal outcomes, perinatal death<sup>69</sup>, ectopic pregnancy<sup>70</sup>, stillbirth<sup>71</sup> to name a few; an average of 10.5% of all pregnant women in Canada (2005-2006)<sup>72</sup> and the United States (2010)<sup>73</sup> smoke. Further, babies born to smoking mothers are often preterm<sup>74</sup> and of low birth-weight, on average 200g lighter per pack per day smoked, than a baby born to a non-smoking mother<sup>75</sup>. The question is which of the 4000 toxins found in cigarette smoke<sup>76</sup> may be affecting the maternal/ placental development of PE?

In this regard, studies have suggested the importance of combustible by-products of cigarette smoke. Women who use smokeless tobacco (ST), or snus, have a slight increased risk of developing PE<sup>64:77</sup>. While ST products have comparable concentrations of many of the addictive (ie nicotine)<sup>78:79</sup> and toxic compounds<sup>76</sup> found in traditional cigarettes, they do not create combustible by-products of cigarettes smoke, such as carbon monoxide (CO)<sup>76</sup> (Table 1-1). It is well known that smoking leads to higher blood and breath concentrations of CO<sup>76</sup>, suggesting that this increased CO may contribute to the lower incidence of PE in women who smoke during pregnancy. In 2004, Kreiser and colleagues<sup>80</sup> published findings indicating that women with gestational HTN or PE have end-tidal breath CO (EtCO) concentrations (1.36 ±0.30ppm) significantly lower than control, normotensive women (1.72±0.42ppm, p=0.001) and non- pregnant women (1.78±0.54ppm, p=0.002)<sup>80</sup>. These results led the authors to conclude that the lower CO concentrations may be a deficient compensatory response to the hypertensive state of PE, or that it may be a primary alteration involved in the pathogenesis of the disorder<sup>80</sup>. These findings also support the idea that the lower incidence of PE among smokers, may in fact be due to the inhalation of CO, potentially replacing a deficient level of the endogenous gas. Our research group has shown that there is an inverse relationship between exposure to environmental ambient CO and the incidence of PE<sup>81</sup>. Maternal data were evaluated retrospectively for all births in Ontario, Canada from 2004- 2009, and ambient CO levels were obtained from the Ontario Ministry of Environment, Air Quality Ontario website<sup>81</sup>. After adjustment for several

**Table 1-1: Constituent differences between cigarette smoke and oral smokeless tobacco (snus) and the development of PE.**

Toxin	Cigarette Smoking		Oral Snus	
		References		References
<b>Over 4000 toxins</b> (ex. Tobacco, N-nitrosamines)	✓	76	✓	76 82
<b>Nicotine</b> (comparable levels)	✓	78;83-86	✓	79;83-86
<b>Combustion by products</b> (ex. CO)	✓	76	<b>NONE</b>	83
<b>Incidence of PE</b>	↓	62;63,64;66;68;77;87;88	↑	62;64;77

confounding factors, the lowest incidence of PE was associated with the highest quartile of CO exposure in ambient air<sup>81</sup>. The idea for use of EtCO measurements as a predictive marker of pathological conditions in pregnancy has been patented as a possible method to determine the onset of a pregnancy complication, such as PE<sup>89</sup>.

## **1.4 Rationale for the hypothesis that the HO/CO system is able to reduce the development or the progression of PE**

### **1.4.1 HO/CO and stage 1 of PE**

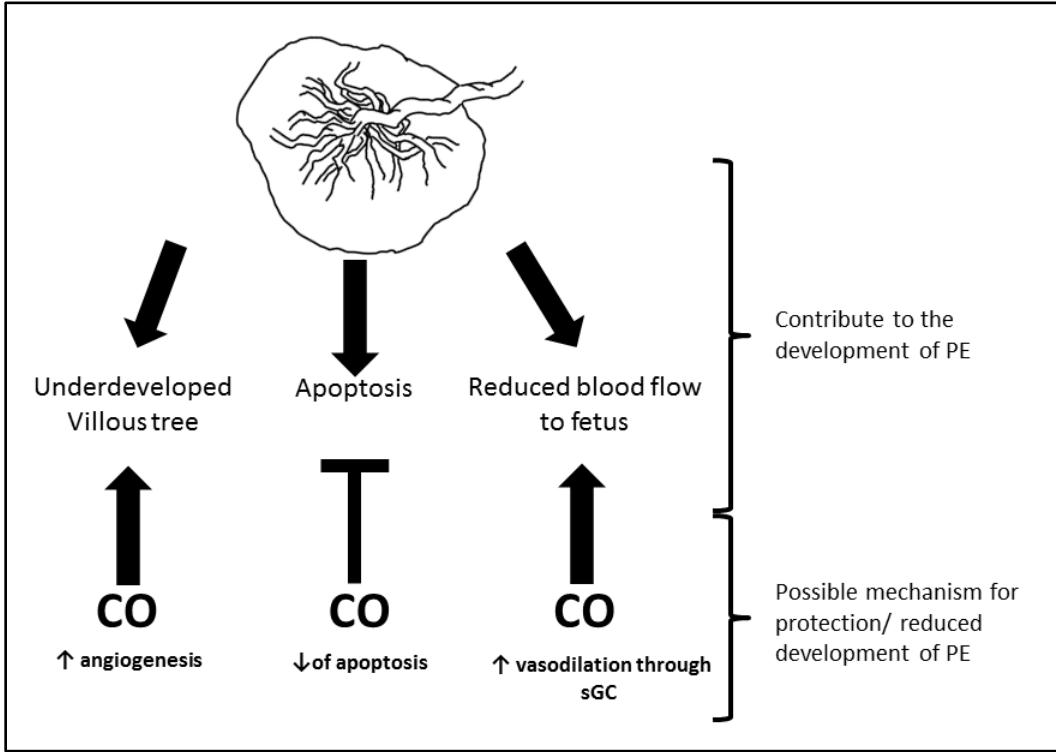
#### **1.4.1.1 HO/CO attenuates placental apoptosis**

The villous syncytiotrophoblast cells lining the intervillous space are especially sensitive to the hypoxia-reoxygenation type of injury, resulting in increased shedding of apo-necrotic syncytiotrophoblast debris into the maternal circulation<sup>11;90</sup>. Indeed, an increased load of trophoblast tissue has been measured in the circulation of women with PE<sup>90;91</sup>. Further, the placentas have also been shown to have more overall placental apoptosis compared to controls<sup>92</sup>, and more specifically to have an increase in syncytiotrophoblast proliferation and apoptosis<sup>93</sup>. In a healthy pregnancy, apoptosis is necessary for normal growth and development, even increasing near term<sup>94</sup>. However, it is the significant increase observed in PE pregnancies that may lead, in part, to the maternal endothelial dysfunction.

Women who smoke cigarettes ( $\geq 10$  cigarettes per day) in pregnancy have a decreased level of apoptosis in the syncytiotrophoblast of their term placenta<sup>95;96</sup>. A decrease in placental trophoblast apoptosis, using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and deoxyribonucleic acid (DNA) fragmentation quantification was observed in term placentas of women who smoke during pregnancy, with an overall decrease in apoptosis from first to third trimester<sup>96</sup>. These two studies used placental tissue exposed to maternal cigarette

smoking throughout pregnancy. However, when placental villous explants were exposed to cigarette smoke extract (CSE) (the bubbling of the smoke from lit cigarettes into media) at increasing concentrations, no difference in cell death was apparent between control and CSE exposure groups<sup>97</sup>. This difference between the *in vivo* and *in vitro* findings may be because of the rapid disappearance of CO in CSE media (Venditti CC and Smith GN, unpublished). Using an *in vitro* model of placental hypoxia-reoxygenation injury, we were able to inhibit syncytiotrophoblast apoptosis and secondary necrosis with CO treatment, using concentrations of CO similar to that measured in the blood of smoking women<sup>98</sup>. Further, in a mouse model of abortion, both the introduction of chromium protoporphyrin (CoPP)<sup>99</sup>, a potent HO inducer, and an adenovirus (Ad) with an HO-1 insert<sup>100</sup>, led to decreased apoptosis through the induction of Bag1 (a marker of anti-apoptosis) mRNA measured at the feto-maternal interface. CoPP also decreased markers of cell death (caspase-3 and phosphorylation of JNK and STAT1) in a reduced utero placental perfusion (RUPP) model in rats<sup>101</sup>. Additionally, induction of HO by CoPP also led to increased phosphorylation and activation of ERK and STAT3, mediators of prosurvival, in the RUPP rat model<sup>101</sup>, resulting in increased fetal survival rates and reduction in the incidence of miscarriage in murine pregnancies<sup>99;100</sup>.

Outside of pregnancy, CO has been shown to have potent anti-apoptotic and cytoprotective effects in several organ systems in both physiological and pathological settings<sup>102-106</sup>. It is possible that the elevated maternal CO levels in women who smoke offer cytoprotection for the syncytiotrophoblast from hypoxia- reoxygenation injury and subsequently limit apoptosis and shedding of placental debris into the maternal circulation of women with PE (Figure 1.2).



**Figure 1.2: Stage 1 of PE: Abnormalities in the development of the placenta that may contribute to the development of PE can be ameliorated by HO/CO system.**

The HO/CO system has been shown to reduce apoptosis in a number of *in vitro* and *in vivo* studies. The HO/CO system is capable of increasing blood flow and thereby nutrients to the placenta or increasing angiogenesis of the placenta, through a pro- angiogenic ratio shift of angiogenic: anti-angiogenic factors. It is possible that these actions contribute to decreased PE incidences in women who smoke while pregnant.

#### 1.4.1.2 HO-1/ CO alteration of the placental Hemodynamics

Smoking in pregnancy affects placental blood flow in a dose dependent manner<sup>107</sup>. Muller *et al.*<sup>108</sup> reported a decrease in uterine systolic/diastolic ratio, and they suggested that it was most likely an increase in blood flow, not velocity; as no change in umbilical arterial systolic/diastolic ratio was noted. Nicotine is one of many chemicals in cigarettes and its effect on the vasculature is one of vasoconstriction<sup>109</sup>, as shown specifically in the isolated perfused placenta by our group<sup>110</sup> and others<sup>15;111</sup>. Using the placental perfusion model, our research group<sup>112</sup> has also shown that CO (at levels both below and well above those measured in the blood of smoking women) was able to elicit a concentration- dependent decrease in placental perfusion pressure. We demonstrated that the vasodilatory properties of CO on the placental vasculature were likely mediated through the soluble guanylyl cyclase (sGC) pathway<sup>112</sup>, as has been reported in other systems of the body<sup>113</sup>. Using 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ), an sGC specific inhibitor, the CO-induced vasodilation was abolished, while the addition of 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (YC-1) augmented the CO-induced decrease in perfusion pressure.<sup>113</sup> In mice, the injection of a CO-releasing molecule (CORM) led to an increase in renal blood flow<sup>114</sup>, a finding that was abolished when blocked with ODQ<sup>114</sup>. Therefore, HO-1 in the placenta may act to increase blood flow through vasodilation of uteroplacental arteries. In isolated human placentas, a hemin- induced HO-1 up-regulation reduced pre-constricted placental artery vascular tension by more than 50%<sup>39</sup>, further supporting the idea that HO-1 may be involved in the regulation of placental vascular resistance and subsequent decrease in maternal uterine vascular tone.

HO activity is increased in many maternal tissues in pregnancy, and it is believed to be an adaptive mechanism for the increased circulating red blood cell mass and erythropoiesis in pregnancy, as this would lead to increased red blood cell turnover and an elevation in free heme<sup>35</sup>. When tin mesoporphyrin (SnMP) (an inhibitor of HO) is administered to pregnant mice, a significant increase in systolic, diastolic and mean arterial pressure (MAP) is observed<sup>35</sup>. In a rat

model of renal HTN, administration of SnMP further exacerbated the elevated blood pressure<sup>115</sup>. The administration of CoPP, an inducer of HO, reduces the MAP in a rat model of placental ischemia<sup>116</sup> and renal HTN<sup>115</sup>. Mice with an HO-1<sup>+/-</sup> genotype are characterized with increased diastolic blood pressure (BP)<sup>56</sup>, further implicating the HO system in the maintenance of normal vascular tone. This action was also seen in mouse fetal umbilical arteries (UMBA), as the addition of SnMP also increases umbilical artery blood velocity acutely, returning to normal within 24 hours (hrs)<sup>56</sup>. Not only does the effect of HO act on maternal BP, but the exposure of mice<sup>117</sup> and rats<sup>118</sup> to CO significantly attenuates HTN, leading to a possible mechanism by which the HO/CO system may reduce the development of PE (Figure 1.2).

## **1.4.2 HO/CO and stage 2 of PE**

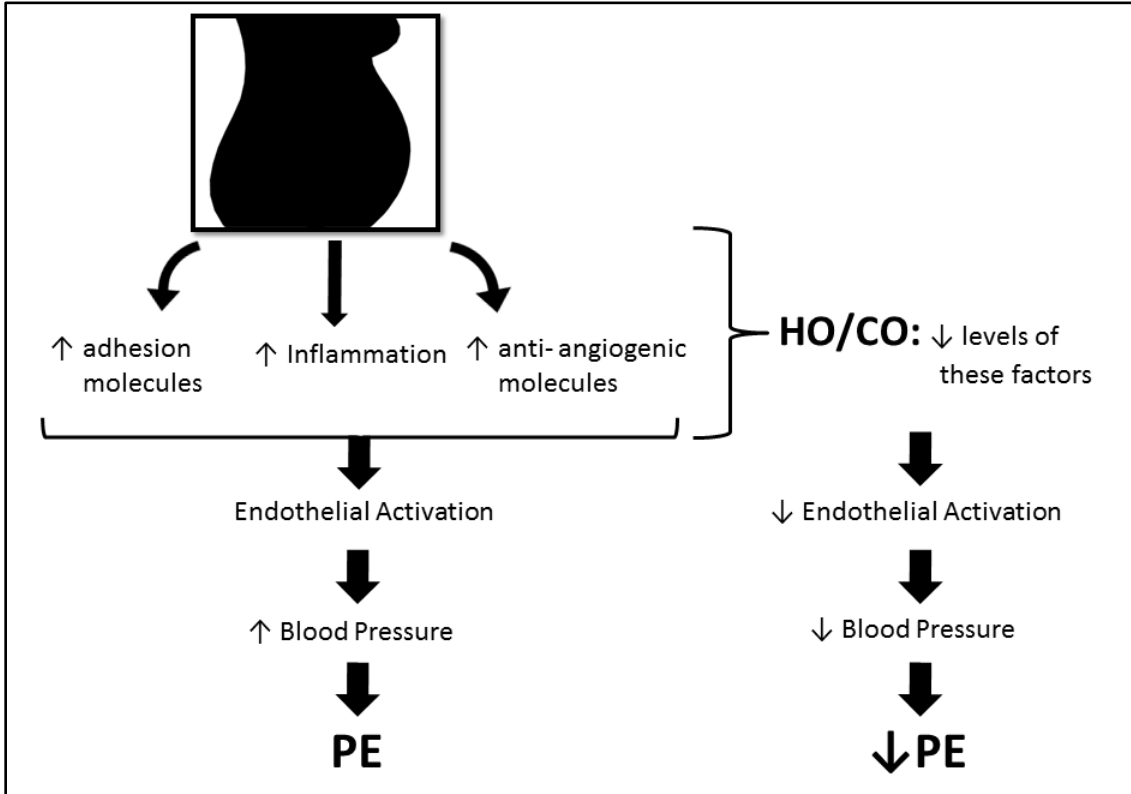
### **1.4.2.1 HO-1/ CO system on the maintenance of vascular adhesion molecules in endothelial injury**

In PE, there is evidence of increased maternal endothelial injury as early as 20 weeks of gestation, suggesting that endothelial dysfunction is a major contributor to the clinical presentation of PE<sup>3</sup>. Among many markers of endothelial dysfunction, both vascular cell adhesion molecule (VCAM)-1<sup>119</sup>, and the intercellular adhesion molecule (ICAM)-1<sup>120</sup> are increased in the serum of women with PE. These two molecules mediate adhesion between endothelial cells and leukocytes. Upon exposure of endothelial cells to inflammatory mediators, cellular adhesion molecules recruit and bind lymphocytes, monocytes and neutrophils in the circulation<sup>121</sup>, which can lead to vascular damage, endothelial cell destruction, membrane lipid peroxidation and increased vascular permeability, through the release of ROS and leukotrienes<sup>122</sup>.

It has been proposed that cigarette smoking may act through the actions of the HO/CO system to blunt the immune system<sup>44</sup>. Song *et al.*<sup>123</sup> stimulated human umbilical vein endothelial cells (HUVECs) with tumor necrosis factor (TNF)- $\alpha$ , inducing an inflammatory response. They

subjected the cells to high concentrations of CORM-3, water-soluble molecules which release CO under physiologic conditions<sup>124</sup>, leading to a down regulation of VCAM-1 protein expression, as shown with western blotting<sup>123</sup>. CORM-3 was also able to induce HO-1 expression levels and when this was blocked using sodium nitroprusside, the same reduction of VCAM-1 was observed, indicating that CO was working independently of HO-1<sup>123</sup>. Zhang *et al.*<sup>125</sup> exposed HUVECs to oxidized low-density lipoprotein, leading to induction of oxidative stress in the cells and up-regulation of both VCAM-1 and ICAM-1 protein levels and macrophage/monocyte chemoattractant protein, also an adhesion molecule of the endothelium. The oxidative stress also led to increased levels of HO-1 activity, as measured by bilirubin levels<sup>125</sup>. Administration of genistein, a soy-based product which further increased HO-1 mRNA, protein and activity levels, reduced the protein levels of all three adhesion molecules. This decrease was augmented by addition of CoPP (an HO-1 inducer) and abrogated by addition of ZnPP, an HO-1 inhibitor<sup>125</sup>. Also using HUVECs, Bergstraesser *et al.*<sup>126</sup> induced inflammatory stress by addition of TNF- $\alpha$ , leading to increased mRNA levels of several adhesion molecules, including VCAM-1 and ICAM-1. The addition of CORM3, which releases CO into the media, led to the decreased mRNA measurement of VCAM-1 and ICAM-1.

Exogenous CO has immunosuppressive effects on macrophages, monocytes and endothelial cells<sup>127;128</sup>. The *in vitro* studies discussed<sup>123;125;126</sup> indicate that vascular endothelial injury, introduced following inflammatory responses and recruitment of adhesion molecules, may be reduced and improved, through the actions of the HO system, and more specifically by CO (Figure 1.3).



**Figure 1.3: Stage 2: Widespread maternal endothelial dysfunction and the effect of the HO/CO system on reducing the incidence of PE.**

An increase in each of adhesion molecules, inflammation and anti- angiogenic molecules have been associated with PE. The HO/CO system has been shown to reduce levels of adhesion molecules, VCAM-1 and I CAM-1, as evidenced by *in vitro* studies of oxidative stress and exposure to HO-1 or CO. The HO/CO system is also associated with an increase in a state of pro-angiogenesis, through the reduction of anti- angiogenic molecules, namely sFlt-1 and the augmentation of angiogenic molecules, such as VEGF and PGF. Each of these actions through the HO/CO system may individually or collectively reduce the incidence / development of PE.

#### **1.4.2.2 HO/CO and inflammation in pregnancy**

Successful human pregnancy is thought to be a state of immunological tolerance<sup>129</sup> and associated with an increase in maternal T regulatory (T-reg) cells, a unique subpopulation of T-cells<sup>130</sup>, that help the fetus, expressing paternal antigens, evade a maternal immune attack<sup>131</sup>. Further, the absence of these cells led to failed gestation, as shown in a mouse model depleted of T-reg cells<sup>131</sup>, and abortion-prone mice also exhibit a diminished number of maternal murine T-reg cells<sup>132</sup>. Data suggest similar mechanisms exist in humans<sup>133</sup>. Zenclussen *et al.* demonstrated decreased HO-1 and HO-2 protein expression by western blotting and immunohistochemistry at the feto-maternal interface of abortion prone mice<sup>134</sup>. They then isolated T-reg cells from spleen and thymus of non-pregnant and gestation day (GD) 14 healthy pregnant mice and injected them intravenously into the abortion prone mice<sup>135</sup>. The T-reg therapy prevented abortion and interestingly, increased HO-1 mRNA levels at the fetal-maternal interface<sup>135</sup>. The inhibition of HO-1 using ZnPP abrogated the protective effects of T-regs<sup>136</sup>, suggesting that T-reg induction and acceptance of a fetus, may function through the HO system.

#### **1.4.2.3 The effect of the HO/CO system on angiogenic markers of pregnancy and their alterations in PE**

The effect of anti-angiogenic molecules has been proposed as a possible contributor to the development of PE<sup>6;18;62</sup>. Angiogenic factors involved in placental vasculogenesis/angiogenesis and maternal adaptation to pregnancy include vascular endothelial growth factor (VEGF) and placental growth factor (PGF)<sup>6;18;137</sup>. Invasive CTBs express VEGF and PGF<sup>138</sup> and these angiogenic factors are thought to play a pivotal role for the success of a normal pregnancy<sup>6</sup>. The levels of both VEGF and PGF are significantly decreased in women diagnosed with PE, as reviewed by Levine and Karumanchi<sup>18</sup>, in both maternal serum<sup>139;140</sup> and placental tissues,<sup>138</sup>. The soluble splice variant of the VEGF receptor 1, also referred to as soluble *fms*-like tyrosine

kinase-1 (sFlt-1) is an anti-angiogenic factor which binds both VEGF and PGF, reducing their interaction with endogenous receptors on the endothelium, and impairing vascular function<sup>18,139,141</sup>. In pregnancies complicated by PE, an increase in both placental<sup>138,139</sup> and maternal serum<sup>139,140,142</sup> levels of sFlt-1 have been reported. Importantly, measured levels of sFlt-1 in maternal serum decrease following delivery<sup>139,142,143</sup>, as would be expected if the main source of increased sFlt-1 were of placental origin<sup>18</sup>. In pregnant rats, the injection of an Ad with the sFlt-1 (AdsFlt-1) construct leads to an increase of sFlt-1 in maternal serum and the development of the maternal signs present in PE patients (HTN and proteinuria)<sup>139,144</sup>. This model has been recreated in mice as well<sup>145-147</sup>. Increasing sFlt-1 levels in rats using mini-osmotic pumps<sup>148</sup> also leads to PE-like signs, as did the RUPP model in rats, whereby maternal sFlt-1 levels were also increased<sup>116</sup>. It has therefore been suggested that decreasing the circulating levels of sFlt-1 could be a possible therapeutic target for the prevention or treatment of PE<sup>149</sup>.

Increasing maternal VEGF, by Ad (AdVEGF)<sup>146</sup>, injections<sup>144</sup> or mini-osmotic pump release<sup>147</sup>, reduces the signs of PE in rodents. Induction of HO-1 by CoPP in rats, normalized sFlt-1-induced HTN, increased VEGF levels and decreased pre-proendothelin-1, a molecule of vascular activation<sup>116</sup>. Using the RUPP model in rats, induction of HO-1 by CoPP altered the sFlt-1/VEGF ratio to one of angiogenesis<sup>116</sup>. Cigarette smoking in pregnancy has been reported to correlate positively with an increase in maternal plasma levels of PGF<sup>150,151</sup> and with a marked decrease in maternal serum sFlt-1 levels<sup>143,150-152</sup>. Mehendale and colleagues<sup>97</sup> showed that exposing placental villous explants from nonsmoking women to CSE, led to a pro-angiogenic state with reduced sFlt-1 production. Further, they reported a trend toward higher PGF levels in the media of these villous explants, at both 48hr and 72hr exposure to the CSE<sup>97</sup>. We have previously demonstrated that protein levels of HO are increased in the placentas (basal plate) of smoking women *in vivo* and in trophoblast cells following an *in vitro* exposure to CSE<sup>153</sup>. To evaluate the *in vitro* effect of CSE, we bubbled the smoke from cigarettes into media and exposed

trophoblast cells (HTR8-svNeo) to differing dilutions of the CSE. In a dose-dependent manner, 0.5%, 1.0% and 2.0% CSE solutions increased the protein levels of HO-1 in the trophoblast cells<sup>153</sup>. Cudmore and colleagues<sup>154</sup> found a decrease in sFlt-1 production when HO-1 was overexpressed in endothelial cells using an Ad with an inserted HO-1 construct. Further, exposure of placental villous explants to VEGF led to an increase in sFlt-1 protein and was further increased following inhibition of HO-1 by SnPP or by electroporation with HO-1 siRNA. The exposure of tissues to CO, through the addition of CO- infused media or CORM-2 in media, led to decreased levels of sFlt-1<sup>154</sup>. George *et al.* have shown that exposure of rat placental villous explants to a hypoxic environment (1% O<sub>2</sub>) leads to a significant increase in sFlt-1 release and superoxide (a marker of oxidative stress)<sup>155</sup>. Both of these markers of injury, sFlt-1 and superoxide, were significantly reduced, and to the same degree, when the tissue media was supplemented with CoPP (an inducer of HO-1) or CORM3 (a CO-releasing molecule)<sup>155</sup>.

As the induction of HO decreases the release of sFlt-1, it would be expected that VEGF bioavailability would increase. In rat vascular smooth muscle cells, a 30% increase in VEGF protein was observed, following the induction of HO-1 by hemin or in the presence of CORMs<sup>156</sup>. The inhibition of HO-1 (by SnPP) led to a decrease in VEGF levels and further decreased the proliferation (26%), migration (46%), tube formation (48%) and capillary outgrowth (30%)<sup>156</sup>. George *et al.*<sup>148</sup> used mini-osmotic pumps to infuse pregnant rats with exogenous sFlt-1 from GD14 to term, inducing PE-like signs (HTN and proteinuria). The induction of HO-1 by injection of CoPP did not alter the maternal sFlt-1 levels, but a significant increase (~50%) in free VEGF levels was reported<sup>148</sup>. They also reported similar results in a RUPP model of placental ischemia in rats, whereby induction of HO-1 (by CoPP) led to a decreased placental protein ratio of sFlt-1: VEGF, a positive shift in the angiogenic profile<sup>116</sup>. Taken together, these data suggest a positive correlation between the HO-1/ CO system and angiogenesis, whereby sFlt-1 is decreased and free VEGF is increased (Figure 1.3).

## 1.5 Hypotheses and objectives

The HO/CO system is important in maintaining a healthy pregnancy. Alterations in this system lead to complications of pregnancy<sup>49</sup>, one of which being PE<sup>50;51</sup>. It is possible that the reduced levels of HO/CO in women with PE contribute to the underlying development of the disorder. The general hypothesis of this thesis is:

*Exogenous exposure to CO will reduce the clinical signs of PE.*

### 1.5.1 Study 1: Exposure to low level CO throughout pregnancy will lead to vasodilatory changes and angiogenic alterations of the uteroplacental unit.

CO is a known vasodilator, like its diatomic cousin nitric oxide<sup>113;157</sup> and functions mechanistically through sGC<sup>113</sup>. Using CO levels similar to those of women who smoke in pregnancy, our group has shown that CO is a vasodilator and decreases placental perfusion pressure through sGC<sup>112</sup>. We hypothesized that exposure to CO *in vivo* would result in vessel dilation of the uteroplacental unit, decreasing blood velocity and providing increased nutrient transfer to the fetuses.

The HO-1/ CO system has been implicated in models of angiogenesis, as *in vitro* exposure of placental explants or HUVECs to inducers of HO-1<sup>154;155</sup> or CORMs<sup>154;155</sup> led to a decrease in sFlt-1 and a concomitant increase in VEGF<sup>148</sup>. We hypothesized that a decrease in maternal plasma and placental sFlt-1 levels with a balanced increase VEGF protein would also occur *in vivo*, contributing to the alterations of the uteroplacental unit following maternal exposure to CO.

Therefore we hypothesized that: *exposure to low level CO throughout pregnancy would lead to vasodilatory changes and angiogenic alterations of the uteroplacental unit.*

**The main objectives were:**

- 1) To determine if vasodilatory changes of the uteroplacental unit occur following exposure to low-level CO
- 2) To measure and quantify the gross changes in the uteroplacental unit following maternal CO exposure.
- 3) To calculate the placental angiogenic effects due to CO exposure, and to measure anti/angiogenic protein levels in maternal plasma and placenta tissue

**1.5.2 Study 2: Exposure of human placental explants to 250ppm CO over a 24hour period will reduce the release of sFlt-1 and increase the level PGF.**

Several research groups have used *in vitro* models to show that the induction of the HO-1/ CO system, by hemin<sup>156</sup>, CoPP<sup>155</sup>, AdHO-1<sup>154</sup>, CO-bubbled media<sup>154</sup>, and CORMs<sup>154-156</sup>, leads to decreased sFlt-1 levels. However, the use of gaseous CO, to our knowledge, has not been used for this purpose. Further *in vivo* experiments in a RUPP model of pregnancy showed that exposure of rats to CoPP, an inducer of HO-1, also decreased sFlt-1 levels<sup>116</sup>. Therefore, we chose to perform this study to evaluate the effects of gaseous CO (250ppm) on placental production and release of sFlt-1.

We hypothesized that: *exposure of human placental explants to CO in ambient air would decrease the release of sFlt-1 from placental explants and increase the angiogenic factor PGF.*

**The main objectives were:**

- 1) To develop a system whereby gaseous CO could be delivered at a consistent and desired level for exposure of explant tissue

- 2) To determine if CO reduces the release of sFlt-1, and sEndoglin (sEng), another anti-angiogenic molecule.
- 3) To determine if CO increases the release or availability of free PGF from explant tissue

### **1.5.3 Study 3: Maternal exposure to 250ppm CO will decrease the clinical signs of PE, HTN and proteinuria, in a mouse model of PE.**

PE is a disorder specific to humans, therefore testing treatment and therapeutics in animal models has been difficult. While several animal models have been created with PE-like signs<sup>158</sup>, we chose to use the AdsFlt-1 mouse model of PE<sup>139</sup>, as the increased sFlt-1 levels lead to HTN, proteinuria and glomerular endotheliosis, similar to what is observed in humans. The first experiment of this thesis showed that exposure of mice to CO led to a shift towards angiogenesis in the sFlt-1/VEGF ratio of placenta and maternal plasma proteins<sup>159</sup>. We hypothesized that this shift would benefit the mice exposed to sFlt-1 in this study and compensate for the increased sFlt-1 levels produced. Further, we hypothesized that a combination of favourable properties of CO would benefit the mice injected with sFlt-1; namely results from the first experiment of this thesis, increased maternal blood flow, increased placental angiogenesis<sup>159</sup>, and previous reports showing that CO leads to decreased inflammatory mediators and decreased apoptosis<sup>32</sup>. In addition, previous reports had shown that induction of the HO-1 system reduced HTN in rats<sup>148</sup>.

Therefore, our hypothesis for this study was: *maternal exposure to low-level CO would prevent the signs of PE, in a mouse model of the disorder.*

#### **The main objectives were:**

- 1) To establish the AdsFlt-1 mouse model of PE in our laboratory, creating the signs of PE, HTN and proteinuria in mice injected with AdsFlt-1

- 2) Expose our mouse model of PE to chronic CO in the latter half of pregnancy and determine the effect on HTN and proteinuria.
- 3) Evaluate histologically, the changes in the renal glomeruli in mice injected with AdsFlt-1± CO.

**1.5.4 Study 4: The exposure of healthy, human volunteers to one hour of 250ppm CO, at two time-points, will increase percent carboxyhemoglobin (%COHb) in blood and EtCO levels, but will maintain levels below those of smoking individuals and within safe regions.**

We and others have shown beneficial physiologic effects of low-level CO exposure *in vitro*<sup>98;112;154;160;161</sup> and *in vivo* using animal models<sup>159;162;163</sup>. In order to translate this information to a clinical setting in the future, a pilot study to evaluate the effects of CO in healthy subjects needs to be completed. To our knowledge, only two previous studies<sup>164;165</sup> have been published whereby humans were exposed to CO, but results on the kinetics of blood and breath CO throughout the study were not reported.

We hypothesized that: *the exposure of volunteers to 250ppm CO at two time-points for a one-hour session each would maintain levels of blood and breath CO below those of heavy smokers.*

**The main objectives were:**

- 1) To determine a dosing regimen that would allow for acute exposure of CO for one hour, with minimal effect on volunteer health or comfort status
- 2) To measure blood and breath CO on an hourly basis following CO exposure
- 3) To measure BP hourly throughout the experiment
- 4) To determine the half-life of CO for future therapeutic studies

**1.5.5 Study 5: Phlebotomy to measure CO should be performed using a syringe, to ensure that the vacuum production of CO in vacutainers does not produce false CO readings**

Concentrations of dissolved CO in blood are very low, 0.19-0.50 ml/100ml<sup>166</sup>. Therefore, even the smallest of differences can lead to significance between groups when CO levels are analyzed. It is imperative that collection of blood and measurement of CO be precise. We chose to evaluate the common method by which blood is drawn in the hospital, into a vacutainer, versus collection by syringe, to determine if the materials present in vacutainers or their rubber caps could contribute to altered measurement of CO levels.

We hypothesize that: *blood collection via vacutainer will result in increased CO concentrations, as measured over time, and in comparison to syringe collection.*

**The main objectives were:**

- 1) To measure and compare levels of CO in vacutainers versus syringe in light and dark environments
- 2) To measure CO levels in vacutainers and syringe over time
- 3) To measure CO levels in blood collected via vacutainer or syringe and in the storage of the blood over time
- 4) To compare blood collection by syringe versus lancet for accuracy in CO measurement and to provide an easy method to obtain blood samples for consecutive CO measurements

## Chapter 2

### **Chronic carbon monoxide inhalation during pregnancy augments uterine artery blood flow and uteroplacental vascular growth in mice**

This chapter was modified from its publication: American Journal of Physiology, Regulatory and Integrative and Comparative Physiology, 305:R939-R948, 2013.

## 2.1 Abstract

End-tidal breath carbon monoxide (CO) is abnormally low in women with preeclampsia (PE), while women smoking during pregnancy have shown an increase in CO levels and a 33% lower incidence of PE. This effect may be in part due to lowered sFlt-1 plasma levels in smokers, and perhaps low-level CO inhalation can attenuate the development of PE in high risk women. Our previous work showed maternal chronic CO exposure (< 300ppm) throughout gestation had no maternal or fetal deleterious effects in mice. Our current study evaluated the uteroplacental vascular effects in CD-1 maternal mice which inhaled CO (250ppm) both chronically, gestation day (GD) 0.5 to 18.5, and acutely, 2.5hrs on each of GD 10.5 and 14.5. We demonstrated, using micro-ultrasound measurements of blood velocity and micro-computed tomography imaging of the uteroplacental vasculature, that chronic maternal exposure to CO doubled uterine artery blood flow and augmented uteroplacental vascular diameters and branching. This finding may be of benefit to women with PE, as they exhibit uteroplacental vascular compromise. The ratio of VEGF protein to its FLT1 receptor was increased in the placenta suggesting a shift to a more angiogenic state, however, maternal circulating levels of VEGF, sFlt-1, and their ratio were not significantly changed. Doppler blood velocities in the maternal uterine artery and fetal umbilical artery and vein were unaltered. This study provides *in vivo* evidence that chronic inhalation of 250ppm CO throughout gestation augments uterine blood flow and uteroplacental vascular growth, changes that may protect against the subsequent development of preeclampsia.

## 2.2 Introduction

Pre-eclampsia (PE) is a complication of pregnancy, characterized by the new onset of hypertension and proteinuria<sup>1;2</sup>. It is a life-threatening disorder, ameliorated only by the removal of the placenta, at which point maternal signs and symptoms usually resolve<sup>1;2</sup>. While the exact etiology of the disorder continues to be elusive, it is believed that PE begins with abnormal placental development, and progresses in some women, when coupled with maternal factors that affect endothelial function<sup>1;2</sup>. Although it is well-known that smoking in pregnancy can cause fetal complications, cigarette smoking continues to be a prominent external factor which reduces the risk of developing PE; several researchers, including us<sup>81;88</sup> have reported similar findings<sup>63;67;167-169</sup>. In fact, cigarette smoking in pregnancy reduces the incidence of PE by as much as 33%<sup>63</sup>, and does so in a dose-dependent manner<sup>64;67;170</sup>. Interestingly, pregnant women who use smokeless tobacco (ie. Snuff) do not have the same reduction in PE as women who smoke cigarettes<sup>64</sup>. The main difference is observed in the combustible elements of cigarette smoking; as nicotine and other components of these tobacco products are similar between them. Carbon monoxide (CO) is one such product of combustion found in cigarettes, increased in blood and end-tidal breath of those who smoke<sup>171</sup>, but decreased in the end-tidal breath systems of women who develop PE<sup>80</sup>.

We have previously shown in CD-1 mice<sup>162</sup>, that maternal chronic exposure to CO (< 300ppm) throughout pregnancy does not negatively affect fetal growth or development. We measured fetal and placental weight, and compared resorption and implantation sites to control animals. In this study, maternal mouse blood-CO levels were comparable to those of women who smoke a pack of cigarettes per day or less, roughly 10% carboxyhemoglobin (%COHb) in the blood or lower. This study implicated the possibility of a dose of CO for further testing the hypothesis of its benefit in reduction in the incidence of PE

The normal development of a placental vascular system is one of complexity and intricate communication with the maternal uterine vasculature. The trophoblast cells of the placenta invade

and remodel the maternal spiral arterioles in the uterine wall, rendering them large conduits for blood, unresponsive to vasoactive factors<sup>1</sup>. In women with PE, this process improperly takes place, affecting the uteroplacental vasculature. Studies utilizing Doppler waveforms in pregnancy have shown a relationship between PE and an increased resistance index (RI) in the uterine artery (UtA) in both early and late gestations<sup>172-174</sup>, possibly leading to the decrease in flow measured in the same vessel<sup>173</sup>. Similar findings were observed in the umbilical artery (UMB A) of fetuses in women with PE, with an increase in RI and pulsatility index (PI)<sup>174</sup>. Women who smoke in pregnancy display no difference in the RI of the UtA compared to non- smoking women<sup>175</sup>, however, in this same group of women, some revealed a diastolic notch, indicative of resistance in the vessel<sup>175</sup>. An increase in RI is also observed in the fetal UMB A of maternal smokers<sup>175</sup>, a finding of negative nature for the developing fetus. A study conducted in pregnant sheep reported similar results when nicotine was administered to maternal sheep<sup>176</sup>, indicating a possible causal role for nicotine in the effects of smoking on the uteroplacental Doppler measurements. In the current study, we chose to evaluate the effects CO on the uteroplacental vascular unit, to determine if similarities with nicotine were observed.

A pulsatile blood velocity is associated with hypoxic areas of the placenta<sup>1:127</sup>, leading to the release of anti- angiogenic factors<sup>177</sup>, one of which, soluble *fms*-like tyrosine kinase-1 (sFlt-1) has come into light as a possible mediator in the development of PE<sup>177;178</sup>. Measured in maternal serum, this molecule is normally elevated in pregnancy, but greatly increased (almost five- fold) in the serum of women with PE<sup>139</sup>; a finding thought to play an important role in the development of PE. Its actions interfere with two very important angiogenic molecules of pregnancy, vascular endothelial growth factor (VEGF)<sup>179</sup> and placental growth factor (PGF)<sup>180</sup>. As a soluble form of the VEGF receptor, sFlt-1 is capable of binding and inhibiting the actions of both VEGF and PGF in pregnancy<sup>181</sup>. Smoking in pregnancy leads to a decrease in plasma levels of maternal sFlt-1 at mid gestation<sup>182</sup>, therefore, we sought to determine if elevated CO levels could be responsible for

this altered measurement, as this action could offer benefit to women with PE.

The objective of the present study was to determine the effect of 250ppm CO on mouse placental development, specifically evaluating changes in placental vascular parameters; protein markers affecting angiogenesis, VEGF and sFlt-1, Doppler ultrasound measurements and gross vascular alterations. The hypothesis is that exposure to low level CO throughout pregnancy will lead to vasodilatory changes and angiogenic alterations of the uteroplacental unit.

## **2.3 Materials and Methods**

All experimental procedures were approved by the Queen's Animal Care University Ethics committee (Smith 2007-052-Or) and carried out according to protocol of the Queen's University animal care committee.

### **2.3.1 Animals and Husbandry**

Female CD-1 mice (8-10 weeks old) were mated with males (6-8 weeks old) of the same strain overnight. The detection of a copulation plug was deemed gestation day (GD) 0.5. Mice were placed in a CO-dosing chamber and allowed food and water *ad libitum* until sacrifice, at GD 14.5. For a total comparison of n- values used for each of the study protocol sections, refer to Table 2-1.

### **2.3.2 Carbon Monoxide Exposure**

CO was administered exogenously to maternal mice, either chronically throughout gestation, (beginning on GD0.5) or acutely (for 2.5 hours on each of GD 10.5 and GD14.5). Doses of 0 and 250ppm CO were administered in a sealed chamber as previously described<sup>162</sup>. CO levels within the chamber were controlled at 250ppm, and verified using gas- size exclusion chromatography. The air measured an average of  $252.24 \pm 14.19$  [ppm  $\pm$  standard deviation (SD)] for chronic CO levels and following the half hour chamber equilibration time,  $249 \pm 13.42$  for acute CO levels. We verified that 30 minutes were sufficient time to produce desired CO levels within the chamber (data not shown).

Three gestational days were used for procedural application; GD0.5 (baseline), GD10.5 (mature placental structure is established)<sup>183</sup> and GD14.5 (placental blood flow velocity is established)<sup>184</sup>. Mice were kept within the chamber at all times that procedures were not taking place. Maternal weight was recorded throughout pregnancy and compared between groups as a

**Table 2-1. Explanation of n-values for each section of the study and used for statistical analysis.**

Procedure	Control	Chronic CO Exposure	Acute CO Exposure
Total Mice Used	17	18	11
Maternal blood CO measurement (GD10.5)	5	5	n/a
Maternal blood CO Measurement (GD14.5)	5	5	3
Doppler Analysis (maternal mice)	12	12	7
Doppler Analysis (fetus number)	48	48	28
Micro- Computed Tomography (maternal mouse number)	5	6	n/a
Micro- Computed Tomography (total placental units imaged)	18	12	n/a
Protein analysis (maternal plasma samples)	12	13	8
Protein analysis (placenta samples)	11	14	13

measure of maternal health. The total number of live fetuses and resorptions per mouse were recorded.

### **2.3.3 Doppler Analysis**

Mice were anesthetized with isoflurane gas (1-3%) and oxygen by face mask during ultrasound procedure (limited to an hour of anesthesia). Maternal heart rate was maintained between 480 and 530 beats per minute, by controlling the amount of isoflurane gas administered to the mouse. Maternal temperature was maintained between 36 and 38 degrees Celsius by a rectal temperature probe. The hair on each mouse's abdomen was removed using a chemical hair remover (Nair) and ultrasound gel was warmed before use.

Control (n=12) and chronically (n=12) CO- exposed mice were imaged using Doppler technology on GD10.5 and GD14.5. Mice exposed to CO acutely (n=7), ultrasound analysis was performed before CO exposure and directly afterwards, on each of GD10.5 and GD14.5.

Using a Vevo 770 Ultrasound (Toronto, ON, Canada), we performed Doppler analysis of blood velocity for each maternal mouse (excluding those used for bloodwork) in the maternal UtA and four matched fetal UMB As and veins (UMB V) (two fetuses from each uterine horn). The maternal UtA blood velocity was measured below the bladder, as a direct branch from the internal iliac artery, as previously described by Mu. J *et al.*<sup>183</sup>. The UMB A and UMB V measurements were taken directly exiting the placenta, as matched vessels. All blood velocity was measured at an angle less than 30 degrees between the Doppler beam and the vessel, using a RMV704 scanhead (Visualsonics, Toronto, Canada) operating at 20-60MHz. Data were collected and saved for analysis at a later date.

Analyses were performed in a blinded fashion using the instrument software package provided with the Vevo 770. For each waveform video, a minimum of 10 measurements were taken for the peak systolic (PSV) and end diastolic blood velocity (EDV). The maternal UtA and

fetal UMB A measurements are presented as  $RI = (PSV - EDV) / PSV$ . Fetal UMB V was compared using peak velocity measurements (cm/s).

#### **2.3.4 Necropsy and injection of contrast agent**

On GD 14.5, following ultrasound analysis, mice were anesthetized by intraperitoneal injection of 10mg/g of 2-2-2 Tribromoethanol (T48402, Sigma Aldrich, Canada). Contrast-infusion procedures were carried out as per Whitely *et al.*<sup>185</sup>, with the following differences. We used 2-2-2 Tribromoethanol to anesthetize our animals. In addition, we used a different medium for creation of the contrast-infused placental specimens. As described by Rennie MY *et al.*<sup>186</sup>, we infused a radio-opaque, silicone rubber X-ray contrast agent (Microfil; Flow Tech, Carver, MA) into the arterial system of the mouse, beginning in the thoracic aorta. The abdomen was opened to allow for visual tracking of the dyed rubber as it filled the arterial system, to ensure that it only filled as far as the labyrinth layer of the placentas; and not the venous system of the maternal mouse. It was left to polymerize for 1hour following proper perfusion of the vessels. During this time, total implantation sites, live fetuses and resorptions were recorded. The entire uterus was then excised and immersed in 4% paraformaldehyde (PFA) for 24hrs at 4 °C. At this point, uterine horns were either transferred to 1X phosphate buffered saline (PBS) for future setting, or immediately set in 1% agar prepared in 4% PFA. Specimens were mounted so as to elongate the uterine horn and expose the placenta and its vasculature to imaging.

#### **2.3.5 Micro-computed (micro- CT) tomography imaging**

All mounted samples were sent to the Toronto Hospital for Sick Children, Mouse Imaging Centre. As previously described<sup>186:187</sup>, three-dimensional images of each placenta were created and blinded for analysis, by the technologist in charge of the micro-CT. Vessel number

and diameter, in addition to diameter length of the intervillous space were measured in a blinded fashion using the Amira Software package (Visage Imaging, San Diego, CA). A minimum of two placental images (one per uterine horn) were saved per mouse for analysis. Maternal UtA diameter was measured in each of the placental units imaged. Ten measurements were taken per image; therefore a total of 20 measurements were averaged per UtA in each mouse. For each placental unit imaged, a minimum of ten measurements of diameter were taken randomly throughout each vessel being analyzed. In each placental unit, the measurements were averaged for each vessel, and this value was compared in each of the respective CO treatment groups. The following arteries were analyzed: UtA, radial arteries, spiral arteries, and canals (See Figure 2.1 for a description of placental layers, and a comparison of control versus treatment groups). We defined radial artery branches and canal branches, as the point at which the vessel began to split into more than one vessel (Figure 2.1). The number and diameter of vessels at this point were also counted. A total of 6 measurements of the diameter of the intervillous space were taken and the mean of these were compared between placentas (Figure 2.1). For those maternal mice who were used in both the ultrasound analyses and the micro-CT analysis, blood flow in the UtA was calculated (control n=8, chronic CO n=5). Maternal mouse UtA mean velocity over a cardiac cycle (MV) was measured and used with the corresponding UtA diameter (d) measurement in the following calculation for blood flow =  $A \cdot MV$ , or Blood flow =  $(\pi \cdot (d/2)^2 \cdot MV)$ .

### **2.3.6 Blood Collection**

Blood was collected from all mice on GD10.5 and GD14.5, but CO levels were only measured in a select group of mice for each experimental group (refer to Table 2-1). Blood was collected from all conscious maternal mice on GD10.5 via the submandibular vein. Using a 5mm lancet (Golden rod, Braintree Scientific, MA), the vein was punctured and 5 drops of blood (roughly 100ul) were collected into microcentrifuge tubes containing 10ul of 1400U/ml sodium

heparin (Sigma Aldrich, H0777) on ice. On GD14.5, upon reaching surgical plane of anesthesia, blood was collected by retro-orbital puncture, using a glass pipette, and transferred to a microcentrifuge tube containing 10ul of 1400U/ml sodium heparin (Sigma Aldrich, H0777), on ice.

### **2.3.7 CO Measurement**

CO was measured in a separate set of mice from those imaged using Doppler ultrasound (Table 2-1). Hemoglobin levels were measured using a Hemocue, Hb 201 (Hemocue, Sweden). Maternal blood was measured for CO level as previously described<sup>162</sup> using gas –solid chromatography (Peak Performer 1, Palo Alto, San Francisco, USA), and blood CO levels were expressed as %COHb.

### **2.3.8 Plasma Separation for ELISA assay**

All blood was centrifuged for 15minutes at 16 000 rpm, and plasma was removed and stored at -80 degrees Celcius until future analysis.

### **Placental Protein measurements**

A random sample of mice (control n=5, chronic CO n=5, acute CO n=6) were not perfused, to allow for placental protein analysis. Placentas were removed from the uterine tissue, immediately frozen in liquid nitrogen and maintained at -80° Celcius. Total placenta numbers used for protein analysis are shown in Table 2-1.

#### *Total Protein Analysis*

Each placenta was homogenized in 1% Triton X, 1XPBS buffer using a dounce

homogenizer. The homogenate was centrifuged at 14000rpm for 20min at 4°C. The supernatant was removed to a fresh microcentrifuge tube and maintained on ice. A 1 in 2 dilution of the sample in the same buffer was prepared and protein levels were measured using a standard CD Lowry Assay (Bio Rad, Sigma). Further dilutions were prepared to ensure each sample was 2ug protein/ul of sample. All sample loading was verified using western blot analysis (data not shown).

### **2.3.9 Enzyme- linked immunosorbent assay (ELISA) analysis**

Measurement of protein level sFlt-1 (Quantikine MVR100, R &D systems, Canada) and free VEGF (Calbiochem, Q1A52 ) was completed using a sandwich ELISA in duplicates, for both maternal plasma and the placental homogenates. The sFlt-1 ELISA is not specific to the soluble VEGF receptor, and also measures the transmembrane VEGF receptor (VEGFR1). In the plasma, the only measured VEGFR should be the soluble form, therefore we refer to this protein as sFlt-1. In the placenta, both soluble and membrane- bound VEGFR1 may be present, therefore we refer to this protein measurement as total VEGFR1. Each ELISA was standardized to its respective control protein supplied with the kit materials. Maternal plasma was diluted 1/5 for the VEGF ELISA and 1/10 for the sFlt-1 ELISA, using the respective diluent provided. Placental homogenates were used at 2ug/ul for the VEGF ELISA (total 100ug of protein loaded per sample in duplicate) and further diluted 1/5for the sFlt-1 ELISA (total of 20ug of protein loaded per sample in duplicate).

### **2.3.10 Statistics**

Statistical analysis of placental vessel diameters and blood flow comparison were completed using a Student's *t*-test; significance identified at  $p < 0.05$ . All remaining statistics were completed using a one way analysis of variance and post- hoc Tukey test (Graphpad Prism

Version 6.0). A p value <0.05 was deemed significant and all results are reported with SD. All n values used in the analysis are shown in Table 2-1.

## **2.4 Results**

### **2.4.1 Maternal CO exposure during pregnancy did not affect fetal resorption number or maternal / fetal weight in comparison to control**

In each of the experimental groups, maternal mice appeared to be healthy throughout gestation, with no difference in the change ( $p>0.05$ ) in maternal weights ( $g \pm SD$ ) from GD0.5 to GD14.5 between the groups (control  $16.4 \pm 1.6$ , chronic CO  $15.4 \pm 1.6$ , acute CO  $15.4 \pm 1.3$ ). Assessment of litter size was determined for each mouse, evaluating the live fetus number, in addition, this number as a fraction of the total implantation sites, and no difference was observed between mice exposed to CO and control (Table 2-2). Further, for each maternal mouse, fetal resorptions were calculated as a fraction of total implantation sites, and again no differences were observed between the groups (Table 2-2). As an indication of CO exposure, levels of CO were calculated as a percentage of total hemoglobin and presented as %COHb. For mice exposed to chronic CO, %COHb levels were significantly higher than control (Table 2-2). Levels of CO in mice exposed to CO acutely were calculated on GD14.5 only, and again were significantly higher than control mice (Table 2-2). Chronic and acute CO exposed mice did not differ in the level of their %COHb levels. Levels up to 14%COHb have been measured in women who smoke while pregnant<sup>188</sup>, and while a direct comparison cannot be made between humans and mice, our study maintains a level of COHb within a close range of pregnant smokers.

**Table 2-2: Maternal plasma CO levels and fetal litter numbers.**

	Maternal Plasma %COHb (mean $\pm$ SD)		Survival Index		Resorptions
	GD10.5 (n=5)	GD14.5 (n=5)	Live fetuses/ total implantation (mean $\pm$ SD)	Live fetuses (mean $\pm$ SD)	Resorptions/ Total implantation (mean $\pm$ SD)
Control	0.53 $\pm$ 0.13	0.82 $\pm$ 0.23	0.94 $\pm$ 0.05	13.1 $\pm$ 2.11	0.06 $\pm$ 0.05
Chronic CO	11.34 $\pm$ 5.58**	13.24 $\pm$ 2.98**	0.92 $\pm$ 0.14	12.5 $\pm$ 1.85	0.09 $\pm$ 0.15
Acute CO	n/a	11.39 $\pm$ 4.02** (n=3)	0.98 $\pm$ 0.07	11.75 $\pm$ 1.84	0.025 $\pm$ 0.07

All values are compared to control and presented as ( $\pm$ SD) (\*p <0.05 and \*\*p<0.001).

#### **2.4.2 Increased vessel diameters of arteries in uteroplacental units of contrast- infused specimens**

Micro-CT images of the placental units allowed for diameter measurements of the vascular tree from the maternal UtA to the placental intervillous space (Figure 2.1). Maternal chronic CO exposure led to an increased vessel diameter in the maternal uterine artery, in addition to the spiral arteries and the branching vessels of the radial arteries and the canals ( $p < 0.05$ ) (Figure 2.2). The intervillous space diameter (mm) was not statistically different ( $p > 0.05$ ) between CO- exposed and control groups,  $5.54 \pm 0.36$  vs.  $5.69 \pm 0.61$  respectively. In addition to vessel diameter, a quantification of vessel number was assessed for radial arteries, canals and each of their branching vessels, Between control and chronic CO- exposed mouse groups, the number ( $\pm$ SD) of radial artery vessels,  $1.75 \pm 0.71$  and  $1.75 \pm 0.45$  respectively and canals,  $3.63 \pm 1.3$  and  $3.08 \pm 1.08$  respectively, were not significantly different ( $p > 0.05$ ). The number of radial artery branches was significantly increased in the chronic CO exposed group ( $11.08 \pm 4.55$ ) versus the control group ( $7.25 \pm 2.44$ ) ( $p < 0.001$ ), as was the number of canal branches, chronic (CO  $7.55 \pm 0.45$ ) versus control ( $2.25 \pm 2.71$ ) ( $p < 0.001$ ).

#### **2.4.3 Uteroplacental blood flow velocity was not different between CO-exposed mice and control, but maternal blood flow was significantly higher in the group exposed to chronic CO**

Blood flow velocity was measured in each of the maternal uterine arteries, in addition to the fetal umbilical arteries and veins. Data are presented for each of these vessels on both GD10.5 and GD14.5, and PSV is displayed in all cases, in addition to RI value for arterial vessels (Table 2-3). Doppler analysis demonstrated no significant change in the PSV or the RI of the maternal UtA or the fetal UMB A of maternal mice exposed to chronic or acute CO compared with control ( $p > 0.05$ ). This finding was true on both GD10.5 and GD14.5, in addition to the acute exposure before and after CO ( $p > 0.05$ ). UMB V peak velocity (cm/s) did not differ between experiments.

**Table 2-3: Doppler blood velocity measurements in the maternal uterine artery and fetal umbilical artery and vein.**

	GD10.5		GD14.5	
	Peak Systolic Velocity (cm/s)	Resistance Index	Peak Systolic Velocity (cm/s)	Resistance Index
<i>Uterine Artery</i>				
Control	17.57 ± 3.71	0.64 ± 0.12	22.05 ± 9.59	0.54 ± 0.04
Chronic CO Exposure	17.57 ± 9.50	0.61 ± 0.04	24.19 ± 7.70	0.55 ± 0.03
Before Acute Exposure to CO	19.57 ± 5.4	0.61 ± 0.08	23.91 ± 6.21	0.57 ± 0.15
After Acute Exposure to CO	20.29 ± 4.1	0.66 ± 0.04	23.49 ± 5.84	0.56 ± 0.11
<i>Umbilical Artery</i>				
Control	3.65 ± 0.63	0.82 ± 0.19	7.07 ± 1.98	0.91 ± 0.40
Chronic CO Exposure	3.20 ± 0.42	0.79 ± 0.04	8.99 ± 2.00	0.97 ± 0.09
Before Acute Exposure to CO	3.32 ± 0.13	0.84 ± 0.04	8.76 ± 0.22	0.97 ± 0.06
After Acute Exposure to CO	3.34 ± 0.19	0.83 ± 0.04	9.19 ± 1.91	0.93 ± 0.09
<i>Umbilical Vein</i>				
Control	1.70 ± 0.27	n/a	3.69 ± 0.78	n/a
Chronic CO Exposure	1.74 ± 0.47	n/a	3.39 ± 0.69	n/a
Before Acute Exposure to CO	1.56 ± 0.26	n/a	4.32 ± 1.69	n/a
After Acute Exposure to CO	1.70 ± 0.83	n/a	4.62 ± 1.00	n/a

All data are presented (±SD). Statistical significance was set at \* p<0.05. Control maternal mice n=12, Chronic CO maternal mice n=12, Acute CO maternal mice n=7. For each maternal mouse imaged, 4 of their respective fetuses were imaged for umbilical artery and vein measurements. PSV= peak systolic velocity and Resistance Index (RI) = (PSV- EDV)/PSV

It is important to note that in order to complete Doppler measurements, mice are anesthetized with isoflurane by nose inhalation. In doing so, the level of maternal CO decreases more quickly than in general room air. Our unpublished data suggest that in normal room air, the half- life for maternal mouse %COHb is 88minutes and for a mouse on isoflurane, the half- life is reduced to 16.5minutes. Special care was taken to ensure that measurements were taken within thirty minutes, to decrease the effect of isoflurane on maternal %COHb.

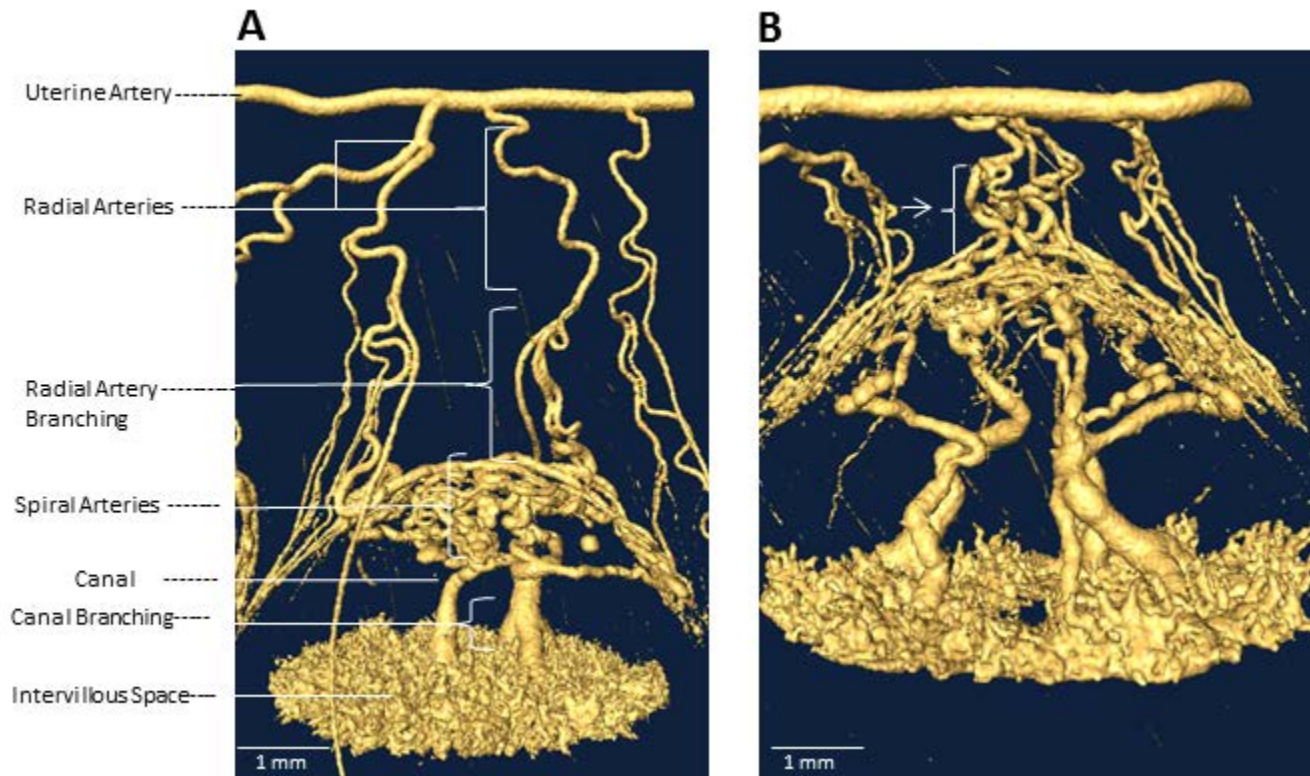
Although maternal blood flow velocity was not different between groups, blood flow calculations led to different results. Calculations for blood flow were possible due to the vessel diameter measurements using the contrast- infused specimens for chronic CO-exposed mice and control. Maternal mouse UtA blood flow was significantly increased in those mice exposed to CO (Figure 2.3).

#### **2.4.4 Placental and maternal plasma protein ratios of sFlt-1 to VEGF are shifted towards an angiogenic balance in CO-exposed animals versus control**

Maternal plasma measurements for both protein VEGF and protein sFlt-1 levels are shown using a box and whisker plot in Figure 2.4 A and B respectively. No difference ( $p > 0.05$ ) was noted between control and chronic or acute CO- exposed mice in VEGF levels (GD10.5 or GD14.5), though a small increasing trend was noted in the CO exposed mice compared to control. Plasma sFlt-1 levels were also not different between the three groups, (GD10.5 or GD14.5), though a trend towards a decrease in protein levels was observed in acute versus control mice. The ratio of sFlt-1 to VEGF was not different between groups on GD10.5, however, on GD14.5, though no change was observed between control vs. chronic CO- exposed mice ( $p > 0.05$ ), a significant decrease ( $p = 0.008$ ) between control and acutely exposed mice was observed (Figure 2.4 C).

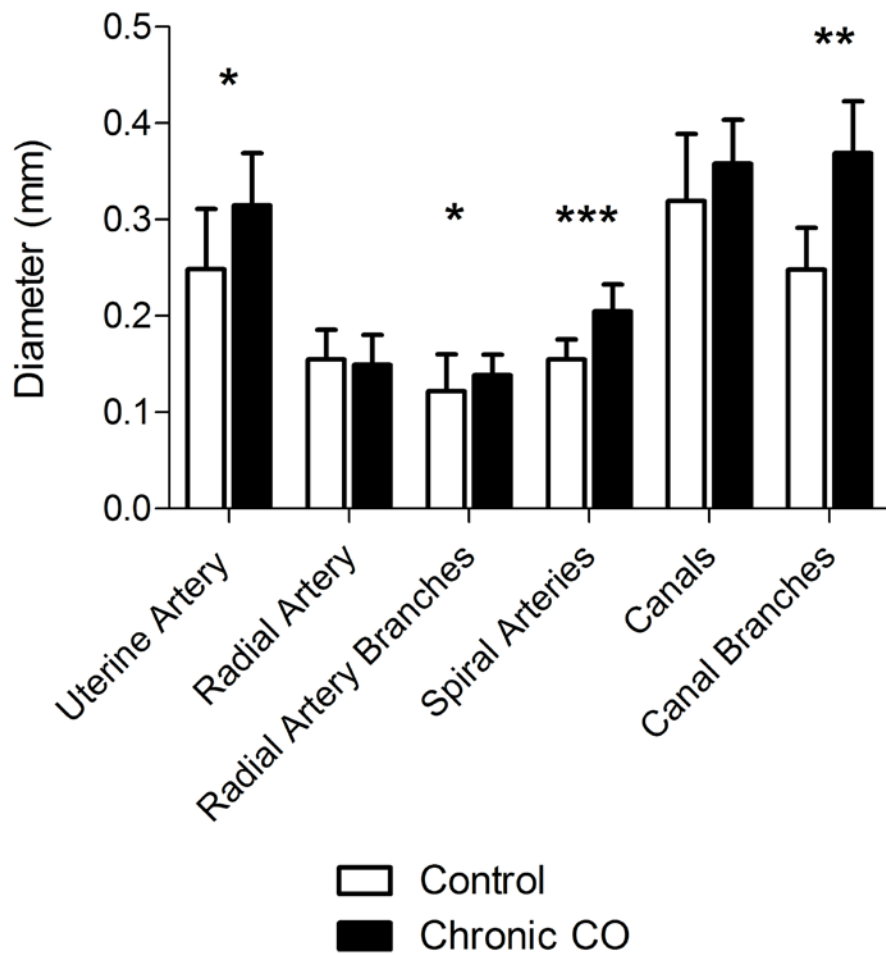
Placental protein levels (Figure 2.5) followed a similar pattern to maternal plasma for each of the respective protein levels. A trend ( $p = 0.092$ ) towards increasing placental VEGF

levels was observed from maternal dams exposed to chronic CO; an increase ( $p= 0.003$ ) was observed in the acutely exposed mice (Figure 2.5A). Though no significance was measured in the total VEGFR1 protein levels (Figure 2.5B), a decreasing trend in protein levels was determined in those mice exposed to acute CO. Finally, the ratio of total VEGFR1: VEGF was significantly lower in both the chronically- exposed ( $p= 0.008$ ) and acutely CO- exposed ( $p=0.0008$ ) maternal mice compared to control (Figure 2.5C).



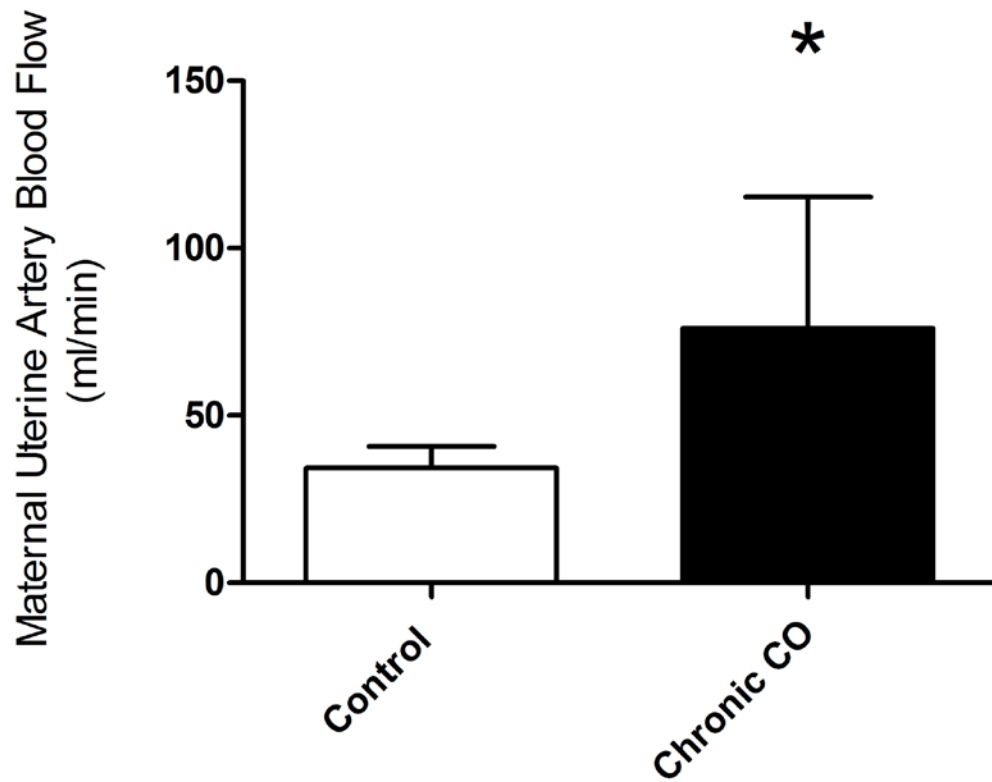
**Figure 2.1: An iso-intensity surface rendering from micro-computed tomography of a CD-1 mouse placenta, GD 14.5, from both a control and maternal CO-exposed mouse.**

A) Representative image of a control placenta, with areas of the placenta identified, as defined in our protocol. B) Representative image of placenta from a chronic CO- exposed maternal mouse. Increased branching of the radial arteries can be seen (white arrow) in comparison to A.



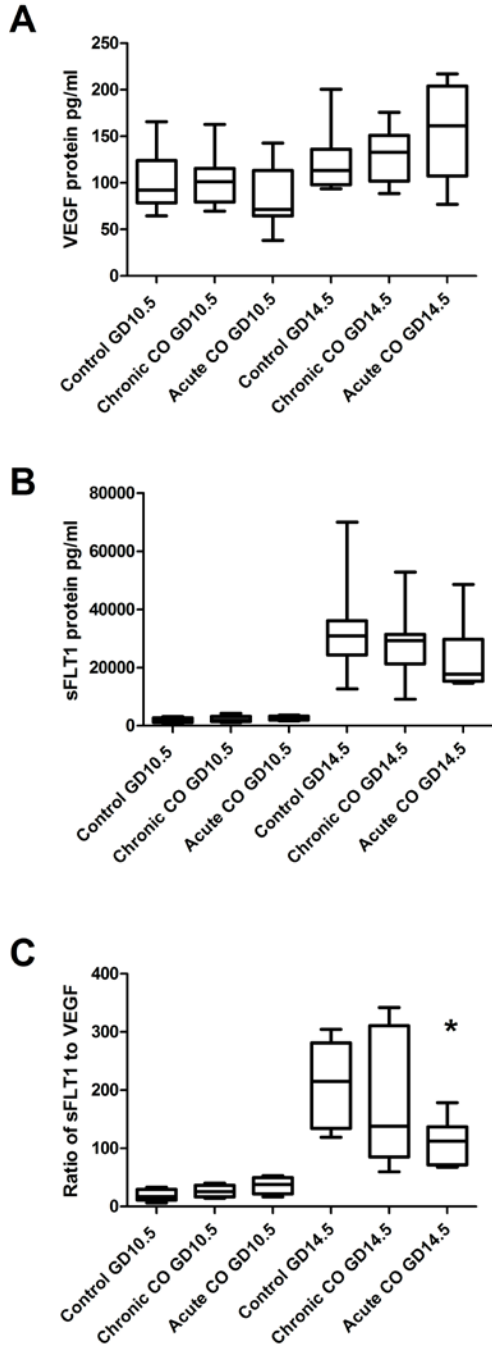
**Figure 2.2 Measurements of placental vessel diameters in those from maternal dams in control groups and those exposed to chronic CO.**

Imaging of the contrast- infused placental specimens was completed by micro- computed tomography and vessel measurements are displayed. Maternal exposure to chronic CO increased the vessel diameter in the uterine artery, the spiral arteries and the branching vessels of the canals and the radial arteries of the uteroplacental unit compared to control (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).



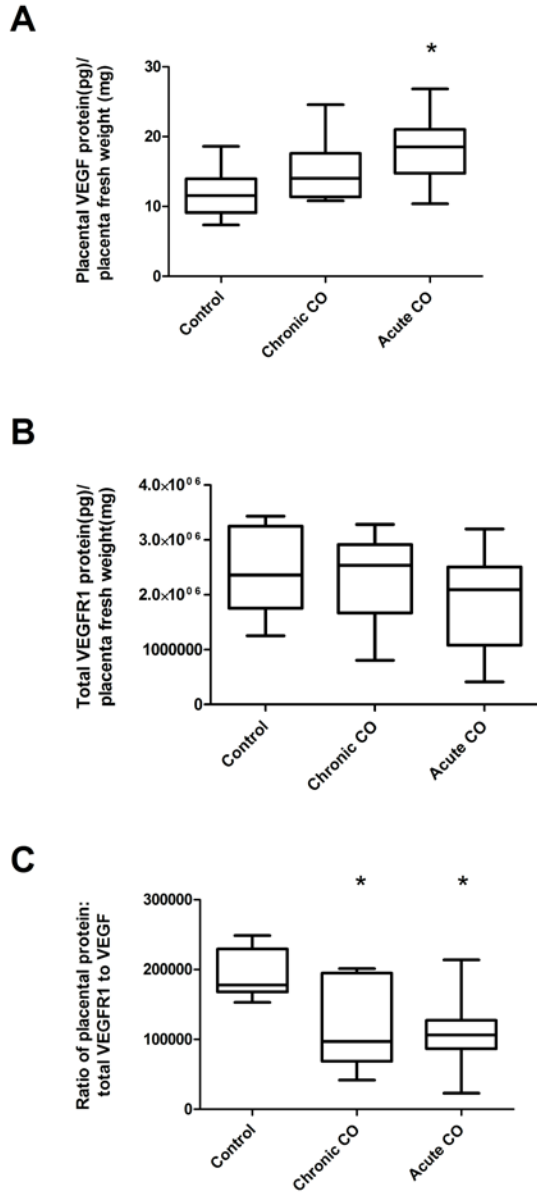
**Figure 2.3: Maternal uterine artery mean blood flow between chronically CO- exposed maternal mice and control mice.**

Using the uteroplacental vessel measurements for uterine artery diameter and the Doppler mean velocity over a cardiac cycle, the mean blood flow was determined. Although Doppler blood flow was not different between the two groups, the maternal uterine artery diameter was increased in those mice exposed to CO, thereby increasing the blood flow in the vessel (\*  $p < 0.05$ ).



**Figure 2.4: Maternal plasma protein levels: vascular endothelial growth factor (VEGF) and its soluble receptor.**

Maternal plasma was collected and compared on GD10.5 and GD14.5 for mice in control, chronic CO-exposed and acute CO-exposed groups. A) Comparison of maternal plasma VEGF protein levels, not significant ( $p > 0.05$ ). B) Comparison of soluble *fms*-like tyrosine kinase -1 (the soluble VEGF receptor, sFlt-1) protein in maternal plasma; no significance between groups ( $p > 0.05$ ). C) Comparison of the ratio of sFlt-1 to VEGF in maternal plasma; significance observed in the acute CO-exposed animals only ( $p < 0.05$ ).



**Figure 2.5: Placental (GD14.5) protein levels for VEGF and its soluble anti- angiogenic receptor.**

A) VEGF protein levels were significantly ( $p < 0.05$ ) different in acutely- exposed animals versus control, but not in chronic CO- exposed animals. B) Levels of total VEGFR1 were not different in any of the groups of mice ( $p > 0.05$ ). C) Both chronic and acute CO- exposed animals resulted in a decrease in anti angiogenic total VEGFR1 to angiogenic (VEGF) protein ( $p < 0.05$ ).

## 2.5 Discussion

PE is a leading cause of maternal morbidity and mortality worldwide<sup>1</sup> and it continues to claim the lives of upwards of 50 000 women a year in developing countries<sup>189</sup>. There is no cure for the disorder itself, until delivery occurs<sup>1</sup>. It has been shown that women with PE have significantly lower end- tidal breath CO levels compared to those with healthy pregnancies<sup>80</sup>. Therefore, it is possible that elevated CO levels in women who smoke during pregnancy may contribute to the reduced risk of developing PE. While this study aimed to evaluate the effects of CO exposure in pregnancy, ultimately as a possible mechanism for the observed reduced incidence of PE in women who smoke, the specific goal of this study was to determine if CO altered the vascular system of the uteroplacental unit.

We used healthy, pregnant CD-1 mice, without induction of PE-like symptoms, to allow for a clear observation of placental alterations due to maternal CO exposure. The CO dose of 250ppm was chosen based on our previous work in CD-1 mice<sup>162</sup> which demonstrated that below 330ppm CO, maternal exposure does not lead to fetal alterations of growth and development. Further, exposure to 250ppm CO led to maternal mouse CO levels of 12.65%COHb, similar to maternal %COHb levels in women who smoke roughly a pack of cigarettes per day during pregnancy; measured in our lab as high as 9.85%COHb (unpublished) and by others as 14%COHb<sup>169</sup>. As well, other research groups have evaluated 250ppm CO in animal models studying its anti- inflammatory effect<sup>190;191</sup> and tissue graft transplants<sup>106;192;193</sup>; for a more complete list of experiments, refer to the review by Motterlini and Otterbein<sup>194</sup>.

We evaluated placental vascular changes on GD 10.5 and GD14.5 in order to test the effect of CO on the placental vascular development (GD10.5- implantation is complete, vascular invasion commences and the blood begins to cross the placenta to the fetus<sup>195;196</sup>, GD 14.5- placental vascular flow velocity is established and the first quiescent phase of fetal vasculature occurs<sup>197</sup>). We demonstrated that maternal chronic exposure to 250ppm CO throughout pregnancy

increases placental vascular branching and vascular diameter. The diameters of almost all uteroplacental vessels were enlarged significantly, while branching was augmented in the radial arteries and the canals, directly above the placental labyrinth. The increase in branching is especially curious, as these vessels (canals and radial arteries) are quite different in vascular character. Radial arteries are very much vascular in nature, while the placental canals are more so channels for blood travel, with no endothelium or smooth muscle. The molecular mechanism of CO on each of these vessels remains to be elucidated. The altered placental growth pattern offers some adaptation control due to an environmental exposure to CO. The micro-CT imaging results suggests that CO- induced remodeling could lead to increased oxygen and nutrient delivery from maternal to fetal vessels. In a pathological pregnancy complication such as PE, the placental nutrient delivery is compromised; increased branching and vessel size could reduce some of the negative effects.

CO has been shown to decrease placental apoptosis and to elevate placental VEGF protein and mRNA<sup>163</sup>. We measured protein levels of total VEGFR1 and VEGF and our data values were similar to those reported in the literature for pregnant mice, for both VEGF<sup>198</sup> and total VEGFR1<sup>145;198</sup>. We confirmed a significant increase in protein VEGF levels in placentas from maternal dams exposed to acute CO.. Perhaps the CO effect is more strongly related to a decreased sFlt-1 release, thus increasing the VEGF bioavailability. Indeed, it has been previously shown that HO-1/ CO pathway is capable of decreasing the sFlt-1 release from human umbilical vein endothelial cells *in vitro*<sup>154</sup> and from rat placental explants in both hypoxic and normoxic conditions<sup>155</sup>. We demonstrated that the ratio of placental total VEGFR1 to VEGF was significantly reduced in both chronic and acutely-exposed animals, indicating a possible mechanism for the placental vessel changes noted. The placentas of women who smoke in pregnancy have been reported as larger in size, with an increase in branching of the vascular tree<sup>199</sup>; the authors suggest that this alteration is an adaptation response, increasing surface area for

nutrient transfer. This adaptive angiogenesis was similar to our findings. It is clear that CO plays a role in angiogenesis, a process necessary for placentation and may account for the CO-induced increases of placental vessel branching in the placentas of women who smoke in pregnancy.

Several researchers have reported on the importance of CO and its parent enzyme heme oxygenase, (HO) in pregnancy<sup>47;153;154;200</sup>. In a recent study<sup>57</sup>, it was shown that without HO-1, placental implantation is compromised, placenta structure is altered and fetal loss is increased significantly. Interestingly, in HO-1 null mice, maternal CO exposure (50ppm) diminished fetal loss and restored the placental trophoblast cell viability; placentas were structurally healthy<sup>57</sup>. In PE, the placenta is compromised at the maternal- fetal interface, where spiral artery remodelling is impaired<sup>2</sup>. It is known that HO-1 is expressed at the maternal- fetal interface and its production of CO through heme catabolism may be important for implantation, placental development and fetal survival (as shown in a murine model)<sup>57</sup>. Perhaps a deficiency in this pathway is a part of the progression of some women to develop PE. Our study provides a possible mechanism whereby the exposure to CO can alter the placental development. Translated to an animal model of PE, CO could potentially ameliorate or attenuate the clinical symptoms of PE.

No significant difference between control and CO –exposed groups was observed in either of the protein levels measured for VEGF and sFlt-1 in maternal plasma. Similar results were observed when the ratio sFlt-1 to VEGF was calculated for chronic CO- exposed maternal mice; however the ratio was significantly lower for those exposed to acute CO. This could be due to a decreased sensitivity in the mice exposed to chronic CO exposure. In a study conducted by George *et al*<sup>148</sup> maternal rat sFlt-1 levels were increased in rats by implanted mini osmotic pumps, with a group of rats also receiving an inducer of the enzyme producing CO. VEGF levels were only increased in rats that were first infused with sFlt-1 levels, followed by induction of the CO system. No alterations were observed when control mice were exposed to increased CO levels. Therefore, it is possible that in our study, exposure to CO would not affect maternal

plasma sFlt-1 or VEGF levels until alterations in normal levels were first induced. One of the goals of using CO as a possible future therapeutic in patients with PE would be to lower plasma sFlt-1 levels. In this regard, future studies would be necessary to investigate the effect of repeat exposure to short term CO levels on maternal plasma sFlt-1.

The Doppler blood velocity findings were surprising, as we expected CO, a known vasodilator, to increase the blood velocity in all vessels measured. It could be, that in control mice, vessels are already maximally dilated and little change would be observed. Perhaps the exposure to CO in pregnancies of complication, such as PE, would offer different results. Though no change was observed, this finding was able to highlight that CO is not responsible for the increased RI and PI in the UMB A observed in the fetal vasculature of maternal smokers. This is of great importance, as an increased RI negatively impacts the nutrient transfer to the fetus.

Though the maternal UtA blood velocity was not different in maternal mice exposed to CO versus control, the blood flow was significantly increased. This makes sense, due to the increased UtA diameter measured in the contrast- infused placentas. The equation for blood flow demonstrates that an increased vessel size with no change in mean blood velocity would lead to an overall increase in flow, or a larger amount of blood travelling per minute. By using CO to augment uterine blood flow rates, it may be possible to reverse or prevent this risk factor, decreasing the development of PE.

In addition to our previous work<sup>162</sup>, others have shown that *in vivo* exposure to low-dose CO or induction of HO-1 is beneficial to both mother and fetus(es). El-Mousleh *et al.*<sup>163</sup> have shown that low dose (50ppm) exposure to CO in pregnancy in early gestation rescues intrauterine growth restriction in mice, in addition to decreasing placental apoptosis, reduces pro-inflammatory cytokines, and exhibits pro-angiogenesis in the placenta. Further, the same research group has demonstrated that IUGR seen in HO-1 null mice can be rescued by maternal exposure to CO<sup>57</sup>. The exact mechanism by which this occurs has not been determined. However, it is

possible that maternal exposure to CO would reduce the oxidative stress experienced by the placenta, potentially decreasing the free radical production and oxidative stress- induced apoptosis as observed in different organ systems<sup>201</sup> and in placental ischemia- reperfusion models<sup>98;155</sup>. Our study did not evaluate the effect of CO on placental oxidative stress, and future work should aim to report on these findings. In addition, George *et al.*<sup>116</sup> have shown that HO-1 induction attenuated ischemia- induced hypertension in rats, and shifted the placental sFlt-1/VEGF ratio to one of increased angiogenesis, previously observed in a rat reduced uterine perfusion pressure placental ischemia model.

There are other potential beneficial effects of both HO-1 and CO in placental development and fetal survival. Several studies have evaluated the effects of HO-1 in pregnancy, and it is clear that this enzyme is necessary for proper implantation and placental development. It would make sense then, that increasing one's CO levels could rescue some of the deleterious effects associated with PE. Perhaps women who smoke cigarettes during pregnancy are able to replace the decreased levels of CO present in women with PE<sup>202</sup>. It is still unclear how, if true, low doses of CO may aid in the decreased incidence of PE, however there are several possible mechanisms for this to take place. In fact, endogenous and exogenous CO has been shown to decrease anti-angiogenic factor release<sup>154</sup>, support angiogenesis<sup>203</sup>, vasodilate blood vessels<sup>112</sup> and suppress inflammation<sup>163</sup>. In placental tissue specifically, CO, at physiologic levels, has shown decreased inflammation and apoptosis<sup>98</sup>, in addition to increased vasodilatory effects<sup>112</sup>. Each of these properties could be helpful in reducing the development of PE.

Unfortunately, smoking does not completely ameliorate the development of PE. Cigarette smoking appears to have less of an effect on the development of PE in more severe forms of the disorder<sup>64</sup> and women who smoke and develop PE are at an increased risk for adverse pregnancy outcomes<sup>65</sup>. As Karumanchi and Levine<sup>204</sup> have postulated, perhaps there is a threshold of angiogenic balance, which when crossed, leads to the development of PE. Further, it is possible

that CO in cigarette smoke reduces the incidence of PE by altering the angiogenic/ anti-angiogenic balance. However, in more severe forms of PE, it would take more of a reduction in the anti-angiogenic molecules to prevent the development of PE. Karumanchi and Levine<sup>204</sup> have further proposed that smoking in pregnancy more frequently reduces the incidence of mild forms of PE, thus increasing the proportion of women who develop more severe forms of PE in women who smoke.

### **Perspectives and Significance**

Our study provides evidence for the effects of CO in pregnancy, and the role it might have in attenuating the signs of PE. We are the first to use placental contrast-infused specimens to evaluate the vascular effects of maternal mouse exogenous CO exposure throughout pregnancy. With a focus on the placental development, we showed that maternal inhalation of 250ppm CO increased angiogenesis in the placenta, in addition to gross vessel size, possibly as a result of the concomitant increase in VEGF compared with VEGFR1. These findings are important, as they provide a possible explanation for the lower incidence of PE among smokers; given that these patients' CO levels are much higher than non- smokers. More importantly, this study supports the possibility of use of CO as a therapeutic in the treatment of PE.

## Chapter 3

# **Carbon monoxide attenuates the release of sFlt-1 from placental villous explants**

### 3.1 Abstract

**Introduction:** Preeclampsia (PE) is a common pregnancy disorder associated with significant maternal and fetal/neonatal morbidity and mortality. The release of placental soluble factors into the maternal circulation is thought to contribute to the widespread maternal endothelial dysfunction; soluble vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) and soluble endoglin (sEng) are two such factors. Inducers of the heme oxygenase (HO) system have been shown to reduce the production of these two factors. The purpose of this study was to determine if carbon monoxide (CO) gas, an endogenous product of the HO system, could reduce the release of sFlt-1 and sEng from term placental explants *in vitro*.

**Methods and results:** Placental chorionic villous samples from term elective caesarian section were plated on netwell culture plates, five 1mm<sup>3</sup> explants per well. Placental villous explants were exposed to either control air (5% O<sub>2</sub>, 5% CO<sub>2</sub> and balance N<sub>2</sub>), or CO air (250ppm CO, 5% O<sub>2</sub>, 5% CO<sub>2</sub> and balance N<sub>2</sub>) and concentrations of tissue sFlt-1, sEng and placental growth factor (PGF) were analyzed over a 24hr time period. sFlt-1 concentrations (pg/mg protein ± SEM) were significantly increased from 6hr (1372 ± 299) to 24hr (3474 ± 486) in standard experimental conditions but exposure to CO decreased (P<0.05) sFlt-1 levels at 24hr exposure (2090 ± 289). No change in sEng (P>0.05) concentrations was observed over time or between the two groups. There was no significant difference (P>0.05) in PGF concentrations between control or CO-exposed groups, though a trend towards increasing protein levels in explants exposed to CO.

**Conclusion:** CO exposure inhibits sFlt-1 release, but not sEng, and offers a possible method that the HO/CO pathway functions to decrease the incidence of PE.

### 3.2 Introduction

Preeclampsia (PE) is a hypertensive disorder specific to pregnancy, diagnosed after the 20<sup>th</sup> week of gestation<sup>13</sup>. It is thought that the disorder is due to incomplete remodeling of the spiral arteries by the trophoblast cells<sup>205</sup>, leading to compromised blood flow to the placenta and the developing fetus<sup>4;13</sup>. In response to placental hypo-perfusion, areas of hypoxic foci develop in the placenta, leading to apoptosis<sup>13</sup>. In addition, the placenta produces soluble factors which are released into the maternal circulation, along with apoptotic placental debris<sup>206</sup>. Once in the circulation, this material adversely affects the maternal vasculature and contributes to the development of widespread endothelial dysfunction<sup>8;206</sup>.

Among the factors released by the placenta is an anti-angiogenic molecule, soluble *fms*-like tyrosine kinase-1 (sFlt-1) or the soluble form of the vascular endothelial growth factor (VEGF) receptor-1, which is increased in the serum and placentas<sup>138;139</sup> of women with PE<sup>139</sup>. This molecule decreases the availability of important angiogenic molecules, VEGF and placental growth factor (PGF), binding to them and decreasing their interaction with endogenous receptors on the endothelium<sup>141</sup>. Not surprisingly, both VEGF and PGF are decreased in maternal serum<sup>139;140</sup> and placenta tissue<sup>138</sup> of women with PE. Both VEGF and PGF are involved in normal placental angiogenesis and vasculogenesis and they are thought to play a pivotal role in the success of a normal pregnancy<sup>6</sup>. Alterations in the balance between these molecules of angiogenesis and anti-angiogenesis led to problems of placental growth and development and contribute to the development of PE.

Another molecule of interest in the development of PE is soluble Endoglin (sEng), thought to be produced by a proteolytic cleavage of the membrane-bound Eng<sup>207</sup>. While Eng is involved in normal vascular remodeling and homeostasis<sup>208</sup>, sEng binds to transforming growth factor (TGF)  $\beta$ 1, inhibiting its interaction with membrane bound Eng<sup>209</sup>, diminishing its vasodilatory effects<sup>207</sup>. Further sEng interferes with endothelial cell proliferation and capillary

formation<sup>207</sup>. The pro-hypertensive properties of sEng suggest a role for this molecule in PE and circulating levels of sEng in women with pre-term PE is significantly increased, greater than four-fold higher than control women<sup>140</sup>.

The heme oxygenase (HO) enzyme has been implicated in the regulation of these anti/angiogenic molecules<sup>154</sup>. This enzyme is found endogenously in most cells of the body to break down the pro-oxidant molecule heme into carbon monoxide (CO), biliverdin and free iron<sup>27</sup>. The HO system is involved in the progression of a healthy pregnancy, as a woman's total HO activity is increased in pregnancy<sup>35</sup> and decreases in expression and protein levels of both HO and CO are associated with complications of pregnancy<sup>49</sup>, including PE<sup>39;50;51</sup>. *In vitro* studies using placental tissue have shown the exposure to cobalt protoporphyrin (CoPP), an HO inducer, or a CO releasing molecule (CORM-3), reduces the increased sFlt-1 release from the tissue following hypoxia<sup>155</sup>. In addition, the inhibition of HO-1 using tin protoporphyrin (SnPP) further exacerbated the sFlt-1 release from placental explants<sup>139</sup>. In reducing the release of sFlt-1 from tissues, a concomitant increase in available angiogenic factors would be expected, and both HO and CO have been able to increase VEGF bioavailability in vascular smooth muscle cells<sup>156</sup>. These studies<sup>139;155</sup> and others<sup>148;156</sup> have suggested a possible role for the HO/CO system in the maintenance of a balance between angiogenic factors and their antiangiogenic counterparts. The objective of this study was to determine the effect of exogenous gaseous CO on placental villous explant production of sFlt, sEng and PGF. The hypothesis is that exposure of human placental explants to CO in ambient air would decrease the release of sFlt-1 from placental explants and increase the angiogenic factor PGF.

### 3.3 Methods

#### Tissue Collection and Explant Dissection

Human placentas (n=6) were obtained from uncomplicated term pregnancies, immediately following elective caesarean section. Women were non- labouring, non- smoking and considered low-risk at the Kingston General Hospital (Kingston, CAN). Five 2.5cm cubes were randomly cut from different areas of the placenta, avoiding calcifications, tears or fibrous material, and the chorionic and basal plate were removed from each piece. The tissue was transferred to the laboratory in a sealed 50ml conical tube containing ice cold, phosphate-buffered saline (PBS).

Chorionic villous explants (5-10mg) each, were dissected in cold PBS, on ice. Tissue pieces were rinsed in a fresh wash of cold PBS and transferred to cold, culture medium, (Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Oakville, ON). Five explants were cultured per well in Costar Netwell supports (Cole Parmer, Anjou Q, CAN) and in 1.2ml of DMEM containing 5% fetal bovine serum and 100ug/ml of streptomycin). Two wells per plate were used, and three plates were used per experimental condition: control air (5% O<sub>2</sub>, 5% CO<sub>2</sub> and balance N<sub>2</sub>, Praxair, Belleville, ON) or CO in air (250ppm CO, 5% O<sub>2</sub>, 5% CO<sub>2</sub> and balance N<sub>2</sub>, Praxair, Belleville, ON). Therefore, for each placenta (n=6), 60 explant samples were cut, 30 of which were incubated in control conditions and 30 in CO exposure conditions. In addition, for each placenta, 10 extra pieces of explant tissue were immediately frozen at -80°C for quantification of lactate dehydrogenase (LDH) activity, as described below.

Explants were maintained at 37°C for the duration of the experiment. At each of the 6, 12 and 24hour (hr) time points, one plate was removed from each of the control and CO chambers. The medium was aliquotted, tissue was blotted and weighed, and all samples were stored at -80°C for future analysis.

## **CO Measurements**

At each of the 6hr and 24hr timepoints, medium and air samples were measured for CO concentrations in both control and CO-exposed groups.

Air samples were collected from the incubator into 30ml syringes, attached to plastic tubing. The syringe was filled and emptied 3 times. At the final filling, the syringe was stoppered. Using a gas-tight syringe, triplicate samples of 200 $\mu$ l (control air) and 10 $\mu$ l (CO air) were added to 2ml amber vials (Sigma- Aldrich, CAN), pre-purged with CO- free air (12% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>, Praxair, Belleville, ON) and stoppered with silicon fitted caps. Samples were analyzed using gas- chromatography and CO levels (ppm) were interpolated from a standard curve created on the same machine using standard gas (10.1ppm) (Praxair, ON), as previously described<sup>162</sup>.

At the 6hr and 24 hr timepoints, when medium samples were aliquotted for storage, duplicate samples (200 $\mu$ l) were added to previously purged (12% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>, Praxair, Belleville, ON) amber vials (Sigma Aldrich, CAN) using a gas-tight syringe (Hamilton, USA). Vials were maintained on ice for a minimum of 30 minutes and analyzed using gas chromatography for CO levels (nL/mL), again interpolated from a standard curve<sup>162</sup>.

## **Lactate Dehydrogenase (LDH) Assay**

To assess tissue viability, LDH concentrations were determined in the sample medium using a commercially available kit (ab65393, Abcam, Cedarlane, ON). The extra explant tissue frozen for this assay was sonicated in 1.2ml of culture medium + 100 $\mu$ l of cell lysis solution on ice, using a cell disrupter (Fisher Scientific Sonic Dismembrator) with a 1/8 inch diameter micro tip probe (3W) until homogeneity was observed in the tubes (<10s). The sonicate was then centrifuged at 14 000rpm at 4°C for 15 minutes, and the supernatant was removed and used as the 100% LDH activity for each placenta. The medium from each tissue well (control= 6,

CO-exposed= 6) was analyzed for LDH activity and compared to the corresponding placenta 100% LDH activity sample.

### **Protein Measurement**

Explant tissue was added to 800ul of PBS (1% Triton X) and sonicated using a cell disrupter (Fisher Scientific Sonic Dismembrator, ON, CAN) with a 1/8 inch diameter micro tip probe (3W) for <10 seconds on ice. Sonicates were centrifuged at 14 000rpm for 15minutes at 4°C, the supernatant was removed and analyzed for total protein content using a standard DC Lowry assay (Biorad, Sigma Aldrich, CAN).

### **sFlt-1, PGF and sEng measurements**

Measurements of protein sFlt-1, PGF and sEng were analyzed in tissue media by sandwich ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. All protein levels were normalized to total tissue protein.

### **Statistical Analysis**

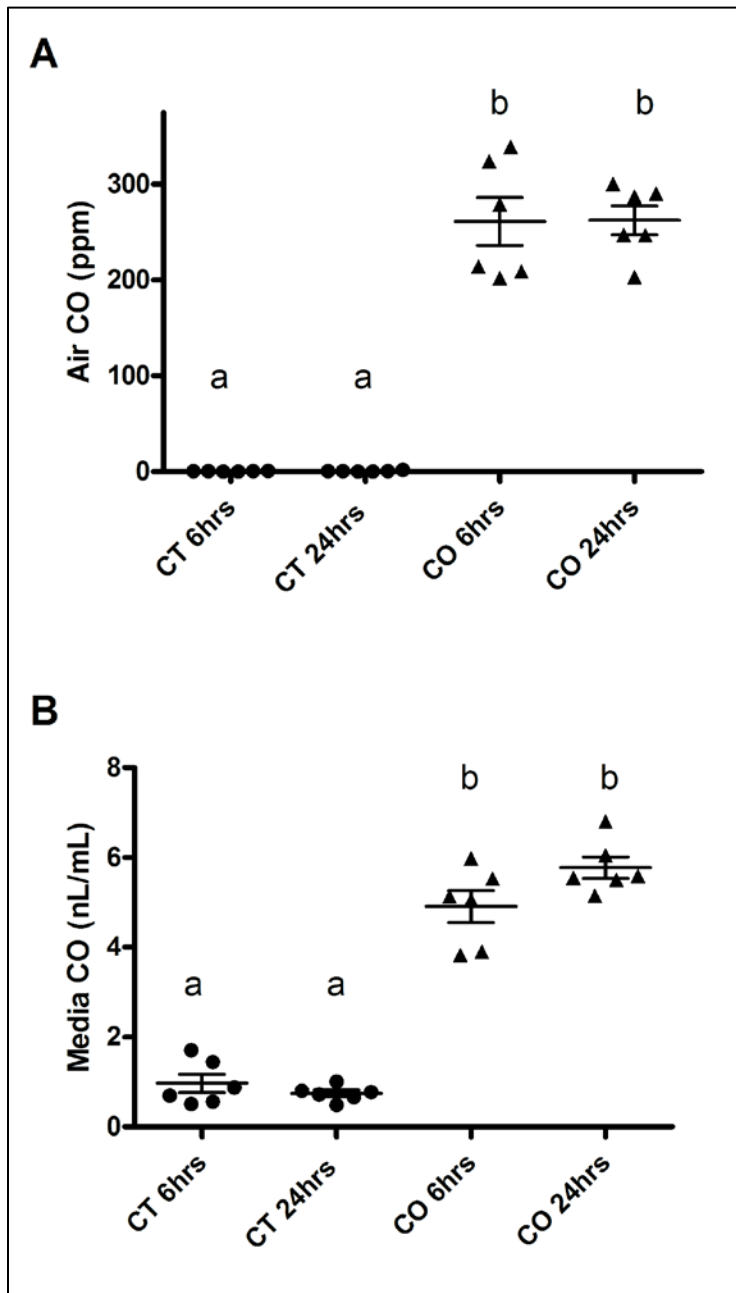
All statistical analysis was completed using Graphpad v5 (Graphpad software, La Jolla, CA) and a one-way ANOVA was used to calculate significance between the groups at the different time points, with the significance threshold at  $P < 0.05$ . All data are presented  $\pm$ SEM.

### 3.4 Results

CO concentrations in both the medium and the air samples were significantly increased ( $P < 0.05$ ) in the CO-exposed groups compared to control (Figure 3.1). The delivery of CO in the incubator was measured (ppm  $\pm$  SD) to be  $261.7 \pm 13.9$ . Cell viability, as measured by LDH release, was not affected over time or by CO exposure (Figure 3.2).

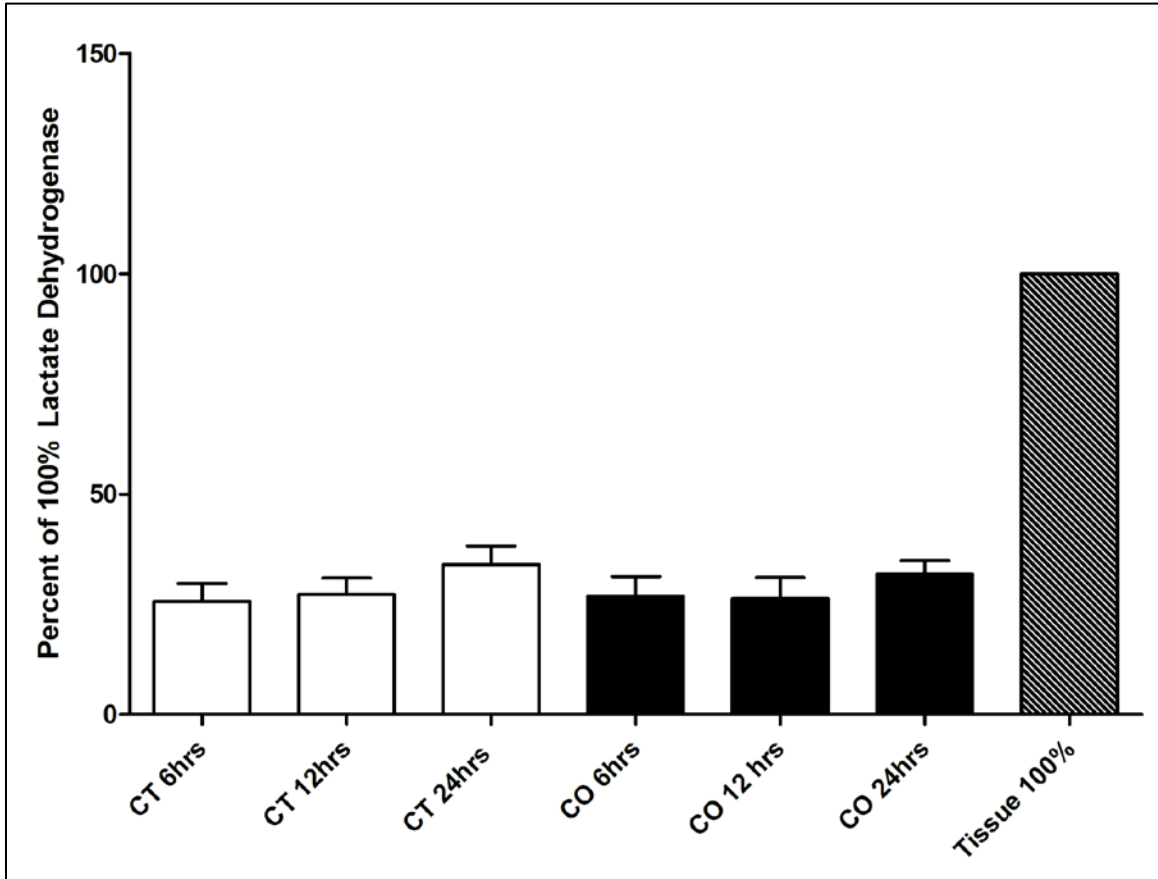
The release of sFlt-1 from the explant tissue increased significantly in the control group from the 6hr to the 24hrs time-point ( $P < 0.05$ ) (Figure 3.3). Exposure to CO attenuated the sFlt-1 release at 24hrs ( $P < 0.05$ ) and was not significantly different from control at either 6 or 12hr time-points ( $P > 0.05$ ). The release of sEng did not change over time in control conditions and was not affected by exposure to CO ( $P > 0.05$ ), Figure 3.3.

Release of PGF was not significantly affected by CO exposure (Figure 3.4).



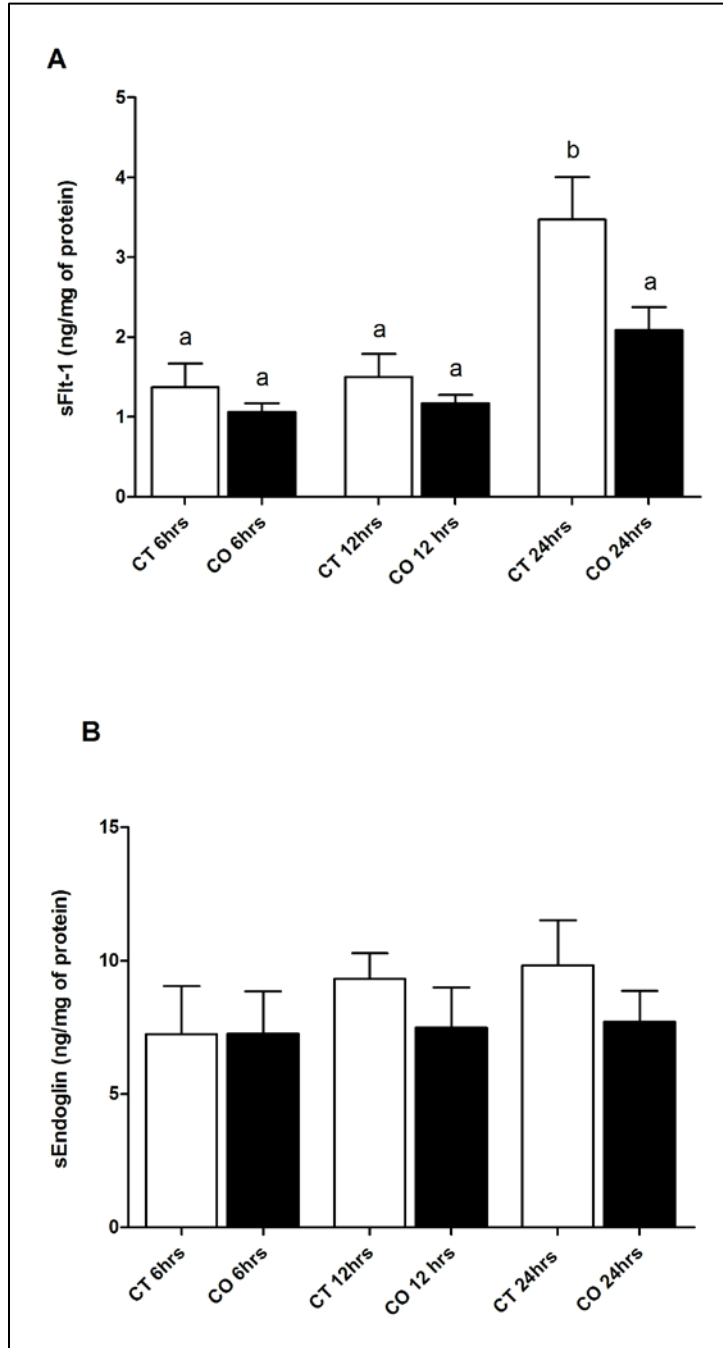
**Figure 3.1: Air and media carbon monoxide (CO) levels for all 6 placentas exposed to control and CO air.**

Air (mean  $\pm$  SEM) (A) and media (mean  $\pm$  SEM) (B) samples were measured for CO levels at 6 and 24hr time-points and significant increases in CO levels were measured compared to control at both time-points ( $P < 0.05$ ). Each dot represents a mean of duplicate samples for media and air samples measured per placenta ( $n=6$ ).



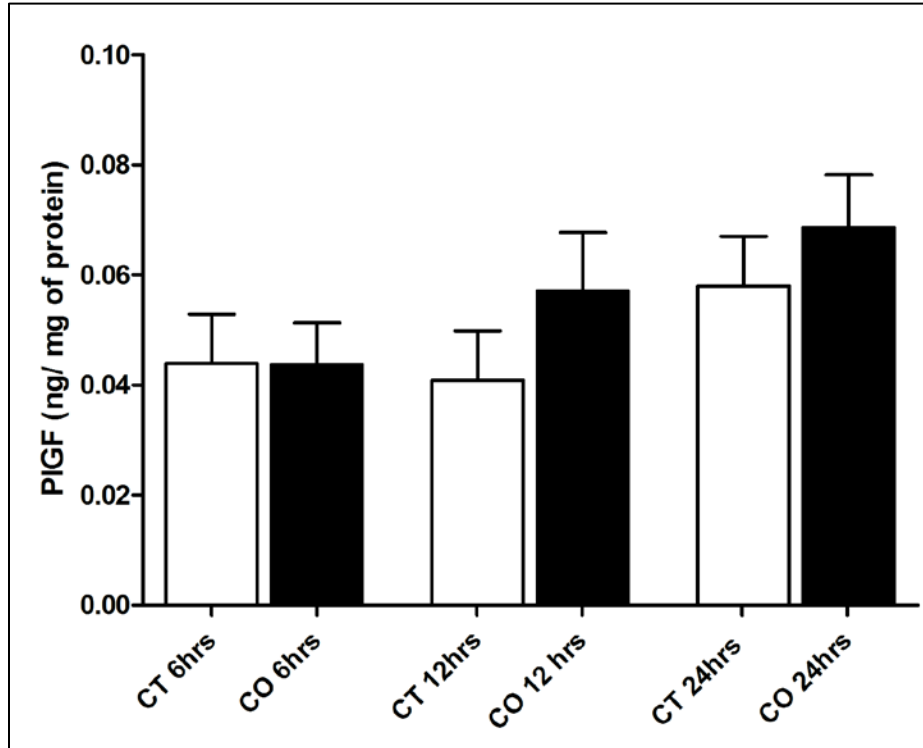
**Figure 3.2: Measurement of cell viability by lactate dehydrogenase.**

Placenta tissue viability was measured by lactate dehydrogenase level and was determined to be not different between groups ( $P > 0.05$ ). All placenta tissue contained significantly less LDH than the 100% control ( $P < 0.05$ ). Each bar represents a mean ( $\pm$  SEM) of duplicate samples for of  $n=6$  placentas.



**Figure 3.3: sFlt-1 and sEng were measured in the medium of explants at each time-point of the experiment.**

A) sFlt-1 (mean  $\pm$  SEM) was increased in control explant medium at 24hrs in comparison to 6hr ( $P < 0.05$ ). Explants exposed to CO did not increase sFlt-1 release over time ( $P > 0.05$ ) and were significantly decreased at 24hrs in comparison to control levels ( $P < 0.05$ ). B) sEng levels were not different between control and CO-exposed explants at any time-point. Each bar represents a mean ( $\pm$  SEM) of duplicate samples for of  $n=6$  placentas.



**Figure 3.4: PGF levels measured in explant medium of control and CO-exposed tissue explants.**

PGF protein was not different between groups at any timepoint ( $P > 0.05$ ). Each bar represents a mean ( $\pm$  SEM) of duplicate samples for of  $n=6$  placentas.

### 3.5 Discussion

While it is generally accepted that placental development is inadequate in PE, it is not known what single or combination of factors leads to the clinical manifestations of PE. The anti-angiogenic molecule sFlt-1 is one possible candidate involved in the progression of the disorder, and is increased in the serum of women with PE<sup>139;140</sup>, in the amniotic fluid<sup>210</sup>, and in the placenta tissue itself<sup>138;139</sup>. Delivery of the fetoplacental unit leads to a rapid decrease in serum sFlt-1 levels within 24hrs<sup>139;210</sup>. Further, a study involving women with preterm PE and extracorporeal apheresis of sFlt-1 resulted in decreased maternal hypertension and proteinuria in parallel<sup>210</sup>. The overexpression of sFlt-1 in both rats<sup>139;144;148</sup> and mice<sup>146;211-213</sup> leads to conditions like PE, increases in blood pressure, proteinuria and endotheliosis of the renal glomeruli. It is hypothesized that sFlt-1 is produced and released into the maternal circulation by the fetoplacental unit, and contributes to the maternal widespread endothelial dysfunction of PE.

Any therapeutic approach to reduce the levels of sFlt-1 could potentially decrease the endothelial dysfunction, or reduce the development or progression of PE. We have shown that the 24hr exposure of placental villous explants to 250ppm CO leads to significant reductions in sFlt-1 protein levels released by the tissue. Similar *in vitro* findings with tissue explants have been reported using HO-1 inducers (CoPP)<sup>155</sup> and statin therapy (shown to increase HO-1 protein)<sup>154</sup>, while the inhibition of HO-1 (by SnPP)<sup>154</sup> augmented the release of sFlt-1. These findings indicate a possible role for the HO system in attenuating the production or release of this molecule. Interestingly, the exposure of placental tissue (rat) to CORMs<sup>155</sup> and human umbilical vein endothelial cells (HUVECs) to medium-infused with gaseous CO<sup>154</sup>, also have shown reductions of sFlt-1 release, in support of the findings of the present study.

The use of CO in PE as a therapeutic was first proposed by our group<sup>127</sup> when it was found that women who smoke cigarettes in pregnancy displayed a reduction in the incidence of PE<sup>62;63</sup> by as much as 33% and in a dose-dependent fashion<sup>64</sup>. The same reduction was not observed in women who used snus, a smokeless form of tobacco, and devoid of combustible

products<sup>64;77</sup>. It was proposed that increased CO levels could be generating beneficial actions and thereby ameliorating the development of PE in some women<sup>204</sup>, and accordingly, the finding that women with PE measure significantly lower end-tidal breath CO levels than women with a healthy pregnancy<sup>80</sup> has also been reported. Our group has recently reported that exposure to ambient environmental CO levels is negatively associated with the incidence of PE, which further agrees with the hypothesis<sup>81</sup>.

At very high levels, CO is a toxic gas; but at low levels of CO, many beneficial physiologic properties have been reported, namely vasodilation, anti- apoptosis, anti- inflammation and angiogenesis<sup>32</sup>. We have previously reported that low levels of CO (250ppm) throughout pregnancy did not lead to maternal or fetal demonstrable effects in mice<sup>162</sup>. Further, when mice were exposed to CO, an increase was observed in uterine artery blood flow, in addition to the arterial branching and diameters of several vessels of the uteroplacental unit<sup>159</sup>. These findings were supplemented with an increased ratio of placental VEGF to sFlt-1<sup>159</sup>. We proposed that CO improved vascular blood flow to the uteroplacental unit through increased vessel diameter size. The maintenance of a well-perfused placenta, may have reduced the secretion of sFlt-1 into the circulation, as this action was proposed to occur in order to increase blood flow to the fetal- placental unit<sup>210</sup>.

The shifted balance in VEGF and sFlt-1 proteins to one of angiogenesis in mice exposed to CO led us to measure the release of angiogenic molecules in this study. No difference was observed in the release of PGF in the tissue explants (Figure 3.4). We speculate that this difference may become significant over a longer period of time, or if the tissue was pre-treated in a hypoxic environment and subsequently exposed to CO. Under normoxic conditions, the exposure of CO may not significantly affect the angiogenic molecule production. This finding was reported in normal pregnant rats exposed to CoPP, an HO-1 inducer, which led to no differences in the VEGF protein levels of maternal plasma or placental tissue<sup>148</sup>. However, a

separate group of animals in this study, continually infused with sFlt-1 using mini-osmotic pumps, was found to have their maternal circulating VEGF levels decreased by 17%. A separate group of animals with similar sFlt-1 injected were injected with CoPP, placental HO-1 was significantly increased and VEGF protein levels were augmented by ~50%<sup>148</sup>.

Lastly, we also chose to evaluate the effect of CO on sEng production in the placental explants, as it is also regarded as an anti-angiogenic molecule. Though it functions through a different pathway than sFlt-1, sEng is also increased in the serum of women with PE<sup>140</sup>. There was no difference in the control and CO-exposed group over time. Others have shown that exposure of HUVECs to adenovirus with HO-1 insert, resulted in significant reductions in medium sEng measurements<sup>154</sup>. Further, HO-1 knockout mice have a significant increase in circulating sEng levels<sup>154</sup>. The reduction in sEng in each of these cases may be reliant on different pathways of the HO system; perhaps through bilirubin or a combination of this product and CO. It is possible that CO- alone is not able to reduce the levels of sEng released from the tissue explants.

We have demonstrated that 24-hr exposure of placental villous explants to gaseous CO reduces the release of sFlt-1, but not sEng. The HO/CO system has been hypothesized as a possible pathway for reducing the development and/or progression of PE, through the reduction of antiangiogenic molecules. We have shown that the exposure of CO alone does not reduce the release of sEng, suggesting another product in the HO system to aid in this process. To our knowledge, we are the first group to explore the HO/CO system in placental explants using gaseous CO. We offer a possible mechanism through which the exposure to cigarette smoke is associated with decreased PE in pregnant women. Further studies into the association of the HO/CO system and PE are warranted.

## Chapter 4

### **Treatment with carbon monoxide prevents the hypertension and proteinuria in an adenovirus sFlt-1 preeclampsia-like mouse model**

#### **4.1 Abstract**

Preeclampsia (PE) remains a leading cause of maternal and neonatal morbidity and mortality worldwide. Smoking cigarettes is associated with a decreased incidence of PE. Based on this observation and previous work, we hypothesize that women who smoke have a lower risk of developing PE because of elevated levels of carbon monoxide (CO) in their blood. The objective of this study was to determine if low-dose CO in ambient air could attenuate the late pregnancy hypertension (HTN) and proteinuria in the Adenovirus (Ad) sFlt-1 PE-like mouse model. Continuous low-dose CO treatment (250ppm) was started on gestation day (GD) 10.5 and maintained until GD17.5. Compared to control and Ad empty vector, AdsFlt-1 mice displayed late- gestation HTN (GD14.5-17.5) ( $P<0.05$ ), proteinuria ( $P<0.05$ ) and reduced Bowman's space which were all prevented with CO treatment. Use of the Ad (with/without sFlt-1) or CO had no effect ( $p>0.05$ ) on litter size, fetal resorption numbers and fetal or placental weights. This study shows for the first time that treatment with CO can prevent HTN and proteinuria in a the AdsFlt-1 mouse model of PE. It provides a possible mechanism for the reduced incidence of PE in smoking women, and supports the possibility of using CO as a future treatment for PE.

## 4.2 Introduction

Preeclampsia (PE) is a serious condition of pregnancy which presents as proteinuria and hypertension (HTN)<sup>214</sup>, and is associated with underlying maternal vascular dysfunction<sup>215</sup>. There is no known cure other than delivery of the feto-placental unit. PE is thought to originate in the placenta, as a result of inadequate implantation, leading to poor placental perfusion and hypoxia<sup>214</sup>. The ischemia increases placental apoptosis and subsequent shedding of placental debris<sup>216</sup> and soluble factors into the maternal circulation, which are thought to contribute to maternal endothelial dysfunction and the clinical signs of PE<sup>217</sup>. Anti-angiogenic molecules are among these factors, as their overproduction leads to an imbalanced angiogenesis and is thought to contribute to the etiology of PE<sup>218</sup>. The best characterized anti-angiogenic molecule relating to PE is soluble *fms*-like tyrosine kinase-1 (sFlt-1), an alternatively spliced, soluble form of the vascular endothelial growth factor (VEGF) receptor 1<sup>219</sup>. It has been measured at increased levels in the placenta<sup>139</sup>, amniotic fluid<sup>220</sup> and maternal serum<sup>139</sup> of women with PE. As an anti-angiogenic protein, sFlt-1 binds to the angiogenic VEGF and placental growth factor (PGF), rendering them inactive<sup>140</sup>. Both free VEGF and PGF are decreased in the circulation of women with PE<sup>139</sup>.

There is no spontaneous animal model of PE and as a result, it has been difficult to determine the pathophysiologic process leading to the development and progression of this disorder. As well, it has limited our ability to develop therapeutic options for the prevention or treatment of PE. Using the association between PE and an imbalance of angiogenic factors, rodent models with PE-like signs (HTN and proteinuria) have been developed through the introduction and over production of sFlt-1 by adenovirus (Ad)<sup>139;145;146;213</sup>. All studies report that late-stage HTN, proteinuria and glomerular damage ensue following the infection with the Ad encoding sFlt-1 (AdsFlt-1)<sup>139;145;146;213</sup>.

A 33% reduced incidence in the development of PE has been associated with smoking cigarettes in pregnancy<sup>62;63</sup> in a dose dependent manner<sup>64</sup>. The same is not true of smokeless

tobacco<sup>64;77</sup>, which led us to hypothesize that carbon monoxide (CO) a combustible product in cigarettes, is the agent conferring reduction of PE<sup>127</sup>. Indeed, it has been shown that women with PE have reduced end- tidal breath CO (EtCO) levels<sup>80</sup>. Further, a negative correlation was determined between increased environmental ambient CO and PE<sup>81</sup>.

We have previously demonstrated *in vitro* that CO is capable of reducing apoptosis in placental villous explants<sup>98</sup> and inducing vasodilation in the isolated perfused placenta<sup>112</sup>, at concentrations similar to those in women who smoke during pregnancy. Further, *in vivo*, we have identified a dose of CO (250ppm) that can be delivered to maternal mice without fetal gross morphological or developmental detriments, and which leads to maternal levels of CO similar to those of smoking women<sup>162</sup>. We have also shown that maternal mouse exposure to this dose of CO results in an increase of utero-placental blood flow and vascularity of the placenta<sup>159</sup>. Therefore, we hypothesized that the use of CO would prevent the development of HTN and proteinuria in the AdsFlt-1 rodent model of PE.

### **4.3 Methods**

#### **Ad replication**

The AdsFlt-1, encoding Flt-1 (1-3) (first generation, E1 and E3 deleted) and Ad empty vector (AdEV) were obtained from Dr. Richard Mulligan at Children's Hospital, Boston, MA. Replication, virion concentration and replication competence were all carried out in our laboratory as described elsewhere<sup>145</sup>. We used a plaque assay<sup>221</sup> to determine infectious virus concentration. We injected different groups of mice with AdsFlt-1 or AdEV at a dose of  $1.25 \times 10^8$  plaque forming units (PFU) of infectious virions. (For an explanation of concentration used in this study), refer to Supplement, Section 4.6).

#### **Animals**

All experimental procedures were approved by the Queen's University Animal Care Ethics committee (Smith-2012-003-Or-A2). Female CD-1 mice (8-10 weeks old), from Charles River Laboratories (Wilmington, MA), were provided with food and water *ad libitum*. Females were mated with males of the same strain overnight and the morning detection of a copulation plug was deemed gestation day (GD) 0.5.

#### **Treatment**

Animals were separated into six groups: Control  $\pm$  CO, AdEV  $\pm$  CO and AdsFlt-1  $\pm$  CO. On gestation day (GD) 7.5, mice in the AdEV or AdsFlt-1 groups were injected with 100ul of  $1.25 \times 10^8$  PFU via their tail vein. They were maintained in a biohazard room for 72hrs and then moved back into our containment room. In order to eliminate effects of the Ad itself, we included the "control  $\pm$  CO" group of mice which did not receive any Ad injections. Animals were sacrificed on GD17.5 using 100ul of 100mg/kg of sodium pentobarbital intraperitoneally (Ceva Sante Animale, Libourne, France).

### **Blood pressure (BP) measurement and CO exposure**

Prior to mating, each mouse was trained daily for two weeks using the Kent Scientific Tail cuff BP device (Conneticut, USA), ensuring a consistent BP reading was maintained over at least three consecutive days. Mice were then mated and BP was measured on each morning until GD17.5. The mean of >5 stable and consistent measurements was taken as a true reading. BP was not measured on GD8.5 -10.5, as mice were maintained in a biohazard room following their Ad injection. On GD10.5 of pregnancy, the group of mice to be exposed to 250ppm CO in ambient air were placed in a dosing chamber until GD17.5, where CO levels were monitored continuously as previously described<sup>162</sup>. These mice were removed from the CO chamber for the daily BP measurements (~20 min).

### **Urine protein: creatinine concentration**

Urine was collected at baseline (GD0.5-1.5) and prior to sacrifice. Mice were placed in separate sterile cages lined with sterile 96 well plates, from which urine was collected and centrifuged at 4000Xg for 10min. The supernatant was removed and stored at -80°C. Urine was diluted 1:2 in distilled water and protein concentration was measured using a Bradford assay (Quickstart Bradford assay, Biorad Mississauga, Canada). Urine was diluted 1:10 in distilled water and creatinine was measured using a standard picrate method (Cayman Chemicals, Michigan, USA).

### **Maternal blood collection and tests**

Maternal blood was collected at early gestation as a baseline via the submandibular vein and prior to sacrifice via the retro- orbital vein. Blood CO measurements were performed using gas- solid chromatography as previously described<sup>162</sup>. Blood was centrifuged at 4000Xg at 4°C for 20min and the plasma was removed and stored at -80°C in aliquots. All plasma sFlt-1

concentrations were measured using a commercially available ELISA kit (MVR100, R&D systems, Minneapolis, MN); a 1/10 dilution (in calibrator diluent) was completed for all mouse baseline plasma, end of gestation plasma for Control  $\pm$ CO and AdEV  $\pm$  CO was diluted 1/10 in calibrator diluent and end of gestation plasma for AdsFlt-1  $\pm$  CO mice were diluted 1/100 in calibrator diluent.

### **Procedures at time of sacrifice (GD17.5)**

The maternal uterine horns were excised; litter number and fetal resorptions were noted. Un-resorbed fetuses and placentas were counted and weighed.

### **Histology (Light Microscopy)**

At the time of sacrifice, mice were perfused with 4% PFA and harvested kidneys and placentas were placed in 4% PFA for 12 hours, paraffin embedded, sectioned at 3 $\mu$ m and stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). Blinded renal H&E sections were imaged at 200X magnification and a minimum of 10 renal corpuscles per kidney were imaged. Using ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), Bowman's capsule and glomeruli were measured in a blinded fashion and from these measurements the volume of the Bowman's space was calculated. The mean of 10 glomeruli measurements per mouse were compared and a minimum of 3 mice per group were used for comparison.

### **Statistics**

All statistical analysis was performed using Graphpad prism v5.0. All data are represented as means  $\pm$ SEM. Mean daily systolic BP was calculated for each mouse, and these measurements were compared by two-way ANOVA. All other data were analyzed using a one

way ANOVA with a post- hoc Tukey test. A probability value less than 0.05 was considered significant.

#### 4.4 Results

We assessed maternal weight change over gestation as a measure of health and this was not different between any of the six groups (Figure 4.1a). No differences between groups were noted in litter size or fetal resorption numbers (Figure 4.1b) or fetal or placental weight (Figure 4.1c). Maternal blood CO levels (%COHb  $\pm$  SEM) were consistent between Control ( $0.61 \pm 0.85$ ), AdEV ( $0.55 \pm 0.04$ ) and AdsFlt-1 ( $0.54 \pm 0.01$ ) groups not exposed to CO and between those mice exposed to CO, Control + CO ( $9.93 \pm 1.06$ ), AdEV + CO ( $10.63 \pm 3.57$ ) and AdsFlt-1 + CO ( $9.90 \pm 3.26$ ). Mouse containment box CO concentrations were confirmed to be continuously delivering  $251.0 \pm 2.5$ ppm for all groups exposed to CO.

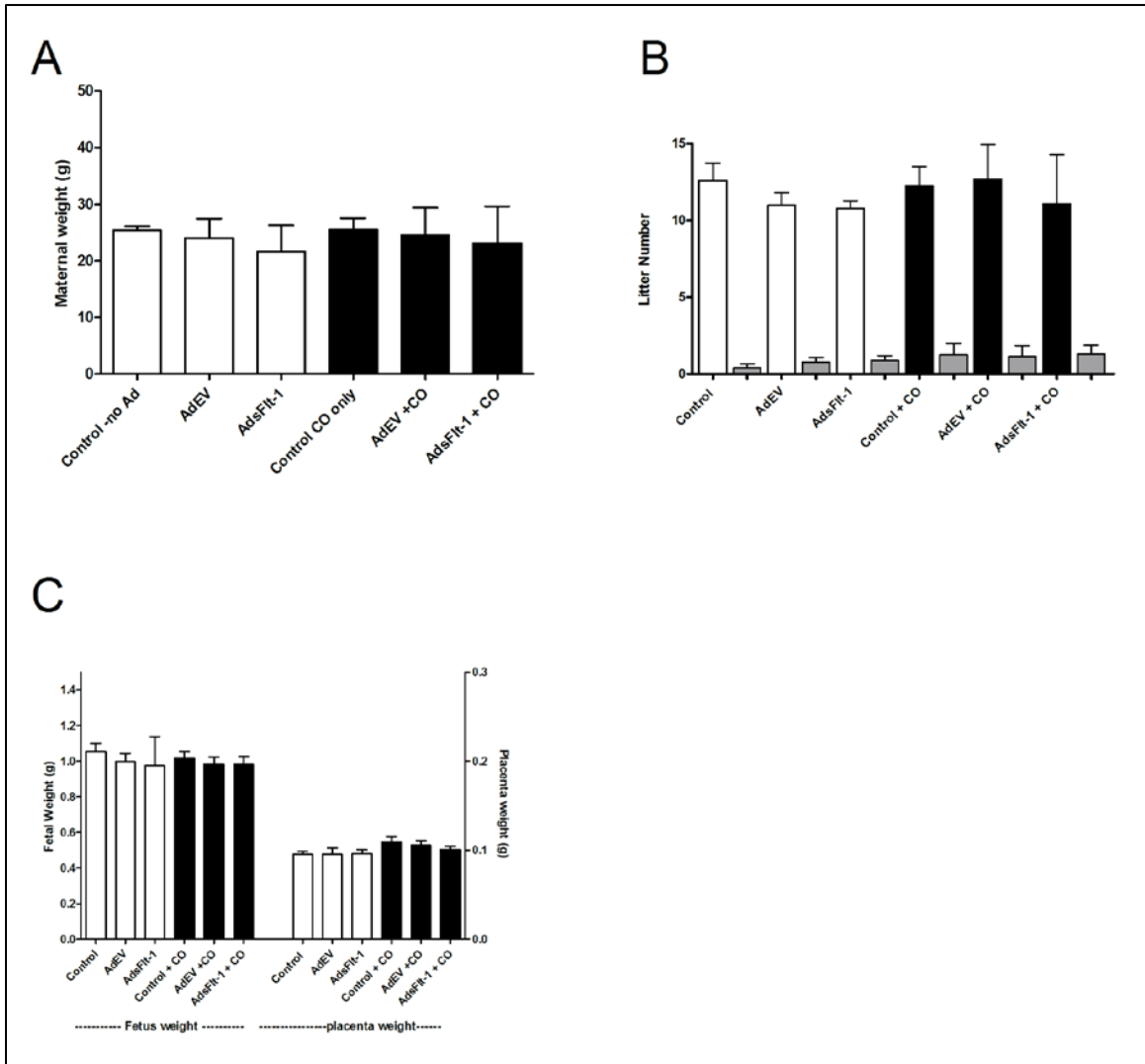
Pregnant animals in each of the control and AdEV groups displayed increases in maternal plasma sFlt-1 concentrations (ng/ml  $\pm$  SEM) from  $0 \pm 1.23$  at E0.5 to  $22.39 \pm 6.46$  and  $33.82 \pm 11.65$ , respectively, at GD17.5 (Figure 4.2). AdsFlt-1 injection resulted in sFlt-1 concentrations of  $234.82 \pm 84.2$ , which was significantly different ( $P < 0.05$ ) from control and AdEV mice. In none of the groups was treatment with CO associated with altered concentrations of sFlt-1 (Figure 4.2).

Control mice and those administered AdEV displayed similar BP with no change across gestation (Figure 4.3). Treatment with CO in these groups did not affect the BP ( $P > 0.05$ ). Mice injected with AdsFlt-1 displayed late-stage HTN, with significant differences from each of control  $\pm$  CO and AdEV  $\pm$  CO ( $P < 0.05$ ) on GD14.5 through 17.5. Treatment of this group with CO completely prevented ( $P < 0.05$ ) the HTN, (Figure 4.3).

Late gestation urine protein: creatinine ratios were significantly increased in AdsFlt-1 injected mice ( $1013.0 \pm 135.4 \mu\text{g}/\text{mg}$ ) compared to control ( $396.0 \pm 56.85 \mu\text{g}/\text{mg}$ ) and AdEV ( $509.9 \pm 60.5 \mu\text{g}/\text{mg}$ ) (Figure 4.4). When treated with CO, AdsFlt-1 mice were found to have reduced urine protein: creatinine ratio ( $597.8 \pm 81.0 \mu\text{g}/\text{mg}$ ) compared to the AdsFlt-1 injected mice without CO treatment, ( $P < 0.05$ ). Mice treated with CO in each of control ( $506.82 \pm$

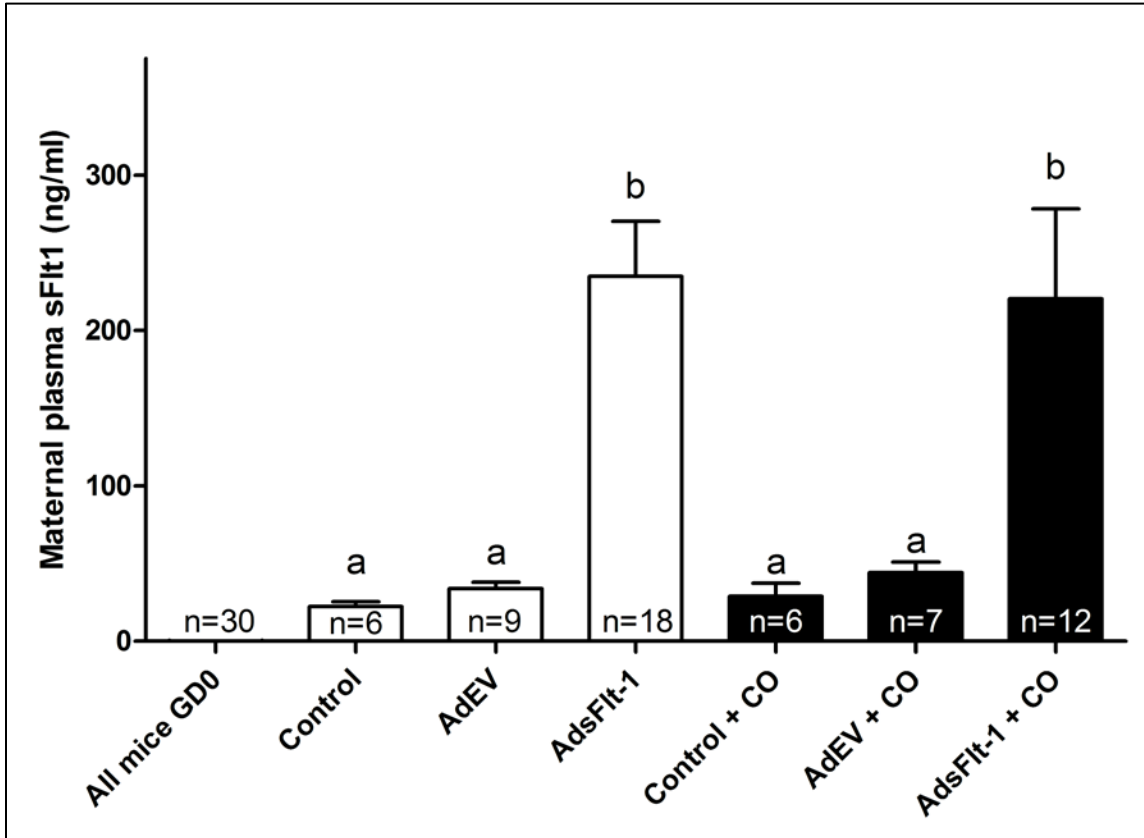
270.6 $\mu$ g/mg) and AdEV (428.96  $\pm$  292.16 $\mu$ g/mg) had urine protein: creatinine levels similar to their respective groups without CO (P>0.05).

Blinded analysis of the renal specimens by light microscopy did not identify differences between mice treated with AdsFlt-1 $\pm$  CO in comparison to all other groups of mice. However, at higher AdsFlt-1 concentrations than used in the current study (data not shown), diffuse glomerular endotheliosis was clearly evident. The blinded measurement of Bowman's space revealed a decrease (P<0.05) in those mice injected with AdsFlt-1 (8.43  $\pm$  1.0nm), in comparison to both control (1.3 $\pm$  2.7nm) and AdEV (1.16  $\pm$  1.15nm) mice (Figure 4.5). This was prevented when AdsFlt-1 injected mice were treated with CO (1.12 $\pm$  2.35nm).



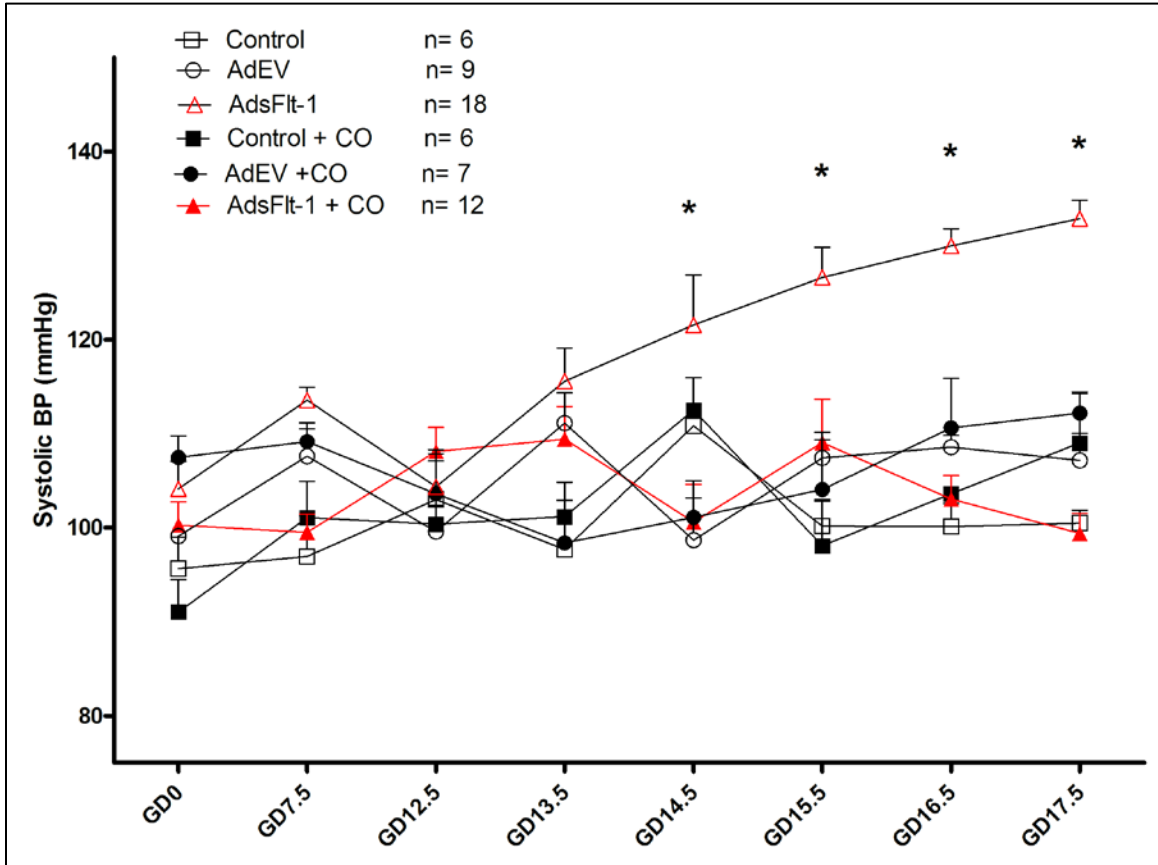
**Figure 4.1: Maternal and fetal health were not negatively affected due to adenovirus (Ad) injection or carbon monoxide (CO) exposure.**

A) Maternal change in weight throughout pregnancy was similar between all groups injected with Ad (white bars) and exposed to CO (black bars) ( $P>0.05$ ). b) No change in litter size, or resorptions (grey bars) were observed among any of the six groups of mice. C) Fetal and placental weight were similar amongst all groups of mice, with no difference due to Ad or CO exposure ( $P>0.05$ ).



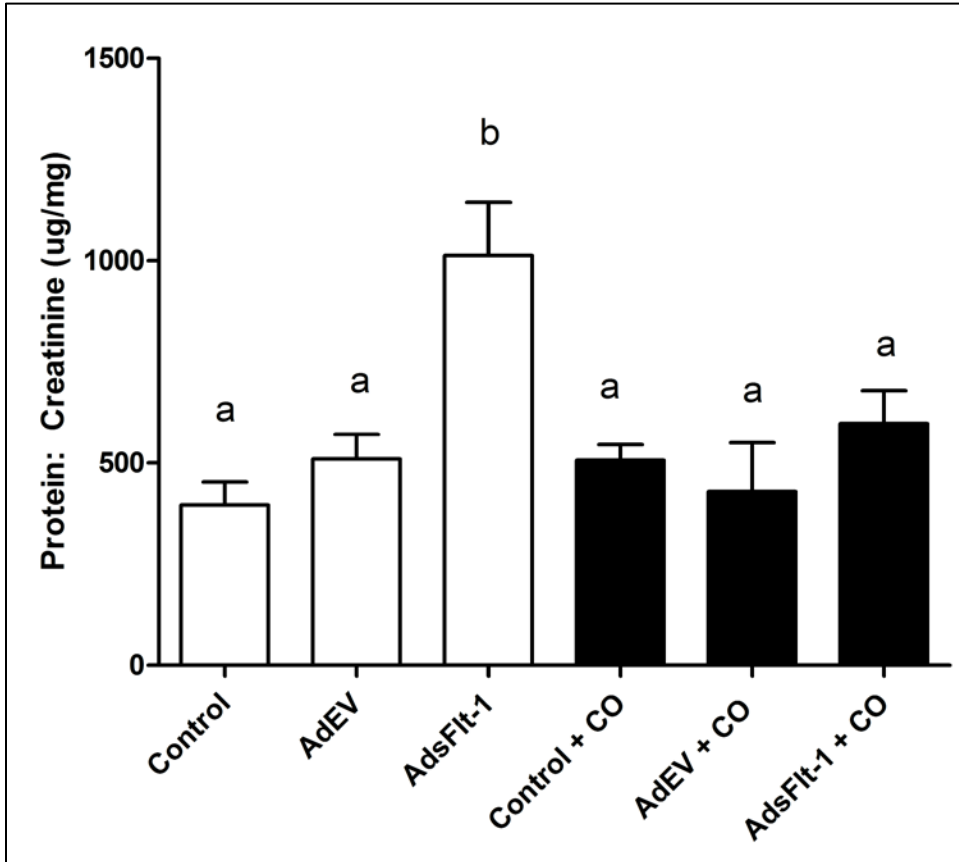
**Figure 4.2: Tail-vein injection of AdsFlt-1 significantly increased maternal plasma sFlt-1 levels compared to control and AdEV groups.**

All maternal mouse sFlt-1 plasma levels (ng/ml  $\pm$  SEM) were  $0 \pm 1.23$  GD0.5 of pregnancy, but at term, both control and AdEV sFlt-1 levels were increased to levels observed in normal pregnancy. Mice injected with AdsFlt-1 measured with significant increases in plasma sFlt-1 levels, and this was not different when mice were exposed to CO. Similar letters are placed above data that was not significantly different.



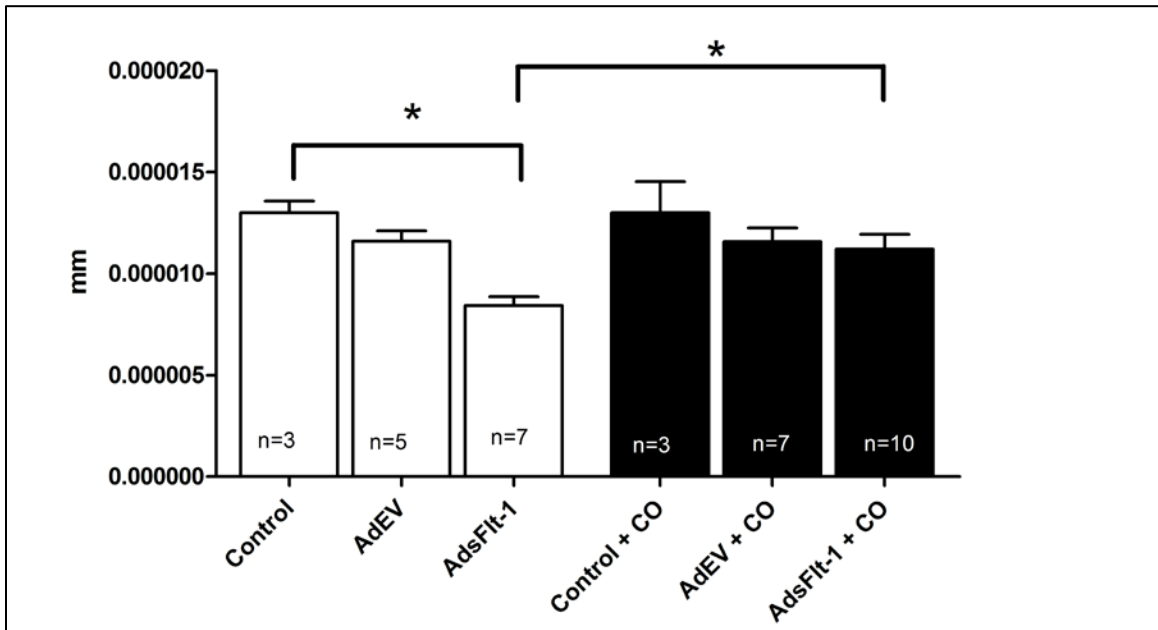
**Figure 4.3: Maternal sFlt-1 –induced hypertension (HTN) in late stage pregnancy is completely normalized when mice are exposed to CO.**

No difference in blood pressure (BP) throughout pregnancy was noted in control  $\pm$ CO or AdEV  $\pm$ CO groups throughout pregnancy. The injection of AdsFlt-1 led to late stage HTN in pregnancy ( $P < 0.05$ ), which was completely attenuated in mice exposed to CO ( $P > 0.05$ ).



**Figure 4.4: AdsFlt-1 injection leads to maternal proteinuria and is attenuated in mice exposed to carbon monoxide (CO).**

Mice injected with AdsFlt-1 displayed significant increases ( $P < 0.05$ ) in urine protein: creatinine ratios as compared to control and AdEV groups of mice. Exposure to CO reduced proteinuria levels to those of mice in both control  $\pm$  CO and AdEV  $\pm$  CO groups. Similar letters are placed above data that was not significantly different.



**Figure 4.5: Bowman's space was significantly reduced in mice injected with AdsFlt-1 in comparison to control mice.**

No difference was observed in the Bowman's space between control  $\pm$  CO and AdEV  $\pm$  CO groups. Mice injected with sFlt-1 displayed significantly reduced renal Bowman's space, in comparison to control mice. Exposure to CO normalized the Bowman's space, so that it was not different ( $P > 0.05$ ) than control  $\pm$  CO, but was significantly increased ( $P < 0.05$ ) compared to mice injected with AdsFlt-1. The n-numbers displayed in the bar graphs represent the number of kidneys analyzed, where each kidney is a mean of 10 glomeruli measured and a mean of those ten are compared in the graph.

## 4.5 Discussion

PE is a disorder unique to humans and as such, treatments are difficult to test, as adequate animal models of PE are few. One such animal model was created through the intravenous injection of AdsFlt-1 in the first trimester of rodent pregnancy<sup>139</sup>. This model increases maternal sFlt-1 protein levels, resulting in increased BP and altered renal histology and function, assessed through increased proteinuria<sup>139</sup>. George *et al.* reduced the sFlt-1 –induced HTN in rats (non-pregnant), by injection of cobalt protoporphyrin (CoPP), an inducer of heme oxygenase (HO)<sup>148</sup>. This endogenous enzyme functions to reduce the pro-oxidant free heme in the body by converting it into bilirubin, an antioxidant molecule<sup>44;222</sup>, free iron and CO, which at low levels has multiple physiologic functions<sup>27</sup>. *In vitro* studies using rat placental explant tissue have reported the reduction in hypoxia-induced sFlt1 levels, through the induction of the HO system by administration of CoPP<sup>155</sup> or administration of CO- releasing molecules (CORMs) directly to placental villous explants<sup>155</sup>. Further, inhibition of HO-1 [by administration of Tin protoporphyrin (SnPP)] or HO-1 siRNA, leads to the augmentation of sFlt-1 release from the tissue. This data suggests that the properties of HO and its product CO could be used as a therapeutic direction to decrease sFlt-1 levels and normalize HTN and proteinuria, leading to a possible prevention or treatment of PE.

Our study used six different groups of mice, control ± CO and AdEV ± CO and AdsFlt-1 ± CO. In the control ± CO and AdEV ± CO groups, similar maternal plasma concentrations of sFlt-1 were found (P<0.05), indicating that the Ad itself and the CO treatment did not affect normal sFlt-1 production. In addition, levels of sFlt-1 in these four groups of mice were in-line with previously reported data for normal pregnancy<sup>145</sup>. The group of mice injected with AdsFlt-1 had a mean sFlt-1 protein: creatinine concentrations (ug/mg± SEM) (1013.0 ± 135.4 ) that were significantly increased compared with AdEV and control mice (P<0.05). These concentrations were similar to AdsFlt-1 injection in rats (215.5 ± 81.2 ng/ml)<sup>139</sup> and CD-1 mice (87.7 ± 4.5)<sup>146</sup>. It is worth noting that our AdsFlt-1 injection was lower than that published in each of the rat<sup>139</sup> or

mouse<sup>146</sup> manuscripts, due to the different methodologies employed in measuring virus particles. Not surprisingly, addition of CO did not alter sFlt-1 concentrations in any of the AdsFlt-1 groups, as the exogenous addition of Ad travels to the liver of the animal and is continuously replicated, producing overexpression of the inserted construct, in this case, sFlt-1<sup>221</sup>.

The injection of AdsFlt-1 resulted in a significant increase in maternal BP in late-gestation compared to mice in the control and AdEV groups. Similar results for hypertensive effects of sFlt-1 injection have been reported in rats<sup>139;144;148</sup> and mice<sup>145-147</sup>. CO treatment prevented the maternal sFlt-1- induced HTN (Figure 4.3), while there was no effect on the AdEV or control groups BP. These findings are corroborated by results shown in rats, following the injection of an HO-1 inducer, CoPP, which significantly reduced the mean arterial pressure induced by sFlt-1 infusion<sup>148</sup>. The HO-1 system has been increasingly studied for the treatment of HTN in numerous forms of the disorder<sup>115;223-225</sup>, and HO-1 likely acts in part through the vasodilatory function of its product CO<sup>32</sup>. While maternal sFlt-1 levels were not reduced, the prevention of HTN with CO treatment indicates that this action is independent of an effect CO might have on sFlt-1 production normally.

Glomerular endotheliosis is one of the hallmarks of PE<sup>14;19</sup>, leading to improper filtration and increased protein in the urine. Though several studies have indicated the clear late- pregnancy development of this renal pathology following treatment of animals with AdsFlt-1<sup>139;144-146</sup>, our study did not reveal consistent glomerular histopathology, when viewed in a blinded fashion. However, injections of higher virion concentrations of AdsFlt-1 in pregnant mice, led to diffuse glomerular endotheliosis, which was clearly evident when reviewed in a blinded fashion (data not shown). The measurement of Bowman's space demonstrated a significant reduction in the AdsFlt-1 group of mice (P<0.05), which was prevented by treatment with CO in comparison to the control and AdEV groups (Figure 4.5). Orsolich *et al.*<sup>226</sup> reported reduction in Bowman's space in mice with diabetic nephropathy, and explained that an expansion of the mesangium<sup>227</sup>

could have been a causal factor, leading to a reduction in capillary surface area available for filtration, and contributing to the development of proteinuria<sup>228</sup>. In the present study, urine protein levels were significantly increased in mice injected with sFlt-1 (Figure 4.4) which was prevented with CO treatment. It is possible that CO prevents the renal dysfunction induced by AdsFlt-1 at the level of the glomerulus, but it may also function through multiple different mechanisms, such as improvement of renal perfusion.

There was no difference observed in the maternal weight change, litter size and fetal resorption numbers with any of the treatments (AdEV, AdsFlt-1, CO) which we used to determine the effect on maternal or fetal health

Manipulation of the HO-1/ CO system is a possible therapeutic target for the prevention or treatment of PE. Women with PE have decreased levels of HO in their placentas, even prior to the development of the HTN and proteinuria<sup>50</sup>. In addition, they have reduced EtCO levels<sup>202</sup>, indicating a role for HO-1/ CO in the development of PE. We have shown that exposure to low-dose CO can increase perfusion in the isolated perfused human placenta<sup>112</sup> and decrease apoptosis secondary to hypoxia/ reoxygenation injury in placental explants<sup>98</sup>. Other research groups have shown that induction of HO-1 (by CoPP) leads to decreased placental apoptosis, potentially through the increased expression of Bag-1 at the feto- maternal interface<sup>99</sup>. These findings offer possible mechanisms for the HO-1/ CO system to function at the level of the placenta and affect some of the findings associated with PE. Further, we have demonstrated that exposure of mice to 250ppm CO throughout pregnancy increases both blood flow and angiogenesis of the uteroplacental unit<sup>159</sup>. Others have shown that treatment with 50ppm CO in early gestation can reduce the incidence of miscarriage in an abortion-prone mouse model<sup>163</sup>. These studies are only a few of many which attempt to determine how the HO-1/ CO system could be implicated in complications of pregnancy, and certainly show positive results in the beneficial use of this system in the treatment of pregnancy complications, like PE.

This study shows for the first time that maternal low-dose treatment with CO can prevent the HTN and proteinuria in a PE-like mouse model induced by AdsFlt-1 injection. A prevention or treatment for PE remains elusive to date, but this study suggests that targeting the HO-1/ CO system offers a promising direction towards a possible therapeutic for PE.

#### 4.6 Supplement: Concentration of AdsFlt-1 used

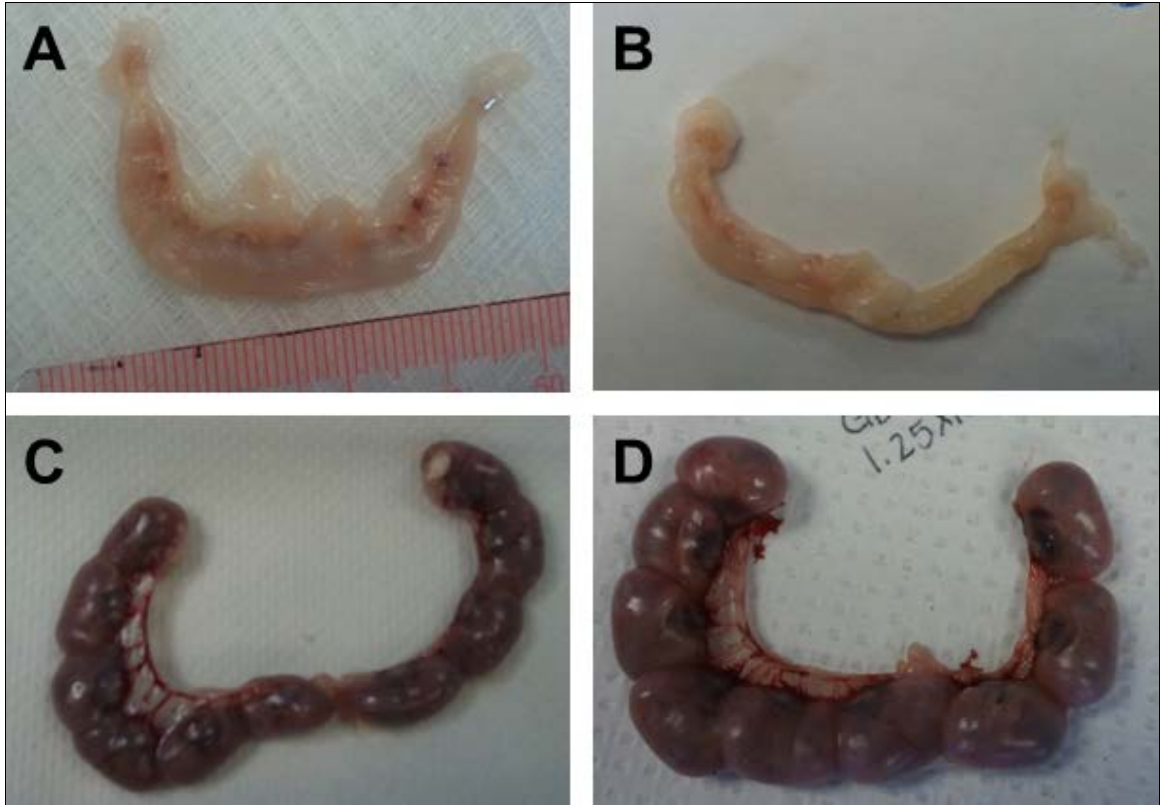
To develop the AdsFlt-1 PE model in our laboratory, we followed protocol by Karumanchi *et al.*<sup>139;229</sup>. The dose used by this group to inject rats was  $1 \times 10^9$  PFU, and we developed concentrations to match this for injection purposes. Others have also used the same dose to inject mice in their laboratories<sup>145;146;213</sup>, which supported the use of this dose concentration. Unfortunately, in our laboratory, the injection of  $1 \times 10^9$  PFU led to complete fetal loss, and on GD17.5, the uterus was resorbed of all fetuses (Figure 4.6A). Using the Vevo 770 ultrasound (Toronto, ON), we determined that fetal death was occurring within 72hrs of AdsFlt-1 injection (data not shown) and no fetuses remained *in utero* at GD17.5 (Figure 4.7). As such, we lowered the dose of injection to  $5 \times 10^8$  PFU. Again, fetal loss was evident and the uterus was resorbed of all fetuses on GD17.5 (Figure 4.6B); no pregnancies were sustained with this dose (Figure 4.7). At  $2.5 \times 10^8$  PFU, 50% of the pregnancies sustained pregnancy (Figure 4.7), with several of the remaining mice undergoing early miscarriage. All mice injected with a concentration of  $1.25 \times 10^8$  PFU were able to sustain pregnancy (Figure 4.6D, Figure 4.7).

We measured sFlt-1 protein levels in the maternal plasma of all mice. Mice injected with  $1 \times 10^9$  PFU led to plasma sFlt-1 levels (ng/ml  $\pm$  SEM) of  $17\,974.8 \pm 10\,742.7$  (Figure 4.8). The AdsFlt-1 protein levels in rats injected with the same dose were 215ng/ml, which is several-fold lower than we measured in our mice. This was evident in each of the subsequent doses,  $5 \times 10^8$  PFU and  $2.5 \times 10^8$  PFU (Figure 4.8). The injection of  $1.25 \times 10^8$  PFU led to maternal sFlt-1 plasma levels of (ng/ml  $\pm$  SEM)  $234.82 \pm 84.2$ , which was more similar to that of rats injected with AdsFlt-1<sup>139</sup> and mice injected with the same concentration<sup>145;146;213</sup>.

We used a plaque assay<sup>221</sup> to determine virion concentration, which is a precise measurement of virion concentration in a sample. This is different than the spectrophotometry method by which other groups determined virion concentration<sup>139;145;146;213</sup>, which is an estimation of virion concentration using a calculation<sup>230</sup>. The different methodologies may have accounted

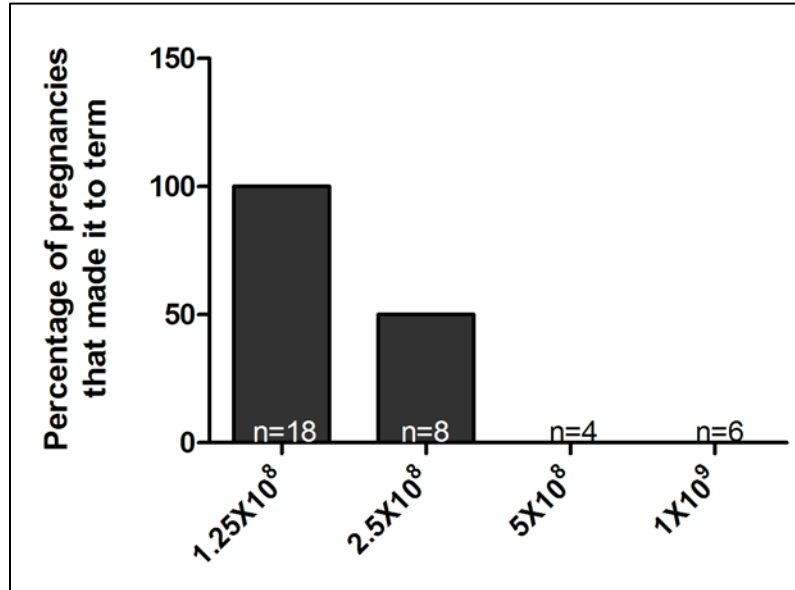
for the fetal loss we experienced with the same virion concentration. Judging by the maternal plasma sFlt-1 level, this is likely the case.

We used  $1.25 \times 10^8$  PFU as the final concentration injected into the mice, and this dose was used for both AdEV and AdsFlt-1 groups.



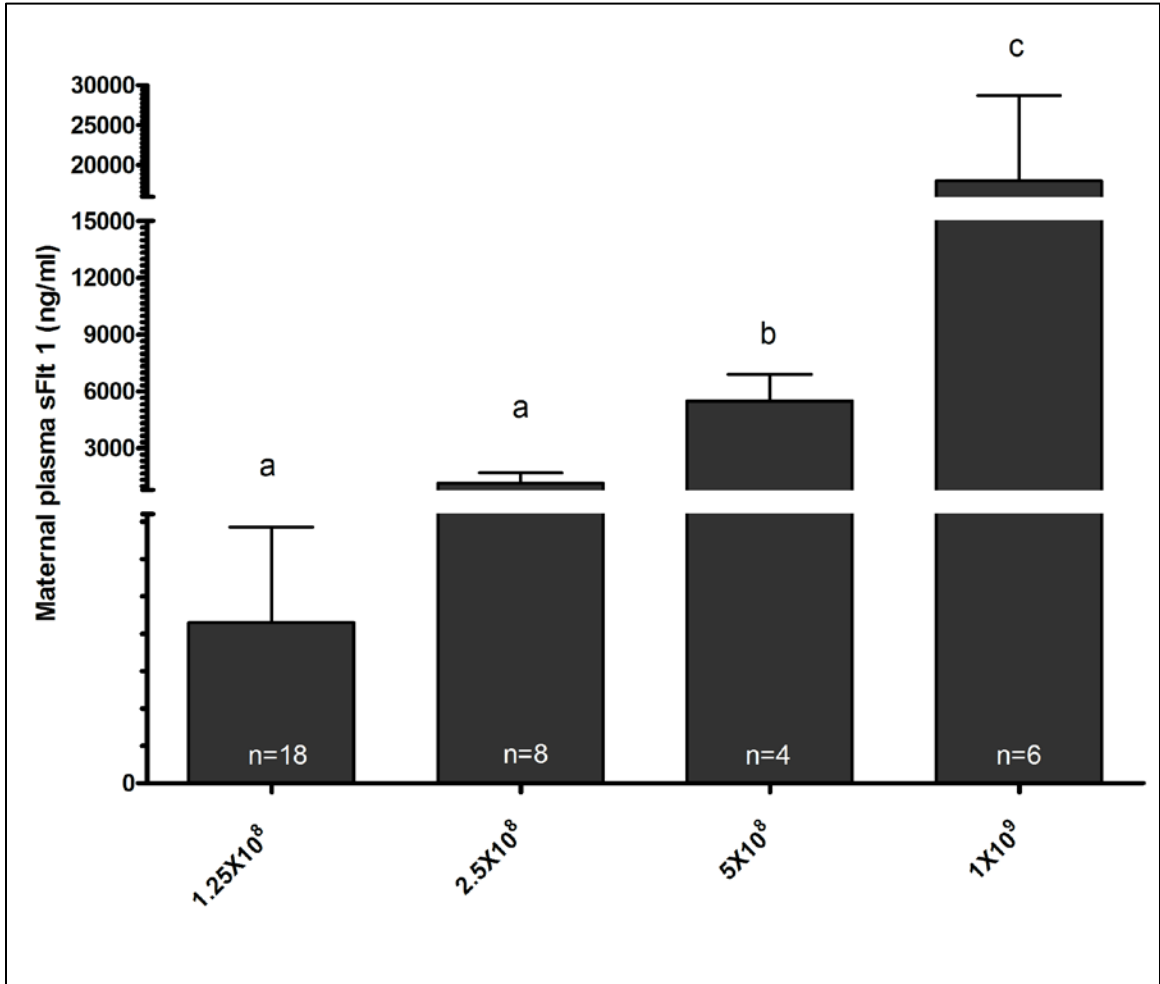
**Figure 4.6: Images of uterine horns excised from pregnant mice injected with AdsFlt-1.**

All uterine horns are from gestation day (GD) 17.5, and are representative images from mice injected with AdsFlt-1 at concentrations of  $1 \times 10^9$  PFU (A),  $5 \times 10^8$  PFU (B),  $2.5 \times 10^8$  PFU (C) and  $1.25 \times 10^8$  PFU (D). Only 50% of the mice injected with  $2.5 \times 10^8$  PFU AdsFlt-1 sustained pregnancy to GD17.5, while the remainder of mice underwent early miscarriage, or resorbed fetuses.



**Figure 4.7: Percentage of mice that sustained pregnancy when injected with AdsFlt-1.**

All groups presented were injected with AdsFlt-1 at the indicated concentrations; n-value is reported for the number of maternal mice injected with AdsFlt-1. No pregnancy was sustained with  $1 \times 10^9$  PFU or  $5 \times 10^8$  PFU injections. Half of the pregnancies were sustained when injected with  $2.5 \times 10^8$  PFU, while all mice sustained pregnancy when injected with the dose of  $1.25 \times 10^8$  PFU.



**Figure 4.8: Maternal plasma protein sFlt-1 concentration following injection of mice with AdsFlt-1.**

The concentration of virions injected into each group of mice is indicated below each bar graph, and number of mice (n) in each group, are indicated on the bar graphs. Mice were not able to sustain pregnancy when injected with either AdsFlt-1  $1 \times 10^9$  PFU or  $5 \times 10^8$  PFU, and both of these groups of mice had significantly increased plasma sFlt-1 levels compared to the mice injected with the lower virion concentration. Only half of the mice injected with AdsFlt-1  $2.5 \times 10^8$  PFU carried their pregnancies through to gestation day 17.5, while all mice injected with AdsFlt-1  $1.25 \times 10^8$  PFU sustained pregnancy until GD17.5. This group of mice measured plasma protein levels of sFlt-1 in the range of previous reported animals injected with AdsFlt-1 and resulting in PE-like signs.

## Chapter 5

**Effects of carbon monoxide (CO) inhalation on human blood and breath**

**CO levels; a pilot study for future therapeutic studies using CO**

## 5.1 Abstract

*In vitro* and *in vivo* studies have shown that the heme oxygenase (HO)/ carbon monoxide (CO) system has many cytoprotective properties and induction of this system has been explored *in vivo* for therapeutic purpose. Exogenous CO can be delivered through a non- rebreather mask to humans, in a non-invasive manner, offering a method for clinical application in the future. Human studies with CO administration are few and the current study aimed to determine the CO kinetics in blood and breath following CO inhalation. Blood pressure (BP), blood carboxyhemoglobin (%COHb) and end-tidal breath CO (EtCO) concentration were measured hourly in ten male and female volunteers following a one hour inhalation of 250ppm CO. %COHb (mean  $\pm$  SD) levels rose significantly ( $P < 0.05$ ) following inhalation, from 0.83%COHb  $\pm$  0.18 at baseline to 6.00%COHb  $\pm$  1.54, and EtCO (mean  $\pm$  SD) followed a similar trend, 2.33ppm  $\pm$  1.22 to 32.67ppm  $\pm$  6.24. During the 4-hour washout phase (the time between CO inhalations), hourly blood and breath CO measurements were taken and the half-life of blood CO was determined to be (hrs  $\pm$  SD) 4.68  $\pm$  1.83. Volunteers inhaled CO for a subsequent hour and both mean %COHb and EtCO levels rose significantly once again, (9.36%COHb  $\pm$  2.14 and 46ppm  $\pm$  6.75, respectively). Blood and breath CO levels normalized 24hrs after the original CO exposure, and were not different from baseline values ( $P > 0.05$ ). No changes ( $P > 0.05$ ) were observed in BP. The results of this study offer valuable half-life information on blood CO levels, in addition to peak levels of breath and blood CO following CO inhalation of 250ppm. This study offers a baseline for future therapeutic studies to use as a reference point.

## 5.2 Introduction

Over the last two decades, therapeutics using gaseous molecules have become popular due to the advantages of administration (inhalation) and their non-invasive application for clinical use<sup>231</sup>. Currently, the use of gas for medical applications includes many low molecular weight molecules (eg. H<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub>O, He, Xe)<sup>232;233</sup>. In addition to these gases, the gasotransmitters, nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H<sub>2</sub>S) have garnered much research interest, due to their anti-inflammatory and vasodilatory properties<sup>127;234</sup>.

Produced endogenously, CO is one of three products formed when heme oxygenase (HO) breaks down the pro-oxidant molecule heme<sup>234</sup>. This reaction occurs in most nucleated cells of the body<sup>27</sup>. While frequently referred to as a toxic gas, which is true at very high concentrations<sup>235</sup>, CO has been shown to possess many beneficial properties at low-levels, including vasodilation, anti-apoptosis, anti-inflammation and angiogenesis<sup>234</sup>.

Extensive preclinical animal studies have evaluated the effects of CO in various disease models and conditions<sup>34;194</sup>, including pregnancy<sup>159;162</sup>. To name a few, studies have reviewed the use of CO in animal models of acute lung injury<sup>236;237</sup>, ischemia/reperfusion injury<sup>238;239</sup>, sepsis<sup>240</sup>, organ transplantation<sup>160;241;242</sup> and recently, we have shown that treatment with CO can reduce the signs of preeclampsia, a pregnancy- specific hypertensive disorder (Chapter 4).

Although beneficial effects have been reported, results from treatment of animal models with CO cannot be directly translated to humans. A study completed in a mouse model of endotoxemia revealed a decreased inflammatory response when mice injected with lipopolysaccharide (LPS) were exposed to 250ppm CO<sup>128</sup>. When translated to a pilot trial in humans, no decrease in inflammatory response was observed when volunteers were injected with LPS and exposed to 500ppm CO<sup>164</sup>. This study is one of few CO-exposure experiments completed in human subjects. Another pilot study was completed in patients with chronic obstructive pulmonary disease (COPD), a disease of inflammation and oxidative stress, where

patients inhaled 100-125ppm CO to investigate the feasibility of reducing inflammation<sup>165</sup>. This study revealed a decrease in inflammatory mediators and offered a possible therapeutic treatment for COPD in the future.

As part of a larger trial, the current study was completed as a pilot project using an open, non-controlled design, on healthy human volunteers as a prelude to the randomized study. The purpose of this study was to determine the breath and blood kinetics of 250ppm CO inhalation in healthy volunteers, and the effect if any, on blood pressure (BP) throughout the experiment.

### **5.3 Methods**

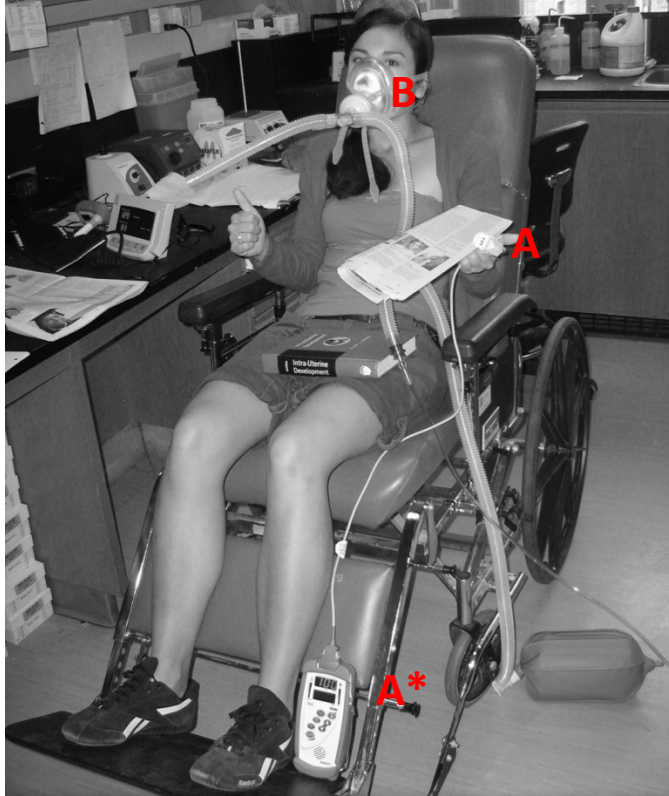
The study was approved by the Queen's University Ethics committee (SURG-194-09). Written and informed consent was obtained from all study participants. Volunteers in this study were recruited from the department of Biomedical and Molecular Sciences, and as such, the age ranges were quite diverse, from 23- 60 years of age.

#### **Experimental design**

Prior to CO exposure, baseline measurements were acquired for each of the following: BP, end-tidal breath CO (EtCO), blood %carboxyhemoglobin (%COHb), and oxygen saturation (%spO<sub>2</sub>). Study participants were fitted with a non- rebreather mask, and inhaled 250ppm CO gas (0.025%CO, 5%CO<sub>2</sub>, 21%O<sub>2</sub>) (Praxair, ON) for one hour (Figure 5.1). Continual monitoring of %spO<sub>2</sub> using a Rad 57 pulse oximeter (Massimo, Irvine, USA) ensured %spO<sub>2</sub> >98% O<sub>2</sub> and frequent questioning of the volunteers' wellbeing occurred throughout the hour-long CO inhalation. BP was measured 30minutes into the inhalation period. Following the CO treatment, the mask was removed from the volunteer and BP, %COHb and EtCO were measured hourly, for 4 hours (hrs), at which point volunteers again received an hour- long treatment with CO, in the same manner as the first dose. Monitoring of the volunteer was maintained and BP was again measured midway through the treatment. Following the second hour of CO inhalation, BP, %COHb and EtCO were measured, 22-24hrs following the beginning of the experiment. At the end of the experiment, a short questionnaire was asked of each patient to gauge their well-being throughout the CO exposure and any discomfort they may have felt.

#### **Procedures**

BP was measured using the BpTRU (VSM MedTech Ltd, Vancouver, Canada). Three



**Figure 5.1: Volunteer setup breathing 250ppm carbon monoxide (CO) for one hour using non-rebreather mask.**

Oxygen levels were measured consistently throughout CO exposure using a pulse oximeter (A\*) attached to a finger spectrophotometer (A). A non- rebreather mask (B) allowed for easy flow of CO and ambient air mixture to be inhaled by the volunteer at a comfortable flow rate.

consecutive measurements were taken to ensure the proper reading was recorded and two similar recordings were averaged for a final value of systolic and diastolic pressure.

Breath CO was measured using a pico Smokerlyzer (Bedfont Scientific, Maidstone, England). This machine is a noninvasive method of measuring breath CO concentrations. The machine's sensitivity is 1ppm and calibration was conducted prior to the study start date.

%spO<sub>2</sub> levels were monitored using a Rad-57 Massimo Rainbow pulse- Oximeter (Massimo, Hanover, Germany). This machine is a noninvasive method of measuring blood O<sub>2</sub> concentration using light spectra and absorption properties. A sensor was placed on the patient's index fingertip. Blood %spO<sub>2</sub> levels were maintained above 98%O<sub>2</sub> throughout the experiment.

Blood was collected via lancet (Onetouch Suresoft lancet, Lifescan, Johnson and Johnson, Canada) into heparin coated capillary tubes (Fisher brand, Cat no. 22-260-950). Blood hemoglobin (Hb) was measured with a Hemocue Hb 201 (Hemocue, Switzerland). Using a gas-tight syringe and repeater system (Hamilton, USA) blood was added to 20µl of 2% sulfosalicylic acid (SSA) (Sigma Aldrich, Cat no. 86193) in a 2ml amber vial (Sigma Aldrich, CAN), previously purged with CO-free air, 21% O<sub>2</sub>/ 5%CO<sub>2</sub>/balance N<sub>2</sub> (Praxair; Kingston, ON). The amount of blood added to the SSA was dependent on the time following CO exposure; before CO exposure, 1.0ul of blood, directly following CO exposure, 0.2- 0.4ul of blood. Vials were completely covered with ice for 60 minutes to allow for CO from the sample to equilibrate with the vial headspace. The lower temperature has been shown to decrease CO leaching from the septum<sup>166</sup>. Following the 60minute incubation period, a gas-solid chromatography (GC) machine (Peak Performer 1 Analyzer, Peak Laboratories, Mountain View, CA) was used to quantify CO concentrations in the head space gas of the blood and plasma vials, as previously described<sup>243</sup>. Blood CO concentration was calculated as a percentage of total body Hb using the following equation:

$$\%COHb = [\text{vol CO} / (\text{Hb} * 1.368)] * 100\% \quad 166;244$$

where 'vol CO' is milliliters of CO bound to 1L of blood, Hb is total Hb concentration in the blood (g/L) and 1.368 is the CO-binding capacity of Hb in milliliters per gram<sup>245</sup>.

Using the first four hrs following CO exposure, the half-life ( $t_{1/2}$ ) for each volunteer was calculated using the following equation:

$$t_{1/2} = [(\log 0.5) / (\log(A_E/A_o))] \cdot \text{time}$$

where  $A_E$  was the %COHb level 4 hrs following CO inhalation,  $A_o$  was the %COHb directly following CO inhalation, and time was set at 4hrs.

### **Statistics**

All statistics were completed using Graphpad Prism v.5.0. Data are presented  $\pm$  standard deviation (SD). A two-way, repeated measured ANOVA was used to analyze all measurements, with a bonferroni post-hoc test and a P-value of  $<0.05$  was considered significant.

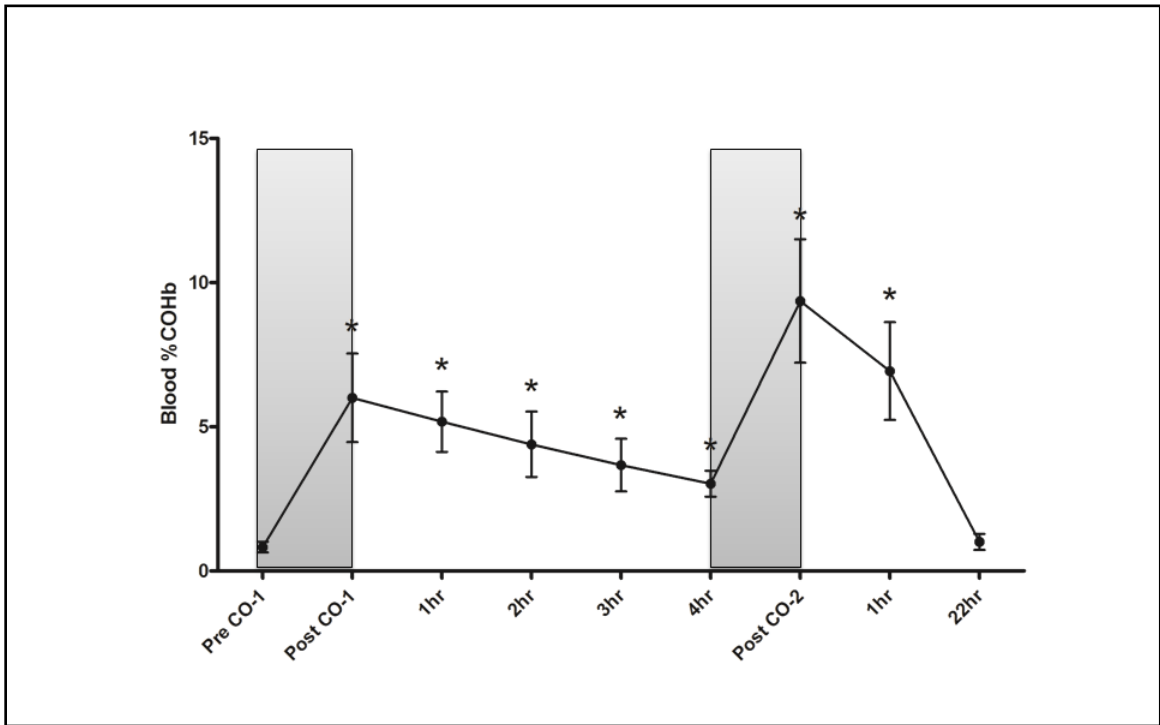
## 5.4 Results

A total of 5 female and 5 male volunteers were recruited in this study. Questionnaire results from all volunteers revealed a general consensus on feeling flushed during CO inhalation and uncomfortable due to the rebreathing mask. No CO inhalation was stopped, as all volunteers indicated that they otherwise felt fine throughout exposure.

Volunteer %spO<sub>2</sub> was maintained above 98% O<sub>2</sub> for all volunteers, even during CO inhalation. At baseline, the mean blood %COHb ± SD was 0.83 ± 0.18. Following CO inhalation, %COHb ± SD levels increased to 6.00 ± 1.54 for all volunteers. After a four- hour washout phase, the average volunteer %COHb levels dropped to 3.30 ± 0.45 and increased to 9.36 ± 2.14 following the second one hour CO inhalation. Measurement 24 hrs after initial treatment demonstrated that %COHb ± SD concentrations had returned to baseline concentrations at 0.80 ± 0.20 (Figure 5.2). The half-life (hrs ± SD) for CO was determined to be 4.68 ± 1.83 hrs for all volunteers.

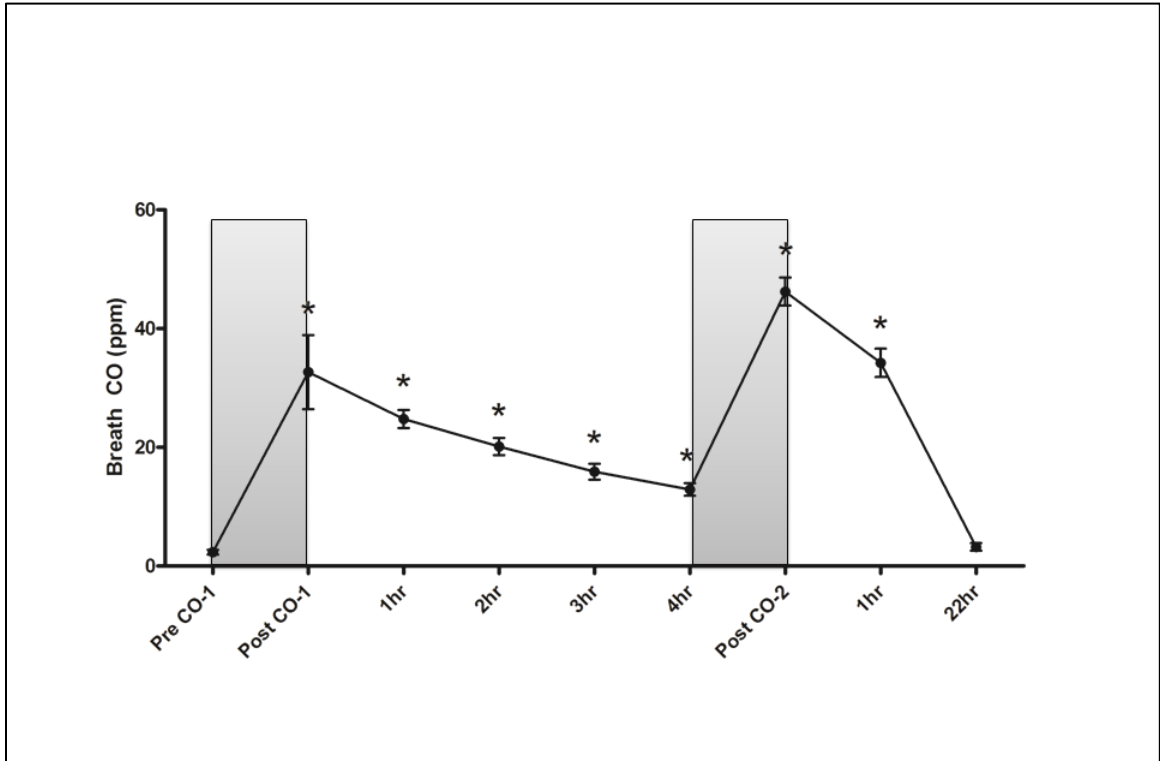
EtCO concentrations followed the same trend as %COHb, with a significant increase (P<0.05) in CO concentrations following CO inhalation, a gradual decrease over the four hour washout period, and a further increase in CO directly after the second CO inhalation (Figure 5.3). All CO levels dropped to baseline 24 hrs after initial exposure. Baseline EtCO (ppm ±SD) levels for all volunteers were 2.33 ± 1.22 and rose to 32.67 ± 6.24 (P<0.05) following one hour CO inhalation. The four hour wash-out period led to a drop in EtCO to (ppm ±SD) 12.8 ± 3.05, which again rose following CO inhalation and surpassed that of the initial CO dose, to 46 ± 6.75 (P<0.05). The EtCO levels at the end of the experiment, 22 hrs after the initial EtCO measurement, were (ppm ±SD) 3.1 ± 1.79 and were not different from the baseline values (P>0.05).

BP measurements are displayed in Figure 5.4. No significant difference from baseline values was observed throughout the experiment. A decrease (not significant,  $P>0.05$ ) in systolic BP was observed at the time of CO inhalation.



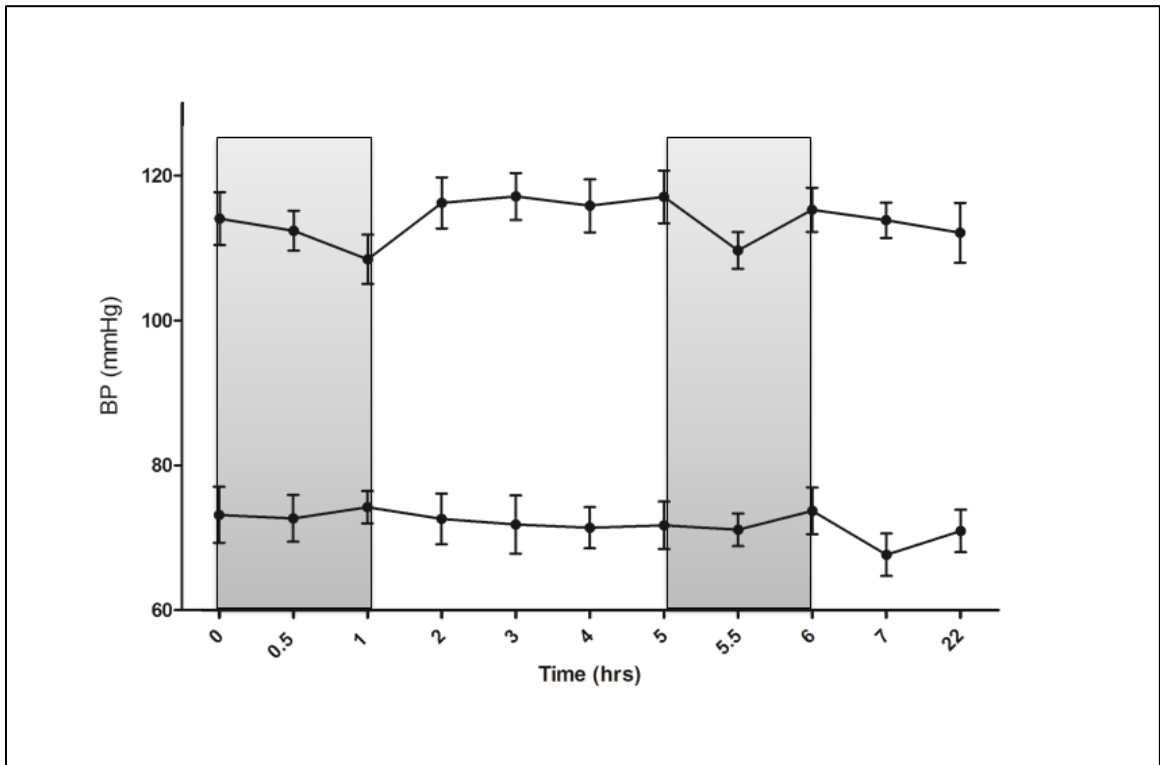
**Figure 5.2: Blood carboxyhemoglobin (%COHb) levels throughout experiment.**

Blood %COHb was measured hourly throughout the experiment. Grey bars indicate the two time intervals when carbon monoxide (CO) exposure took place. Significance compared to initial baseline CO measurement is indicated by an asterisk (\*)  $P < 0.05$ .



**Figure 5.3: End- tidal breath carbon monoxide (CO) levels throughout the experiment.**

End- tidal breath CO (EtCO) levels ( $\pm$ SD) were measured hourly throughout the experiment and follow a similar trend as observed with blood CO levels. Grey bars indicate the two, one-hour CO exposures for volunteers. Each EtCO level is compared to baseline EtCO and considered significant at  $P < 0.05$ . At the end of the experiment, 22hours following the initial CO exposure, volunteer breath CO levels returned to normal ( $P > 0.05$ ).



**Figure 5.4: Mean blood pressure (BP) measurements for all volunteers throughout the experiment.**

No significance ( $P > 0.05$ ) was determined for any BP measurement in comparison to baseline values. Grey bars indicate the two, one hour time-points when CO was inhaled by volunteers.

## 5.5 Discussion

The results of this study provide valuable information on the kinetics of low-dose, 250ppm CO inhalation on human %COHb and EtCO levels over time. This inhalation dose did not result in any demonstrable health effects to the volunteers, throughout exposure or after it.

The current work evaluated the effect of CO exposure in both female and male groups (n=10). The changes in %COHb and EtCO concentrations were similar. The CO measurements allowed for the determination of %COHb half-life, calculated to be  $4.68 \pm 1.83$ hrs. This is in range with previous work, reporting the half-life to be 4-6hours when breathing ambient air<sup>246</sup> and 1.23-2.47hours when breathing 100% O<sub>2</sub><sup>247</sup>. Although the age range for the study participants was quite diverse, the results were similar amongst all subjects, indicating that the findings are translatable to many different age groups for future work. A previous study has also reported that half-life determination is independent of age or race<sup>247</sup>.

Changes in the CO concentrations in blood (%COHb) and breath (EtCO) were similar, as both led to an increase following CO inhalation and a considerable drop in values over the 4 hour washout phase. This information is important for future studies, in evaluating the dosing regimen of CO inhalation, and to ensure that toxic levels of CO are not reached. The highest blood CO level achieved in this study was 7.99%COHb in the first CO exposure and 12.43%COHb following the second CO inhalation. These values are similar to those of smoking individuals (up to 14%COHb)<sup>248</sup>, but below levels associated with symptoms of clinical toxicity (>20%COHb)<sup>249</sup>. This provides a basis for future use of 250ppm CO in studies evaluating the therapeutic potential of CO.

The EtCO levels resulted in a similar change to the blood %COHb levels. Our group (Venditti and Smith, unpublished data) and others<sup>248;250</sup> have shown that EtCO levels correlate well with blood %COHb, thus the similarities in changes were not surprising. The current data

provide a possible method by which to easily measure CO concentrations during clinical trials to evaluate CO concentrations quickly and frequently within a subject.

There were no significant differences noted in the BP measurements in our subjects. While CO is a known vasodilator<sup>251</sup>, and functions in a similar fashion to NO<sup>252</sup>, the vasodilatory effects were perhaps not enough to affect normal BP, but may be evident in a state of hypertension. This will need to be determined in future studies involving human subjects.

The HO/CO system has become of great interest for therapeutic potential in numerous diseases as reviewed by Ryter and Choi<sup>234</sup> and Foresti<sup>253</sup>. Although numerous studies have been completed *in vitro* and *in vivo*, the translation of these results to human clinical data is not directly possible. We have completed one of few studies evaluating the effect of CO on human volunteers and offer results on human kinetics in blood and breath following CO inhalation to 250ppm. This study offers baseline information for females and males regarding blood %COHb and EtCO levels, in addition to alterations following two, one hour CO exposures. The results from this study provide information for future work using CO as a therapeutic.

## Chapter 6

**Collection of blood by vacutainer introduces increased carbon monoxide levels when measured by gas chromatography**

## 6.1 Abstract

**Introduction:** Measurement of carbon monoxide (CO) by gas chromatography (GC) is a precise and very specific method to assess the concentration of gas in air or liquid, including blood.

Blood collection and storage can affect the measured CO levels, and we aimed to assess whether heparin vacutainer tubes emit false readings of CO, and further, whether blood collection in vacutainers was the best method to measure CO levels in blood.

**Methods:** All CO levels were analyzed using GC. Vacutainers were evaluated for CO levels when exposed to light versus dark conditions over a period of ten days. The CO levels in the air of vacutainer tubes were measured for levels of CO from 0 to 40 days. Blood CO levels were calculated and compared using each of a vacutainer, syringe and lancet.

**Results:** Vacutainers exposed to light led to significantly higher ( $P<0.05$ ) air CO levels than those measured in vacutainers maintained in a dark environment. Further, CO levels were significantly increased ( $P<0.05$ ) over time (day 21 and 40) in empty vacutainers, originally purged with CO-free air. Blood collection by vacutainer led to increased CO levels ( $P<0.05$ ) at all time-points (days 0, 1, 30 and 120) compared to those measured by syringe. Blood collection by lancet proved no different in CO measurement than that of a syringe ( $P>0.05$ ) and offers an alternative method to blood collection for precise CO measurement.

**Conclusion:** While vacutainers offer an easy method for blood collection, researchers must be aware of the increased CO measurement in samples collected this way. A syringe or a lancet are two easy alternate options for more precise measurements.

## 6.2 Introduction

Once considered a molecule only of toxic nature, carbon monoxide (CO) has recently come into light as a cytoprotective agent at low levels, possessing properties in anti-inflammation<sup>128</sup>, anti-apoptosis<sup>98;254</sup>, maintenance of vascular tone<sup>112</sup>, and protection from ischemia-reperfusion injury<sup>98</sup>. It is endogenously produced through the breakdown of heme by heme oxygenase (HO), where three end-products are produced in equimolar amounts: CO, iron and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase<sup>44</sup>. All three end products, though cytotoxic at high concentrations, possess beneficial properties at low levels; bilirubin possesses anti-oxidant properties<sup>255</sup> and has been correlated with a lower incidence of familial cardiovascular disease<sup>256</sup>, iron upregulates the production of ferritin, an iron-chelating molecule<sup>257</sup>, and carbon monoxide (CO), as listed above. The importance of HO in the body is unparalleled, as it has been found in every nucleated cell of the body<sup>257</sup>, in addition to its role in many pathological diseases, such as cancer tumours<sup>258;259</sup>, malaria<sup>260</sup>, Parkinson's disease<sup>261</sup> and disorders of angiogenesis<sup>154</sup>, to name a few.

In the body, CO is bound to hemoglobin (Hb), forming carboxyhemoglobin (COHb). Measurement of this molecule allows for not only knowledge of CO levels, but in addition, indirect measurement of HO activity as well. As research around these molecules has increased tremendously over recent years<sup>27;32;262-266</sup>, proper measurement and technique allows for improvement of results and literature comparison. Several methods to determine total CO bound to Hb have been reported<sup>258;267-269</sup>, while a new method, gas chromatography (GC)<sup>166;270</sup>, offers more precise and specific recognition of CO levels, and this has been described elsewhere<sup>166</sup>. While this method was first described by Collison *et al.*<sup>271</sup>, it has since been modified and improved by Vreman *et al.* in several reports<sup>166;267;270</sup>. This group identified a potential error in blood CO levels using EDTA-containing vacutainer tubes<sup>166</sup>. Further, they identified a proper time and temperature storage for blood sample incubation prior to measurement<sup>166</sup>.

Recently our laboratory began measuring CO levels in human blood as part of a larger research study. We identified a potential error in the blood %COHb levels we were acquiring when collected in heparin vacutainer tubes. We sought to determine what levels of CO are found in heparin vacutainers with and without blood present, and further to determine the most precise and easily conducted method for measuring blood CO levels in our patient volunteers. We hypothesized that blood collection via vacutainer will result in increased CO concentrations as measured over time, and in comparison to syringe collection. We developed a more precise and consistent measurement procedure for collection and handling of blood samples.

## **6.3 Methods**

### **The effect of light on CO- level measurement in a vacutainer tube**

Ten 6ml heparin vacutainer tubes (Becton Dickinson, B367878, USA) were purged with CO-free air (21% O<sub>2</sub>/5% CO<sub>2</sub>/ Balance N<sub>2</sub>) (Praxair, Kingston, ON) which had been passed through a Hopcalite catalytic converter at 100°C (Trace Analytical, Menlo Park, CA 94025). For a total of ten days, five vacutainers were exposed to normal light, while five were maintained in the dark (wrapped in tin foil and hidden from light). Following the incubation time period, 2ml of CO-free air (21% O<sub>2</sub>/5% CO<sub>2</sub>/ Balance N<sub>2</sub>) (Praxair, Kingston, ON) was added to each vacutainer using a 3ml syringe (Fisher scientific, Cat. No 14-817-26) and 22 gauge needle. Using a gas-tight syringe and repeater system (Hamilton, USA), triplicate samples (100ul) of air were measured for each vacutainer by GC as previously described<sup>166</sup> (and explained briefly below), and converted to total parts per million (ppm).

### **Measuring CO levels in a vacutainer**

The vacuum was removed from twenty- five 6ml heparin vacutainer tubes (Becton Dickinson, B367878, USA) by adding CO-free gas (21% O<sub>2</sub>/5% CO<sub>2</sub>/ Balance N<sub>2</sub>) (Praxair, Kingston, ON) to each tube using a syringe (Fisher scientific, Cat. No 14-817-26) and 22 gauge needle. All vacutainers were maintained in the dark (wrapped in tin foil) for the remainder of the experiment. On each of day 1(24hrs later), day 6, day 10, day 21 and day 40, five vacutainers were removed, total CO was measured in the vacutainer and reported as ppm.

### **Time comparison of blood CO level in a vacuainter versus a syringe**

We sought to determine if CO levels differed in human blood, when collected via vacutainer or directly into a pre-heparinized syringe. As part of a larger study (OBGY-165-06), blood samples were collected from ten non-smoking volunteers by venipuncture, and each were

collected in both a 6ml heparin vacutainer (Becton Dickinson, B367878, USA) and a 3ml syringe (Fisher scientific, Cat. No 14-817-26) containing 50ul of 14.3U/ml of sodium heparin (Sigma Aldrich, H0777) and maintained on ice. Within ten minutes of blood collection, Hb was measured using a Hemocue Hb 201 (Hemocue, Switzerland). Blood was stored at 4°C, previously shown to remain stable for up to two months<sup>166</sup> or measured for CO levels immediately, as previously described<sup>166</sup>.

Briefly, 2ml amber vials (Sigma Aldrich) were sealed with open top caps (Chromatographic Specialties, C223710C) and 8mm silica septa (Chromatographic Specialties, C13302). Each vial contained 20ul of 2% sulfosalicylic acid (SSA) (Sigma Aldrich, Cat no. 86193) and were purged with 21% O<sub>2</sub>/5% CO<sub>2</sub>/ Balance N<sub>2</sub> air (Praxair, Kingston ON). Using a gas-tight syringe and repeater system (Hamilton, USA), 1.0ul of blood was added to the SSA in each vial and shaken briefly to ensure total mixing. Each vial (including the cap) was covered in ice, as this has been shown to decrease CO leaching from the vial and maintain CO stability<sup>166</sup> and incubated for 30- 60minutes to allow for CO from the sample to equilibrate with the headspace of the vial. The vial air was then measured for CO using GC as previously described<sup>272</sup>. Blood CO level was calculated as a percentage of total body Hb using the following equation:

$$\% \text{COHb} = [\text{vol CO} / (\text{Hb} * 1.368)] * 100\% \quad ^{166}$$

where 'vol CO' is milliliters of CO bound to 1L of blood, Hb is total Hb concentration in the blood (g/L) and 1.368 is the CO-binding capacity of Hb in milliliters per gram<sup>245</sup>.

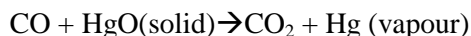
On each of day 6, 10, 21 and 40, the same procedure was completed as the initial blood samples and CO levels were measured for each volunteer.

### **Measurement of blood CO levels using a lancet versus a syringe**

In addition to venipuncture samples from each of the 10 volunteers, a small blood sample was also collected using a lancet and collected into heparinized capillary tubes (Fisher brand, Cat no. 22-260-950). The blood was transferred to a sterile, Eppendorf 0.2ml microcentrifuge tube (Fisher Scientific, Cat. No. 05-402-93) using a rubber pipette bulb. Blood was analyzed in the same manner as described above.

### **Gas analysis**

A GC (Peak Performer 1 Analyzer, Peak Laboratories, Mountain View, CA) was used for all CO measurements. Amber vials (2ml) containing sample to be analyzed were placed on the injection port and using a silica based column (60/80 Mole Sieve 13X Column), the machine collected all headspace gas from the vial at 20ml/min and separated the gases present based on pore sizes within the column. The detector housed mercuric oxide (HgO) and allowed for a reaction with CO to occur:



where Hg is mercury. The Hg vapour was able to pass out of the HgO bed and past an ultraviolet photometer with a specific absorbance of Hg at 254nm. The change in energy due to the Hg vapour present was directly proportional to the amount of CO in the headspace and caused a peak value to be recorded based on the light disturbance. The blank CO values obtained were subtracted from all sample CO levels. A standard curve was produced by injecting increasing amounts (0-500uL, by 50ul increments) of CO standard gas (10.1 parts per million (ppm); Scott Specialty Gases, Troy, MI) into a previously purged vial with 21% O<sub>2</sub>/ 5% CO<sub>2</sub>/ balance N<sub>2</sub> (Praxair, Kingston, ON) and reading these samples with the GC. Sample values were interpolated from the standard curve to calculate CO levels.

## **Statistics**

All statistics were completed using Graphpad 5.0 (Graphpad software, CA) and a p-value <0.05 was deemed significant. A student's t-test was used for both the light vs. dark experiment and the syringe vs. lancet experiment. For comparison of CO levels over time, for both the vacutainer- only and the vacutainer vs. syringe experiment, an analysis of variance using a post-hoc Tukey test was used.

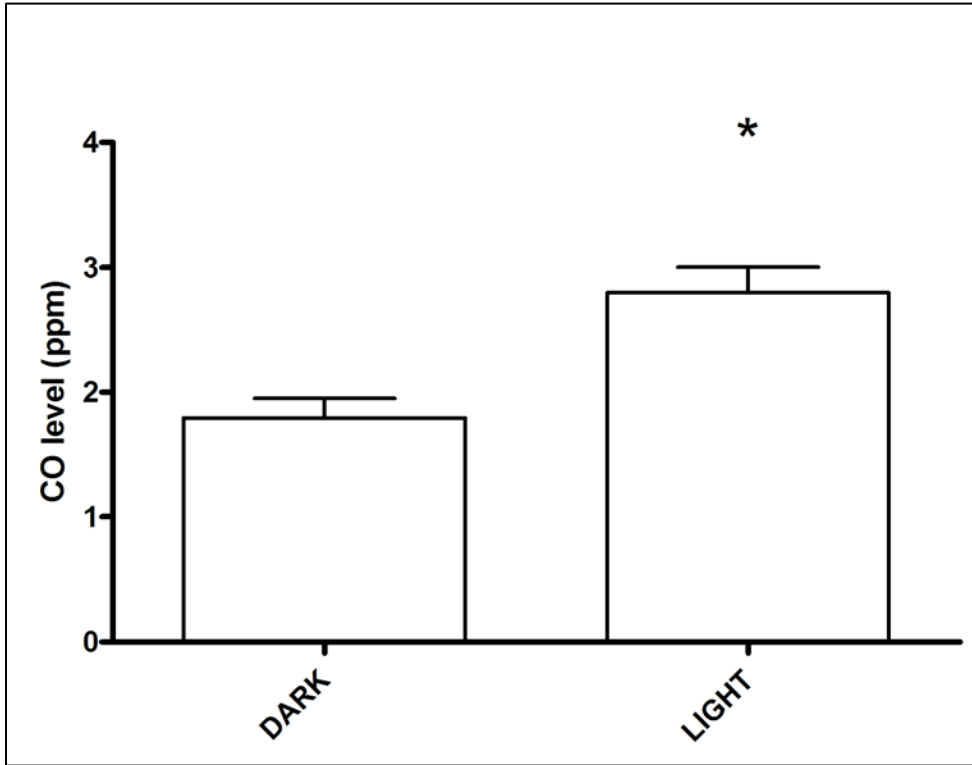
## 6.4 Results

Following ten days of light exposure, CO vacutainer levels were significantly higher ( $P < 0.05$ ) than those from vacutainer tubes maintained in the dark (Figure 6.1). CO levels were 1.5X higher when vacutainers were incubated in the light versus dark environments.

While we found that incubating vacutainers in the dark would reduce CO level measurements, we sought to determine if CO levels would be maintained relatively constant over time. Vacutainer tubes were kept at 4°C and in the dark, but still, over time, CO levels in air were increased (Figure 6.2) and significantly different ( $P < 0.05$ ) on both day 21 and 40 from the first day of analysis.

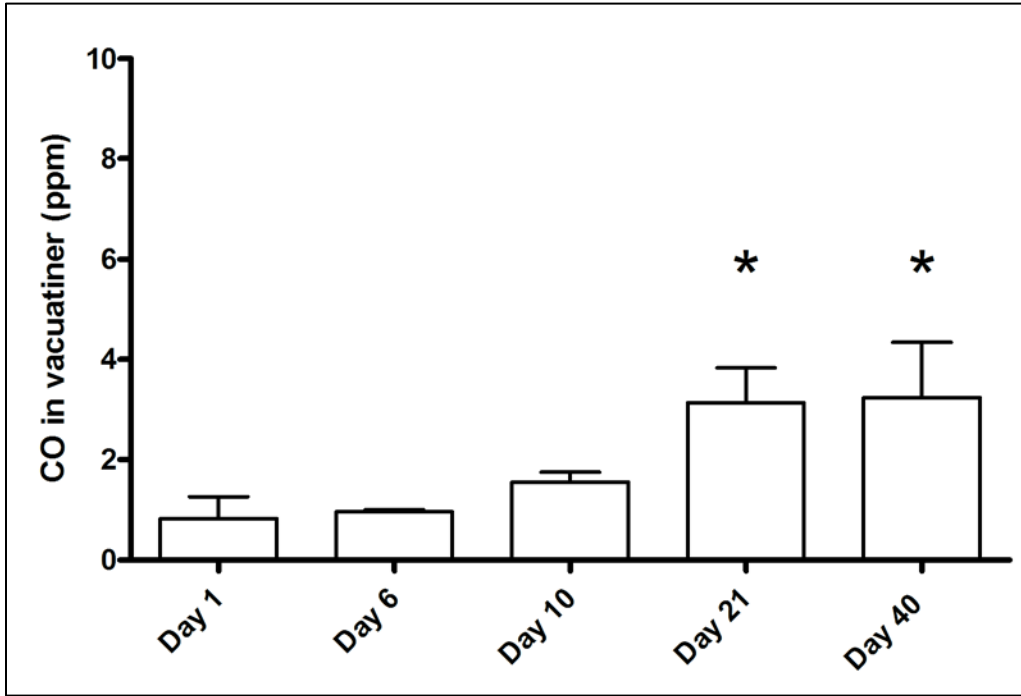
Figure 6.3 displays the CO levels from Day 0 to Day 120 post blood collection. CO levels remained stable (not significantly different,  $P > 0.05$ ) throughout the experiment when collected via syringe, but were significantly lower ( $P < 0.05$ ) than those collected via vacutainer at all timepoints. At each timepoint CO levels were 1.5 to 2.5 times lower using the syringe method compared to the vacutainer. Further, vacutainer CO levels significantly increased ( $P < 0.05$ ) at day 120, compared to other timepoints for either the vacutainer or syringe CO levels.

As the amount of blood required to measure CO is extremely minimal,  $\leq 1.0\mu\text{l}$ , we collected blood using a lancet, assuming that volunteers would agree to a finger poke more readily than by normal phlebotomy. This has been tested in neonates using a heel stick and was proven accurate by Vreman *et al.*<sup>166</sup>. We measured CO levels in 5 volunteers, comparing both the syringe and lancet method, and determined there to be no difference between the two ( $P > 0.05$ ), as shown in Figure 6.4.



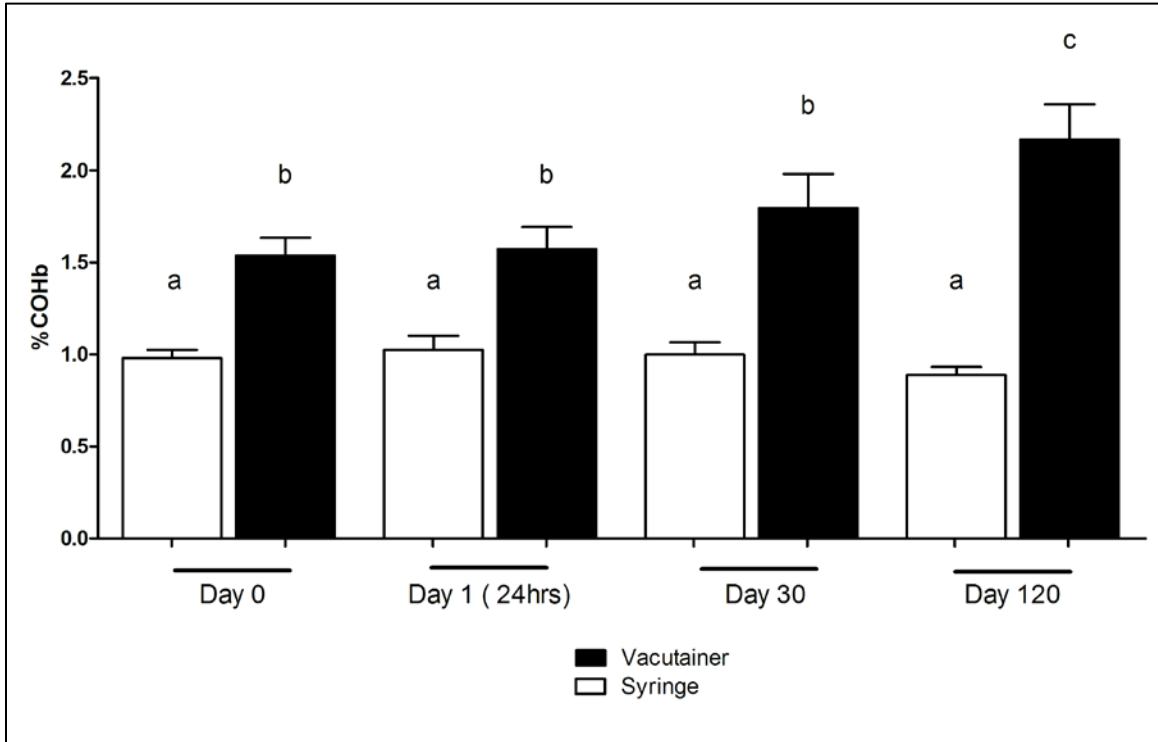
**Figure 6.1: Comparison of vacutainer incubation measuring carbon monoxide (CO) accumulation in dark versus light environments.**

The exposure of vacutainers to light, produced increased CO within the vacutainer -maintained air in comparison to vacutainers maintained in the dark (\*  $P < 0.05$ ).



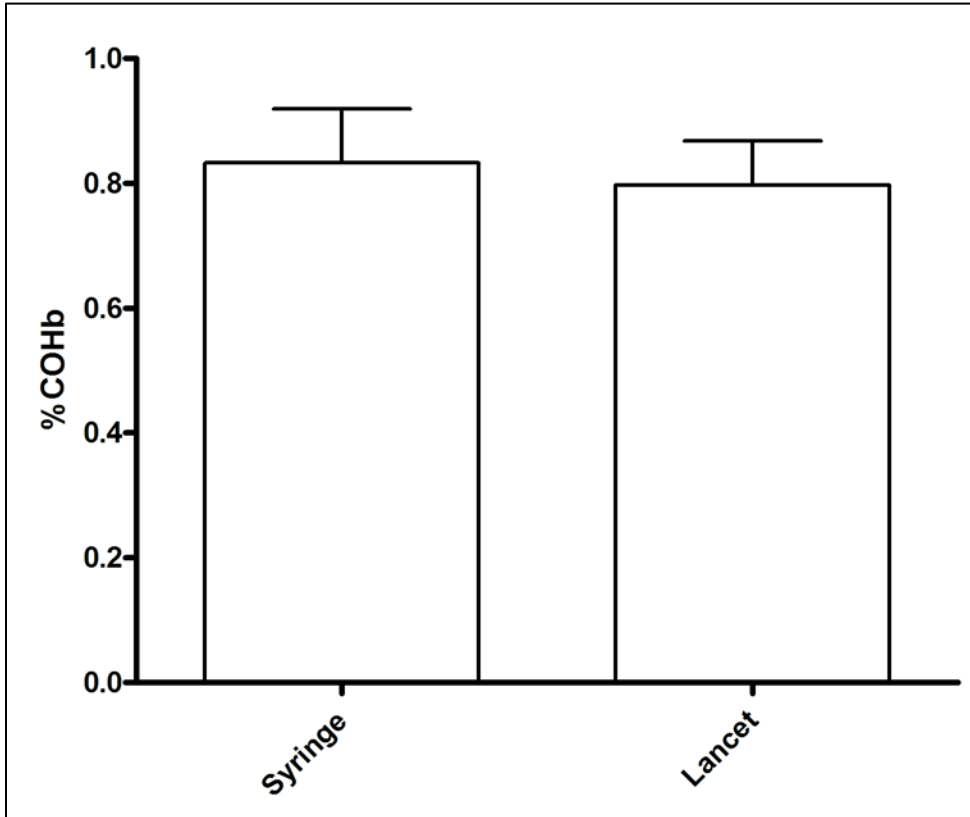
**Figure 6.2: Comparison of carbon monoxide (CO) levels over time in heparin- vacutainer tubes.**

For each time-point, five different vacutainers were measured for CO levels. All vacutainers were maintained in the dark, and over time, the CO levels measured within the vacutainers increased, becoming significant above day 21 (\*P<0.05).



**Figure 6.3: Comparison of carbon monoxide (CO) levels in blood obtained by vacutainer and syringe methodology.**

The collection of blood by syringe and vacutainer was compared for CO level, in addition to CO accumulation over time. Blood was collected from ten subjects using both collection techniques. CO levels were consistently higher with vacutainer collection in comparison to syringe ( $P < 0.05$ ). At each time point, percent carboxyhemoglobin (%COHb) levels maintained stable using the syringe methodology ( $P > 0.05$ ). Vacutainer blood %COHb levels were similar from day 0 to day 30 ( $P > 0.05$ ), but were increased significantly at 120 days following collection ( $P < 0.05$ ). Similar data is represented with matching letters in the graph. Differences are noted with dissimilar letters.



**Figure 6.4: Comparison of carbon monoxide (CO) measured in blood obtained by lancet or syringe blood collection.**

There was no difference in the percent carboxyhemoglobin (%COHb)  $\pm$  SD levels measured in ten volunteers, using either the syringe or lancet blood collection ( $P>0.05$ ).

## 6.5 Discussion

GC is the method of choice when measuring %COHb due to its specificity and selectivity for the gas<sup>270</sup>. In comparison to other methods, such as spectrophotometry, GC allows for use of smaller volumes of blood and is able to measure very low proportions of CO bound to Hb<sup>270</sup>. While patient blood samples are most often collected using vacutainer tubes, the measurement of CO in these tubes displays increased %COHb, as shown in this study and others<sup>270</sup>. This study aimed to determine if vacutainers increased the measurement of CO levels in blood samples. Further, we sought to determine a blood collection method which can be completed with ease and can decrease the level of false CO measurement.

When conducting human studies, blood collection may occur at random time intervals by hospital staff, leading to sample retrieval from research personnel at inconsistent timepoints. We found that samples were often maintained at room temperature or exposed to light following collection, and examined if this could affect the blood %COHb samples. Indeed, Vreman and colleagues<sup>166;270</sup> previously reported that maintaining samples at 4°C ensures stable %COHb readings for up to two months. While they also reported that samples should be maintained in the dark, we showed that exposure to light over time leads to increased production of CO, even in the absence of a blood sample (Figure 6.1).

While exposure to light was shown to alter the vacutainer CO level, we further hypothesized that CO levels would increase over time, perhaps due to possible photooxidation of organic compounds within the rubber vacutainer stopper<sup>273</sup>, but this remains to be elucidated. The experiment we conducted was performed in the dark and at 4°C, however, it was found that CO levels increased over time in the air samples of vacutainers (Figure 6.2). While the CO measured was in air-only, the increase was substantial, almost two-fold by day 21, indicating that CO should be measured in samples before 21 days post collection, and ideally as soon as possible. CO air levels were stable until day 10 of the experiment, but whether or not this would translate

into blood CO levels was unknown. Therefore, we chose to test CO levels bound to Hb in ten volunteers and compared collection methodology by vacutainer and syringe.

Collection of blood by vacutainer produced stable CO readings from day 1 to day 30 post blood venipuncture (Figure 6.3) and this was in line with the previous experiment we conducted with only air, as CO levels began to rise as early as 21 days following the beginning of the experiment (Figure 6.2). Interestingly, although measurements were stable (Figure 6.3), they were statistically higher than those measured with a syringe. In addition, syringe %COHb measurements were not different from day 1 to day 120 post venipuncture, indicating that collection by this method would allow for up to 4 months of consistent readings (Figure 6.3). Importantly, should collection only be possible by vacutainer tube, researchers should be aware of increased %COHb measurements and should be sure to include this in study methodology, as it may alter precision and significance of data.

Lastly, as an alternate to venipuncture, we conclude that blood collection by lancet and capillary tube collection is a reliable and alternate tool for %COHb measurement (Figure 6.4). It can be completed immediately and without a necessary phlebotomist, thus offering a methodology for any researcher to use. This would eliminate the possible problem of storage (light, temperature) from blood collection to researcher retrieval and allow for more timely measurement of the sample.

The study of the HO system and pathologies is quickly becoming one of great interest to researchers around the globe. More consistent and standardized measurement of CO would allow for better comparison of results between groups and elimination of handling error. While completing our own research in both humans and animals, we determined a range of %COHb levels for smoking and non-smoking humans, in addition to control mice and those exposed to exogenous CO, acutely<sup>159</sup> and chronically<sup>159;162</sup>. Table 6-1 displays a reference of these %COHb

**Table 6-1: Reference Table for blood volume and matched percent carboxyhemoglobin (%COHb) values measured in human and mouse subjects.**

	Amount of blood in vial (µl)	%COHb	Reference
<b>Humans</b>			
Non- smoker	1.0	0.5- 1.2	<sup>270</sup>
Smoker	0.2- 0.4	Up to 14	<sup>274</sup>
Exposure to 250ppm CO (one hour)	0.2	<8.0	Chapter 5
Exposure to 250ppm CO (two hours)	0.2	<12.5	Chapter 5
<b>Mice</b>			
		<b>%COHb ± SD</b>	
0ppm	1.0	1.3 ± 0.3	159;162
25- 100ppm	0.6- 0.8	2.1 ± 0.6- 9.1 ± 1.4	
150-200ppm	0.4-0.6	11.4 ± 1.9- 12.2 ± 1.9	
250ppm	0.2	13.5± 2.1	
>300ppm	0.2	Up to 15.0	

Levels, in addition to the amount of blood used when utilizing a GC for CO measurement, as [er the protocol outlined in this study.

The use of GC for %COHb measurement is one of high sensitivity (up to 1 part per billion, or nL/L) and specificity. It is highly accurate and more specific and time efficient in comparison to several different methods of CO measurement<sup>275:276</sup>. Measuring blood CO levels using a GC greatly strengthens results, allowing for more precise measurements and therefore the ability to detect even subtle changes in experimental groups. We have shown that collection of blood by heparin vacutainer and measurement of CO in these samples leads to an increase in measured CO levels and offer two alternate measurement procedures.

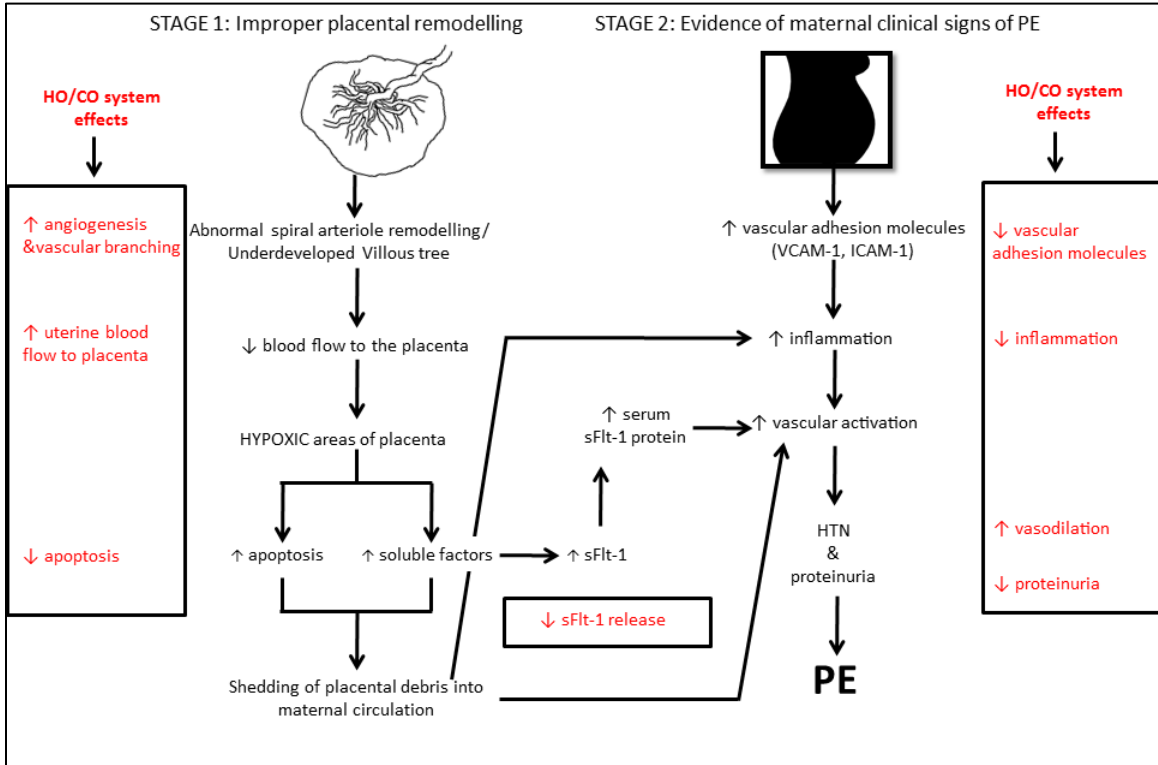
## Chapter 7

### **General Discussion**

## 7.1 General Summary

Over 2000 years ago, eclampsia was first described as: *seizures occurring during pregnancy which resolve following delivery*<sup>277</sup>. It is now clear, that Pre- eclampsia (PE), the prelude to eclampsia, is not simply pregnancy- induced hypertension (HTN) and proteinuria<sup>278</sup>. Appreciation for the spectrum of the disorder has led to a considerable increase in our understanding of PE over the last quarter century<sup>278</sup>. Unfortunately, there remains immense amounts of information that we do not know about the disorder, including, importantly, its etiology<sup>278</sup>. PE continues to affect 5-7% of all pregnancies worldwide, increasing the risk of severe obstetric complications by as much as 25%<sup>279</sup>, with a significant risk of morbidity and mortality among mothers and their fetuses<sup>5:189</sup>. Although research advances have been made, the treatment of the disorder remains the same, with the only known cure being delivery of the fetoplacental unit.

The theory for a two stage disorder was first described by Redman *et al.*<sup>7</sup> in 1991, suggesting that the development of PE begins early in gestation, with the incomplete remodeling of the maternal uterine spiral arteries (Stage 1), and becomes evident late in pregnancy, with the clinical signs of HTN and proteinuria (Stage 2). The dysfunctional placenta produces and secretes factors into the maternal circulation, contributing to the widespread endothelial dysfunction that leads to the maternal disorder<sup>278</sup>(Figure 7.1). One protein identified in excess in maternal plasma of women with PE, is soluble *fms*-like tyrosine kinase-1 (sFlt-1)<sup>139</sup>, and is over produced by the placenta in women with the disorder<sup>139</sup>. Importantly, sFlt-1 in maternal serum decreases following delivery<sup>139</sup>, as would be expected if the main source of sFlt-1 was of placental origin<sup>18</sup>. Further, increasing the levels of sFlt-1 in animal models of pregnancy can lead to the development of the signs of PE, HTN and proteinuria<sup>139:144-148</sup>. Numerous researchers have studied sFlt-1, evaluating its effect on angiogenic proteins<sup>18:139:141</sup>, the vascular endothelium<sup>148</sup> and extracorporeal removal of maternal serum sFlt-1 as a potential therapeutic<sup>210</sup>.



**Figure 7.1: Outline of the two-stage model of preeclampsia (PE) and the possible areas that the heme oxygenase (HO)/ carbon monoxide (CO) system may be able to reduce or prevent the development of the disorder.**

The progression of PE is outlined in black writing, with the improper placental development producing factors that spill into the maternal circulation and contribute to maternal endothelial dysfunction. This placental insufficiency, in conjunction with maternal vascular dysfunction, can lead to the development of PE in some women. The HO/CO system has been shown to alter several of the pathways leading to development of PE, many of which are highlighted here in red and which have been described in this thesis.

Women who smoke in pregnancy have a reduced incidence of PE<sup>62;63</sup>. Further, women with PE have reduced levels of carbon monoxide (CO) in their breath<sup>80</sup>, and it is hypothesized that this combustible product, increased in the breath of smoking women<sup>76</sup>, could be a potential therapeutic for the disorder. While this may seem paradoxical, as CO at high concentrations is known to be toxic<sup>235</sup>, low concentration CO leads to vasodilation, reduction of both inflammation and apoptosis, and increased angiogenesis<sup>32</sup>, all of which may be beneficial in treating PE (Figure 7.1). Further, in patients with PE, HO-1 protein levels in the placenta are significantly lower than those of healthy pregnant women<sup>39</sup>. Studies have evaluated inducers/ inhibitors of HO-1<sup>39;115;116;154</sup>, CO-releasing molecules (CORMs)<sup>123;154;155;192</sup>, and CO-bubbled media<sup>154</sup>, as potential therapeutic agents both *in vitro* and *in vivo*, however, studies using CO gas directly as a therapeutic *in vivo* is lacking<sup>160;161;163-165</sup>.

As a gasotransmitter, CO is one of three unique molecules (nitric oxide (NO) and hydrogen sulfide (H<sub>2</sub>S) being the other two), which are synthesized in the body, and differ from classic signaling molecules<sup>27;280</sup>. Gasotransmitters are small molecules of gas, permeable to membranes and whose actions can be mimicked by exogenous administration<sup>280</sup>. This is important in using CO as a therapeutic, as endogenous increases in CO can be mimicked by exogenous administration, as well as through upregulation of the HO enzyme. The ease of gaseous CO administration makes it a good target for possible use as a therapeutic. On the other hand, the toxicity of the gas at high concentrations<sup>235</sup> leads to the necessity for studies evaluating the safety of the gas at lower concentrations.

In this regard, the aim of this thesis was to evaluate the effect of low-dose (250ppm) gaseous CO as a potential therapeutic for PE. We evaluated exposure to CO in animal models of pregnancy, in placental villous explants and in healthy human volunteers. Specifically, the results show that i) 250ppm CO exposure in pregnancy increases uteroplacental blood flow and placental angiogenesis (Chapter 2), iii) 250ppm CO can reduce the production/ release of sFlt-1 from

placental villous explants (Chapter 3) iii) exposure to 250ppm CO can prevent the development of HTN and proteinuria in a mouse model of PE (Chapter 4). The data completed in humans, revealed that iv) administration of 250ppm CO to healthy human volunteers leads to levels of blood and breath CO below those of smoking individuals with a half -life of approximately 4.5 hours (Chapter 5) and finally v) collection of blood for CO measurement should be completed using syringe or lancet to avoid errors in CO quantification (Chapter 6).

## **7.2 Effect of maternal CO exposure on the vasculature of the uteroplacental unit**

CO is a known vasodilator<sup>264</sup> which functions through soluble guanylyl cyclase (sGC)<sup>112;157</sup>. We have previously shown that levels of CO, similar to those of women who smoke in pregnancy, led to vasodilation and decreased perfusion pressure in the isolated human placenta<sup>112</sup>. In Chapter 2, we performed an experiment whereby we hypothesized that maternal exposure to low-dose CO in pregnancy would lead to vasodilation of the uteroplacental unit and subsequent increased blood flow. The results demonstrated that blood velocity in the uterine artery (UtA) or umbilical artery (UMB A)/vein (UMB V) did not change following exposure to CO. However, micro-computed tomography (micro CT) measurements of the UtA revealed an increased diameter, and quantitative results indicated a resultant increase in blood flow. Further, increased angiogenesis of the placental unit was evident through micro CT imaging, and indicated branching of the radial arteries and placental canals. These alterations suggest a means by which CO exposure could lead to remodeling of the placental unit and an increased nutrient transfer to the fetus. Indeed, it has been shown that the placentas of women who smoke in pregnancy are larger in volume<sup>281</sup> and size<sup>199</sup> compared to non- smokers. Pfarrer *et al.*<sup>199</sup> prepared plastic casts of fetal capillaries within placental cotyledons of four smoking women (>20 cigarettes per day). Using scanning electron microscopy, they observed that the density of the terminal capillary

convolutes was increased, as was the branching of the convolutes (adaptive angiogenesis), and they hypothesized that possible compensatory mechanisms were aimed at increasing the oxygen and nutrient exchange surface area of the placenta<sup>199</sup>. It is possible that the elevated CO concentration in women who smoke accounts for this increased angiogenesis and branching of the placental units. Further, it offers a potential mechanism by which CO can increase nutrient transfer in a compromised pregnancy, such as PE (Figure 7.1).

### **7.3 CO exposure alters the balance of maternal and placental anti-angiogenic/ angiogenic molecules**

The anti-angiogenic molecule, sFlt-1, has become a protein of significant interest in PE over the last decade<sup>282;282-284</sup>. It is increased in maternal serum and the placenta of women with PE, but decreases following delivery<sup>139</sup>. Women who smoke in pregnancy have decreased sFlt-1 in their serum<sup>143;150-152</sup>, and a matched increase in placental growth factor (PGF)<sup>150;151</sup>. *In vitro* studies have shown that HO-1 induction can block the release of sFlt-1 from human umbilical vein endothelial cells (HUVECs)<sup>154</sup> and rat placental explants<sup>155</sup>. While CO-bubbled media<sup>154</sup> and CORMs<sup>155</sup> have been studied to test the effect of CO on sFlt-1 production; to our knowledge, the use of gaseous CO on sFlt-1 release has not been studied. We have shown that exposure of human placental explants to 250ppm CO over a 24hr period, leads to significant reduction in sFlt-1 release in comparison to CO-free air (Chapter 3). This supports the *in vivo* results (Chapter 2), whereby the ratio of sFlt-1: vascular endothelial growth factor (VEGF) was decreased in the placentas of pregnant mice exposed to 250ppm CO. This would indicate a shift towards angiogenesis following CO exposure, and could explain the increased placenta arterial branching observed in mice exposed to chronic CO (Chapter 2). Others have shown similar results with manipulation of the HO-1 enzyme. George *et al.*<sup>148</sup> infused pregnant rats with recombinant sFlt-1 from gestation day (GD) 14 to GD19 using implanted mini-osmotic pumps. This procedure increased maternal circulating sFlt-1 levels almost two-fold and decreased unbound VEGF by

17% on GD18<sup>148</sup>. In a subset of animals with sFlt-1 administration, the researchers injected CoPP (an HO-1 inducer) on GD14 of pregnancy which led to an increase in VEGF levels by 50%<sup>148</sup>. A subsequent study using a reduced uterine perfusion pressure (RUPP) model in rats, demonstrated that induction of HO-1 (by CoPP) altered the ratio of sFlt-1/VEGF to one of angiogenesis<sup>116</sup>. It is clear that the HO-1/CO system can alter the anti-angiogenic (sFlt-1)/ angiogenic (VEGF and PGF) balance both *in vitro* (Chapter 3) and *in vivo* (Chapter 4), and suggests a mechanism by which women who smoke in pregnancy display a reduced incidence of PE (Figure 7.1).

#### **7.4 Exposure to CO in pregnancy, reduces the HTN and proteinuria in a mouse model of PE**

sFlt-1 is known to negatively affect the endothelium and contribute to endothelial dysfunction in PE<sup>283</sup>. Not surprisingly, increasing sFlt-1 levels in rodent pregnancy leads to maternal clinical signs of PE; HTN and proteinuria<sup>139;144;145;147;148</sup>. Manipulation of the HO/CO system is hypothesized to decrease the signs of PE, possibly through a combination of the vasodilatory, angiogenic, anti-apoptotic and anti-inflammatory actions of CO<sup>32</sup>. Studies have shown that inhibition of HO-1 by SnMP, leads to increased MAP in pregnant rats<sup>265</sup> and induction of HO-1 by CoPP in a RUPP model in rats, attenuates the placental ischemia- induced HTN<sup>116</sup>. Additionally, the induction of the HO-1 system, through CoPP, can reduce an sFlt-1-induced increase in MAP<sup>148</sup>. In Chapter 4, we created a PE-like model in mice by injection of AdsFlt-1, similar to what was originally created in rats<sup>139</sup>. By then exposing maternal mice to 250ppm CO in ambient air continuously, we prevented the HTN and proteinuria. Further, we observed normalization of renal changes in the mice treated with CO. This finding further supports the possibility of the HO/CO system as a possible therapeutic for women with PE.

## 7.5 Manipulation of the HO/CO system as a therapeutic for PE

Over the last decade, research on the HO/CO system in disease and therapeutics has generated much interest. CO is well known for its toxic nature at high concentrations; CO is lethal when inhaled in air at levels above 30 000 ppm, or leading to COHb levels above 50%<sup>235</sup>. While extremely high CO exposure is toxic, low- concentration CO exposure is now thought to have potential for therapeutic use<sup>285</sup>. The findings presented in Chapters 2, 3 and 4 of this thesis provide promising results in support of the use of CO as a therapeutic in PE.

Low amounts of CO are produced endogenously in nucleated cells of the body<sup>27</sup>, leading to endogenous levels of 0.5-1.5%COHb<sup>166</sup>. Smoking cigarettes leads to the production of CO through combustion of cigarette constituents, leading to concentrations in the range of 400ppm at the alveolar membrane<sup>286</sup> and resulting in blood levels up to 17% COHb<sup>287</sup>. Using these %COHb levels as a guide, research into the use of CO as a therapeutic must ensure blood concentrations of CO are at or below concentrations found in women who smoke, and well below toxic concentrations.

In Chapter 5, we examined the effect of 250ppm CO inhaled over one hour on volunteer %COHb level, breath CO and BP. The half- life of CO was determined to be  $4.68 \pm 1.83$  hrs, with the highest CO level in blood and breath being 12.0%COHb and 53.5ppm, respectively. Importantly, the collection of blood for CO measurement was done without the use of a standard vacutainer. We provide evidence in Chapter 6, which revealed that the use of vacutainers introduce error in blood CO quantification. We also demonstrated that the use of a lancet could provide enough blood for analysis and allow for precise CO measurement, without any differences from syringe collection. This allowed for an acceptable methodology to be used to collect blood from volunteers treated with CO (Chapter 5), in order to measure blood CO concentrations on an hourly basis. The treatment of volunteers with 250ppm CO provides the basis for further studies on the use of exogenous CO as a therapeutic in human disease states.

Pregnancy imparts an added factor into the equation; the fetus. CO passively crosses the placenta and it is well known that fetal hemoglobin has a much higher affinity for CO than that of the mother<sup>274</sup>. Longo *et al.*<sup>288</sup> measured CO concentrations in pregnant ewes and their fetuses following maternal exposure to CO (30-300ppm) and reported that fetal %COHb levels rise much more slowly than those of their mother, but over time, reach a level 15-20% higher, with a much longer half-life in the fetus (7hrs) compared to their mother (2hrs)<sup>288</sup>. This information is important to keep in mind when testing therapeutic potentials of the HO/CO system in pregnancy.

It is possible that manipulation of the HO system and/or administration of exogenous CO may be used in the future as a therapeutic, and a few researchers have published findings in support of using this system as a potential therapeutic for pregnancy complications<sup>5;44;46;149;289</sup>. Clinical studies of CO in a pregnant population would only be possible following extensive studies demonstrating its safety. As a start, we have tested the effect of maternal chronic CO exposure in ambient air in pregnant mice, showing that exposures below 300ppm CO do not result in any demonstrable fetal or maternal adverse effects<sup>162</sup>. As studies have demonstrated that the exposure of pregnant mice to CO in ambient air can reduce fetal growth restriction and miscarriages<sup>57;163</sup> and increase blood flow to the uteroplacental unit (Chapter 2), the potential for use of CO in pregnancy is not one without merit.

### ***Current pilot studies manipulating the HO system for PE prevention or treatment***

#### ***Statins and PE***

Statins are plasma lipid- lowering drugs<sup>290</sup> that offer protection for cardiovascular disease<sup>291</sup>. Statins have been shown to control endothelial dysfunction and inflammation, mediate anti-proliferation, modulate the immune system, and act through antioxidant<sup>292;293</sup> and angiogenic mechanisms<sup>294</sup>. *In vitro* studies with aortic smooth muscle cells<sup>295</sup> and human endothelial cells<sup>296</sup>, as well as *in vivo* research using mice<sup>297;298</sup> have shown that statins are able to induce

transcription and translation of HO-1. In addition, a study in mice by Muchova *et al.*<sup>297</sup> identified that the daily injection of both atorvastatin or rosuvastatin (5mg/kg) over a period of 2-3 weeks, leads to an increase in tissue- specific HO-1 activity, as measured by CO (using gas chromatography) and bilirubin levels (using high performance liquid chromatography). In three different models of PE, the introduction of pravastatin by injection<sup>298;299</sup>, or water infused with the statin<sup>300</sup>, led to decreased HTN and proteinuria. Pravastatin prevented vascular dysfunction and upregulated normal vasodilatory mediators in mice<sup>300</sup>. These findings indicate a possible mechanism by which statins may confer their protective effects, through HO-1 and its production of CO, and offer a possible role for statins in the treatment of PE; further reviewed by Costantine and Clearly<sup>301</sup>.

At this time, two studies involving statins are being conducted in pregnant women. At the Eunice Kennedy Shriver National Institute of Child Health and Human Development, researchers started a double blinded placebo controlled pilot trial to collect pharmacokinetic data and preliminary maternal –fetal safety data for pravastatin in pregnancy<sup>302</sup>. A pilot study for the StAmP trial, (Statins to Ameliorate early-onset Pre-eclampsia) is also being conducted in the United Kingdom, in a multi-center, double-blind, randomized, placebo-controlled trial<sup>303</sup>. To be eligible for the study, women must be between 24+0 and 31+6 weeks gestation, with a singleton pregnancy, diagnosed with early- onset PE, capable of maintaining pregnancy for 48hrs and carrying a viable fetus. When recruited, women are randomly allocated to receive either oral pravastatin (40mg) or placebo, once daily, until delivery. The primary outcome of the study is to evaluate the effect of statins on sFlt-1 at 48hrs post randomization, with several secondary measures also being evaluated, including BP, proteinuria, total hospital stay and neonatal outcomes, to name a few. These two studies will provide insight in the possible use of statins for the treatment of PE, and allow for development of future therapeutics to modulate the HO/CO system further.

The use of pharmacological drugs, such as Statins, to upregulate the HO system may have added unwanted side effects in pregnancy. The use of statins in pregnancy is contraindicated and designated as pregnancy category X by the US Food and Drug Administration. Although studies indicate that use of statin therapy in first trimester is safe and does not lead to increased fetal abnormalities<sup>304-306</sup>, the lowering of cholesterol may be an unwanted effect in pregnancy. The use of very strict controlled doses of gaseous CO directly, which are also increased with the use of statins, could be associated with less unwanted side effects.

## **7.6 Summary**

We have identified that exogenous gaseous CO treatment (250ppm) to pregnant mice does not demonstrate fetal or maternal adverse health effects, but alters placental angiogenesis and increases uterine blood flow, presumably increasing nutrient transfer to the fetus (Chapter 2). A shifted anti- angiogenic / angiogenic balance towards one of angiogenesis (Chapter 2 and Chapter 3) is thought to contribute to the prevention of PE-signs, HTN and proteinuria, following exposure to CO (Chapter 4). Exposure of human volunteers to 250ppm CO allowed for the calculation of half-life for CO in blood and measurement of %COHb and EtCO levels, which can be used for future therapeutic studies with CO. The studies of this thesis have contributed to the knowledge regarding the HO/CO system and pregnancy and provide novel findings in support of the use of CO as a therapeutic and a potential treatment for PE.

## 7.7 Future Directions

The research in this thesis has provided several findings regarding the effects of CO on pregnancy and importantly, the prevention of HTN and proteinuria in a PE-like mouse model. While several conclusions were reached, the results from each Chapter produced more inquiries, leading to a number of possible experiments for the future. This thesis identifies six important areas for future research:

- a) Testing the effect of intermittent CO exposure to maternal mice, more similar to the exposure of cigarette smoke in pregnancy
- b) Characterizing the effect of chronic and acute CO on vascular reactivity during pregnancy
- c)
  - i) Determining if exposure to CO in pregnant mice will lead to hypoxia injury in the mother, fetus or placenta
  - ii) Determining if the exposure of placental explants to CO following pre-exposure of hypoxic conditions will attenuate the induced markers of oxidative stress and sFlt-1
- d) Testing inducers of HO-1 and CORMs in pregnant mice to evaluate their effects in pregnancy in comparison to gaseous CO, and to better characterize the mechanism of HO-1 induction in pregnant mice
- e) Using different animal models of PE to test the effect of CO on preventing, reducing, or reversing the pathophysiological signs of the disorder, and divulging possible mechanisms through which this occurs
- f) Behavioural studies in mouse offspring whose mothers were exposed to CO *in utero*

**a) Objective:** *To test the effect of maternal intermittent CO exposure throughout pregnancy on vascular alterations, as this exposure is more similar to cigarette smoking CO exposure*

**Hypothesis:** *Pulsatile maternal CO exposure in pregnancy will lead to more pronounced effects than chronic CO on maternal and placental vasculature, as an adaptation to the gas will not occur.*

Chronic CO exposure in pregnancy led to several vascular alterations in the uteroplacental unit; increased blood flow, angiogenesis and vessel diameter (Chapter 2). The acute maternal exposure of CO revealed more pronounced alterations in the placental sFlt-1/VEGF protein levels than mice exposed to CO chronically (Chapter 2). It is possible that chronic exposure allows for maternal adaptation to the gas and compensatory mechanisms to normalize CO effects. Further, the use of CO as a therapeutic would not be completed by chronic inhalation of the gas in pregnancy, and may be more similar to a pulsatile inhalation. Therefore, the results from this future study would offer results and information for future therapeutic use.

**b) Objective:** *To characterize the effect of chronic CO on vascular reactivity during pregnancy*

**Hypothesis:** *CO exposure will lead to increased vasodilatory responses in the vascular endothelium of both uterine and mesenteric arteries*

In CD-1 mice, the chronic maternal exposure to CO throughout pregnancy led to an increase in uterine artery blood flow and an increased diameter of several vessels of the uteroplacental unit (Chapter 2). Although blood velocity was not altered, vascular changes were evident, and this should be explored further. Studies using wire myography<sup>307</sup> may be able to test the effect of CO on vascular reactivity. In a normal mouse pregnancy, endothelium-dependent relaxation is enhanced in both uterine and mesenteric arteries, while endothelium-independent relaxation is enhanced in pregnant uterine arteries only<sup>308</sup>. The uterine and mesenteric arteries

could be removed following the exposure of CO chronically in pregnancy. *Ex vivo* wire myography could test the effects of endothelium- dependent (methacholine) and independent (sodium nitroprusside) relaxation responses to determine if CO affects long term endothelium alterations. This experiment may also help to elucidate the reduction in HTN that was observed when mice were subjected to CO in a PE-mouse model (Chapter 4).

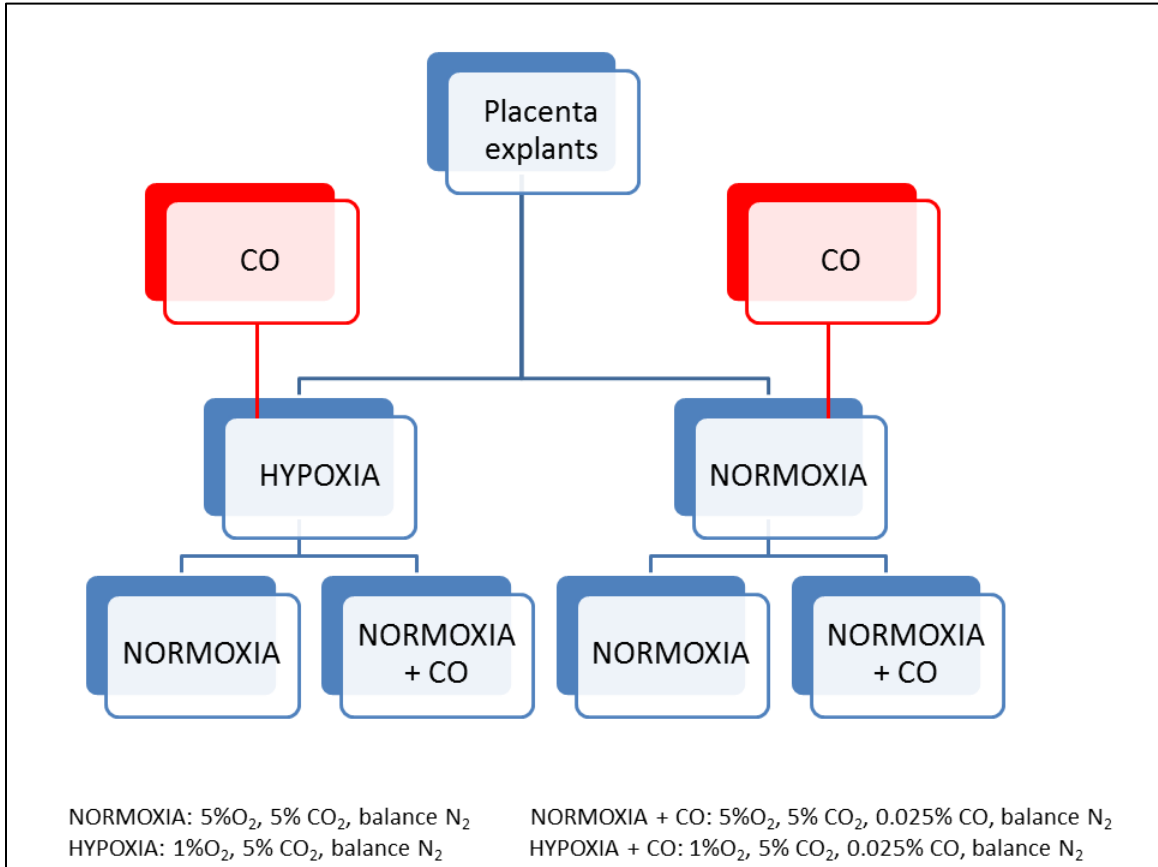
**c) Objective: i)** *To determine if pregnant mouse exposure to 250ppm CO leads to hypoxic injury in the mother, fetus or placenta ii) To determine if CO exposure to placental explants can reduce the increased sFlt-1 production and oxidative stress induced by pre-exposure to hypoxic conditions*

**Hypothesis: i)** *The use of low-level CO will not lead to hypoxic injury in the maternal mouse or fetal/ placental unit ii) and will reduce the markers of oxidative stress and sFlt-1 in a placental explant model, first exposed to hypoxic conditions*

At high levels, exposure to CO leads to a reduction in the O<sub>2</sub> binding capacity of hemoglobin and possible hypoxic insult<sup>309</sup>. Exposure of male mice to 3000ppm CO for 3 hrs was lethal; it caused rapid acidosis, a profound reduction in blood pressure, and asystole<sup>310</sup>. The exposure of male mice to 1000ppm for 3hours was not lethal and did not lead to hypoxic injury in the cardiac muscle, as evidenced by a lack of hypoxyprobe staining (a method of staining hypoxic areas in tissue) in histological analysis in comparison to mice injected with 100% CO<sup>310</sup>. Others have used this probe in pregnancy to test for hypoxia injury as well<sup>311-313</sup>. Hypoxyprobe methodology works by injection of pimonidazole hydrochloride (Hypoxyprobe-1; Natural Pharmacia, Burlington, MA) into mice at a concentration of 60 mg/kg prior to sacrifice. Pimonidazole is a compound that selectively binds thiol groups in proteins of cells exposed to hypoxia (pO<sub>2</sub><10mmHg). A monoclonal anti-pimonidazole antibody (Hypoxyprobe) is used to detect the areas of hypoxia in the tissue of interest<sup>311</sup>. Staining of maternal and uteroplacental

tissue with hypoxyprobe following CO exposure, will determine whether low- level CO exposure leads to hypoxia in either the mother or fetus.

ii) The *in vitro* experiment presented in Chapter 3 provided the first study for placental explant exposure to gaseous CO. This study revealed alterations in sFlt-1 release, but no effect on PGF, or sEng. While surprising, it is possible that CO does not confer effects on these molecules without a previous insult, as observed in rat explants exposed to CoPP, an HO-1 inducer<sup>148</sup>. A follow-up study would expose placental explants to hypoxic conditions, followed by CO exposure, to determine the effect on each of VEGF, sFlt-1, PGF and sEng. Further, exposing explants to CO before hypoxia conditions should also be evaluated for the effect on the proteins of interest (Figure 7.2).



**Figure 7.2: Outline for possible *in vitro* placental explant experiment.**

The primary experiment (blue boxes) would evaluate the effect of hypoxia, followed by CO, on the release of proteins pertaining to PE: VEGF, PGF, sFlt-1 and sEng. The secondary experiment (red boxes), would subject explants to CO prior to hypoxic and normoxic conditions, in order to determine if CO could alter the release of proteins in that manner.

**d) Objective:** *To test inducers of HO-1 or CORMs in pregnant mice to evaluate their effects in pregnancy in comparison to gaseous CO*

**Hypothesis:** *If inducers of HO-1 or CORMs up-regulate maternal CO levels in mice to the same level as mice exposed to gaseous CO, which I hypothesize that they will, then similar alterations in vascularity and proteins of angiogenesis will occur.*

Many researchers have used inducers of HO-1, such as CoPP<sup>99;101;125;148</sup> and CORMs<sup>114</sup> in pregnancy, but to our knowledge, no study has also measured the maternal CO concentration. This study would compare levels of maternal blood CO from mice injected with CoPP or CORMs to blood CO levels of mice exposed to exogenous gas CO<sup>162</sup>. The exposure of mice to CoPP or CORMs would also allow for the study of possible adverse effects which could not be identified with the sole use of CO exposure.

**e) Objective:** *To test the effect of CO exposure in different animal models of PE to support the current findings in the AdsFlt-1 model of PE, and to test the effect of CO on prevention, reduction and reversal of pathophysiologic signs of PE*

**Hypothesis:** *Maternal CO exposure will prevent and reverse the signs of HTN, proteinuria and glomerular alterations in all models of PE*

In Chapter 3, we identified that CO prevented the HTN and proteinuria in a PE-like model of pregnancy. It would be ideal to show the same results in different models of PE. The RUPP model of pregnancy would be one such model, as it is associated with an increase in maternal BP and placental superoxide, in addition to altering the angiogenic protein balance of the placenta<sup>116</sup>. These alterations were all normalized using CoPP, an inducer of HO-1<sup>116</sup>. Maternal exposure to gaseous CO would evaluate if this gas could replace HO-1 induction, by ameliorating the outcomes of RUPP. A second ideal model would be the C1q<sup>-/-</sup> mice, as they

recapitulate the signs of PE: HTN, albuminuria, glomerular endotheliosis, increased sFlt-1 and decreased VEGF<sup>299</sup>. Testing the effect of maternal CO administration in this model would allow for further evaluation of CO as a possible therapeutic in PE. It would also be of value to test the use of HO-1 inducers, such as CoPP or CORMs in the AdsFlt-1 model of PE, in order to determine if these molecules could also reduce the HTN and proteinuria in this model of PE.

One of the key soluble factors being evaluated for its role in PE is sFlt-1. It is increased in maternal serum and placentas in women with PE and decreases following delivery of the fetoplacental unit<sup>139</sup>. We have shown that chronic CO exposure (250ppm) in mice alters the angiogenic balance towards one of angiogenesis in maternal plasma and placenta tissue<sup>159</sup>. *In vitro*, we showed that placental explants exposed to 250ppm CO reduced the release of sFlt-1 into the media, in comparison to control conditions (Chapter 3). We were not able to evaluate the effect of CO on maternal sFlt-1 levels in our PE- like model in mice (Chapter 4), as sFlt-1 was continuously expressed in the liver following Ad injection<sup>221</sup>. It would be ideal to test the hypothesis that CO can reduce sFlt-1 in different models of PE, such as RUPP<sup>116</sup> and Ciq-/-<sup>299</sup>. In addition, future studies should evaluate the effect of CO on PGF, another angiogenic protein affected by increased sFlt-1 levels, and sEng, a different anti-angiogenic molecule, increased in the serum of women with PE. *In vitro*, AdHO-1 is capable of reducing hypoxia –induced sEng release from HUVECs<sup>154</sup>, and CORM-2 is capable of decreasing sFlt-1 release from HUVECs<sup>154</sup>. Therefore, there is evidence for a role of CO in the alteration of these proteins and a possible pathway by which CO may act therapeutically to treat the signs of PE.

**f) Objective:** *To conduct behavioural studies in mouse offspring whose mothers were exposed to CO, in order to determine if fetal CO exposure in utero, leads to fetal neurological defects*

**Hypothesis:** *Low-level CO exposure in utero will not affect fetal neurological development and no differences will be determined between control and CO- exposed fetuses*

We previously conducted a dose-response study, whereby we exposed maternal dams to chronic CO inhalation throughout pregnancy, at different doses of CO. We identified that above 300ppm CO, demonstrable fetal effects were observed, in terms of reduced fetal weight, decreased litter size and increased resorptions<sup>162</sup>. We examined fetuses histologically for alterations of all organs, including the brain, and did not determine any effect below 300ppm maternal CO exposure<sup>162</sup>. In order to determine if CO exposure *in utero* affects fetal neurological development, we would need to conduct behavioural testing. The most common test used for behavioural testing is an open field test<sup>314</sup>, whereby exploratory behavioural and anxiety level are measured. To test for spatial learning and memory, the Biel-maze could be used<sup>315</sup>. This is similar to the well-known Morris water maze<sup>316</sup> but conducted out of water, which is thought to induce anxiety itself. Further, object recognition testing<sup>317</sup> could also test learning by the amount of exploration of new objects in a mouse's environment. The comparison of fetuses born to mothers exposed to CO and those born to mothers who were not, would elucidate differences, if any, due to CO exposure.

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