CARBON MONOXIDE AND PREGNANCY: A SEARCH FOR A POSSIBLE THERAPEUTIC IN THE TREATMENT OF PRE-ECLAMPSIA

by

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Abstract

Pre-eclampsia (PE) is a pregnancy disorder that affects roughly 5-7% of all pregnancies and is a leading cause of both maternal and fetal/neonatal morbidity and mortality. With no present cure for the disease, researchers are interested in the lower incidence of PE observed among the cigarette smoking pregnant population. However, women who use smokeless tobacco do not experience the same decreased incidence of PE, leading to hypothesis of protection against PE from the largest combustible product of cigarette smoke, carbon monoxide (CO). Studies evaluated levels of CO in PE women and found that they were statistically lower than those of healthy pregnancy. Researchers have found CO to possess many cytoprotective and regulatory properties and specifically within the placenta, it has been found to increase perfusion pressure, decrease oxidative stress, decreases ischemia/reperfusion induced apoptosis and maintain endothelial functioning. The idea for use of CO as a possible therapeutic for PE has thus become a real possibility.

This study determined CO levels in pregnant women ± smoking as well as in PE women±smoking, as to discover a possible therapeutic range for future treatments. The best correlated automated CO measurement device with blood CO levels was determined, for use in future clinical studies. This thesis also sought a possible CO delivery concentration, in order to achieve the CO levels observed in the human correlation study. A threshold level of maternal CO exposure in a murine animal model was found, for which fetal and maternal negative toxicities were not observed. The results of this thesis lend a few more pieces to the complicated puzzle involving CO and PE and offer another step toward the possibility of a therapeutic treatment/prevention using this gaseous molecule.
Co-Authorship

The author performed all experiments described in this thesis, with the supervision of Dr. Graeme Smith and the assistance of Richard Casselman and Dr. Henk Vreman. Clinical subjects were recruited and samples collected with the help of Dr. Graeme Smith, Michelle Roddy, Roberta Faroldi, Lizy Kodiattu, Heather Ramshaw and numerous nurses at Kingston General Hospital. Histological slide preparation was performed by John Dacosta.
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To all my friends, thank you for keeping me grounded and for sharing so many memories with me over the past years. Thanks you for reminding me to take breaks and to have fun. Karly, thank you for being the best friend that everyone hopes for. Veronique, thanks for allowing me to vent about my frustrations to you consistently and for always being available for a trip to White Mountain.

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# Table of Contents

Abstract............................................................................................................................................ii
Co-Authorship ....................................................................................................................................iii
Acknowledgements.........................................................................................................................iv
Table of Contents............................................................................................................................vi
List of Figures ....................................................................................................................................vi
List of Tables .....................................................................................................................................viii
List of Abbreviations ......................................................................................................................xi

Chapter 1 Introduction ..................................................................................................................... 1
  1.1 Carbon Monoxide and Pre-eclampsia ................................................................................... 1
    1.1.1 Pre-eclampsia ........................................................................................................... 1
    1.1.2 Carbon Monoxide and Pre-eclampsia ............................................................................. 2
    1.1.3 Hypothesis for Carbon Monoxide’s Role in Attenuating PE .......................................... 3
  1.2 Pregnancy and Carbon Monoxide.......................................................................................... 6
    1.2.1 Carbon Monoxide Levels in Pregnant Women............................................................... 6
    1.2.2 Carbon Monoxide and its Roles in Pregnancy .............................................................. 7
    1.2.3 Carbon Monoxide and the Transplacental Passage ....................................................... 11
    1.2.4 Smoking and Pregnancy: Fetal Effects Observed ......................................................... 13
  1.3 Endogenous Carbon Monoxide Levels ................................................................................ 15
    1.3.1 Tissue Production and Metabolism of Carbon Monoxide ............................................ 15
    1.3.2 Heme Breakdown by Heme Oxygenase ....................................................................... 16
    1.3.3 Similarities with Nitric Oxide ....................................................................................... 18
  1.4 Carbon Monoxide and Human Exogenous Exposure .......................................................... 19
    1.4.1 Characteristics and Environmental Carbon Monoxide Exposure ................................. 19
    1.4.2 Carbon Monoxide Transfer to Hemoglobin ................................................................. 20
    1.4.3 Elimination of Carbon Monoxide in the Body ................................................................. 25
    1.4.4 Carbon Monoxide in the Blood ..................................................................................... 25
    1.4.5 Intracellular Effects of Carbon Monoxide ..................................................................... 28
    1.4.6 Hypotheses and Objectives ........................................................................................... 30

Chapter 2 Carbon Monoxide Measurement using Gas Chromatography ...................................... 33

Chapter 3 A Comparison of Biological Carbon Monoxide Levels in Pregnant Women ± Smoking, including those with Pre-eclampsia .......................................................... 35
List of Figures

Figure 1-1 Schematic diagram of the activation of sGC by either NO or CO, leading to vessel relaxation. ........................................................................................................................................................................... 10
Figure 1-2 The movement of CO within the mammalian body. ................................................................................................................................. 22
Figure 1-3 Heme porphyrin ring binding with O₂ or CO. ....................................................................................................................................................... 23
Figure 1-4 The O₂ dissociation curve and the effect of CO on O₂ binding .................................................................................................................. 27
Figure 2-1 Schematic diagram of gas-solid chromatography device..................................................................................................................................... 34
Figure 3-1 The CO pulse oximeter device ................................................................................................................................................................. 43
Figure 3-2 The expiratory end-tidal breath CO analyzer ............................................................................................................................................... 44
Figure 3-3 Maternal CO levels measured in pregnant women using three different techniques, end-tidal breath pulse oximetry and blood sampling......................................................................................................................... 50
Figure 3-4 Correlations between automated measurement devices of CO and blood %COHb levels .................................................................................................................................................................................. 51
Figure 3-5 Maternal urine cotinine concentrations vs. all three measured CO levels ................................................................. 52
Figure 3-6 Reported cigarettes smoked/day in relation to all CO measurements and urine cotinine levels.................................................................................................................................................................................................. 53
Figure 4-1 The CO chamber delivery system ................................................................................................................................................................. 71
Figure 4-2 Air humidifier and sensor .............................................................................................................................................................................. 72
Figure 4-3 Representation of fetal resorption and abnormality characterization .................................................................................................. 73
Figure 4-4 Placental locations reviewed for apoptosis ............................................................................................................................................. 74
Figure 4-5 Dose response of maternal and fetal CO levels and Hb to increasing maternal exogenous CO exposure ........................................................................................................................................................................... 79
Figure 4-6 Comparison of the mean fetal and placental mass per litter for each CO exposure level vs. control......................................................................................................................................................... 81
Figure 4-7 Comparison of fetal litter size to maternal CO exposure .................................................................................................................................................................................................. 82
Figure 4-8 Maternal tissue CO levels measured in the heart, liver, lungs, kidney and brain for each CO exposure .................................................................................................................................................................................................. 83
Figure 4-9 Maternal placenta and spleen tissue CO levels with increasing CO exposure ........................................................................................................ 84
Figure 4-10 Representative comparison of placenta morphology using H&E staining for each maternal CO dose .............................................................................................................................................................................. 85
Figure 4-11 Representative comparison of brain morphology using H&E staining for each maternal CO dose .............................................................................................................................................................................. 86
Figure 4-12 Representative comparison of heart ventricular morphology using H&E staining for each maternal CO dose. ................................................................................................................. 87
Figure 4-13 Apoptotic index for fetal brain, heart, placental junction and labyrinth for each CO experiment. .................................................................................................................................... 88
Figure 5-1 Representative figure for ultrasound analysis of blood flow through the umbilical artery. ........................................................................................................................................ 105
Figure 5-2 Evaluation of flow velocity in maternal and fetal blood vessels following maternal CO exposure. .................................................................................................................................. 106
List of Tables

Table 1-1 The range of differing human Hb levels ................................................................. 24
Table 1-2 Affinity levels of CO to specific hemoproteins relative to O\textsubscript{2} binding, ............ 29
Table 3-1 Average CO and cotinine levels in recruitment categories: NTN ± smoking and PE ± smoking......................................................................................................................... 48
Table 3-2 Assessment of smoking levels in NTS volunteers...................................................... 49
Table 4-1 Average gas chromatography measured CO levels and GO-Link humidity measured per CO experiment.................................................................................................................. 78
Table 4-2 The effect of maternal CO exposure on total number of resorptions and abnormalities. .................................................................................................................................................. 80
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<td>analysis of variance</td>
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<tr>
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<tr>
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<td>LM</td>
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<td>normotensive non-smoking</td>
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<td>oxyhemoglobin</td>
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<td>probability</td>
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<td>phosphate- buffered saline</td>
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<td>partial pressure of carbon monoxide</td>
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<td>pre-eclamptic non-smoking</td>
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<td>reactive oxygen species</td>
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<td>soluble guanylyl cyclase</td>
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<td>sulfosalicylic acid</td>
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<tr>
<td>STBM</td>
<td>syncytiotrophoblastic microparticles</td>
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<tr>
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<td>mediated dUTP nick end labelling</td>
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<td>TdT</td>
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Chapter 1
Introduction

1.1 Carbon Monoxide and Pre-eclampsia

1.1.1 Pre-eclampsia

One of the leading causes of maternal and fetal/neonatal morbidity and mortality is pre-eclampsia (PE), affecting 5-7% of all pregnancies worldwide. Displaying clinical symptoms of hypertension (>140/90mmHg) and proteinuria (>300mg/24hr), this syndrome usually presents in the third trimester. The current cure for the disease is delivery of the placenta and thereby the fetus as well; thus a diagnosis earlier in gestation could lead to premature delivery of the fetus and further complications. The exact cause of the disease is still unknown but it is thought that many etiologies lead to its development. Women with underlying hypertension or other chronic illness, such as renal disease, diabetes and autoimmune disease are at an increased risk of developing PE. Other factors contributing to this increased risk include young maternal age, advanced maternal age, high body mass index, multiple gestations, a history of PE, multifetal gestation and women who are carriers of certain inherited metabolic disorders.

Pre-eclampsia is a two-stage condition, as it originates in the placenta with poor perfusion and progresses in some, but not all women, into a maternal syndrome of PE. This syndrome is seen more commonly in women with large placentas, including multifetal gestations, as the excess placental tissue cannot be adequately perfused. In a normal pregnancy, a mechanism known as a “physiological change” occurs. The maternal uterine spiral arteries supplying the intervillous space undergo progressive remodelling and modification; extravillous cytotrophoblast invade and replace the endothelium and blood vessels. This remodelling allows for a transition from high resistance, low flow blood vessels to low resistance, high flow blood vessels, unresponsive to
vasoactive stimuli. In PE, a failure of spiral artery transformation has been well documented, along with a shallow trophoblast invasion into the decidual tissue of the uterus. This decreased perfusion lends to areas of hypoxia in the placenta, increasing free radical oxygen species (ROS) production and local and systemic oxidative stress. Further, the syncytiotrophoblast lining the intervillous space has been shown to be at an increased risk of apoptosis and necrosis due to oxidative stress, leading to shedding of the cellular debris into the circulation, known as syncytiotrophoblast microfragments (STBMs). Women with PE tend to have an increase of STBMs in their circulation. The increase in STBMs induces an inflammatory response and vascular endothelial dysfunction, increasing sensitivity to pressor agents, exhibition and activation of a coagulation cascade and a loss of vascular integrity that are present in PE patients. Maternal endothelial dysfunction is directly responsible for the characteristic signs and symptoms used to diagnose this disorder: elevated blood pressure and proteinuria.

Presently, the only cure is to remove the problematic organ; the placenta. PE can cause life threatening maternal conditions, including abruptio placentae with disseminated intravascular coagulopathy, cerebral encephalopathy or hemorrhage, pulmonary edema, hepatic failure and acute renal failure. Fetal effects can include preterm birth, reduced fetal growth, hypoxia acidosis and perinatal death.

1.1.2 Carbon Monoxide and Pre-eclampsia

With no present treatment or prevention for PE, the puzzling relationship between smoking and the incidence of this disease is of interest to many researchers. It was found that women who smoke cigarettes during pregnancy are at a 32% reduced risk of developing PE, compared to non-smoking pregnant women. This inverse relationship was found to be dose related and was
further confirmed by a different group, who measured cotinine levels as an assessment of smoking exposure. This same decrease is not observed in women who use snuff during pregnancy, lending to the hypothesis that a combustible product in cigarette smoke, possibly carbon monoxide (CO), is responsible for the decreased incidence of PE. In a study conducted by Baum et al., end-tidal breath CO levels were measured in pregnant women, with or without PE, and interestingly, women with PE were found to have statistically lower end-tidal CO levels. This study did not include women who smoke during pregnancy, however a study conducted by Zhang and colleagues demonstrated that cigarette smoking was associated with a reduced risk of hypertension in pregnancy. Pre-term birth as well as term uterine contractions, have also been associated with very low end-tidal CO levels, possibly drawing a link between intrinsic decreased CO levels and increased uterine activity. Lower breath end-tidal CO in women with PE, along with a decreased incidence of the disease in women who smoke cigarettes (and therefore have increased levels of CO), lend support to the notion that moderately elevated levels of CO may offer an advantage in terms of the risk of PE.

### 1.1.3 Hypothesis for Carbon Monoxide's Role in Attenuating PE

It is difficult to determine exactly how CO may aid in the attenuation of PE, but a number of ideas have been proposed based on CO’s numerous properties and effects. Four main hypotheses will be discussed in relation to the similarity of CO with nitric oxide (NO) and thus its effect on vascular tissues in the placenta, CO and trophoblast invasion, CO and apoptosis and CO and inflammation of the placenta.

It is well known that NO plays a pivotal role in the maintenance of endothelial function, as a potent vasodilator, as well as an inhibitor of both platelet and neutrophil activation. While
controversy exists over the up/down regulation of NO in PE patients\textsuperscript{28-31}, literature is suggestive that any NO present, reacts with free radicals in the circulation and forms the ROS peroxynitrite\textsuperscript{31,32}, further leading to the diseased state. With similar properties as NO, CO has been shown capable of vasodilating blood vessels through the activation of soluble guanylyl cyclase (sGC)\textsuperscript{33}, as well as decreasing an inflammatory response\textsuperscript{34}. As a stable gas, CO does not react with circulating ROS as in the case of NO\textsuperscript{35}. The potential exists then, for CO to replace the diminished NO observed in PE patients and thus restore several regulatory functions of the endothelium. Perhaps this restoration by CO is the reason behind the lower incidence of PE found in cigarette smokers.

A few research groups including our own, have examined the effect of exogenous CO on blood vessels in the placenta\textsuperscript{36-38}. Carbon monoxide was shown to be capable of vasodilatation, in both the anchoring villi\textsuperscript{39} and placental resistance vessels\textsuperscript{37}. In this way, CO may not only dilate spiral arterioles, but may also increase intra-placental and feto-placental blood flow\textsuperscript{23}. Although the spiral arteries of PE women are unmodified blood vessels, perhaps the increased CO levels in those women who smoke are capable of inducing a somewhat constant blood flow across the placenta, thus reducing the hypoxia/reoxygenation (H/R) occurrence that is seen in PE\textsuperscript{23}.

The modification of the spiral arteries in a normal pregnancy occurs around the 13\textsuperscript{th} week of gestation, with implantation beginning around 2-3 weeks. In this time, the uterine cavity is relatively hypoxic, with a partial pressure of oxygen (pO\textsubscript{2}) of 10-20mmHg\textsuperscript{40,41}, suggesting this environment be the optimal condition for invasion and remodelling of the spiral arteries\textsuperscript{23}. Perhaps it is the up-regulation of hypoxia-induced gene products that aid in this process\textsuperscript{23}. Women who smoke cigarettes during pregnancy are relatively hypoxic compared to non-smokers,
due to both a decrease in binding sites available for oxygen ($O_2$) on the hemoglobin (Hb) molecule, as well as the increased affinity for $O_2$ that Hb incurs following the binding of CO. In this way, $O_2$ delivery to all tissues is decreased $^{42}$, including endometrial tissue surrounding the implantation site. It is possible that women who smoke during pregnancy are at a reduced risk of developing PE due to a presence of a more hypoxic uterine environment leading to improved placentation.

A PE placenta has been reviewed as an inflammatory environment, akin to one observed in an allograft rejection $^1$. Activated macrophages and uterine natural killer cells $^{43}$ infiltrate both the spiral arteries and the decidua, releasing a number of compounds that have been shown capable of decreasing trophoblast invasiveness and initiating apoptosis $^{44,45}$. Recently, exogenous CO administration has been evaluated as a possible protective measure used in reducing transplantation and allograft rejection in patients. By either incubating organs in environments of high CO, or by inducing an over expression of heme oxygenase (HO), which further up regulates CO, the organ transplantation success rate increased $^{46-48}$. Carbon monoxide was shown capable of reducing the inflammatory response as well as tissue apoptosis $^{49}$, in conjunction with increasing perfusion of the organs $^{46,48}$. It is possible that increased CO levels in pregnant smoking women may be able to diminish the inflammatory response observed and protect cytотrophoblast cells from the pro-apoptotic signals initiated by the activated macrophages $^{23}$.

Lastly, CO’s anti-apoptotic properties have been studied extensively, and in relation to PE, could decrease the H/R insult that affects localized areas of the placenta. In PE, apoptotic shedding of the syncytiotrophoblast cells and ROS enter the maternal circulation following repeated H/R
insult \(^{14,17}\) and this apoptotic cascade in the syncytiotrophoblast cells has been studied using villous explant models \(^{18}\). Also, cellular markers of apoptosis have been measured in maternal serum and placental tissue in women with PE \(^{50}\). As CO has already been shown to inhibit the apoptotic cascade in a number of cell culture and tissue preparation experiments \(^{51-54}\), it may then be functioning in the same manner in the placenta for women with elevated CO levels, such as pregnant smokers. Using term placental villous explants, our laboratory subjected this tissue to CO-infused media at levels 5-6X higher than that found in the blood of women who smoked greater than a pack a day during pregnancy. Following \textit{in vitro} H/R treatment, those tissues that were also subjected to CO displayed a 60% lower incidence of apoptosis than those that were not. This experiment indicated that CO’s potent anti-apoptotic properties in the human placenta could hold therapeutic potential in the management of PE \(^{55}\).

1.2 Pregnancy and Carbon Monoxide

1.2.1 Carbon Monoxide Levels in Pregnant Women

A number of hematological changes occur over pregnancy to account for the newly growing fetus. As such, the levels of endogenous CO production and thus carboxyhemoglobin (COHb), the bound form of CO with Hb in the circulation, levels in pregnant women differ from those who are not pregnant. According to Longo LD, CO levels in pregnancy are almost double those of non pregnant women, however, within four days post partum, these levels reduce to normal \(^{56}\), most likely due to the loss of pregnancy-induced blood volume increase post partum \(^{57}\).

Pregnancy induces an increase in erythrocyte production \(^{57}\), allowing for a large turnover in Hb by HO and in turn, roughly a 30-40% increase in CO levels \(^{58}\). The growing fetus, with its own
endogenous CO production, contributes about 15% of the maternal CO level \(^{58}\). The majority of endogenous pregnancy CO production is attributed to the increase in progesterone levels and women in the luteal phase of their menstrual cycle were shown to have higher levels of CO \(^{58,58,59}\), probably from progesterone’s role in stimulating the induction of hepatic microsomal enzymes \(^{60}\). As pregnancy leads to a large increase in progesterone level by the placenta, pregnant women also increase their levels of CO presumably by the same mechanism. A study conducted by Delivori-Papdopoulus \(^{58}\) showed that upon administration of progesterone, increased CO production rates are observed.

1.2.2 Carbon Monoxide and its Roles in Pregnancy

The effect of CO on the hemodynamic control of the placenta has been the focus of a few research studies \(^{36-39}\). Since the placenta lacks innervation \(^{61}\), it depends on local or circulating vasoactive substances for vascular effects. In order to increase O\(_2\) and nutrient delivery to the fetus, term arterial vessels of the fetoplacental circulation are maintained at near maximal dilation \(^{62}\). Carbon monoxide, with similar vasodilating properties to NO \(^{63}\), was shown to operate as a vasodilator in the placental vasculature \(^{64}\). Like NO, CO can activate sGC \(^{65}\) through the activation of cyclic guanosine monophosphate (cGMP) (Figure 1-1). Two forms of sGC have been located in the placenta, endothelial derived (sGC\(_{\alpha_1\beta_1}\)), as well as another detected in placenta homogenates (sGC\(_{\alpha_2\beta_1}\)) \(^{66}\) and unique to only a few other organs \(^{67}\). Specifically, the \(\alpha_2\) subunit of sGC was located within the syncytiotrophoblast villous vessels \(^{68}\), where HO has also been reported \(^{64}\). Interestingly, debate persists over the existence of nitric oxide synthase (NOS), an enzyme responsible for the production of NO, in different regions of the placenta and myometrium \(^{69-71}\). In the uterus, an increase in myometrial cGMP levels during pregnancy has
been associated with the uterine quiescence \(^{72}\) and this idea has been supported by animal studies \(^{73,74}\). Through activation of sGC, NO has been studied in the NO-cGMP pathway in myometrial quiescence during pregnancy and in the onset of labour \(^{39,74}\).

In the placenta, expression of HO has been studied extensively. An abundance of both HO-1 (constitutive HO) and HO-2 (inducible HO) messenger ribonucleic acid (mRNA) were measured in human placental homogenates \(^{75}\) and both were shown to increase with advancing gestation \(^{76,77}\). Protein levels in different areas of the placenta have also been studied to a large degree, showing a wide distribution of HO concentration values, as well as alterations due to gestational age \(^{64,75,77,78}\). Using a dually perfused cotyledon placenta, inhibition of HO revealed an increase in perfusion pressure \(^{64}\), while exogenous CO administration demonstrated an ability to relax pre-constricted anchoring villi \(^{39}\). In accordance with these results, Acevedo and Ahmed were able to inhibit both spontaneous and oxytocin-induced myometrial contraction with the use of hemin, an HO inducer \(^{79}\). Due to its actions on the sGC pathway, as well as the knowledge of increased HO mRNA and protein expression in the placenta, CO has shown the ability to both increase placental perfusion and intervillous volume and blood flow.

It has been proposed that HO-2 and endothelial NOS (eNOS), both constitutively expressed, may have similar physiological roles within the body \(^{80}\). Within the syncytiotrophoblast, eNOS is believed to have three physiological targets: the intervillous space, autocrine effects on trophoblast function and paracrine interactions with villous core components \(^{81}\). It also inhibits platelet aggregation and leukocyte adhesion \(^{81}\), as does CO \(^{82,83}\). As both HO-2 and eNOS are found in syncytiotrophoblast, they may have complimentary roles. Carbon monoxide would then
aid with maternal blood flow into the intervillous space and a diminished immune response to the feto-placental allograft \(^8^1\). In accordance with this hypothesis, HO-2 was found to be significantly higher in early pregnancy compared to late gestation \(^6^4\)- indicating a possible role for CO in establishing blood flow from the spiral arteries to the intervillous space \(^8^1\). Cultured trophoblast cells from first trimester placenta have been shown to be capable of producing CO \(^7^6\), also lending to the possibility of CO’s aid in trophoblast penetration of spiral arteries.
Figure 1-1 Schematic diagram of the activation of sGC by either NO or CO, leading to vessel relaxation.
1.2.3 Carbon Monoxide and the Transplacental Passage

It is well known that CO in maternal blood can cross the placenta by passive diffusion\textsuperscript{56,84}. However, some researchers also believe that facilitated diffusion may aid in this process\textsuperscript{85,86}. While fetal Hb has a higher affinity for CO than maternal Hb, in a pregnant non smoker, the difference in %COHb levels between the two is not significant\textsuperscript{87}. Normal fetal %COHb levels are 0.7-2.5%, lending to a ratio of fetal to maternal COHb of 0.6 to 1.6\textsuperscript{56}. In pregnant women who smoke cigarettes, fetal and maternal CO levels are significantly different, with a much higher level in the fetus\textsuperscript{87}.

Both Longo et al\textsuperscript{88} and Hill et al.\textsuperscript{89} have reported on the kinetics of exposure to CO in both maternal and fetal systems in the ewe. By subjecting pregnant ewes to acute or chronic levels of CO (30 to 300ppm), they were able to measure the adaptive responses in both maternal and fetal CO levels over time. In acute exposure, maternal %COHb increased rapidly to a peak and subsequently dropped following exposure. Fetal %COHb displayed a much slower rate of increase, matched maternal levels and kept rising until roughly double those of maternal %COHb. With an exposure of 30 to 100ppm, maternal %COHb increased rapidly in the first 2-3 hours and marked a plateau at 7-8 hours. Fetal CO levels showed little increase in the first hour following exposure, but then began to increase over the next 4-5 hours; very slowly in comparison to the maternal rise. Equilibrium was reached at 36-48 hours; with fetal %COHb levels 15-20% higher than those of their respective mothers. Based on these calculations, the half-life elimination of CO was calculated as 2 hours for the mothers and 7 hours for the fetus.

There exists a range in severity of outcomes in pregnant women who are subjected to acute or chronic levels of toxic CO levels. The age and health condition of the woman plays a major role
in her ability to deal with the CO effects. In acute CO poisoning, the fetal outcome is proportional to the severity of CO exposure and the COHb levels in the mother\textsuperscript{90}. In chronic exposures, the CO levels may be lower than that of acute poisoning, but still induce fetal toxicity when the mother does not seem to be affected\textsuperscript{91}. It is important to note that the neonatal rate of CO uptake is more rapid than that of an adult, due to an infant’s greater rate of minute ventilation calculated on a per weight basis\textsuperscript{56}.

Premature delivery has been correlated with increased ambient CO levels\textsuperscript{92,93}, however, other particles and gases in the atmosphere may also contribute to this correlation and thus this “cause and effect” finding must be reviewed further. In the same way, women who smoke cigarettes during pregnancy are at an increased risk for premature delivery\textsuperscript{94} and although CO levels are high in this group of women, numerous other chemicals are present in cigarettes that may contribute to this finding, rather than the CO level. Carbon monoxide toxicity in the fetus is mainly attributed to tissue hypoxia and the direct effects of CO on hemoproteins in the fetal body. The greater affinity of fetal Hb for CO relative to maternal Hb, also attributes to the higher levels of CO toxicity in the fetus\textsuperscript{84}.

Animal studies have shown that CO exposure in the first two trimesters of pregnancy has been associated with significant intrauterine growth restriction\textsuperscript{95,96} most likely due to prenatal hypoxia effects. In pregnant women exposed to CO, the gestational age, the CO level and the duration of exposure all contribute to the effects the toxic gas may have on the fetus. Women living at high altitudes, and thus in a hypoxic condition for the duration of pregnancy\textsuperscript{97} are at an increased risk for CO poisoning as it potentiates the already hypoxic condition on the fetus.
Carbon monoxide poisoning in pregnancy occurs at levels of maternal %COHb greater than 20% \(^{56}\) with a corresponding critical CO level for the fetus of 60 %COHb \(^{98}\). Curtis et al. \(^{99}\) documented CO poisoning cases in pregnant women as a) mother and fetus died, b) mother survival and fetal death, or c) mother and fetus survival. In the cases of maternal death, %COHb levels varied from 48 to 95%, while fetal %COHb ranged from 0 to 25%. The low levels of fetal CO were undoubtedly due to acute CO exposure. Among mothers who bore stillborn infants, fetal %COHb levels ranged from 20 to 49%. In the cases of both mother and fetal survival, CO levels were not measured. In all three categories, mothers displayed classical symptoms of CO poisoning: dizziness, headache, nausea, visual disturbances and confusion.

At the first sign of CO poisoning (>20%COHb), hyperbaric O\(_2\) treatment is imperative \(^{100}\). In pregnancy, this treatment is especially important if the CO poisoning lends to a loss of consciousness in the mother \(^{101}\). Although the rate of CO elimination from adults has been documented \(^{102}\), that of the fetus has not been measured \(^{56}\). With the administration of 100% O\(_2\) in a pressurized system, the half-life of CO from maternal Hb would decrease from a normal 2 to 3 hours to 0.75 hours \(^{56}\). As noted above in the pregnant ewe study, the fetal half life of CO is much longer than that of their mothers and thus duration of treatment would need to be several times that necessary to reduce the maternal COHb to an acceptable CO level \(^{103}\). As a rule of thumb, in order to decrease fetal %COHb levels to below 3 to 4 %, pregnant women should be exposed to 100% O\(_2\) five times longer than that necessary to reduce her own COHb level to a specific value \(^{56}\).

**1.2.4 Smoking and Pregnancy: Fetal Effects Observed**

Maternal cigarette smoking continues today, despite the known adverse pregnancy and fetal outcomes. Due to cigarette smoking in pregnancy, the fetus has an estimated 5% reduction in
growth rate\textsuperscript{104}, as well as a birth weight roughly 200g lighter (per pack per day) than a fetus born to a non-smoking mother\textsuperscript{105}. In addition, maternal cigarette smoking has been associated with an increase in fetal morbidity\textsuperscript{24,106} sudden infant death syndrome\textsuperscript{107}, spontaneous abortions\textsuperscript{34,76,108}, childhood cancers\textsuperscript{109}, ectopic pregnancies and an increase in the incidence of placental abruption and previa\textsuperscript{110,111}. The effects of smoking are dependent on the dose of the drug taken and the gestational age of pregnancy, as they may be reversed and prevented if smoking is stopped\textsuperscript{48}. It is essential to note that the cigarette smoke not only impacts fetal growth specifically, but early placentation as well.

The constituents of a cigarette are numerous with over 4000 chemicals reported\textsuperscript{112}. The majority of compounds present in the smoke of a cigarette are produced during the combustion process\textsuperscript{113}. One cigarette’s smoke contains an average of 40 000ppm CO by volume\textsuperscript{112} lending to an alveolar CO concentration of roughly 400 to 500ppm\textsuperscript{56}. Someone residing in a smoke filled room, a second-hand smoker, may be exposed to levels of CO from 25 to 100ppm\textsuperscript{56}. The CO production varies with the cigarette’s size, temperature of combustion, number of puffs and the length the cigarette is smoked\textsuperscript{56}. The constituents of tobacco smoke have been researched in great detail, with nicotine, tar and CO identified as the major end-products; 0.8, 9 and 1 mg/ cigarette respectively\textsuperscript{114}. Although any one of the compounds found in a cigarette may lend to the deleterious effects observed in pregnancy, an abundance of research has focused on the effects of nicotine, polycyclic aromatic hydrocarbons and thiocyanates; all of which have been characterized with an ability to cross the placenta\textsuperscript{115-117}. 
To avoid the combustible products formed when cigarettes are lit, smokeless tobacco has been created and is popular among certain populations [113]. These products (i.e. snuff) contain equivalent levels of nicotine to those of cigarettes [118], but have no combustible end products, such as tar and CO. Interestingly, women who use these products during pregnancy, as opposed to smoking cigarettes, are not devoid of the adverse outcomes discussed above. A study conducted by Krishna, K, showed that women who chewed tobacco during pregnancy still delivered babies which were 100-200g lighter than those born to women who did not smoke or chew tobacco at all [84]. It is still unclear as to which product(s) in cigarettes leads to the negative effects during pregnancy, and this conundrum continues to be an area of interest in the research community.

1.3 Endogenous Carbon Monoxide Levels

1.3.1 Tissue Production and Metabolism of Carbon Monoxide

The predominant endogenous source of CO, (approximately 79%) occurs through the natural degradation process of Hb, whereby heme is enzymatically catabolized by HO [44,45] and the transformation of protoporphyrin into bilirubin liberates an atom of CO [100]. Further heme-derived CO is produced by the turnover of heme from other hemoproteins, such as sGC, NOS [119], myoglobin (Mb), cytochromes, peroxidases and catalase, contributing approximately 20-25% to the CO generation [120]. Lastly, metabolic processes, such as photooxidation [121], lipid peroxidation [121], xenobiotic activity and bacterial activity [122] contribute up to 14% of all CO produced in the body [123-126]. Blood disorders which cause hemolysis (such as thalassemia), can increase CO levels drastically in the body [119].
The production of CO in a normal human male is upwards of 42ml of CO per hour\textsuperscript{100}. In women, the level is identical to that of men during the follicular phase of her menstrual cycle, but almost doubles in the luteal phase\textsuperscript{58,59}. It is thought that the increased production of progesterone at this time lends to higher CO levels, as progesterone is an inducer of hepatic microsomal enzymes\textsuperscript{60}.

1.3.2 Heme Breakdown by Heme Oxygenase
The greatest source of CO production in the body is through the enzymatic breakdown of heme, mainly occurring in the reticulo-endothelial system of the liver and spleen\textsuperscript{127}. This process is completed by the enzyme HO, producing three catalytic end products: CO, biliverdin and free iron (Fe\textsuperscript{2+}). The Hb from senescing red blood cells (RBCs) is the predominant source of heme\textsuperscript{119}. It is the availability of this substrate that determines the rate of CO production, rather than the HO level in a cell\textsuperscript{128,129}.

Three isoforms of HO exist, an inducible form, HO-1, a constitutive form, HO-2 and the least active isoform, HO-3\textsuperscript{130}. HO-1, often referred to as a ‘stress protein’ is expressed in all nucleated mammalian cells\textsuperscript{131} and is induced by a number of stimuli, including but not limited to: cytokines, endotoxins, hyperthermia, hypoxia, hyperoxia, shock, ischemia/reperfusion, and oxidants\textsuperscript{130,132,133}. HO-2 has been shown to contribute to the regulation of basal heme metabolism and is found in a number of tissues in the body\textsuperscript{130,132}. HO-3 is the least characterized of the three isoforms, with very little activity expressed to date\textsuperscript{108,130}. 
The breakdown of heme proteins in such cases as hemorrhage, hemolysis or cell damage causes release of the heme moiety and can be detrimental. Free heme can both cause peroxidation of lipid membranes in cells or become a toxic source of Fe$^{2+}$, which can generate hydroxyl radicals or become highly reactive iron-oxygen compounds. HO is classified as an antioxidant enzyme, as it removes free heme from the system, which itself can undergo auto-oxidation to form superoxide and hydrogen peroxide. These two products can further aid in the production of ROS. Although HO-1 requires O$_2$ to catalyze the breakdown of heme, in moderate to severe hypoxic conditions, it remains active, due to its relatively low Michaelis-menten ($k_m$) value. The HO system has also been implicated as a part of the cell defense mechanisms against stressors such as: heat shock, heavy metals, ROS, lipopolysaccharide and other inflammatory processes.

The three end products of heme degradation by HO are CO, biliverdin and Fe$^{2+}$, and at high concentrations, all three possess cytotoxic capabilities. However, at the low endogenous quantities produced by HO, they all have been shown to possess cytoprotective properties. Carbon monoxide has been shown to induce vasodilation, inhibition of platelet aggregation and inhibition of apoptotic and inflammatory cascades. Biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase, demonstrates intense anti-oxidant properties. In fact, studies have correlated a higher level of bilirubin and biliverdin levels with a lower incidence of cancer and cardiovascular disease. Free iron, released from the heme molecule itself is capable of inducing a generation of free radicals. This production is reduced by the actions of HO, which interacts with intracellular iron pumps and upregulates the production of ferritin, an iron-chelating molecule. The presence of CO (endogenous or exogenous) bound to ferrous heme also limits Fe$^{2+}$ from participating in redox cycling.
Through both CO’s and HO’s actions, the release of Fe^{2+} is reduced and therefore limits the ROS levels.

A plethora of information surrounding HO and its catalytic end products exists in the literature to date. Ranging from its anti-oxidant, anti-inflammatory and anti-apoptotic actions, to its hemodynamic control, HO has become an enzyme of great interest in a number of different systems. In addition, it has also become a popular enzyme of study in pathological conditions.

1.3.3 Similarities with Nitric Oxide

Carbon monoxide, like NO, functions as a physiological messenger molecule\textsuperscript{153,154}. Both molecules activate sGC to produce increased levels of cGMP, although NO is a more potent activator\textsuperscript{155}. It is unknown if both molecules bind the same site on the heme moiety of sGC\textsuperscript{54}, but the resulting action is similar, lending to vasodilation, platelet aggregation\textsuperscript{63,156}, neurological processes\textsuperscript{157} and an improved survival rate of transplanted organs\textsuperscript{158}.

Studies have shown that CO can influence signals and activities of NO\textsuperscript{159,160}, specifically, it can induce NO production at low levels and inactivate NOS by binding to it at higher levels\textsuperscript{59,127,161-163}. It has also been shown that at low levels of CO, the strong oxidant peroxynitrate is produced in platelets and vascular cells\textsuperscript{127,159}. At the same time, the reverse is also true, as NO can induce CO production through HO-1 mRNA and protein upregulation\textsuperscript{52,164}. 
Both NO and CO form complexes with Hb, however, NO can bind both ferrous and ferric forms of heme. Interestingly, the dissociation from Hb is much slower for CO than both NO and O\textsubscript{2}, therefore, over time, CO can replace NO from ferrous heme. Most studies evaluating these kinetics are performed at very high CO concentrations and so it is difficult to ensure that the same is true at low endogenous CO levels. Under hypoxic conditions, the generation of NO and CO differ slightly. Both NOS and HO-1 require O\textsubscript{2} to function, however the higher K\textsubscript{m} for NOS renders it more susceptible to the effects of hypoxia and thus increases the production of CO over NO in such cases.

1.4 Carbon Monoxide and Human Exogenous Exposure

1.4.1 Characteristics and Environmental Carbon Monoxide Exposure
Carbon monoxide is an odourless, colourless gas which has been deemed the silent killer due to its cytotoxic capabilities following inhalation at high concentrations. This gas was first discovered in 1857 by a French physiologist, Claude Bernard, when he determined its asphyxiating capabilities, but for decades thereafter it eluded scientists, as it was thought that no biological relevance could be found from exploring the physiological roles of such a toxic gas. Today there exist a number of new emerging studies which have shown beneficial properties of CO relating to human health.

CO is a diatomic molecule of low molecular weight (m.w. 28.01) that occurs naturally in a gaseous form under atmospheric temperature and pressure. It is formed both naturally and by human activities and it is relatively insoluble in aqueous media (2.3ml/100ml at 23°C) as well as
in organic solvents\textsuperscript{167,168}. Although CO is combustible, it is chemically stable under physiologic conditions\textsuperscript{127}. Normal ambient air CO levels are quite low, ranging from 0.04ppm in suburban areas to 3 or 4ppm in greatly populated urban areas\textsuperscript{169}. This background concentration of CO is primarily a result of oxidation of hydrocarbons, such as methane or the partial combustion of organic molecules and is produced in large quantities at sites of volcanoes, forest fires, plant metabolism and oceanic activity\textsuperscript{35}. The higher concentrations of atmospheric CO in urban areas are mainly due to the combustion of fuel in an incomplete manner, namely: vehicle emissions and heat or power production\textsuperscript{169}. Occupational Health and Safety has deemed workplace CO maximums to reach 50ppm for a maximum 8hr work shift\textsuperscript{170}.

1.4.2 Carbon Monoxide Transfer to Hemoglobin

Human CO levels are a combination of endogenous production as well as exogenous exposure (Figure 1-2). Once inhaled, CO diffuses across the alveolar-capillary membrane quite rapidly depending on alveolar gas volume, ventilation, and the concentration of hemoglobin (Hb) in the pulmonary capillaries\textsuperscript{127}. It primarily competes with O\textsubscript{2} to bind with the heme porphyrin ring in Hb, forming COHb (Figure 1-3), while less than two percent of the CO in the body remains unbound\textsuperscript{171}. To reach Hb in the RBC, CO must diffuse across the alveolar-capillary membrane, through the plasma, across the RBC membrane into the stroma, where the reaction with Hb can take place (Figure 1-2). This process is rapid due to a differential of CO partial pressures across the membrane. As CO binds with Hb, it maintains a low partial pressure in the blood and subsequently furthers the diffusion of CO into the blood. Environmental CO pressure is usually higher than that within the body, therefore CO uptake is proportionally faster than its elimination\textsuperscript{169}. Exercise increases gas exchange efficiency and decreases pO\textsubscript{2}, thus promoting CO uptake\textsuperscript{172}. 

20
Hemoglobin is a metalloprotein found in RBCs. In humans, it is made of 4 subunits, each with a protein chain and a non-protein heme group. The protein chains are alpha helical and fold into a globin shape, forming a middle pocket where the heme group can bind. Heme itself is a heterocyclic ring, a porphyrin ring with an Fe\(^{2+}\) atom held in the center. If Fe\(^{2+}\) is in its ferrous state, both \(O_2\) and its competitor CO can bind. However, in its ferric state, the whole structure is referred to as methemoglobin and is rendered incapable of binding either substrate\(^{170}\).

There are a number of differently structured Hb molecules, based on the arrangement of the four protein chains types: alpha, beta, gamma and delta. The most common form of Hb in adults is HbA, with 2 alpha and 2 beta subunits non-covalently bound. Alternate coordinations of subunits lead to differing affinities for \(O_2\) and Hb structure complications. Fetal Hb, HbF, consists of 2 alpha and 2 gamma subunits and binds \(O_2\) with greater affinity than adult Hb\(^{87}\). Due to the lower \(p\,O_2\) in the fetal circulation, this affinity allows for the fetus to obtain \(O_2\) from the maternal circulation. The general Hb levels in humans vary, see Table 1-1.
Figure 1-2 The movement of CO within the mammalian body. Arrows represent the movement of CO. Exogenous sources allow for CO to enter the body through the lungs, cross the alveolar capillary membrane and travel into the circulatory system where the binding with Hb in RBCs quickly occurs. Carbon monoxide can also bind with heme proteins in tissues. Endogenous production of CO is mainly from the breakdown of both Hb and heme proteins by HO.
Figure 1-3 Heme porphyrin ring binding with O₂ or CO. Both O₂ and CO bind to the Fe²⁺ center of a heme molecule and thus compete for the same binding location. Also attached the Fe²⁺ atom is a functional group (R) and a globin molecule (G).
Table 1-1 The range of differing human Hb levels.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hemoglobin range (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>130-170</td>
</tr>
<tr>
<td>Females</td>
<td>120-150</td>
</tr>
<tr>
<td>Children</td>
<td>110-160</td>
</tr>
<tr>
<td>Pregnant Woman</td>
<td>100-120</td>
</tr>
</tbody>
</table>
1.4.3 Elimination of Carbon Monoxide in the Body

In order to eliminate CO from the body, CO must reach the lungs via COHb in the blood, exchange with O₂ and be exhaled. The half-life of CO elimination from blood with inhalation of normal air varies on an individual basis. Landaw reported a half life of 3 to 5h for %COHb levels of 2 to 10%, while Peterson and Stewart demonstrated a range of 2 to 6.5h for slightly increased initial COHb concentrations. Between men and women, differences exist as well, with non-smoking men displaying a half life of 4.5h versus 3.2h in non-smoking women. Inhalation of pure O₂ (100%) can decrease the half life to 0.5-1hr, or roughly by 75% at atmospheric pressure. Administration of 5% carbon dioxide (CO₂) can help to accelerate CO washout by increasing ventilation rates, however, hyperbaric O₂ treatment is the most effective in displacing CO and is used in cases of CO poisoning. Hyperbaric chambers are able to: a) increase dissolved O₂ concentrations, accelerate the dissociation of CO from all hemoproteins in the body and shift the oxyhemoglobin (O₂Hb) curve (Figure 1-4) to the right, allowing for increased liberation of O₂ to the tissues. A great deal of debate exists on the clinical value of %COHb level compared with toxicity of CO poisoning.

1.4.4 Carbon Monoxide in the Blood

To form COHb, at least one of the four Fe²⁺ atoms in Hb must bind to a CO molecule at the expense of an O₂ molecule. The reaction is stable and reversible and occurs in the following manner:

\[ \text{HbO}_2 + \text{CO} \rightarrow \text{COHb} + \text{O}_2 \]
Both the binding and elimination of CO with Hb is slower than that of O₂ and its chemical affinity for Hb is roughly 200 times greater than that of O₂\textsuperscript{178,179}. The equilibrium constant or M value, is known as the Haldane coefficient\textsuperscript{127}. The steady state relationship between the partial pressures of CO, O₂ and M is referred to as the Haldane expression\textsuperscript{127,178}:

\[
\frac{\text{COHb}}{\text{O}_2\text{Hb}} = M \frac{\text{pCO}}{\text{pO}_2}
\]

where COHb is carboxyhemoglobin, O₂Hb is oxyhemoglobin and pCO and pO₂ are the partial pressures of CO and O₂ respectively. Therefore, at low partial pressures of O₂, or at high partial pressures of CO, COHb concentrations will increase.

The binding of CO to Hb causes two main effects on the available O₂ for the body. Not only does CO decrease the number of binding sites available for O₂, but it also changes the allosteric structure of the molecule, such that Hb’s affinity for O₂ increases\textsuperscript{177}. In doing so, increased COHb levels shifts the O₂Hb dissociation curve to the left and transforms its usual sigmoidal shape into a near rectangular hyperbola\textsuperscript{177} (Figure 1-4). The shift especially affects the steep part of the curve, where the critical range of O₂ release to the tissues takes place. With high exposure to CO, this process lends to a state of hypoxemia, even in the presence of the normal pO₂ levels.

In both males and females, CO levels fluctuate continuously, depending on physiologic conditions or environmental exposure. On average, non-smoking CO levels are usually below 1%COHb\textsuperscript{180}, but can increase to 1-2% COHb in urban areas\textsuperscript{176}. Moderate smokers of tobacco cigarettes have increased CO levels of 5-6%COHb and heavier smokers from 10- 19%COHb\textsuperscript{176}. Carbon monoxide is related to hypoxia or O₂ deprivation and thus can cause symptoms of throbbing headaches and shortness of breath with levels of 10-30% COHb\textsuperscript{176}. Above 50%
Figure 1-4 The $O_2$ dissociation curve and the effect of CO on $O_2$ binding. Due to $O_2$’s cooperative binding properties, a sigmoid binding curve represents its binding with Hb. Hemoglobin can release more $O_2$ in the tissues (where $pO_2$ is low) and can bind $O_2$ in the lungs (where $pO_2$ is high). As CO binds with Hb, it not only removes binding sites available for $O_2$ (thus decreasing the percent saturation), but also alters Hb’s affinity for $O_2$, and allows less release to the tissues.
COHb, seizures, coma, cardiovascular toxicity, respiratory failure and even death, can be expected \(^\text{176}\).

### 1.4.5 Intracellular Effects of Carbon Monoxide

For many years, it was assumed that cellular effects of CO were unimportant, as Hb’s high affinity for CO would act as a buffer, removing free CO from the circulation and eliminating the possible reactions with tissues from occurring \(^\text{127}\). Recently, a poor correlation was found between COHb levels and the signs/symptoms of CO poisoning \(^\text{121}\) and with the lingering toxic effects following COHb elimination \(^\text{127,181}\). It has been proposed that long after CO has been removed from the blood, it is still present in the tissues and can further cause toxic effects in the body.

Both CO and O\(_2\) react with a multitude of reduced transition metals, particularly heme Fe\(^{2+}\), for which molecular O\(_2\) is the preferred ligand \(^\text{51}\). This binding is defined by the Warburg partition coefficient- the ratio of CO to O\(_2\) whereby half the binding sites are occupied by CO \(^\text{127}\). In mammalian tissues these include: Hb \(^\text{182}\), Mb \(^\text{183}\), cytochrome c oxidase \(^\text{184}\), cytochrome P-450 \(^\text{185}\), sGC \(^\text{83}\), dopamine \(\beta\) hydroxylase \(^\text{186}\) and tryptophan oxygenase \(^\text{187}\). It is well known that of all these hemoproteins, CO reacts most tightly with Hb and secondly with Mb, thus leaving free levels of CO so low, that the binding to other hemoproteins is likely insignificant \(^\text{184,188}\), see Table 1-2 for relative affinities. These metalloproteins contain either an Fe\(^{2+}\) or copper center at their active binding sites, where CO competes with O\(_2\) to form a complex. CO is removed by an excess competition of molecular O\(_2\) or by oxygenation of CO to CO\(_2\) \(^\text{174,189}\), although the latter reaction occurs in very small amounts \(^\text{189}\).
Table 1-2 Affinity levels of CO to specific hemoproteins relative to O_2 binding,

<table>
<thead>
<tr>
<th>Protein</th>
<th>CO affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>200-250</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>20-25</td>
</tr>
<tr>
<td>Cytochrome aa3</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
1.4.6 Hypotheses and Objectives

**Women who smoke in pregnancy have significantly higher CO levels than women who do not smoke in pregnancy**

Of the 4000 chemicals in a cigarette, one of its leading combustible products is CO\(^{110}\). One cigarette contains an average of 40 000 ppm CO\(^{110}\), which leads to an alveolar range of 400-500 ppm\(^{54}\). Thus, a woman who smokes during pregnancy is exposed to a significantly higher level of CO than a non-smoking pregnant woman. It is therefore hypothesized that CO levels measured in smoking pregnant women will be significantly higher than those of non-smoking pregnant women.

*The handheld breathalyzer will correlate more strongly with blood CO measurements than the pulse CO-oximeter*

To date, the most accurate method of measuring CO concentrations is through blood sampling. Two other methods of measurement have been devised, the end-tidal breath analyzer and the co-oximeter. These two handheld devices are able to produce a CO concentration result within a 1 minute time period and are therefore very beneficial for rapid testing, specifically in hospital use. As both devices measure CO levels differently, it is necessary to determine which one more accurately correlates with blood CO measurement. Studies have been completed with both the end-tidal breathalyzer\(^{190,191}\) and the co-oximeter\(^{125,192}\), however to our knowledge a direct comparison between the two has not been published.

The end-tidal breathalyzer measures the amount of CO in one’s breath, which should correlate well with the amount of CO in one’s body; CO exhaled is proportional to the amount present in
the body. The co-oximeter on the other hand, performs calculations based on absorption principles of both COHb and O₂Hb in the capillary bed of a finger. Although this device has been proven quite accurate at toxic levels of CO, it has shown to produce error at normal physiologic CO levels, based on blood pH levels and O₂ saturation and temperature. Therefore, it is hypothesized that the direct measurement of the end-tidal breathalyzer will more accurately correlate with the blood CO measurement than the pulse co-oximeter.

**Maternal murine exposure to CO will cause toxic fetal effects at or above 150ppm CO**

As CO is often referred to as a toxic gas, exposure of any sort has been associated with negative results. More specifically, exposure during pregnancy has been an area of much concern, as the effects on fetal development are greatly unknown. Many researchers have attempted to explore the levels at which CO exposure causes maternal and fetal toxicity using different animal models, however, each of their delivery methods, species of animal, exposure time and toxicity criteria differ, making it difficult to directly compare the conflicting results from each of the studies. Chronic exposures of 90 and 180ppm of CO to maternal rabbits proved to cause an increase in neonatal mortalities and a decrease in birthweight. An increase in resorptions was also observed with CO exposures to CF-1 mice at levels of 250ppm for GD6-15 and a significant increase in fetal mortality was shown for CD-1 mice at levels of 500ppm exposure on GD 8-18. As our study will be exposing the maternal mice to chronic CO for a longer time period than the other studies (GD1 to GD17) and using a much more sophisticated gas exposure system, we hypothesize that at levels at or above 150ppm, we will begin to see toxic fetal effects.

The recent discovery of CO’s physiologic capabilities has spurred research into the roles of CO in the human body. It is known that CO can dilate blood vessels, decrease inflammation and
decrease apoptosis\textsuperscript{47}. Specific to pregnancy, CO has been shown to decrease perfusion pressure and thus increase perfusion of the placenta\textsuperscript{37}.

We hypothesize that low levels of CO exposure (below 100ppm) will not cause toxic effects to either the maternal mouse or its fetuses. At these levels, it is thought that CO’s main physiologic roles will occur and potentially produce a beneficial effect on fetal development. Perhaps CO will increase perfusion of the placenta and further increase nutrient transfer to the fetus, thereby increasing fetal growth.

Lastly, it is clear that the toxic effects of CO cannot be ignored, as it is well known that CO competes with O\textsubscript{2} for binding sites on the Hb molecule\textsuperscript{42} and can therefore induce a state of hypoxia at higher exposure levels. Thus, we hypothesize that levels above 150ppm will cause a hypoxic environment for the fetus and cause a negative effect on their growth. It is thought that a decrease in mass and litter size, as well as an increase in fetal resorptions will be observed in a dose dependent manner following this maternal CO exposure level.

**Specific Objectives**

In order to examine the hypotheses mentioned above, the following objectives were undertaken:

a) Determine the CO concentration in both smoking and non-smoking pregnant woman

b) Compare the CO measurements of two rapid, handheld automated CO measuring devices with blood CO levels

c) Expose pregnant mice to different chronic CO exposures and assess the level of fetal toxicity based on the CO concentration given
Chapter 2

Carbon Monoxide Measurement using Gas Chromatography

Carbon monoxide concentrations were measured using a gas-solid chromatography machine (GC) (Peak Performer 1 Analyzer, Peak Laboratories, Mountain View, CA) (Figure 2-1). 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 heated column (105°C) housed mercuric oxide (HgO) and allowed for a reaction with CO to occur:

\[ \text{CO} + \text{HgO(solid)} \rightarrow \text{CO}_2 + \text{Hg (vapour)} \]

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 values (0-500uL) of CO standard gas (10.1 parts per million (ppm); Scott Specialty Gases, Troy, MI) into a previously purged vial with 21%O2/ 5% CO2/ balance N2 (Praxair, Kingston, ON) and reading these samples with the GC. Sample values were interpolated from the standard curve to calculate CO levels. Blood CO measurements were presented as nL CO/uL sample volume; also equivalent to ml CO/L sample volume. Tissue CO measurements were presented as pmol/ul sample volume and air sample CO measurements were presented as ul/L or ppm.
Figure 2-1 Schematic diagram of gas-solid chromatography device. The valve remains closed in order to allow carrier gas (free of CO, at a rate of 20ml/min) to bypass the valve and enter the column, ensuring no CO is present in the column. The valve then opens and allows the carrier gas to enter the sampling headspace for 20 seconds. Subsequently, the valve closes again, headspace gas is injected into the column with the carrier gas, where CO is separated and sent into the detector. A reaction with HgO occurs and releases Hg vapour, detectable at 254nm. The change in energy due to the Hg vapour present is directly proportional to the amount of CO in the headspace and causes a peak value to be recorded based on the light disturbance.
Chapter 3
A Comparison of Biological Carbon Monoxide Levels in Pregnant Women± Smoking, including those with Pre-eclampsia

3.1 Abstract

Objective: Women who smoke cigarettes during pregnancy have a decreased risk of developing pre-eclampsia (PE). Those who smoke cigarettes have increased levels of carbon monoxide (CO), while women with PE have been reported to have lower end-tidal CO levels. This study sought to compare CO levels in normotensive pregnant women ± smoking (NTN, NTS), as well as pre-eclamptic women ± smoking (PEN, PES), in order to find a potential treatment dose for PE in the future. Further, this study compared two automated CO measuring devices against blood carboxyhemoglobin (COHb) values, to find a rapid test machine for future study purposes.

Methods: Pregnant women (>24 weeks) were recruited prior to their elective caesarian section, during an in-hospital stay or at their clinical obstetrical appointment. Levels of CO were measured using an end-tidal breath analyzer, a CO-oximetry device and through blood sampling, using a gas-solid chromatography machine. Urine was collected for a cotinine measurement.

Results: A total of 74 volunteers were recruited into the study; 46 NTN, 24 NTS, 1 PEN and 1 PES. With a range of 1.5-9.85 blood %COHb, the NTS group had significantly higher (p<0.0001) CO levels compared to NTN with all three measurement methods, as well as in urine cotinine levels. Although no statistical analysis was conducted, CO levels in PE patients were lower than both NTN and NTS. The end-tidal breath analyzer proved to be best correlated with blood CO measurement (r = 0.8524), while that of the CO-Oximeter was much lower in correlation (r = 0.4644). Significant correlations were observed between urine cotinine measurements and both breath and blood, r = 0.7478 and r = 0.8059, respectively. Reported
cigarettes smoked correlated with CO-oximeter levels and were not significantly correlated with either breath or blood CO levels.

**Conclusion:** Our findings are suggestive that a possible range for future CO treatment could be 1.5 to 9.85\%COHb. We found that both blood CO and end-tidal breath CO measurements correlated well with urine cotinine levels and not with reported cigarette number, thus we suggest that all clinical studies involving CO rely on a quantitative measurement of smoking level, rather than number of cigarettes smoked per day. Lastly, we propose an alternative method to blood CO measurement for rapid in-hospital measurement of CO, the end-tidal breath CO analyzer.
3.2 Introduction

One of the leading causes of maternal and fetal/neonatal morbidity and mortality is pre-eclampsia (PE), affecting 5-7% of all pregnancies worldwide. It is a two-stage disease, as it originates in the placenta with poor perfusion and progresses in some, but not all women, into the maternal syndrome of PE. In a normal pregnancy, a mechanism known as a “physiological change” occurs to allow for better nutrient transfer to the fetus. Maternal uterine spiral arteries are remodelled and transitioned from high resistance, low flow blood vessels to low resistance, high flow blood vessels, unresponsive to vasoactive stimuli. In PE, a failure of spiral artery transformation has been well documented, leading to decreased placental perfusion.

Maternal endothelial dysfunction is directly responsible for the characteristic signs and symptoms used to diagnose this disorder: elevated blood pressure and proteinuria. Presently, the only cure is to remove the problematic organ; the placenta.

With no present treatment or prevention for PE, the puzzling inverse relationship between smoking and the development of this disease is of great appeal to many researchers. A recent review demonstrated that women who smoke cigarettes during pregnancy are at a 32% reduced risk of developing PE, compared to non-smoking pregnant women. This same decrease was not observed in women who use snuff during pregnancy, a smokeless form of tobacco and led to the hypothesis that a combustible product in cigarette smoke, possibly carbon monoxide (CO), was responsible for the decreased incidence of PE. Further, Baum et al. measured end-tidal breath CO levels in pregnant women with or without PE, and interestingly, women with PE were found to have statistically lower end-tidal CO levels.
At low concentrations, CO has proved involvement in a number of physiologic functions, such as smooth muscle relaxation and vascular tone control\textsuperscript{33,36}, platelet aggregation\textsuperscript{156}, anti-apoptotic and anti-inflammatory effects\textsuperscript{111,147}. Endogenously, CO is an end result of the breakdown of a heme moiety by the enzyme heme oxygenase (HO), found in numerous organ tissues\textsuperscript{130}, including areas of the placenta and umbilical cord\textsuperscript{64,75,199}.

In the body, CO competes with oxygen (O\textsubscript{2}) for its binding sites on hemoglobin (Hb) to form carboxyhemoglobin (COHb) and travels via the circulatory system. Regular physiologic levels of CO are roughly 0-1.5\% COHb\textsuperscript{125}, but can increase to 1-2\% COHb in urban areas\textsuperscript{176}. Tobacco cigarette smokers have increased levels of CO, ranging from 5-6\% COHb for moderate smokers to 10-19\% COHb or higher, for heavy smokers\textsuperscript{176}. As pregnancy imparts a number of changes on the female body, alterations in CO concentrations are also observed, increasing endogenous levels roughly 30-40\%\textsuperscript{56}.

The purpose of our study was to compare the levels of CO between non smoking and smoking pregnant women and to evaluate, where possible, CO levels in PE women \pm smoking. Carbon monoxide levels were to be compared using 3 different methods of measurement. We hoped to characterize a spectrum of CO in which pregnant women are regularly exposed, as a possible range of therapeutic use in the future treatment of PE.
3.3 Methods

Patient Selection

Pregnant women (>24 weeks gestation) with a singleton baby were eligible for the study. Women were classified into one of four groups: PE ± smoking and normotensive ± smoking (PENS, PES, NTN, NTS). Pre-eclampsia was defined as a blood pressure greater than 140/90 and a protein level greater than 300mg over 24hr. For those women who smoked, self-reported smoking levels, time since last cigarette and number of years having smoked were all recorded. Urine cotinine levels were measured to obtain a quantitative indicator of amount smoked.

Patient Recruitment

This research study was approved by Queen’s University research ethics board (OBGY-165-06). Patients were recruited by research personnel prior to an elective caesarean section, during an inpatient stay or during a clinical visit with their obstetrician. The study was discussed at length with each potential subject, after which they were invited to be a participant. If the subject agreed, two consent forms were signed by both the patient and the research assistant; the patient received a copy and the other was retained for record purposes.

Sample Collection

For each recruited patient, 3 different CO samples were obtained (Pulse Oximeter reading, End-tidal CO level, blood COHb percentage) as well as a urine sample. Below are the explanations for each procedure.
A) CO Pulse Oximeter (Rad-57 Masimo Rainbow Set Pulse CO Oximeter: Hanover, Germany) (Figure 3-1). This machine is a noninvasive method of measuring artillery blood CO concentration using light spectra and absorption properties. A sensor was placed on the patient’s index fingertip (right or left hand), which was directly connected to the Rad-57 instrument via a cable. A pair of light emitting diodes sent both red visible light (660nm) and infrared light (940nm) through capillary beds of the fingertip and a photodiode received the unabsorbed light. By relating the comparison of O$_2$Hb, which predominantly absorbs light at 940nm and reduced hemoglobin, which absorbs light at 660nm, an algorithm estimates oxygen saturation. Carboxyhemoglobin absorbs light at 660nm and not 940nm, which further allows the co-oximeter to calculate the %COHb in the blood of a pulsatile cycle. The machine’s sensitivity is 1%COHb. Calibration was conducted prior to the study start date by the manufacturer.

b) Expiratory End-Tidal Breath CO analyzer (pico Smokerlyzer) (Figure 3-2). This machine is a noninvasive method of measuring breath CO levels. Each patient was asked to hold their breath for 15 seconds and subsequently exhale completely into the disposable cardboard tube on the end of the breathalyzer. A unidirectional flow turbine is located at the end of the attachment tube on the pico CO machine. The number of rotations is proportional to the volume of air that passes through the transducer. The rotation frequency is proportional to the flow rate. The light emitting diodes present produce infrared beams, interrupted by the vane of the turbine twice per rotation and sensed by phototransistors. Since CO absorbs light at a specific wavelength, the amount of CO present alters the light transmission and allows for a calculation reading in parts per million (ppm). The machine’s sensitivity is 1ppm and calibration was conducted prior to the study start date.
c) **Blood Collection**: Maternal venous blood was drawn from each patient into a 3ml syringe, containing 50ul of 14.3U/ml sodium heparin (Sigma Aldrich, H0777). Hemoglobin was measured within 10 minutes of blood collection, using a Hemocue Hb 201 (Hemocue, Switzerland). The remaining blood was kept on ice until processed for CO measurement (within one hour of collection). According to Vreman H.J, blood maintained at 4°C can be stored for up to two months without changing significantly the %COHb.\(^{125}\)

**Blood CO Sampling Analysis**

Amber 2ml vials (Sigma- Aldrich Ltd) sealed with open top caps (Chromatographic Specialties, C223710C) and 8mm silica septa (Chromatographic Specialties, C13302) and containing 20ul of sulfosalycylic acid (SSA) (Sigma Aldrich, Cat no. 86193) were purged with 21% O2/ 5%CO2/ balance N2 (Praxair; Kingston, ON). A set of triplicate vials were prepared with only SSA to serve as blank measurements. A small amount of blood (1ml) was transferred from the syringe to a microcentrifuge tube. In triplicate vials, blood was added to the SSA liquid using a gas-tight syringe and repeater system (Hamilton, USA). Vials each contained 1ul of blood for samples from non-smoking women and 0.4ul of blood for blood samples from smoking women. Vials and their caps were covered with ice for 60 minutes to allow for CO from the sample to equilibrate with the vial headspace. The lower temperature has been proven to decrease CO leaching from the septum.\(^{119}\)

Following the 60 minute incubation period, a gas-solid chromatography (GC) machine (Peak Performer 1 Analyzer, Peak Laboratories, Mountain View, CA) (Chapter 2 and Figure 2-1) was used to quantify CO concentrations in the head space gas of the blood and plasma vials.
**COHb calculation:**

In the human body, the majority of CO present in blood is bound to Hb. As such, CO levels measured were presented as percent COHb (%COHb) values, using the following equation:

\[
\%\text{COHb} = \left[ \frac{\text{vol CO}}{(\text{Hb} \times 1.368)} \right] \times 100\% 
\]

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 hemoglobin in milliliters per gram.

d) Urine collection

Urine (>10ml) was obtained at the time of recruitment for measurement of cotinine levels. All urine samples were stored in sterile plastic containers at -80°C for a maximum of one year. Samples were analyzed using a solid phase Cotinine ELISA test (Calbiotech, CA, USA).

**Data Analysis**

Statistical analysis was completed only on data sets with NTN and NTS, as both PENS and PES did not have enough patient data to generate accurate statistics. Thus, although data may be shown for PENS and PES, it is strictly for analysis of trends, as it could not be statistically analyzed as a group vs. control levels. All data sets were analyzed for Gaussian distribution using the D’Agostino and Pearson omnibus normality test. Differences between groups (NTN and NTS) for each of the four measurements (End-tidal breath CO, Oximeter CO, Blood CO and Urine cotinine) were computed using an unpaired student’s t-test with Welch’s correction. As all data was normally distributed, data sets were analyzed using Pearson product-moment correlation coefficient (r). Statistical significance was set at p<0.05 for all tests performed.
Figure 3-1 The CO pulse oximeter device. (Rad -57 Masimo Rainbow Set Pulse CO Oximeter: Hanover, Germany)
Figure 3-2 The expiratory end-tidal breath CO analyzer (pico+ smokerlyzer, Bedfont Scientific).
3.4 Results
This study included a total of 74 patients, with 46 NTN control pregnant women, 24 NTS pregnant women, 3 PENS pregnant women and 1 PES woman. These numbers do not include the 8 volunteers who were excluded from the study for such reasons as: failure to return for appointment (1), premature delivery (2), decision to decline study at follow-up appointment (1) and inaccurate sample collection (4).

The mean (±SD) age of the women in the study was 29.3 (±5.15) years with a mean (±SD) gestational age of 34.0 (±4.21). Women recruited prior to a caesarian section numbered 1, women recruited during a hospital stay numbered 5 and all other volunteers were recruited at their routine clinical obstetrical appointment. All data sets were found to be normally distributed using the D’Agostino Omnibus test.

Measurement of CO
Average CO levels are displayed in Table 3-1 for all volunteer groups and corresponding with each of the three CO measurement devices, as well as urine cotinine. Control NTN volunteers had significantly lower CO levels than NTS with all three measurement devices (p<0.001), as well as significantly lower urine cotinine levels than NTS (p<0.0001). PENS displayed the lowest mean CO levels using both end-tidal breath CO and blood %COHb measurements, while average oximeter PENS %COHb was higher than both NTN and PES values. The range of CO levels in all four groups of volunteer women is shown in Figure 3-4. Both end-tidal breath CO levels and blood %COHb showed NTS with the highest CO levels amongst the volunteers (Figure 3-4 A, B), while oximetry %COHb appeared to display much more variation in CO readings amongst the groups studied (Figure 3-4C).
Comparison of CO measuring devices

The most accurate measurement of CO levels is through blood sampling using gas-solid chromatography. Figure 3-5 separately correlates end-tidal breath CO and oximetry %COHb with blood %COHb, displaying significant correlation coefficients of $r = 0.8524$ (p<0.0001) and $r= 0.4644$ (p<0.0001) respectively.

Assessment of Smoking

Table 3-2 lists the relevant smoking data for all NTS volunteers, number of cigarettes smoked per day, number of years having smoked cigarettes, elapsed time since last cigarette. In conjunction with reported number of cigarettes smoked, we measured urine cotinine levels, a stable metabolite of nicotine, as a direct indicator of the amount of cigarettes smoked. Urine cotinine levels were also measured in the groups of women who reported not smoking, or having quit as a result of pregnancy (Figure 3-6). The strongest correlation with urine cotinine levels was with blood %COHb, $r = 0.8059$ (p<0.0001), while a correlation of $r=0.7478$ (p<0.0001) and 0.2636 (p<0.05) were measurements for end-tidal breath and oximeter CO correlations with urine cotinine respectively.

Reported Smoking Levels

The reported number of cigarettes smoked per day by NTS women were correlated with all measurement CO measurement devices, as well as urine cotinine levels (Figure 3-7). The most highly correlated data was between oximeter %COHb and reported cigarettes smoked, $r = 0.5968$ (p<0.01) (Figure 3-7 A), followed by reported cigarettes smoked per day vs. end-tidal breath CO
$r = 0.4663 \ (p<0.05)$. Correlations between blood %COHb and urine cotinine versus reported cigarettes smoked were not significant $r = 0.2019 \ (p>0.05)$ and $r = 0.038 \ (p>0.05)$. 
Table 3-1  Average CO and cotinine levels in recruitment categories: NTN ± smoking and PE ± smoking. Using three CO measurement methodologies, end-tidal breath, pulse oximetry and blood sampling, CO was compared for each group sampled. Levels of maternal urine cotinine were also presented as a quantitative measurement of smoking levels. Data is displayed as mean (SD).

<table>
<thead>
<tr>
<th></th>
<th>NTN (n=46)</th>
<th>NTS (n=24)</th>
<th>PENS (n=3)</th>
<th>PES (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End-tidal Breath</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ppm)</td>
<td>1.85 (0.89)</td>
<td>15.42 * (7.77)</td>
<td>1.33 (0.58)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Oximeter Reading</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% COHb)</td>
<td>1.72 (1.26)</td>
<td>3.96 * (2.54)</td>
<td>2 (1.74)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Blood (% COHb)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.01 (0.36)</td>
<td>4.05 * (1.91)</td>
<td>0.84 (0.093)</td>
<td>1.15</td>
</tr>
<tr>
<td><strong>Urine Cotinine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>0.96 (2.75)</td>
<td>12 126 * (8871.30)</td>
<td>0 (n/a)</td>
<td>517</td>
</tr>
</tbody>
</table>

Unpaired student’s t test with Welch’s correction for NTS vs. NTN data, *p<0.0001
Table 3-2 Assessment of smoking levels in NTS volunteers.

<table>
<thead>
<tr>
<th></th>
<th>Elapsed Time Since Last Cigarette (hours)</th>
<th>Years Having Smoked Cigarettes</th>
<th>Number of Cigarettes Smoked per Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>1.85</td>
<td>9.32</td>
<td>10.29</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.38</td>
<td>5.2</td>
<td>5.37</td>
</tr>
<tr>
<td>Range</td>
<td>0.16- 4</td>
<td>0.67- 20</td>
<td>4-20</td>
</tr>
</tbody>
</table>
Figure 3-3 Maternal CO levels measured in pregnant women using three different techniques, end-tidal breath pulse oximetry and blood sampling. Carbon monoxide levels measured for all four groups of pregnant patients (NTN ± smoking and PE ± smoking) using end-tidal breath CO (A) and blood %COHb (B) displayed a similar trend for NTN, PENS and PES sampling. Oximeter values (C) did not present in the same fashion, with more scattered and varied results for all groups of women.
Figure 3-4 Correlations between automated measurement devices of CO and blood %COHb levels. Comparisons of both end-tidal breath CO (A) and oximeter CO (B) were made with blood %COHb levels, with significant Pearson product-moment correlation coefficients of $r = 0.8524$ ($p<0.0001$) and $r = 0.4644$ ($p<0.0001$) respectively.
Figure 3-5 Maternal urine cotinine concentrations vs. all three measured CO levels. Comparison of maternal urine cotinine levels vs. end-tidal breath CO (ppm) (A), pulse oximetry (%COHb) (B) and blood (%COHb) (C). Pearson product-moment correlation coefficients are \( r = 0.7478 \) (\( p < 0.0001 \)), \( r = 0.2636 \) (\( p < 0.05 \)) and \( r = 0.8059 \) (\( p < 0.0001 \)) respectively.
Figure 3-6 Reported cigarettes smoked/day in relation to all CO measurements and urine cotinine levels. Carbon monoxide measurements of breath end-tidal CO (A), pulse oximeter %COHb (B), blood %COHb (C) and urine cotinine (D) in relation to reported cigarettes smoked per day. Pearson product-moment correlation coefficients are $r = 0.5968$ ($p<0.01$), $0.4663$ ($p<0.05$), $0.2019$ ($p>0.05$) and $0.038$ ($p>0.05$) respectively.
3.5 Discussion

CO has been hypothesized to play a role in the decreased incidence of PE observed among pregnant smoking women. This study demonstrated that CO levels in smoking pregnant women are significantly elevated compared to non smoking pregnant women. The CO measurements may have been impacted by factors that we will address here. Firstly, in the smoking population, the time since each volunteer’s last cigarette (Table 3-2) would have affected the measured CO levels. The half-life of CO is roughly 2.5 hours, therefore those volunteers at the higher end of the range would have already begun to eliminate CO and may have expressed lower levels than their realistic values. Secondly, the brand of cigarettes (light vs. regular) may have altered the CO levels measured, although it was not possible to correct for this factor.

The concentration of CO measured in our four groups of pregnant women, NTN ± smoking and PE ± smoking, proved to have an overall range of oximeter 1- 9 %COHb, end-tidal breath 1-34ppm and blood 0.33-9.85% COHb, although these extreme end values were not matched between devices. Blood CO levels for a normal, non smoking individual are between 0 and 1.5% COHb, while it is known that pregnancy increases CO levels by 30-40% due to hematological changes that occur, as well as the addition of fetal endogenous CO production. In smoking individuals, these CO levels can rise dose dependently, and have been measured as the high range for heavy smokers to be between 19% and 22.4% COHb. Our blood CO level range demonstrates that our smoking population does not adequately represent a heavy smoking pregnant population and therefore underestimates the extreme high-end CO level found in some smoking pregnant women. Our study sought to determine a range of CO levels for possible future treatment in PE patients and it is unlikely that CO levels comparable to very heavy
smokers would be used for this reason. Therefore, the smoking population that was measured here provides a suitable range of comparison for this purpose.

End-tidal breath CO levels can vary depending on the device used, as methodology and specificity is different for each design\textsuperscript{190,201}. These devices are however, non invasive and much more convenient to use than laborious blood sampling. A few groups have evaluated specific end-tidal breath CO devices for efficacy and have found them to accurately measure CO levels\textsuperscript{190,191,202}. One group has evaluated end-tidal CO levels in pregnant woman with relation to uterine contractions\textsuperscript{25}, pregnancy-induced hypertension and PE\textsuperscript{19} and gestational hypertension and PE\textsuperscript{203}. Each study has found end-tidal CO levels to be lower in each of their study group populations compared to controls. Although our sample size was small (n=3), the mean end-tidal breath CO level for our PENS volunteers was lowest amongst all four groups measured (Table 3-2). As CO has been shown to possess vasodilatory properties with human placental blood vessels\textsuperscript{39,64}, the lower CO levels observed in these studies may be attributing to the increased contractility of the uterus or the higher blood pressure observed in PE.

Maternal cigarette smoking is associated with a number of adverse maternal and fetal outcomes in pregnancy\textsuperscript{110,204}. The paradoxical effect of cigarette smoking during pregnancy and the reduced incidence of PE has long puzzled investigators\textsuperscript{24,106}. Many hypotheses have been explored in order to attempt an explanation for this relationship, although none have been substantiated by large amounts of basic research. Investigators have speculated that a lower incidence of pregnancy- induced hypertension among smokers may be attributed to a nicotine inhibition of thromboxane A2 production\textsuperscript{205}, while others have explored the idea of a reduced immune
response or an induction of liver enzymes that could metabolize endothelial toxins produced by the placenta. Others still have considered the possibility that as chronic smoking desensitizes the endothelium, it could reduce the responsivity to acute utero-placental hemodynamic changes from the vascular injury characteristic of PE. In light of the increased levels of CO in smoking women compared to non smokers, perhaps PE protection is mediated through its actions. Through CO’s anti-inflammatory and anti-apoptotic properties, it may also be capable of optimizing trophoblast mediated transformation of uterine spiral arterioles, which is underdeveloped or non existent in woman with PE. The vasodilatory effects of CO and specifically in the placenta may help smoking women to minimize the placental oxidative stress that is observed in PE through increased placental perfusion.

Unfortunately, there still exists a small population of women, who despite cigarette smoking in pregnancy, still go on to develop PE. This population is still much smaller on a percent basis versus the non smoking population, as well as occurring at a later gestational age for onset of severe disease versus the non smoking PE population. A few other studies also examined the effects of smoking on the clinical manifestations of PE as well. Both Cnattingius and colleagues, as well as Lindqvist and Marshal found a reduced risk in developing PE for women who smoke cigarettes during pregnancy, however found that there was no difference in the severity of the disease between each group. A study conducted by Marcoux and colleagues agreed with the latter result of those studies. Our sole PES volunteer demonstrated much lower CO levels with all three measurement devices compared to the NTS group. Perhaps the CO levels in smoking PE women are lower because the available CO has been utilized in damaged tissues in an attempt to attenuate the pathological occurrence of PE (inflammation, apoptosis). The available CO may also be decreased following its use to improve placental perfusion in areas of
oxidative stress found in PE. As our sample size only included one patient, it is clear that further studies investigating the specific levels of CO in this group of women is warranted.

Carbon monoxide levels can be measured using three different methods, end-tidal breath, oximetry or blood sampling, all of which were completed in this study. To date, the blood COHb sampling by GC is considered to be a reference method for CO analysis. This procedure, although proven to be extremely accurate, is time consuming and fairly complicated, thus making it impractical for rapid in-hospital measurements. In this way, either of the two automated, handheld devices, end-tidal breath or pulse-oximetry, would be of more convenience. Our study evaluated the correlation accuracy of these two devices with blood %COHb levels in pregnant women.

Correlations with both automated CO devices were made with blood %COHb levels (Figure 3-5). A much stronger correlation was observed between end-tidal breath CO and blood %COHb (Figure 3-5A). End-tidal breath CO is a dependent measurement on %COHb. With increasing %COHb, elimination can only occur by exhalation and thus would increase end-tidal CO levels as well. Although the correlation coefficient was not as high as some other devices have proven to be, the positive and significant correlation still shows a useful compatibility with blood CO measurement. On the other hand, pulse oximetry devices have also measured high correlation coefficients with %COHb, however, these studies did not compare low CO level values (<5%), which is more representative of a non-smoking population. A study conducted by Majoney J.J et al. showed that a large amount of error is introduced at levels below 5%COHb, and may not be useful for clinical applications requiring accurate measurements in the
normal CO range (<2.5%)\textsuperscript{193}. Hampson NB showed that pulse oximetry devices in severe CO poisoning cases can also over-estimate the %COHb level\textsuperscript{212}. A number of interfering substances and conditions can affect the oximetry devices, such as temperature\textsuperscript{192}, bilirubin\textsuperscript{213}, methemoglobin\textsuperscript{214}, pH and oxygen saturation\textsuperscript{194}. Therefore, for clinical settings, where low CO levels may be measured, the end-tidal breathalyzer would be the more appropriate automated device to use versus the pulse oximeter.

In order to verify smoking levels quantitatively, urine cotinine levels were analyzed for each study subject. The levels of cotinine measured were in agreement with other studies\textsuperscript{215-217}. Correlations with each of the CO measurement methods proved to be highest for blood %COHb, followed by end-tidal breath CO levels (Figure 3-6 A, C). One would expect these two measurements to correlate best with urine cotinine as the correlation data showed that end-tidal breath CO levels correlated best with blood %COHb levels (Figure 3-5 A).

Lastly, our study correlated the number of reported cigarettes smoked with each of the three CO measurement devices, as well as with urine cotinine levels. A number of studies have used reported cigarettes smoked as an indicator of passive, mild or heavy smoker\textsuperscript{21,24,201,216}. Our results showed that a significant correlation was observed between reported cigarettes smoked and oximetry %COHb levels, $r = 0.5968$ (Figure 3-7A) and a lower significance observed for end-tidal breath CO $r = 0.4663$ (Figure 3-7B). No significant correlation was observed between reported cigarettes smoked and %COHb levels (Figure 3-7C) or urine cotinine levels (Figure 3-7D). This finding is interesting, as blood %COHb is recognized as a reference CO measurement and urine cotinine is the best biomarker used as a quantitative measurement of tobacco
cigarette smoking\textsuperscript{21,218,219}. As cigarette smoking during pregnancy is viewed as a socially
unacceptable behaviour, perhaps these women sought to mask their level of smoking by altering
the number of reported cigarettes they revealed to us, as shown in a previous study\textsuperscript{220}.

Alternatively, elapsed time since last cigarette may have affected the results, as cotinine has a half
life of 16 to 19 hours\textsuperscript{221}, while CO has a half life of 2.5 hours\textsuperscript{88}. Thus, some women may have
decreased \%COHb levels, while maintaining a high urine cotinine measurement. However, the
low correlation we calculated between oximetry \%COHb and blood \%COHb measurement in this
study (Figure 3-5B), showing an inaccurate oximetry \%COHb level, lends to a more plausible
explanation for under-reported cigarette levels and inaccurate values for both measurements.
This finding is important for future clinical work, to ensure an analysis of urine cotinine levels is
performed as a direct indicator of tobacco smoke exposure in conjunction with reported cigarette
smoking.

The outcomes of this study are two-fold. A physiologic range of CO levels was determined across
pregnant non-smoking and smoking woman. A possible therapeutic dose may be used within this
range for future treatment of PE patients. Secondly, the end-tidal breath analyzer was found to be
correlated more strongly with the reference blood \%COHb levels than the oximetry device,
making it the more appropriate automated device for future clinical CO studies.
Chapter 4
Determining a Threshold for Exogenous Carbon Monoxide Exposure in a Pregnant Murine Animal Model

4.1 Abstract

Introduction: Impaired placental perfusion secondary to incomplete spiral artery remodelling leads to the syndrome of pre-eclampsia. Carbon monoxide (CO) has been shown to cause vasodilation in the placental blood vessels, which is suggestive of a possible role for CO in vasorelaxation of the pre-eclamptic spiral arterioles.

Objective: The purpose of this study was to determine the level of CO that could be administered to a pregnant mouse without adverse maternal or fetal effects.

Methods: Pregnant CD1 mice were subjected to constant levels of CO (0 to 400ppm) from conception to the 17th day of gestation. Maternal and fetal blood percent carboxyhemoglobin (%COHb) levels were measured using gas-solid chromatography (GC) and compared to those of human maternal non-smoking and smoking COHb levels; roughly 0.5% and 5-15% COHb respectively. Gross and histological analysis of fetal and placental morphology and apoptotic index were compared for each CO concentration. Litter size, fetal mass and placental mass were compared for each CO level vs. control. Maternal organ tissues were also analyzed for CO concentration using GC.

Results: The ranges of maternal and fetal CO blood measurements were 1.12-15.6 %COHb and 1.0-28.6 %COHb, respectively. Placental mass was not significantly affected by CO levels, however litter size and fetal mass were significantly decreased vs. control at 400ppm. Histological analysis of morphology proved no difference with any CO exposure in fetal or placental tissues. The apoptotic index in fetal brain, heart, placental labyrinth and junctional zone
was highest at 400ppm, with significance only in heart and brain tissues. Significance of abnormalities and resorptions was seen at 400ppm vs. control. Maternal tissue CO content was highest in placenta, spleen and heart.

**Conclusions:** This experiment displayed a threshold for maternal CO exposure at 400ppm as significance vs. control was observed in each of fetal weight, litter size, resorptions and abnormalities and heart and brain apoptotic index. Significance was also observed in maternal tissue CO levels at 400ppm in most tissues analyzed.
4.2 Introduction
Carbon monoxide (CO) has been deemed the silent killer due to its cytotoxic capabilities following inhalation at high concentrations. However, CO is endogenously produced at low concentrations, and at these levels, it is involved in a number of normal physiologic functions: smooth muscle cell relaxation and control of vascular tone, platelet aggregation, anti-inflammatory and anti-apoptotic events. The enzyme responsible for endogenous CO production is heme oxygenase (HO), a natural enzyme found throughout the body that breaks down the molecule heme. It produces regulatory levels of CO in a number of tissues, including most abundantly in muscle, heart, liver, spleen, and kidney. Heme oxygenase has been isolated from human placental and umbilical cord tissues, lending to research into the roles of the HO/CO system in pregnancy.

The body adapts to an exogenous inhaled exposure of CO by “mopping up” the gaseous molecule at the alveolar capillary membrane, where CO combines with Hb to form carboxyhemoglobin (COHb). Carbon monoxide can bind with a number of molecules throughout the body containing a heme moiety such as hemoglobin (Hb), myoglobin (Mb), cytochromes and soluble guanylyl cyclase (sGC), in direct competition with molecular oxygen (O\textsubscript{2}) for the binding sites. In the case of Hb, the binding of CO shifts the O\textsubscript{2} dissociation curve to the left, limiting the release of O\textsubscript{2} to the tissues.

In pregnancy, CO readily crosses the placenta and is free to combine with fetal Hb and tissue heme moieties. Longo and Hill used pregnant sheep to show that there exists a lag period from the maternal CO exposure to the fetal CO rise, however, over time fetal CO levels will
match and even surpass those of their mother. Fetal hypoxia may result at high levels of maternal CO exposure, causing developmental challenges, however, it is unclear at what CO concentration a maternal exposure becomes a fetal threat. Studies focusing on the aftermath of maternal CO poisoning have documented fetal death at COHb levels of 60% \( ^{98} \) corresponding with maternal levels of greater than 20% COHb \(^{56}\).

With minimal chronic animal CO studies in the literature, there are only a few that have looked at the effects of chronic CO exposure in pregnancy; and these still are among different species and head conflicting results. At levels above 1000 ppm CO, with differing time exposures, studies in Wistar rats \(^{195,223}\), rabbits \(^{196}\) and mice \(^{197}\) displayed decreased fetal weights and an increase in fetal deaths, abortions and resorptions. These CO levels lead to toxic CO poisoning and were well above the resulting effects of CO exposure from cigarette smoke. A study conducted by Choi and Oh \(^{198}\) observed the effects of 750 ppm CO on pregnant Sprague-Dawley rats for 3 h/day on gestational day (GD) 7, 8 or 9. An increase in abortions, stillbirths, and skeletal abnormalities, as well as a decrease in birth weight was recorded. Of important note are the studies conducted at 500 ppm and below, at which levels of COHb in the animals are closer to those of smoking pregnant women. Astrup et al \(^{224}\) subjected rabbits to 90 or 180 ppm from the day of mating to the day before parturition and reported maternal COHb levels of 8-9% and 16-18% respectively. The number of neonatal mortalities increased with CO exposure in conjunction with a decrease in birth weight. Schwetz and colleagues \(^{103}\) treated CF-1 mice with 250 ppm CO for 7 or 24 h/day on GD 6-15, lending to a COHb levels of 10-11%. An increase in resorptions and birth weight were observed with 7 h/day exposure, however a decrease in birth weight was observed with 24 h/day exposure \(^{103}\). Lastly, a study with CD-1 mice chronically exposed to 150, 250 and 500 ppm CO on
GD 8-18, reported significant fetal mortality observed only at 500ppm, with no effect recorded on implantation site number\textsuperscript{225}.

The aim of the present study was to investigate the whole body chronic CO exposure of CD-1 pregnant mice, in a highly regulated system of CO dosing from GD1 to GD17. We sought to evaluate both the maternal and fetal effects due to the CO treatment and to find a level of chronic CO exposure with which a negative impact was not observed.
4.3 Methods

Animals and Husbandry

Female (6-8 weeks old) CD1 mice were purchased from Charles River, USA and mated with males (5-7 weeks old) of the same strain. Timed matings were performed overnight and the morning detection of a vaginal plug was designated GD1. Females were weighed and placed into a CO chamber (Figure 4-1) on GD1, where levels of CO administration were monitored continuously. All the animal handleings and experimental procedures were carried out according to the University Animal Care Committee of Queen’s University. The study was approved by the Queen’s University Ethics Committee (REB no. Smith 2007-052-R1)

Carbon Monoxide Concentrations

Levels of CO administration were 0, 25, 60, 100, 150, 200 250, 300 and 400ppm. For each experiment, a minimum of nine pregnant female mice were given a distinct level of chronic CO, from GD1 to GD17. Carbon monoxide was administered to the female mice using a regulated CO chamber created in our laboratory (Figure 4-1) where stable CO concentrations could be finely controlled using a computerized system and verified using GC (Chapter 2). Room air was collected using a compressor (Figure 4-1, A) and passed through a Norgren air dryer (Littleton, CO USA) (Figure 4-1 B). The dry air was mixed with 10% CO gas (Praxair, Kingston, ON) (Figure 4-1 C) through separate flow meters (Alicat Scientific, Tucson, AZ USA) (Figure 4-1 D) and adjusted to the specific CO concentration required, using a Gas Mixing software Program (Qubit Systems Inc) (Figure 4-1 E). The air was bubbled through distilled water (Figure 4-2 A), reintroducing humidity levels of 40-50%, (animal care regulations) and monitored using a GO-Link humidity sensor (Figure 4-2 B). The air continued into a tightly sealed plastic aquarium,
(Miracle’s Aquarium, Kingston, ON) (Figure 4-1F) in which the mouse cage was placed and exhausted out of the chamber into the room’s air filter system (Figure 4-1G). With a flow of 7000ml/min, the air was changed over a minimum of 10 times per hour within the chamber. Food and water were provided *ad libitum*. The cage was changed twice weekly by research personnel only. At this time, the CO administration was turned off (20 minute maximum). Air samples were collected at minimum twice weekly from the aquarium air port (Figure 4-1H) and measured using a gas-solid chromatography machine (Peak Performer 1, Palo Alto, San Francisco, USA) (Chapter 2), ensuring CO levels matched the desired concentrations.

**Experimental Procedure**

Female mice were sacrificed on GD17 by intraperitoneal injection of 10mg/g of Tribromoethanol (Avertin). Upon reaching a surgical plane of anesthesia, maternal blood was drawn by retro-orbital blood collection using a glass pipette. The blood was added to a microcentrifuge tube containing 10ul of 14.3U/ml sodium heparin (Sigma Aldrich, H0777) and placed immediately on ice (see blood procedure). Mice were then perfused (gravity perfusion) through the left ventricle for 15 minutes with either phosphate buffered saline (PBS) (seven mice/experiment) or 4% paraformaldehyde (two mice/experiment). In the case of repeat experiments, there was an increase in number of mice used for each of the PFA and PBS perfused procedures.

**Blood Procedure**

Hemoglobin was measured within 5 minutes of blood collection using a Hemocue Hb 201 (Hemocue, Sweden). Amber vials (2ml) (Sigma- Aldrich Ltd) capped (Chromatographic Specialties C223710C) with 8mm silica septa (Chromatographic Specialties, C13302) and containing 20ul of 2% sulfosalicylic acid (SSA) (Sigma Aldrich, Cat no. 86193) were purged
with 21% O2/5% CO2/balance N2 (Praxair, Kingston, ON). Between 0.4 ul and 1 ul of blood (depending on the level of CO administration) was added using a gas tight Hamilton syringe and repeater system (Hamilton, USA). Triplicate vials per blood sample were prepared and set on ice for 60-120 min. Carbon monoxide levels were read using a GC (Peak Performer 1, Palo Alto, San Francisco, USA) and expressed as a percentage of total Hb using the following equation:

\[
\%\text{COHb} = \left( \frac{\text{vol CO}}{(\text{Hb} \times 1.368)} \right) \times 100% \]

where “vol CO” is milliliters of CO bound to 1 L of blood, Hb is total Hb concentration in the blood (g/L) and 1.368 is the CO-binding capacity of hemoglobin in millilitres per gram.

**PBS perfused mice Fetal results**

Following a 15 minute perfusion, the uterus was dissected out and all fetuses and their respective placentas were removed and weighed. The mean per litter was recorded and this number was used for statistical analysis. Litter size was also noted per mouse. All fetuses and placentas were examined for gross morphological abnormalities. Fetal death was reported as early gestational demise (resorbed) or as a late gestational death or growth anomaly (abnormality) (Figure 4-3). Resorbed fetuses were classified as a non distinct placenta from fetal structures, while an abnormality was defined as a distinct structural placenta and fetus, however, a re-absorption of fetal tissue.

**Fetal Blood Collection**

Three random fetuses were decapitated and blood was collected using a heparin-coated capillary tube (Fisher brand, Cat no. 22-260-950). The blood from the three fetuses was pooled into a
microcentrifuge tube and followed the same blood processing as previously described (see Blood Procedure).

**Tissue CO Levels**

Sections of maternal organs (brain, liver, lung, kidney, spleen, heart) as well as one placenta section were collected and placed on ice immediately. Upon weighing the tissues (10-150mg), each was added to four times its mass in distilled water (ml). The tissues were sonicated on ice using a cell disruptor (Fisher Scientific Sonic Dismembrator) with a 1/8 inch diameter micro tip probe (3W) until homogeneity was observed in the tubes (<10s). Tissue was prepared immediately for CO measurement, stored at 4°C for measurement within 24 hours, or frozen at -20°C for measurement within 48 hours.

Tissue preparation was performed as per methodology explained by Vreman HJ *et al.* Briefly, amber vials (2ml) were prepared with 5ul of 30% SSA (Sigma Aldrich, Cat no. 86193) and enough water to make the total volume following tissue addition add up to 40ul. A set of blank vials was also prepared, substituting distilled water for the sonicate addition. All vials were sealed with screw caps containing silica septa (Chromatographic Specialties, C13302). The vials were purged with 21%O2/5%CO2/balance N2 (Praxair, Kingston, ON) and tissue sonicates (5-15ul) were added to the vial liquid layer using a Hamilton repeater syringe system (Hamilton, USA). All vials were briefly shaken prior to ice incubation for a minimum of 60min (caps fully covered in ice, to decrease CO leaching), at which time CO was released from the sonicate into the vial headspace. This headspace was then measured for CO levels using gas-solid chromatography (Chapter 2). Blank vial CO levels were subtracted from the tissue CO levels and all sample concentrations were expressed as pmol of CO/mg fresh weight (FW).
4% PFA perfused mice
Two mice per CO concentration experiment were perfused with 4% PFA. Following perfusion, the uterus was removed. Cutting between implantation sites, three embryos were removed and stored in 4% PFA for 24 hours; subsequently transferred to 70% ethanol until processing (7days – 60days). Tissues were embedded in paraffin according to standard procedures. Special care was taken to place all organs in similar orientation prior to embedding and tissues were sectioned (4um thickness).

Paraffin embedded slide staining
Each embryo (6 per CO dose experiment) was sectioned and placed on slides. One slide was stained with hematoxylin and eosin (H&E) for general morphology analysis. Fetal brains (parietal lobe), fetal heart tissue (ventricle) and fetal placentas (mainly labyrinth) were analyzed for histological morphometry changes compared to control. Three random pictures were taken using a Nikon Eclipse E800 Light Microscope and Q capture software in each of the parietal lobe (200X magnification), the heart ventricular tissue (100X magnification), the whole placenta ( 10X and 40X magnification) as well as the placenta labyrinth (200X magnification). The brain and heart tissues were analyzed for change in cell shape or size. The placentas were analyzed for relative proportions of labyrinth versus junctional zone and alterations in cell shape and size.

A second slide per embryo was stained for apoptosis using a Promega Tunel Colorimetric Kit (Fisher, Canada). Procedure was followed as the kit described. In addition, an Avidin/Biotin blocking kit (SP2001, Vector Laboratories) was also used to eliminate non-specific binding of biotin/avidin system reagents. Negative controls were incubated without terminal deoxynucleotidyl transferase (TdT), while positive controls were incubated with TdT following
incubation with a deoxyribonuclease agent (DNase) (D5319, Sigma Aldrich). A third control was tested following DNase treatment, without TdT, to ensure apoptotic nuclei were not detected by another reagent. A Mayer’s hematoxylin counter stain was completed subsequent to the Tunel staining. All sections were mounted with permount (Fisher Scientific, SP15-100).

All sections were blinded by a third party and observed using a Nikon microscope at a magnification of 40X. Images of the TUNEL (brown) and the hematoxylin (purple) were captured at three randomly selected fields for each of the tissue locations: fetal left ventricle, parietal lobe, placental junctional zones and labyrinth zones (Figure 4-4). Images were captured using Quatro Pro 5 software. TUNEL positive nuclei (apoptotic nuclei) and hematoxylin stained nuclei (total nuclei) were counted using Windows Paint. The apoptotic index in each section was calculated as a percentage of TUNEL stained nuclei divided by the total number of hematoxylin stained nuclei.

**Statistical Analysis**

Dose response CO effects on each of maternal and fetal %COHb and Hb were analyzed using linear regression analysis, as explained by Elashoff JD. All other analyses were completed using a one way analysis of variance with a post-hoc Dunnett’s multiple comparison test. A p-value less than 0.05 was deemed significant for all tests.
Figure 4-1. The CO chamber delivery system. A: Air compressor, B: Air Dryer system, C: 10% CO gas, D: Flow meters, E: Gas mixing software, F: Aquarium chamber G: Outlet air tubing H: CO Air port
Figure 4-2 Air humidifier and sensor. A: Distilled water air humidifier B: GO-Link humidifier sensor.
Figure 4-3 Representation of fetal resorption and abnormality characterization. A typical fetal resorption is shown A, with no distinction between a primitive placenta or fetus. A fetal abnormality (abortion or growth anomaly) is shown in B, where it is clear that the fetus is necrotic, but a distinction can still be made between the placenta and the fetus. A normal fetus at GD17 is shown in C.
Figure 4-4 Placental locations reviewed for apoptosis. The three white “X” areas were deemed as junctional zone apoptosis. The three black “X” areas were deemed as labyrinth zone apoptosis.
4.4 Results

Daily air samples from the mouse chamber were measured for CO levels using a gas-solid chromatography (GC) machine and these mean levels are expressed as CO ppm (SD) in Table 4-1. Humidity levels were consistently measured and recorded every 30 minutes for each CO experiment. These averaged levels are expressed as humidity (SD) in Table 4-1. Temperature in the CO chamber was also monitored daily and maintained at 25°C.

A positive correlation was observed between maternal and fetal %COHb levels (Figure 4-5 A), as well as maternal and fetal Hb levels (Figure 4-5 B) with increasing CO exposure. All were deemed significant with linear regression analysis and a slope greater than zero, maternal %COHb p=0.0004, fetal %COHb p=0.0004, maternal Hb p<0.0001 and fetal Hb p=0.0076. With each administration of CO exposure, fetal %COHb was consistently higher than the matched maternal %COHb level, a mean (SD) of 1.8X (0.2) that of maternal levels.

Mean fetal mass did not differ compared to control with CO concentrations of 25ppm to 300ppm (Figure 4-7A). At 400ppm CO, fetal mass was significantly lower than control fetal mass (p<0.05). Placental mass was not found to be significantly different than the control with any of the CO doses (p>0.05) (Figure 4-7B). Fetal litter size followed the same trend as mean fetal mass, with significance only observed at 400ppm compared to control (p<0.05).
Fetal resorptions and abnormalities are presented as (values/total implantation sites)*100% (Table 4-2). A positive trend is observed with both resorptions and abnormalities with increasing CO concentrations, however in both cases, significance is only observed at 400ppm, (p<0.05).

Figure 4-9 displays the CO levels found in each of the tissues heart, liver, lung, kidney and brain, for each maternal CO exposure. A significant increase in the heart CO level vs. control was found at 100, 150, 200, 250, 300 and 400ppm (p<0.05). In the liver, a significant increase in CO levels was not observed until 250ppm, but continued to increase significantly until 400ppm (p<0.05). Both the lung and kidney showed significant increases in CO levels at 400ppm only (p<0.05), while the brain displayed a significant increase in CO level vs. control at 250ppm and 300ppm (p<0.05), but not at 400ppm. In Figure 4-10, CO levels in both the placenta and spleen are shown separately from the previous tissues, as these two tissues contained at least 4X the CO level. The placenta and spleen CO levels were both significantly different from control above and including 60ppm (p<0.05).

Histological analysis of specific fetal tissues did not show any morphological differences between those exposed to CO and controls. In Figure 4-11, representative placentas for CO doses of 0ppm, 250ppm and 400ppm CO are shown. Analysis was actually performed on at least 3 placentas per CO experiment. The tissues were reviewed for spiral arteriole modification changes, implantation site changes and overall placenta morphology. No observable differences were noted in any of the CO dose tissues vs. the control. Figure 4-12 displays a representative parietal lobe section (200X magnification) for each of the CO exposures as well as a control placenta. Analysis was performed on at least 3 brains per CO experiment and no morphological differences
were noted in comparison with the control samples. In Figure 4-13, representative sections of the left ventricle myometrial tissue (100X magnification) for each CO dose as well as a control are shown. Again, analysis was performed on at least 3 ventricular tissues per CO experiment and no morphological differences were noted compared to control tissue.

As a measure of cell death due to CO exposure, apoptotic cells were counted and expressed as a percentage of total cells per field. For each tissue examined, at least 3 fetuses were used for analysis. Three separate fields per tissue, per fetus were examined and used to calculate the mean apoptotic index. In Figure 4-14A, significance in fetal brain apoptosis is observed at 400ppm compared to control level apoptosis, with a 3% increase (p<0.05). Figure 4-14 B displays a significant increase (5.5%) in ventricular tissue apoptosis for 400ppm compared to control exposure (p<0.05). In both placental junction (maternal/fetal interface) and labyrinth, Figure 4-14 C and D respectively, no significant apoptosis was observed compared to control, however the highest apoptosis was noted at 400ppm in each case.
Table 4-1 Average gas chromatography measured CO levels and GO-Link humidity measured per CO experiment. Air samples were taken daily from the CO chamber and measured using the gas chromatography machine to ensure CO levels matched those desired and set with the computer software. All average calculated samples (Standard deviations) per CO experiment are listed. Humidity samples were recorded every 30 minutes continuously throughout each CO experiment. Averages (standard deviations) per CO experiment are listed.

<table>
<thead>
<tr>
<th>CO (ppm)</th>
<th>Average CO (SD)</th>
<th>Average Humidity (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.51 (0.40)</td>
<td>48.04 (4.05)</td>
</tr>
<tr>
<td>25</td>
<td>24.65 (1.01)</td>
<td>51.35 (3.30)</td>
</tr>
<tr>
<td>60</td>
<td>59.6 (1.60)</td>
<td>51.71 (3.60)</td>
</tr>
<tr>
<td>100</td>
<td>100.09 (2.92)</td>
<td>52.21 (4.56)</td>
</tr>
<tr>
<td>150</td>
<td>150.03 (.57)</td>
<td>49.47 (4.06)</td>
</tr>
<tr>
<td>200</td>
<td>199.93 (5.12)</td>
<td>49.06 (2.20)</td>
</tr>
<tr>
<td>250</td>
<td>251.44 (4.78)</td>
<td>53.86 (3.15)</td>
</tr>
<tr>
<td>300</td>
<td>304.94 (4.35)</td>
<td>49.08 (4.88)</td>
</tr>
<tr>
<td>400</td>
<td>403.17 (4.12)</td>
<td>48.00 (2.79)</td>
</tr>
</tbody>
</table>
Figure 4-5 Dose response of maternal and fetal CO levels and Hb to increasing maternal exogenous CO exposure. A positive trend was observed with both maternal and fetal blood %COHb vs. CO concentration exposure (A). The slope of the line significantly deviated from zero in both cases, maternal %COHb p=0.0004 and fetal %COHb p=0.0004. A positive trend was observed with both maternal and fetal Hb vs. CO concentration exposure (B). The slope of the line significantly deviated from zero in both cases, maternal Hb p<0.0001 and fetal Hb p=0.0076.
Table 4-2 The effect of maternal CO exposure on total number of resorptions and abnormalities. An increase in both resorptions and abnormalities was observed with increasing CO, however significance was only observed at 400ppm in comparison to the control values.

<table>
<thead>
<tr>
<th>CO (ppm)</th>
<th>No. of Litters</th>
<th>Total Implant.</th>
<th>Total No. Resorptions</th>
<th>% Mean Resorp./Implant. sites (SD)</th>
<th>Total abnorm. Fetal Develop.</th>
<th>% Mean of Abnorm./Implant sites (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>116</td>
<td>1</td>
<td>0.48 (1.8)</td>
<td>1</td>
<td>0.5 (2.1)</td>
</tr>
<tr>
<td>25</td>
<td>18</td>
<td>191</td>
<td>3</td>
<td>1.6 (4.5)</td>
<td>3</td>
<td>1.5 (4.2)</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
<td>214</td>
<td>4</td>
<td>2.2 (5.4)</td>
<td>5</td>
<td>2.3 (3.6)</td>
</tr>
<tr>
<td>100</td>
<td>9</td>
<td>129</td>
<td>2</td>
<td>1.4 (2.8)</td>
<td>2</td>
<td>2.0 (5.4)</td>
</tr>
<tr>
<td>150</td>
<td>9</td>
<td>126</td>
<td>1</td>
<td>1.0 (2.7)</td>
<td>2</td>
<td>1.3 (3.9)</td>
</tr>
<tr>
<td>200</td>
<td>9</td>
<td>92</td>
<td>4</td>
<td>3.1 (4.9)</td>
<td>2</td>
<td>2.1 (4.2)</td>
</tr>
<tr>
<td>250</td>
<td>8</td>
<td>103</td>
<td>3</td>
<td>2.6 (4.0)</td>
<td>1</td>
<td>1.1 (3.2)</td>
</tr>
<tr>
<td>300</td>
<td>9</td>
<td>125</td>
<td>9</td>
<td>6.3 (9.6)</td>
<td>5</td>
<td>4.3 (8.7)</td>
</tr>
<tr>
<td>400</td>
<td>6</td>
<td>83</td>
<td>7</td>
<td>13 (8.3) *</td>
<td>26</td>
<td>31.5 (2.7) *</td>
</tr>
</tbody>
</table>

Statistical Analysis performed with an ANOVA and post hoc Dunnett test, * p<0.05.
Figure 4-6 Comparison of the mean fetal and placental mass per litter for each CO exposure level vs. control. An increase in mean fetal mass was observed at 25ppm compared to control, although this was not significant (p>0.05)(A). For each of the CO exposures 60ppm to 300ppm, no significant difference was observed between mean fetal mass and mean control fetal mass (p>0.05). At 400ppm CO exposure, there was a significant decrease in mean fetal mass vs. control (* p<0.05). There was no significant difference at any of the CO exposures vs. control for mean placental mass (p>0.05).
Figure 4-7 Comparison of fetal litter size to maternal CO exposure. Litter size was variable across experiments and no observable trend was noted compared to the control group. Significance compared to control was only noted at 400ppm (p<0.05).
Figure 4-8 Maternal tissue CO levels measured in the heart, liver, lungs, kidney and brain for each CO exposure. Significance was observed in the heart tissue at all CO exposures above and including 100ppm. In the liver, significance was measured above and including 200ppm. The kidney and the lung were only significant compared to control CO levels at 400ppm, while the brain proved significant elevations in CO levels at 250ppm and 300ppm only (* p<0.05).
Figure 4-9  Maternal placenta and spleen tissue CO levels with increasing CO exposure. Significance was observed in both placenta and spleen above and including 60ppm CO exposure, compared to control levels (* p<0.05).
Figure 4-10 Representative comparison of placenta morphology using H&E staining for each maternal CO dose. A representative placenta is shown at 10X magnification for CO exposures 0ppm-A, 250ppm-B and 400ppm-C-I). The areas in boxes were then imaged at 40X magnification and present as 0ppm-A1, 250ppm-B1 and 400ppm-C1. No observable differences were observed in the spiral arteries, the implantation layer or the overall placenta, between the CO exposure groups when compared to control placentas.
Figure 4-11 Representative comparison of brain morphology using H&E staining for each maternal CO dose. A representative brain was imaged at 200X magnification for each CO exposure (0ppm-A, 25ppm-B, 60ppm-C, 100ppm-D, 150ppm-E, 200ppm-F, 250ppm-G, 300ppm-H and 400ppm-I). No differences were observed between the CO exposure groups when compared to control brains.
Figure 4-12 Representative comparison of heart ventricular morphology using H&E staining for each maternal CO dose. A representative ventricle myocardium (green line) was imaged at 200X magnification for each CO exposure (0ppm-A, 25ppm-B, 60ppm-C, 100ppm-D, 150ppm-E, 200ppm-F, 250ppm-G, 300ppm-H and 400ppm-I). No differences were observed between the CO exposure groups when compared to control ventricles.
Figure 4-13 Apoptotic index for fetal brain, heart, placental junction and labyrinth for each CO experiment. Each separate fetal tissue was analyzed for apoptosis in three sections and a mean per fetus was calculated. For each CO concentration, a mean of 3 to 5 fetal tissue means was calculated and this value is presented as a point on the graphs. Statistical significance was observed at 400ppm for the fetal brain (A) and fetal heart tissues (B), with a higher index observed in the fetal heart tissue. Variation existed in the placental junction (C) and labyrinth (D) apoptosis counts across CO levels, the highest apoptotic index was observed at 400ppm in the placental labyrinth. No statistical significance was found for any CO level compared to control in the placental junction or labyrinth. (Statistical significance was calculated with an ANOVA and post hoc Dunnett’s multiple comparison test, *p<0.05)
4.5 Discussion
A number of studies have evaluated the effects of CO exposure on pregnancy, however a direct comparison of these studies is difficult, as several parameters vary between them. Firstly, the method of delivery of CO (or even cigarette smoke) can vary from whole body \(^{227-229}\) to nose-only inhalation \(^{225,230-232}\). Time exposure (acute vs. chronic) varies; daily time intervals \(^{196}\), specific days of gestation \(^{225,233}\) or constant exposure \(^{224}\). The dosing levels are consistently different between experiments, with many studies evaluating the extreme CO levels (1000ppm to 10 000ppm) \(^{196,197,223}\), well above the range of toxicity. Lastly, the chosen species of animal used in each experiment is variable and can lead to varying results even with the same CO exposures.

Our study’s aim was to use a highly regulated CO chamber system (designed by our own laboratory) to expose pregnant mice chronically (GD-1 to GD-17) to CO levels that would mimic those of smoking women (<500ppm).

Maternal cigarette smoking is the most common method by which biological CO levels are increased \(^{225}\). Cigarette smoke contains an average of 40 000ppm CO by volume, and during the smoking process it becomes diluted in air, leaving the alveoli to actually see roughly 400-500ppm \(^{234}\). It is well known that CO can cross the placental barrier and therefore in pregnancy, CO can affect the fetal partial pressure of O\(_2\) (pO\(_2\)) \(^{84}\).

In the present study, the highest dose of CO (400ppm) yielded a mean maternal %COHb level of 15.6 (Figure 4-5A) with a significant dose response of maternal %COHb to CO exposure, \(p=0.0004\). Women who heavily smoke cigarettes during pregnancy have a calculated CO level of up to 22.4%COHb \(^{190}\), confirming that our study CO doses caused %COHb levels similar to those of smoking women. It is important to note however, that in comparison with women who
intermittently smoke, and thus are affected by peaks and troughs of high CO exposure, these mice were subjected to CO levels on a constant basis. Also of importance are the elevated ventilation and elimination rates, along with increased CO uptake present in smaller mammals (in this case mice) compared to humans. Thus, the %COHb values can not be evaluated directly, but can be used as representative values to compare the mouse model with the effects in pregnant women.

As an adaptation response to increased CO levels (as well as hypoxia), it is well documented that mammals will increase both Hb concentration, as well as hematocrit ratio in order to increase the O2-carrying capacity of the blood. In Figure 4-5B, a significant dose response is observed with maternal Hb to increasing CO exposure levels, p<0.0001. In the pregnant human, Hb levels are roughly 100-120g/L and one would expect a similar increase to occur as was observed with murine Hb levels.

Fetal %COHb levels and Hb levels followed the same trend as maternal values, with a significant dose response over CO exposure observed in each, p=0.0004 and p=0.0076 respectively. Interestingly, fetal blood %COHb was 1.8X higher than matched maternal values, which is in agreement with a study conducted in pregnant sheep, as well as human studies (using umbilical blood as a fetal representative value). The Hb levels of both maternal and fetal systems differ, and thus their affinities for CO do as well. The affinity of fetal Hb for CO is established to be higher than that of the maternal system. This, coupled with the lower pO2 in capillary blood of the fetus vs. maternal system, would add to the effect of increased fetal %COHb/maternal %COHb. Maternal COHb ratios depend on CO exposure, fetal and maternal endogenous CO.
production by heme catabolism, elimination rate and the maternal Hb affinity. In the fetus, however, %COHb depends on the partial pressure of CO (pCO) in the maternal circulation, fetal endogenous production, CO elimination by placental exchange and fetal Hb affinity for CO. The maternal system may increase its ventilation rate in order to eliminate higher than normal CO levels, while the fetus is unable to do so and is entirely dependent on the maternal system to decrease CO levels; thus there is a lag time between maternal and fetal %COHb levels returning to normal.

There is some discrepancy when reviewing the effects of CO exposure on fetal birth weight. It is well established that pregnant women who smoke cigarettes will give birth to a neonate roughly 200g lighter (per pack per day) than a fetus born to a non-smoking mother. It is unclear whether or not CO is the culprit in cigarette smoke that induces the decrease in birth weight. Wouters EJM and colleague and Soothill PW and colleagues studied the human effects of increased fetal COHb on fetal weight with data in both cases that was not convincing for a simple cause-effect relationship. Our study found a slight increase in fetal weight at 25ppm (although not significant) followed by a significant decrease in birth weight compared to control at 400ppm CO (Figure 4-6), which is in agreement with a study performed in pregnant sprague dawley rats exposed to 480ppm CO. Perhaps the increased placental perfusion effects of CO aided in increased nutrient transfer and helped to increase fetal growth until a toxic effect was observed at 400ppm. Other studies have also found a decrease in birth weight with increased CO exposure, however the species of animal, the CO exposure level and time interval differed among all studies. Singh and Scott found that subjecting pregnant CD-1 mice (on GD 7 to GD 18) to CO exposures from 0-500ppm, lead to a significant decrease in fetal weight as early as 125ppm. As this study’s dosing regime was different than our own study, it is difficult to
compare the two directly. Perhaps the fetus is better suited to adapt to CO level exposures when subjected from conception, rather than a system shock mid way through pregnancy. It would appear that Singh and Scott performed a study that examined the effects of accidental CO poisoning throughout pregnancy, rather than low smoking CO levels.

Interestingly placental weight was not significantly different than the control at any of the CO doses. Bissonnette and Wickham reported that placental CO diffusing capacity increased significantly with increased gestational age and correlated with fetal weight but not placental weight. Thus, as CO levels increased across our experiment, no correlation would have been observed with placental weight.

Our litter size (number of healthy fetuses) measurements are consistent with fetal birth weight, with a significant decrease observed at 400ppm CO. This was unexpected, as fetal litter size and fetal weight are generally negatively correlated with each other. Litter size was not shown to be affected by CO exposure at doses of 150ppm in rats or 200ppm in rats, while a reported a 3400ppm exposure in rats did significantly decrease litter size. Our data is coherent with the increase in both resorptions and abnormalities observed at 400ppm. Thus, perhaps litter size appeared to be smaller due to significant late gestational deaths and abnormalities which occurred. An increase in resorptions and abnormalities above 180ppm maternal exposure was observed in a number of animal models, although CO exposure levels and dosing schedules were different amongst them. Our study showed abnormalities to be higher in the 400ppm group as compared to resorptions and this may have been due to the increase in diffusing
capacity in the placenta with increasing gestational age\textsuperscript{85}, thus a more deleterious effect in the latter portion of pregnancy.

Tissue CO levels showed that the gas was not only “mopped up” by the heme moiety found in Hb, but also by the heme containing molecules found throughout tissue. In comparison to a study conducted by Vreman \textit{et al.}\textsuperscript{119}, our tissue CO levels at control CO exposures closely approximated the values expressed in their study. Although the study involved exposure of a rat to 500ppm CO followed by subsequent tissue CO measurement, the same was not conducted with a mouse and therefore no comparison of tissue CO levels exists for our experiment. In the current study, both the spleen and the placenta were the most difficult tissues to perfuse as their open circulation did not allow for entire blanching of blood from the tissue. This would explain the much higher CO levels (Figure 4-9) compared to the other tissues sampled (Figure 4-8). A dose response was observed in both tissues. Of the other five tissues measured, the heart expressed the highest CO levels and was significantly increased compared to control above 60ppm. This could be due to the high levels of Mb, a heme containing protein with an affinity for CO second to Hb, present in the heart\textsuperscript{127}. While an increase in CO levels was observed in each of the liver, lung and kidney, it is unclear as to why a peak CO measurement was observed at 250ppm CO in the brain. To our knowledge, there is only one other study that evaluated the levels of CO in tissue, which was eluted to earlier\textsuperscript{119}. Thus, it is not possible to determine a threshold of toxicity per tissue based on the literature available to date.

No differences were observed in any of the fetal tissues analyzed for morphological changes compared to control. This finding was confirmed by a General Pathologist in the department.
The same was not true in the apoptotic index, as both an increase in brain and heart apoptosis were observed at 400ppm. A known adaptation response to increased CO levels is an increase in fetal heart weight (cardiomegaly), despite a reduced body weight at birth. It is possible that increased CO above a certain threshold induces a hypoxic environment that leads to an increase in heart tissue death. In order to account for the growing number of apoptotic cells, the heart tissue may attempt to increase its cell count number or cell volume, thus lending to an increased size. Studies have shown that the removal of a rat neonate from an environment of CO (200ppm) after birth returned the neonatal heart to a normal size most likely due to a reduced cell volume, rather than cell number.

Neurological consequences as a result of fetal CO exposure are of great interest to researchers. Our study showed a significant increase in fetal brain apoptosis at 400ppm compared to control. It is unclear as to why an increase was first observed at 100ppm CO exposure, however subsequent increases in doses did not match or rise in apoptosis counts. To our knowledge no other study has specifically studied the apoptosis counts in fetal brain tissue following prenatal maternal CO exposure. Piantadosi and colleagues exposed young male rats to toxic levels of CO (2500ppm) for 30 minutes and reported levels of apoptosis in cerebral cortex and hippocampus compared to control. Studies have evaluated the neurobehavioural effects of prenatal CO exposure (150ppm) in rats and have reported a decrease in locomotion response, in locomotor response to L-Dopa, in homing behaviours and in the development of geotaxis decrease in retention deficits. Morris et al. reported that a 250ppm prenatal CO exposure in piglets decreased their fetal activity in an open field. Perhaps the apoptosis seen in brain tissue in our fetal mice is associated with the neurobehavioural effects observed in these aforementioned...
studies and the increased cell death observed at 100ppm may be important to review further, as neurobehavioural changes have been seen as early as 150ppm exposure.

In this study, we showed that at levels of 400ppm maternal CO exposure, a number of toxic results were noted. Maternal and fetal %COHb as well as Hb values were maximal, fetal birth weight and litter size were significantly decreased compared to control and resorptions and abnormalities were significantly increased compared to control. Tissue CO levels were maximal at 400ppm, while apoptosis in both the heart and the brain tissue were significantly elevated as well. These results indicate a threshold level of CO exposure in the murine animal model at 400ppm.
Chapter 5
General Discussion

5.1 Main findings of Thesis
The contents of this thesis evaluated the gaseous molecule CO in relation to pregnancy. Specifically, the answers to three main questions were sought after:

1. What are the biological levels of CO in human pregnancy, including women who smoke cigarettes, as well as those complicated by PE?

2. What is the threshold level of exogenous maternal CO exposure in the murine animal model?

3. Which automated CO measuring device correlates best with the gold standard in CO measurement, blood sampling, in order to find an accurate and rapid CO measuring device for hospital use?

The objectives were all completed and the results will be further discussed, specifically in relation to PE.

5.2 Biologic CO Levels: a Step Towards a Potential PE Therapeutic
For decades it has been recognized that women who smoke cigarettes during pregnancy are at a reduced risk in developing PE \(^{205,254-256}\). The problem was in identifying what substance in cigarette smoke (of which there are over 4000) \(^{112}\) was the reason for the protection. A Swedish study reported that the use of snuff (a smokeless form of tobacco) did not produce the same decreased incidence of PE \(^{22}\) and led to the idea that the combustible gas CO, produced while smoking cigarettes, may in fact offer a so called “protective” effect against the disease. To
further lend support to the hypothesis, a study conducted by Baum and colleagues found that women with PE had significantly lower end-tidal CO levels than those without the disease.

The main pathophysiological effects of PE were discussed at length in Chapter 1, however they will be briefly reviewed here. Several insults contribute to what is known about the development of the disease, including inadequate spiral artery remodelling in the decidua, decreased and pulsatile blood flow to the placenta, placental oxidative stress leading to apoptosis and necrosis, shedding of placental debris and maternal endothelial dysfunction. As CO has been identified as offering possible protection from the disease in smoking pregnant women, a few in vitro studies have looked at the possible roles in which it may be useful. CO functions similarly to its diatomic cousin NO, and so it is possible that CO may take the place of this molecule in women with PE, as NO’s function has been found to be reduced in these cases. Carbon monoxide has also been shown to increase vasodilation in the placenta and resistance vessels, as well as decrease placental perfusion pressure. Further, CO has shown the ability to decrease inflammation and apoptosis and necrosis. Therefore, in a number of separate studies, CO has shown the ability to decrease several of the etiologies associated with PE and further implicates that idea of using this molecule as a potential therapeutic.

In order to assess what levels of CO may be used as possible treatment for PE, biological levels of CO needed to be identified in normotensive pregnant women ± smoking as well as PE ± smoking. We were able to measure the CO levels in 74 pregnant women, although the levels in PENS (n=3) as well as PES (n=1) were too small to analyze statistically. The measurements in PE patients did however allow us to observe the lower %COHb levels in PENS vs. NTNS women
as well as the lower %COHb levels in the sole PES vs. the NTS group, which would be in line with the study conducted by Baum and colleagues. However, the values may have simply been a result of low sample numbers and would need to be further explored. As expected, the levels of blood CO in NTN women were significantly lower than those in NTS women and ranged from 0.33- 1.8 %COHb. The range of CO levels in smoking women was 1.5- 9.85 %COHb. Literature shows that maternal cigarette smokers can have levels up to 22.4%COHb, thus our results do not represent the possible upper margin of CO levels in pregnant smoking women. However, the range observed in this study can be used as a guideline for future CO studies.

5.2.1 Maternal CO Exposure and the Effects on the Developing Fetus
In the event that CO is used as a therapeutic in the future, achieving the specified maternal levels of CO in pregnant women to match those of NTS would involve exposure to a certain level of exogenous CO. The specific level of CO was unclear and was tested using a mouse model of pregnancy. The effects of the exogenous maternal CO exposures on the developing fetus were also studied, in order to determine a threshold of CO exposure.

Levels of CO administration in this study ranged from 0-400ppm, where maternal mice were exposed in a whole body chamber to CO chronically. In comparison to smoking women who experience peaks and troughs of high CO levels, our chronic experiment represented the extreme case effect of each CO dose administered. In the maternal system, %COHb levels increased in a dose dependent manner with increasing CO doses. In comparison with the human pregnancy study, a %COHb similar to that observed with the highest value NTS patient (9.85%COHb), was
found between 100ppm and 150ppm CO exposure. Animal models can not be compared directly with human data, however, the CO levels were good indicators for relative comparisons.

Although a positive finding has been reported with increased cigarette smoking and a decreased incidence of PE, there exists a plethora of literature outlining the negative effects of cigarette smoke on pregnancy. To name a few, cigarette smoking has been found to decrease fetal birth weight\textsuperscript{105,239,240} and has been associated with an increase in fetal morbidity\textsuperscript{24,106} sudden infant death syndrome\textsuperscript{107}, spontaneous abortions and placental abruption and previa\textsuperscript{110,111}. Our study was able to review a few of the fetal effects due solely to CO exposure, eliminating the numerous chemicals also introduced when a mother lights up a cigarette. Firstly, fetal \%COHb was found to increase in a dose dependent manner and was also observed to be 1.8X that of the matched maternal levels. This finding is in agreement with other researchers\textsuperscript{87,88,241} and is important to note when reviewing the possible doses of future CO treatment. The fetus is thought to be more susceptible to the negative effects of CO exposure, mainly due to its lower pO\textsubscript{2} and thus an already established leftward shift of the oxyhemoglobin (O\textsubscript{2}Hb) dissociation curve\textsuperscript{56}. With increased CO, the pO2 levels would decrease even further and shift the O\textsubscript{2}Hb dissociation curve more so in the leftward direction. This would further attenuate the ability of O\textsubscript{2} delivery to the fetal tissues and produce a state of hypoxia. It is unclear exactly at which level of maternal or fetal CO level this hypoxic state is found, although at levels of 400ppm, our study showed an increase in apoptosis in both the fetal heart and the fetal brain tissues, as well as an increase in fetal resorptions and fetal abnormalities, perhaps indicative of a decrease in O\textsubscript{2} levels.
We found no statistical differences in fetal weight or litter size based upon CO exposure until 400ppm, at which point a significant decrease was observed. At this level of CO maternal %COHb levels were 15.6% CO and therefore were higher than even our highest human smoking patient (9.85%COHb). This further confirmed that our human NTS range of CO levels was adequate to determine a possible therapeutic dose for PE. Comparatively, the biological threshold in humans that is identified as having non detrimental effects is 15-20% COHb and again our CO level dose is below this level.

This study was able to conclude that a threshold level of CO exposure in pregnant mice exists at 400ppm, at which point fetal toxicities were noted. At levels of 100-150ppm, maternal %COHb levels match those of human moderate smokers, with no significant differences in the fetuses versus controls. In this way, this level of CO offers a possible treatment dose for future follow-up studies.

5.3 Identification of a Rapid and Accurate Automated CO Measurement Device

The current reference method for CO measurement is through blood sampling and is a time consuming and tedious process. It is impractical to use this method for rapid testing of CO levels in patients, specifically with the desire to ensure safe levels of treatment CO administration in future studies. To aid with this dilemma, two automated CO measurement devices were tested for accuracy compared with blood CO sampling on the 74 pregnant women recruited.
Although each of the automated devices tested for a different CO measurement, (end-tidal breath CO vs. pulse %COHb oximetry CO), they were each expected to correlate well with blood %COHb. The end-tidal breath CO device proved to have a higher correlation with blood %COHb, with a coefficient of \( r=0.8523 \) compared to \( r=0.4644 \) of the oximetry device. Both machines were calibrated by their respective manufacturers. This study identified the end-tidal breath analyzer as the more accurate automated device between the two sampled, as well as being well correlated with blood sampling. Future studies involving clinical CO work may adopt this device as an accurate measurement tool in order to decrease testing time restraints and to obtain rapid results.

5.4 CO use as a Therapeutic

Carbon monoxide has been reported to correlate well with cases of both severe stress and survival \(^{258,259}\). An increase in CO levels was measured in critically ill patients with cardiac disease, those on dialysis \(^{260}\), and those with sepsis \(^{146}\). Perhaps each of the patients’ own body systems were attempting to increase CO levels in order to aid with the less than healthy state they were in, likely through the HO/CO system. In the case of survivors, it is possible that their systems were able to produce enough CO to decrease the negative effects of their illness. An \textit{in vitro} lung epithelial study showed that through HO-1 over expression and/or CO gas treatment to lung epithelial cells, a resistance to oxidative damage, apoptosis and ischemic lung injury was observed \(^{261}\).

Recently, allograft rejection has been diminished and even inhibited with the use of CO \(^{111,148,199,262}\). Inadequate perfusion, increased inflammation, oxidative stress and necrosis are all
akin to an allograft rejection \(^{47}\) and CO has been shown to ameliorate a number of these issues. Ischemia/reperfusion injury was attenuated in cardiac rejection transplants by exposure of the tissue to CO \(^{263}\). An interesting study looked at the survival of transplanted organs taken from patients having died in CO poisoning events \(^{264}\). In these clinical cases, the transplanted patients had overall satisfactory recovery, indicating that CO-poisoned hearts were still adequate for transplant survival. In other allograft improvement studies, successful therapeutic approaches using CO gas have used organ harvesting with CO or recipient CO inhalation \(^{111}\), CO releasing compounds \(^{265}\) or the upregulation of CO production by an over expression of HO \(^{262,265}\).

As CO has currently been used with clinical work, such as allograft rejections, the possibility for CO use as a therapeutic for PE is even more encouraging. The delivery of CO to patients with PE by any of the systems used for allograft transplant studies seems plausible, however these would need to be further studied to evaluate which method is associated with the best maternal and fetal response.

5.5 Final Conclusions

The results of this thesis have indicated a range of biologic CO levels in pregnant human women that may be useful in determining a future CO dose as a therapeutic in the treatment of PE. Further, we determined which automated device was best suited to measure CO rapidly and accurately compared to blood sampling; as a more convenient method for hospital use. Lastly, a threshold of maternal CO exposure levels was determined, at which point fetal toxic effects were observed. These conclusions will be of benefit for future studies that continue to test the beneficial effects of CO against the etiologies of PE.
Future Studies
The results of this thesis answered a few important questions regarding CO and PE, however, they have also produced far more inquiries, leading to a number of possible experiments in the future. Additional studies in both endogenous production and exogenous exposure to CO are warranted, in order to further elucidate the protective nature that CO seems to offer against PE.

Our study evaluated biological CO levels in pregnant women and was able to determine a range of CO in those who smoked cigarettes that may be useful for a future target therapeutic range in PE patients. Our n values in both of the PE groups (PENS and PES), however, were not high enough to perform statistical analyses and therefore, it would be interesting to continue the study specifically to increase the recruitment of volunteers with PE. In this way, we could confirm the trends that were evaluated in the present study, where CO levels were lower than even NTN women.

The second study performed in this thesis was able to determine a threshold level (400ppm) of exogenous CO exposure in a pregnant murine animal model. At levels of 100 and 150ppm, maternal CO levels matched those of women who smoke during their pregnancy, while no negative fetal effects were observed. A future experiment may repeat this study with CO levels of 60ppm to 150ppm, while evaluating a number of different parameters. For instance, measuring the effect of blood pressure using radio telemetry devices throughout pregnancy, would offer a very useful indication of maternal adaptive responses to the CO treatment. Concurrently, blood flow analysis with ultrasound technology, could allow for further analysis of the effects on the circulatory system within vessels of the uterus, umbilical cord, and placenta.
(spiral and intra-placental arteries). We conducted a pilot ultra-sound study to evaluate the circulatory effects on one mouse from each of the CO exposures (25, 60, 250, 300 and 400ppm) and specifically to determine the best vessels for analysis, based on fetal size at GD17. Figure 5-1 displays a representative ultrasound image, measuring blood flow velocity through an umbilical artery. The aim would be to use nine mice per experiment (the maximum number to fit in the CO chamber box) and ultrasound each mouse throughout gestation, in each of the vessels listed above. This would allow for matched measurements within mice, at different GD times, as well as a comparative analysis between CO exposures. Our primitive data (Figure 5-2) was not able to draw any conclusions as to the vasculature effect of CO due to the small n value (n=1) per CO experiment. Larger sample sizes will undoubtedly lead to interesting data about the vascular effects of CO on pregnancy.

Lastly, a spontaneous genetic mouse model of PE was reported by Davisson and colleagues with elevated blood pressure and urine protein levels similar to PE in humans. It would be interesting to expose this model to levels of CO that we propose as a possible therapeutic dose and to evaluate the effects on blood pressure and protein levels.
Figure 5-1 Representative figure for ultrasound analysis of blood flow through the umbilical artery. Each peak represents the velocity of blood flow at maximal contraction of the blood vessel. A mean of five peaks is used as a representative value in determining the actual blood flow through each vessel.
Figure 5-2 Evaluation of flow velocity in maternal and fetal blood vessels following maternal CO exposure. For each CO level, one mouse was analyzed for blood flow velocity. Bar graphs for uterine artery (uterine A) and umbilical vein (UV) represent a mean of five velocity peaks for each mouse. Bar graphs for intraplacental artery (IP A) and umbilical vein (UV) represent a mean of five velocity peaks for each of three measured areas per mouse. An increasing trend was observed in the maternal uterine A from 25ppm to 300ppm, but dropped in velocity at 400ppm. No observable trend was established in any of the other blood vessel flow measurements.


92. Wilhelm M, Ritz B. Local variations in CO and particulate air pollution and adverse birth outcomes in Los Angeles County, California, USA. Environ Health Perspect 2005;113:1212-1221.


123


