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CORM-A1 treatment leads to increased carbon monoxide in pregnant mice

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1. Introduction

Preeclampsia (PE) is a leading cause of maternal and neonatal morbidity and mortality, affecting 5–8% of pregnancies worldwide [1]. Studies have shown that women who smoke cigarettes (but not smokeless tobacco products), have a 33% lower incidence of PE [2–4]. Gaseous carbon monoxide (CO), when inhaled as a by-product of cigarette combustion, leads to elevated serum carboxyhemoglobin (COHb) in smokers [5]. Pregnant women who go on to develop PE have significantly reduced levels of breath end tidal CO (EtCO) compared to women who have had uncomplicated pregnancies [6]. Based on these findings, it is suggested that the reduction in PE risk associated with smoking may be due, in part, to CO exposure.

CO is toxic at high doses; with most exposures a result of accidental inhalation [7]. The dangers associated with high levels of CO arise from its preferential binding to hemoglobin (Hb), with an affinity 220 times greater than that of oxygen [8]; CO binds circulating Hb and reduces the oxygen carrying capacity [7,8], and at high levels, ultimately leads to hypoxia. Lesser known is the role of CO as a physiological signaling molecule [9] involved in control of normal vascular tone [10–13], as well as having anti-inflammatory [14], anti-apoptotic [15,16], and cytoprotective activity [17]. CO is produced endogenously as a by-product of the catalytic degradation of heme by the heme oxygenase (HO) enzyme [18]. HO-1, the inducible isoform of the enzyme, is expressed throughout the body, including the placenta [19], and is up-regulated under conditions of cellular stress [18].

Imbalance in the HO/CO system has been observed in PE [20] and this axis may be a promising target for the development of a therapeutic for PE [9,21–25]. Use of CO in cell culture and animal models has been shown to reduce inflammation [26], apoptosis [16], and increase vasodilation and angiogenesis of blood vessels in the placenta [27]; properties that could be beneficial in the treatment of PE. It has been previously shown that 250 ppm gaseous CO in pregnant CD-1 mice produces COHb similar to women who smoke during pregnancy (10–13%) [28], with no demonstrable adverse effects on the pregnancy outcome [28]. Also, chronic CO inhalation was able to prevent hypertension, proteinuria and renal changes in a murine model of PE [29].

The use of gaseous CO as a therapeutic has significant challenges in regards to optimizing a method of delivery that results in accurate dosing [24]. **C**arbon mon**o**xide-**r**eleasing **m**olecules (CORMs) have been developed with these challenges in mind, and may provide a more feasible alternative to deliver CO *in vivo* in controlled doses. These molecules have received attention for their pharmaceutical applications as they only moderately increase COHb, and as such, may have similar beneficial effects to inhaled CO with a lower risk of toxicity or adverse health outcomes. CORMs have been developed with different chemical and physiological properties, including solubility, half-life, and mechanism of CO release, including temperature, pH, or light-sensitivity [30–32]. CORMs have been studied in a number of animal models of disease, including inflammation [26], ischemia-reperfusion injury [33,34], and renal vascular response [35]. One such study investigated the effects of CORM-3 in a PE rat model demonstrating that use of CORM-3 in late gestation attenuated hypertension, postulated to be through increased glomerular filtration rate (GFR) [36]. Although there is evidence showing the effectiveness of CORMs on disease outcomes, very little has been reported on their use and safety during pregnancy. This work is critical if we wish to employ the use of CORMs as a source of exogenous CO in the development of therapeutics for pregnancy complications, such as PE.

CORM-A1, a water-soluble, slow-releasing CO donor, has been previously shown to have hypotensive and vasodilatory effects *in vivo* [37]. We hypothesize that the use of CORM-A1 in normotensive mice at

Abbreviations: PE, pre-eclampsia; CO, carbon monoxide; COHb, carboxyhemoglobin; EtCO, end-tidal carbon monoxide; Hb, hemoglobin; HO, heme-oxygenase; CORM, carbon monoxide-releasing molecule; GFR, glomerular filtration rate; CORM-A1, carbon monoxide-releasing molecule-A1; BP, blood pressure; HR, heart rate; UACC, university animal care committee; GD, gestational day; iCORM-A1, inactive CORM-A1; IP, intraperitoneal; IV, intravenous; °C, degrees celcius; EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; SSA, sulfosalycylic acid; EGD, early gestational demise; LGD, late gestational death; SD, standard deviation; ANOVA, analysis of variance; SBP, systolic blood pressure

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mid-gestation will increase COHb and decrease blood pressure (BP) transiently without any embryotoxic effects or adverse pregnancy outcomes. The specific aims of this study were to a) quantify increases in systemic COHb and alterations in BP and heart rate (HR) following treatment with varying doses of CORM-A1, and b) measure pregnancy outcomes, including implantation sites, live pups, early and late resorptions, fetal weight and placental weight.

2. Methods

2.1. Animals and husbandry

All experimental procedures were approved and carried out according to protocols of the University Animal Care Committee (UACC) at Queen's University (REB #2016-1641), in compliance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Female CD-1 mice (5–7 weeks, Charles River, USA) were mated overnight with males (> 8 weeks old) of the same strain. The detection of a copulation plug the following morning was deemed gestational day (GD) 0.5. All mice were provided food and water *ad libitum*. Mice were housed in a 12 h light:dark cycle.

2.2. Treatment with CORM-A1

CORM-A1 was chosen as the CO donor drug for this study as it is water-soluble, releases CO under physiological conditions, and has the longest CO dissociation rate of all commercially available CORMs. On GD10.5 mice were randomly allocated to one of six groups; four treatment groups (single dose of 5 mg/kg, 8 mg/kg, 10 mg/kg CORM-A1 (Sigma-Aldrich, MO, USA), or daily treatment with 5 mg/kg CORM-A1 on GD10.5-12.5) and two control groups (saline or inactive CORM-A1 (iCORM-A1)). CORM-A1 treatment doses were selected based on previous *in vivo* studies measuring beneficial effect of CORM-2, CORM-3, or CORM-A1 on disease outcomes. Most literature cited doses of CORMs ranged from 2 to 40 mg/kg [17,26,33,34,36,38,39], including the doses of 5 mg/kg [36], 8 mg/kg [26,33,34], and 10 mg/kg [39] (delivered by intravenous (IV) or intraperitoneal (IP) injections) that we selected for the current study. The subset of mice treated with 5 mg/kg CORM-A1 on GD10.5-12.5 were used to determine if daily administration of CORM-A1 would have any additive effect on systemic %COHb. The treatment window of GD10.5-12.5 was selected based on the suggested time frame for the establishment of placental circulation. It has been shown in murine pregnancy on GD9.5 that there is a relationship between trophoblasts and decidual vessel epithelium and continued angiogenesis in the decidua at the maternal/fetal interface [40]. CORM-A1 was solubilized in sterile saline (0.9% NaCl, BioShop, ON, Canada) immediately prior to treatment. The inactive form (iCORM-A1) was prepared by exposing 10 mg/kg CORM-A1 to room air for a minimum of 24 h prior to use, allowing CO to dissociate from the solution, as previously described [38]. Treatments for dosing studies were delivered via IP injection. A different subset of mice were used to obtain BP measurements. These mice received iCORM-A1 (5 mg/kg) or CORM-A1 (5 mg/kg) daily on GD10.5-12.5 by tail vein IV injection (in order to accommodate injections while mice were in BP restraint).

2.3. BP measurement

Starting on GD0.5, BP and HR were measured daily using the CODA volume-pressure recording tail cuff BP system (Kent Scientific, CT, USA), with recordings monitored and calculated using CODA4.1 software. During all BP measurements, mice were placed in a restraint and body temperature was maintained at 32–35 °C using a warming platform. Average time in the restraint was 15.21 ± 3.34 min and 17.79 ± 2.99 min pre- and post-treatment respectively. For BP to be considered representative, a minimum of 5 successful recordings were required in each daily BP session per mouse. The mean of all successful measurements (≥5) on that day was taken as a true reading. BP was recorded daily from GD0.5–17.5.

2.4. Blood collection for CO quantification

Blood (50–100 μL) was collected via submandibular puncture into tubes containing ethylenediaminetetraacetic acid (EDTA) (BioShop, ON, Canada), stored on ice and processed within 10 min of collection. Samples from 5 control mice were collected prior to treatment to establish a baseline of endogenous CO levels. Samples (50 μL) collected from all mice on days of iCORM-A1 or CORM-A1 treatment were at timed intervals post-injection. At each time point (15–90 min) 5–8 mice were used to collect samples, however the same mice did not provide samples at every time point due to blood collection restrictions from the UACC based on total mouse blood volume. For mice receiving CORM-A1 daily on GD10.5-12.5, blood samples were collected only at 15, 30 and 45 min post-injection (n = 5).

2.5. Measurement of %COHb

Blood CO was determined by gas chromatography (GC) as previously described [41,42]. Hb was quantified in duplicate using a Hemocue Hb201 (Hemocue, Sweden). Amber vials (2 mL, Sigma-Aldrich, MO, USA) capped with 8 mm silica septa (Chromatographic Specialties, C223710C, ON, Canada) and filled with 20 μL of 2% sulfosalicylic acid (SSA) (Sigma-Aldrich, Cat#86193, MO, USA) were prepared. Blood (0.2 μL–1.0 μL, based on CORM-A1 dose and time post-injection) was added into vials using a gas-tight Hamilton syringe and repeater system (Hamilton, USA). Vials were stored on ice for 45–180 min before analysis using head-space GC (Peak Laboratories, CA, USA). %COHb was calculated using the measured Hb and the CO-binding capacity of Hb [42].

2.6. Experimental procedures at the time of sacrifice

Mice were anaesthetized on GD17.5 by IP injection of 110 mg/kg sodium pentobarbitol (Ceva Santé Animale, McGill University, Canada). Each uterine horn was excised and the number of implantation sites was recorded. The number of live fetuses, early gestational demise (EGD), and late gestational deaths (LGD) was recorded for each litter. Wet weights were recorded for each fetus and matched placenta.

2.7. Statistical analysis

%COHb measurements are presented as mean ± standard deviation (SD) and were analyzed using Kruskal-Wallis test with Dunn's test for multiple comparisons. Comparisons between treatment groups at 15-min post-CORM-A1 or iCORM-A1 injection were analysed using 2-way analysis-of-variance (ANOVA). Data on all pregnancy outcomes (implantation sites, live fetuses, EGD, LGD, fetal weight, and placental weight) were expressed using box and whisker plots, graphed using the median and 5-95th percentiles. Pregnancy outcome data were described as mean ± SD and analyzed by Kruskal-Wallis test. Raw BP and HR data was collected by CODA4.1 software and expressed at mean ± SD, and analyzed by multiple T-test using Holm-Sidak correction. All analyses were completed using GraphPad Prism v6.0 with a significance of $p < 0.05$.

3. Results

3.1. CORM-A1 increases COHb in pregnant mice

COHb was measured for 90 min following CORM-A1 treatment in pregnant dams on GD10.5 (Fig. 1). In both the saline (0.61 ± 0.09%, n = 5) and iCORM-A1 (1.36 ± 0.91%, n = 6) control groups there was