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Maternal hypoxia and caffeine exposure depress fetal cardiovascular function during primary organogenesis

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Abstract

Aims—Hypoxia is known to influence cardiovascular (CV) function, in part, through adenosine receptor activation. We have shown in a mouse model that during primary cardiac morphogenesis, acute maternal hypoxia negatively affects fetal heart rate, and recurrent maternal caffeine exposure reduces fetal cardiac output (CO) and down-regulates fetal adenosine A_{2A} receptor gene expression. In the present study, we investigated whether maternal caffeine dosing exacerbates the fetal CV response to acute maternal hypoxia during the primary morphogenesis period.

Methods—Gestational day 11.5 pregnant mice were exposed to hypoxia (45 second duration followed by 10 minutes of recovery and repeated 3 times) while simultaneously monitoring maternal and fetal CO using high-resolution echocardiography.

Results—Following maternal hypoxia exposure, maternal CO transiently decreased and then returned to pre-hypoxia baseline values. In contrast to a uniform maternal cardiac response to each exposure to hypoxia, the fetal CO recovery time to the baseline decreased, and CO rebounded above baseline following the 2nd and 3rd episodes of maternal hypoxia. Maternal caffeine treatment inhibited the fetal CO recovery to maternal hypoxia by lengthening the time to CO recovery and eliminating the CO rebound post-recovery. Selective treatment with an adenosine A_{2A} receptor antagonist, but not an adenosine A₁ receptor antagonist, reproduced the altered fetal CO response to maternal hypoxia created by caffeine exposure.

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Disclosure

There is no financial conflict of interest related to the current research.

Conclusions—Results suggest an additive negative effect of maternal caffeine on the fetal CV response to acute maternal hypoxia, potentially mediated via adenosine A_{2A} receptor inhibition during primary cardiovascular morphogenesis.

Keywords

adenosine receptor; caffeine; fetus; hemodynamics; hypoxia

Introduction

The period of primary cardiovascular (CV) morphogenesis is also time of greatest vulnerability to epigenetic events (1). During this critical developmental window the fetal CV system is the first functioning organ to transport oxygen and nutrients between the utero-placental and feto-placental circulations (2). A broad range of developmental errors can occur during this time and the investigation of CV morphogenesis and adaptation reveals significant epigenetic plasticity, supporting the important role of epigenetic events on developmental fate (3). Maladaptation in response to these epigenetic events results in deviations from the normal trajectory of fetal development.

Maternal hypoxia is a well-known factor that affects developing fetal CV function, major organ development including the brain, heart, liver, and kidney, and can cause intrauterine growth restriction (IUGR) (4–6). We previously reported in a mouse model that acute maternal hypoxia affects fetal heart rate during primary CV morphogenesis (7). We have also reported that modest maternal caffeine exposure in a mouse model acutely impairs fetal CV function during this critical developmental window and recurrent maternal caffeine exposure produces intrauterine growth restriction. The negative effect of caffeine exposure on fetal CV function is mediated by caffeine inhibition of the adenosine A_{2A} receptor (8).

Adenosine and adenosine receptors play a crucial role in cellular and tissue response to hypoxia (9). For example, adenosine mediates local blood flow regulation during ischemia and hypoxia. Plasma adenosine concentration is usually higher in the fetus than in adults (10, 11). Recent studies have shown that fetal adenosine levels increase under various stress conditions, such as hypoxia or placental dysfunction (12, 13). The elevation of umbilical venous plasma adenosine levels has also been demonstrated in newborns after vaginal delivery (10) and in fetuses with intrauterine growth restriction (11). These findings suggest that adenosine and adenosine receptor function play an integral role in the developing fetal stress response to hypoxia. If adenosine modulates the fetal hemodynamic response to hypoxia then adenosine antagonists, including caffeine, may have a negative adaptive effect during the response to hypoxic conditions. The cellular effects of adenosine are mediated *via* G protein-coupled membrane-bound receptors. At present, four distinct adenosine receptors subtypes, A₁, A_{2A}, A_{2B}, and A₃, have been cloned and characterized in several species (14–17). Of these subtypes, the A₁ and A_{2A} receptors are activated at low adenosine concentrations and A₁ adenosine receptors mediate the negative dromotropic, chronotropic, and inotropic adenosine effects (14, 15). Activation of adenosine A_{2A} and perhaps A_{2B} receptors triggers vasodilatation (14, 16, 17). Many studies suggested that adenosine is potentially beneficial for salvaging ischemic and/or reperfused myocardium. These properties include vasodilatation, β -adrenergic antagonism (18, 19), and glycolytic stimulation (20).

The plasma concentration of adenosine in the fetus is usually higher than in adults (10, 11, 21) and adenosine concentration in the placenta increases 100-fold during delivery (22). Intrauterine hypoxia and acidosis increase the plasma level of adenosine in the fetus and the placenta (10–12). Kubonoya *et al.* reported that plasma adenosine level increased cyclically

with intermittent umbilical cord occlusion in fetal sheep and they speculated that adenosine may have a protective effect against hypoxia (23). Adenosine is a vasodilator in most vascular beds, but is a vasoconstrictor of renal glomerular afferent arteriole and placental circulation (16). Read *et al.* speculated that release of adenosine by local ischemia in the placenta contributes to the ensuing vasoconstrictor response, directing blood to the placental villi that have higher oxygen tensions where its vasodilator effect could help sustain adequate blood flow (21).

Previous reports regarding the action of adenosine on the fetal CV system during the period of primary morphogenesis are limited to those of studies performed *in vitro*. Xu *et al.* showed that the affinity of the A_{2A} receptor was 60-fold higher than that of the A_{2B} receptor in ventricular cells cultured from 14-day chick embryos and that adenosine and N-ethyladenosine-5'-uronic acid (NECA), capable of activating both the adenosine A_{2A} and A_{2B} receptors, caused a greater increase in the contractile amplitude (24). Previous studies showed that A₁ receptor agonist reduced heart rate in a dose-dependent manner and A₁ receptor antagonist increased heart rate in a murine fetuses from GD 8 and older (25–27). Revkees *et al.* suggested that adenosine may be a regulator of cardiac cell division during early development (26).

In a recent large prospective cohort study, it has been shown that caffeine consumption during pregnancy was associated with an increased risk of fetal growth restriction and this association continued throughout pregnancy (28). Furthermore, Weng *et al.* demonstrated an elevated risk of miscarriage associated with caffeine consumption during pregnancy in their prospective cohort study (29).

These studies, including our own, suggest that negative fetal CV function by acute maternal hypoxia, which could be induced by anaerobic exercises, smoking, and/or even emotional events, may be exacerbated by additive caffeine exposure (ingestion of caffeinated foods and drinks). Therefore, in the present study, we investigated whether the embryo/fetal CV response to the acute maternal hypoxia is negatively impacted by additive maternal caffeine exposure or by exposure to adenosine receptor inhibitors that may recapitulate the caffeine effect.

Materials and Methods

Animals

Eight- to 12-week-old virgin female CD-1 mice were mated with CD-1 males (2:1) overnight and were examined for copulatory plugs the next morning. We defined gestational day (GD) 0.5 as noon of that day. Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh.

Experimental Protocol

Saline (control group), caffeine (10 mg/kg per dose, caffeine group), the adenosine A₁ receptor antagonist 8-cyclopentyl-1, 3-dimethylxanthine (4.8 mg/kg per dose, CPT group), or the adenosine A_{2A} receptor antagonist 3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (3.0 mg/kg per dose, MSX-3 group) was administered to CD-1 pregnant mice on GD 11.5 by subcutaneous injection. Caffeine dose was determined based on our previous study of maternal caffeine exposure mimicking regular daily human caffeine ingestion (8). Selective adenosine receptor antagonist doses are the minimal effective doses required to inhibit adenosine receptor (8). Each group consisted of seven dams. Thirty minutes after drug administration, dams were subjected to hypoxia using 100% nitrogen for 45 seconds and the changes in maternal and fetal hemodynamics were measured simultaneously using two echocardiography systems. The maternal hypoxia

stress was repeated three times at intervals of 10 minutes, and maternal and fetal hemodynamics were continuously monitored for a total of 40 minutes.

Drugs

All drugs were dissolved in sterile saline (with a few drops of 0.1 N NaOH for CPT and MSX-3; final pH adjusted to 7.4) and administered subcutaneously in a volume of 0.01 ml/g of maternal body weight. The dose of the adenosine receptor antagonists was chosen according to the previous report by Schindler *et al.*(30) which is the same dosage as in our previous study (8). Maternal serum caffeine concentrations measured in our previous study at 30 and 60 min after administration of 10 mg/kg of caffeine were 1.1 ± 0.27 (n=4) and 1.1 ± 0.25 (n=5) $\mu\text{g/ml}$, respectively (8).

Echocardiography

Standard transthoracic (dam) and maternal transabdominal (fetus) echocardiography were performed simultaneously using two ultrasound imaging systems. We used a high frequency ultrasound biomicroscope (Vevo 660; VisualSonics, Toronto, ON, Canada) with a 40-MHz mechanical sector transducer to measure fetal dorsal aortic flow, and an Acuson Sequoia C256 system with a 13-MHz linear ultrasonic transducer (15L8; Acuson Corporation, Mountain View, CA) to measure maternal pulmonary arterial flow. Pregnant mice were anesthetized with isoflurane gas (2% isoflurane for 1 minute induction and 1.5 to 1.7% for maintenance with 100% oxygen gas) and restrained in the supine position. Maternal blood pressure was monitored using a tail cuff blood pressure system (XBP 1000; Kent Scientific, Torrington, CT). The ambient temperature was maintained at approximately 37°C with a temperature controller (THM 1000; Visual Sonics) and a heating lamp (8). We measured the maternal heart rate (HR), the velocity-time integral (VTI, cm) of pulmonary flow from pulsed-Doppler velocity tracings, and main pulmonary artery diameter (D, cm) from B-mode images. We calculated maternal cardiac output (CO, ml/min) as $\text{CO} = \pi(D/2)^2 \times \text{VTI} \times \text{HR}$. We chose a single fetus in order to visualize the fetus in the longitudinal axis of the body (sagittal view), which enables us to monitor both ventricle and developing dorsal aorta within an acceptable angle (less than 15 degrees between the longitudinal axis of the dorsal aorta and Doppler ultrasound beam) to estimate blood flow. The fetal CO ($\mu\text{l/minute}$) was calculated from pulsed-Doppler velocity waveforms and M-mode images of the dorsal aorta applying the same formula for maternal CO calculation.

Statistical Analysis

Data are expressed as means \pm SE. Maternal and fetal hemodynamic data from different treatments were analyzed using two-way repeated analysis of variance (ANOVA) with the Bonferroni *post hoc* test.

Results

Baseline maternal and fetal heart rate (HR), stroke volume (SV), and cardiac output (CO)

The baseline maternal and fetal HR, SV, and CO were not different among control, caffeine, adenosine A₁ receptor antagonist (CPT), and adenosine A_{2A} antagonist (MSX-3) treated groups before hypoxia exposure (Table 1).

Maternal hemodynamics

In the control group, maternal HR, SV, and CO decreased suddenly approximately 30 seconds (s) after the beginning of the first exposure to hypoxia and were restored rapidly to approximately 80% of the baseline within 60s after hypoxia and then gradually recovered to baseline thereafter (Figure 1). Similarly, maternal hemodynamics recovered rapidly to close

to the baseline within 60s after the second and third periods of hypoxia with a decrease to approximately 80% of baseline followed by a gradual recovery to baseline. There were no statistical differences in maternal heart rate, stroke volume, or CO, between the control group, caffeine group, and MSX-3 group. However, CPT group did display the delayed heart rate reduction during the second and third periods of hypoxia ($P < 0.05$ vs. Control, ANOVA). There were no significant differences in maternal blood pressure among the four maternal groups (Figure 2).

Fetal hemodynamics

A decrease in fetal HR, SV β , and CO in response to maternal hypoxia appeared slowly after the decrease in the maternal heart rate, stroke volume and CO, respectively. The maximum drop was found approximately 3 minutes after the start of the hypoxia (Figure 3). In the control group, repeated hypoxia resulted in a shortening of the recovery time. The recovery time to baseline after the second and third exposures to hypoxia was statistically shorter than the recovery time after initial hypoxia (Figure 4). Repeated hypoxia also resulted in the blood flow exceeding the baseline following recovery. The CO overshoot was caused by an increase in fetal heart rate over baseline in addition to the restoration of stroke volume to the baseline (Figure 3). There were no significant differences in the lowest fetal CO after hypoxia between the control group and the caffeine or selective adenosine A_{2A} receptor antagonist, MSX-3, group. However, recovery was negatively impacted and no restoration to baseline occurred in either the caffeine or MSX-3 groups. There were statistical differences in the fetal CO during the recovery periods between the control and the caffeine group or MSX-3 group (Figure 3C). The differences in fetal CO in the caffeine and MSX-3 groups were caused by the combination of the lower stroke volumes and lower heart rates. In addition, shortening of the recovery time and overshoot in CO in response to repeated hypoxia were not observed in the caffeine or MSX-3 groups (Figure 3C, Figure 4). No significant differences between the control, caffeine, and MSX-3 groups were found in the 10-minute mean percentage change in fetal CO after the first hypoxia. However, the 10-minute mean percentage changes in recovery time in the caffeine and MSX-3 groups were less than those in the control group after the second and third exposures to hypoxia (Figure 4). In the CPT, selective adenosine A₁ receptor antagonist, group, the decrease in fetal heart rate and stroke volume were less than those in the control group (Figure 3A and 3B); as a result, the drop in fetal CO in the CPT group was significantly less than that in the control group (Figure 3C). Mean percentage decrease in fetal CO of CPT treated group 10 minutes after the first hypoxia was less than the control group (Figure 5). Control and CPT groups did not change the fetal CO from baseline after 2nd and 3rd hypoxia exposure, whereas the fetal CO of caffeine and MSX3 groups remained decreased (Figure 5).

Discussion

Our previous studies in a mouse animal model showed that developing fetal CV function is vulnerable to transient maternal hypoxia (7), and a modest amount of maternal caffeine exposure has adverse effects on developing fetal CV function mediated by adenosine A_{2A} receptor inhibition by caffeine (8). Maternal caffeine exposure induces variable reduction in regional fetal arterial blood flow (-46% for the internal carotid artery, -25% for the dorsal aorta, and -15% for the umbilical artery on GD 11.5) suggesting that regional changes in blood flow may be due to regional variations in vascular resistance (8). In the present study, we evaluated the effects of adenosine antagonists on the early fetal CV system during intermittent maternal hypoxia. It is speculated that the intermittent maternal hypoxia increases the adenosine concentration in the fetoplacental circulation. Hence, it was expected that maternal hypoxia might trigger increased maternal and/or fetal adenosine to modulate the effects of hypoxia on fetal CV function. In the present study, an initial

exposure to maternal hypoxia was shown to negatively impact fetal hemodynamics. However, repeated episodes of maternal hypoxia accelerated the recovery of fetal cardiac output to the baseline and resulted in overshoot beyond the baseline. The results of inhibition of A_{2A} receptor, which blunted the recurrent hypoxia mediated recovery, suggest that adenosine is likely to exert favorable effects on the fetal CV system via A_{2A} receptor activation (Figure 6A). Caffeine retarded the recovery of fetal dorsal aortic flow following hypoxia, similar to the effects of treatment with an A_{2A} receptor antagonist (Figure 6B). Although caffeine is a non-selective competitive antagonist of adenosine receptors, the results of the present study suggested that caffeine has a negative effect on fetal CV function via A_{2A} receptor inhibition during maternal hypoxia.

We have previously shown that adenosine A₁ receptor inhibition showed no effect on fetal CV function without hypoxic stress (8). However, in the present study, A₁ receptor inhibition significantly prevented the decrease in fetal cardiac output induced by maternal hypoxia. This difference may be due to the action of adenosine A₁ receptor inhibition on the maternal response to hypoxia, and the effects of A₁ receptor inhibition on fetal heart rate without hypoxia were in agreement with those reported previously (25, 27). Our results of CPT treatment suggest that adenosine A₁ receptor antagonists might be of use in cases of fetal distress in the setting of maternal hypoxia and caffeine exposure. However, adenosine functions as a counterregulatory hormone in the myocardium by decreasing work and thereby protecting the myocardium from ischemia (31). Further studies are required to confirm the possible efficacy of A₁ receptor antagonists in fetal distress.

Previous studies showed that adenosine A₁ and A_{2A} receptors are present and regulates fetal CV function from early organogenesis period (8, 25), and the caffeine and CPT (and MSX-3 may be) can cross the placenta (32). However, it remains to be investigated whether maternal hypoxia and caffeine exposure effects on developing fetal CV function are mediated by adenosine receptors in the maternal utero-placental circulation and/or the fetoplacental circulation, and further studies are warranted to define the underlying mechanisms by which maternal hypoxia and caffeine exposure effect developing fetal CV function and growth.

In conclusion, we demonstrated the *in vivo* response of the early developing fetus to combined maternal hypoxia and caffeine exposure and our results suggest that caffeine exposure affects fetal CV response to the acute maternal hypoxia via adenosine A_{2A} receptor inhibition during the critical period of primary CV morphogenesis.

Acknowledgments

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Abbreviations

CV	cardiovascular
GD	gestational day
CPT	8-cyclopentyl-1, 3-dimethylxanthine
MSX-3	3-(3-hydroxypropyl)-8-(m-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt
CO	cardiac output

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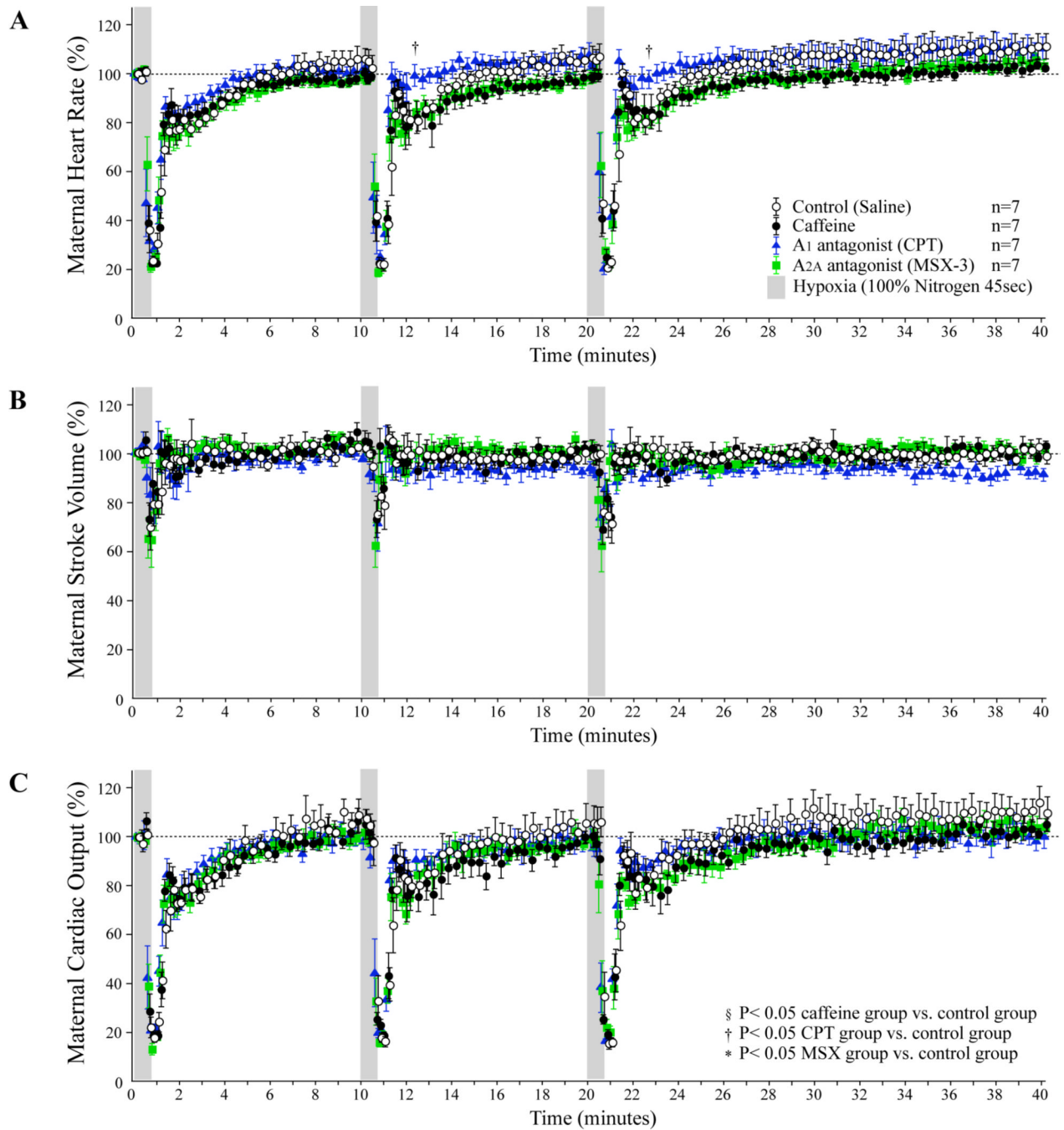


Figure 1. Maternal hemodynamic effects of repeated maternal hypoxia with adenosine receptor antagonists

Dams were repeatedly exposed to 100% nitrogen for 45 seconds followed by a 10 to 20 minute recovery period. (A): Maternal heart rate, (B): Maternal stroke volume, (C): Maternal cardiac output.

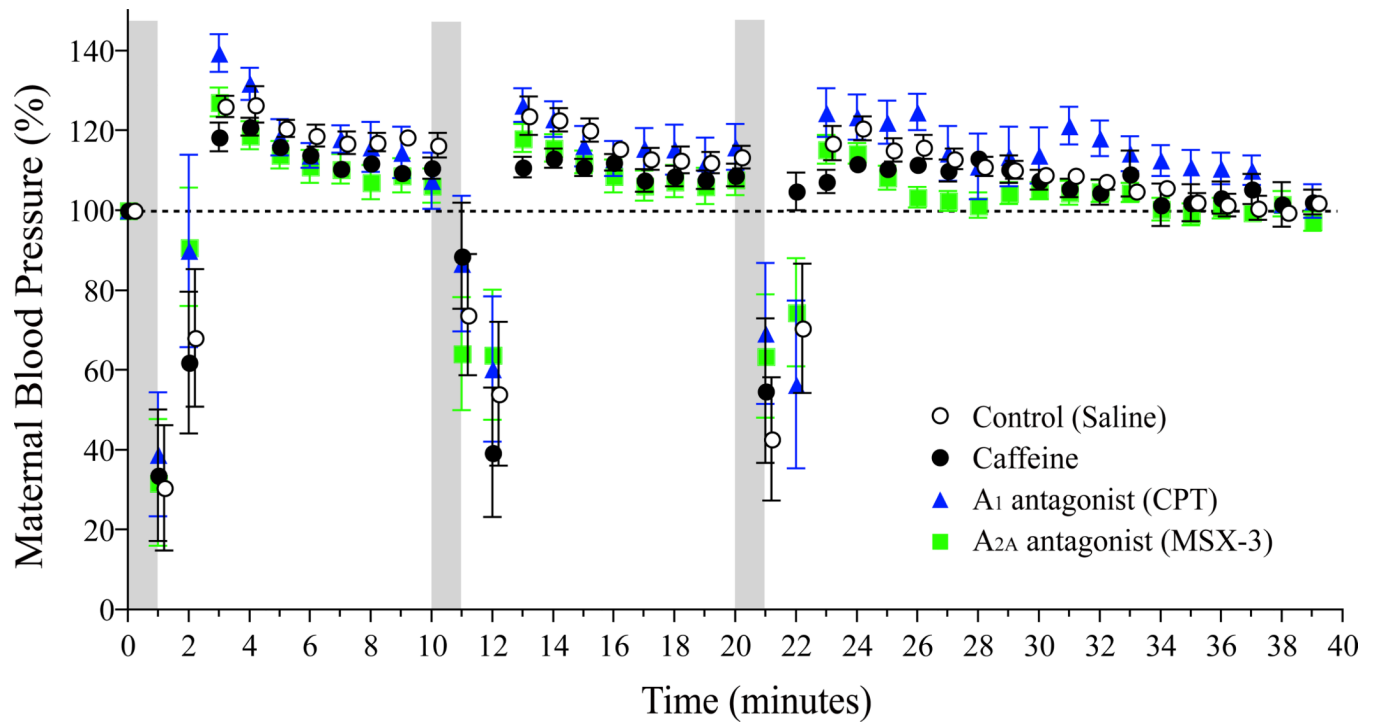


Figure 2. Maternal blood pressure responses to hypoxia, caffeine, and Adenosine receptor antagonist treatments
 Maternal blood pressure was consecutively measured using a tail cuff blood pressure system. There were no significant differences in one-minute mean maternal blood pressure among the four groups.

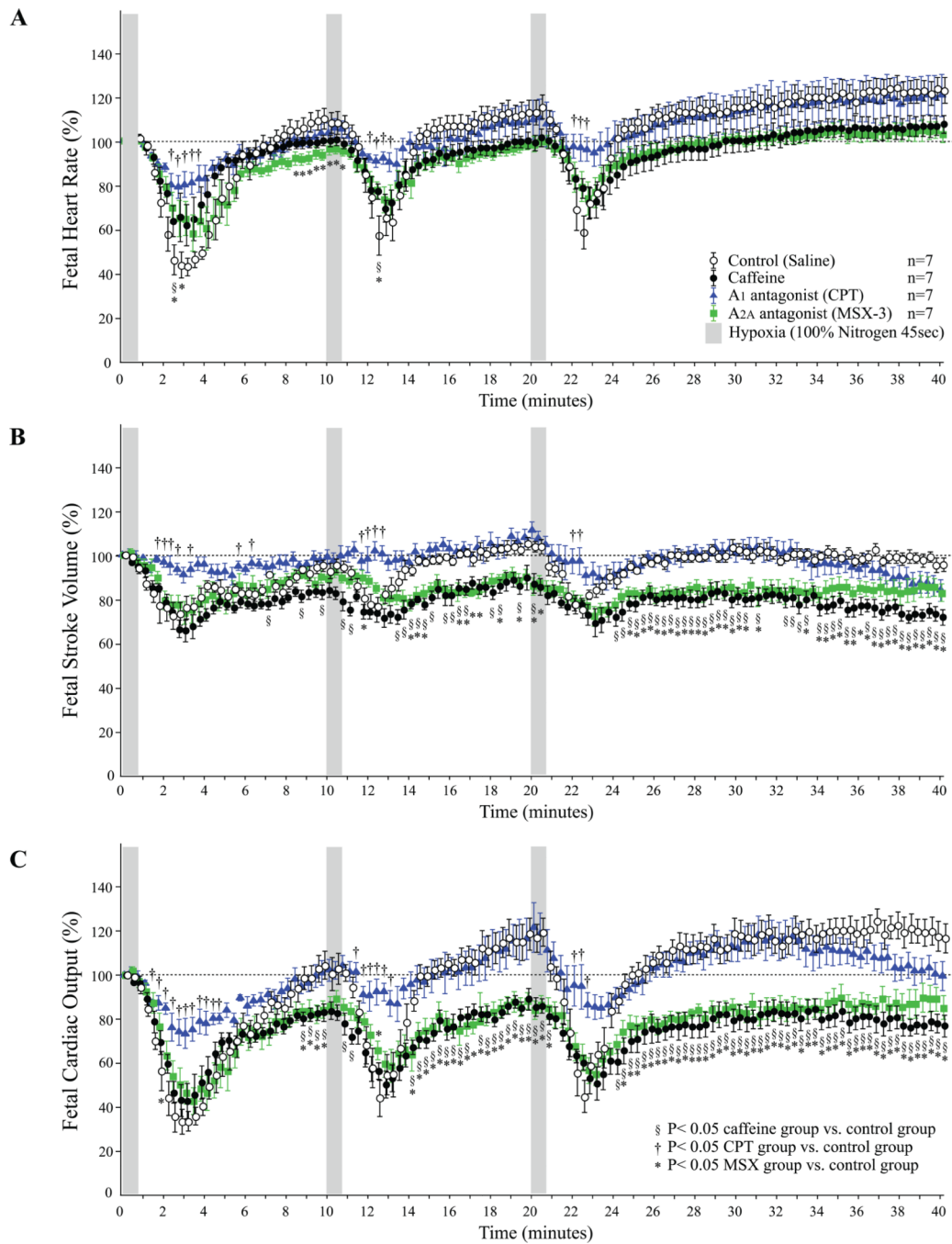


Figure 3. Fetal hemodynamic effects of repeated maternal hypoxia with adenosine receptor antagonists

Dams were repeatedly exposed to 100% nitrogen for 45 seconds followed by a 10 to 20 minute recovery period. (A): Fetal heart rate, (B): Fetal stroke volume, (C): Fetal cardiac output.

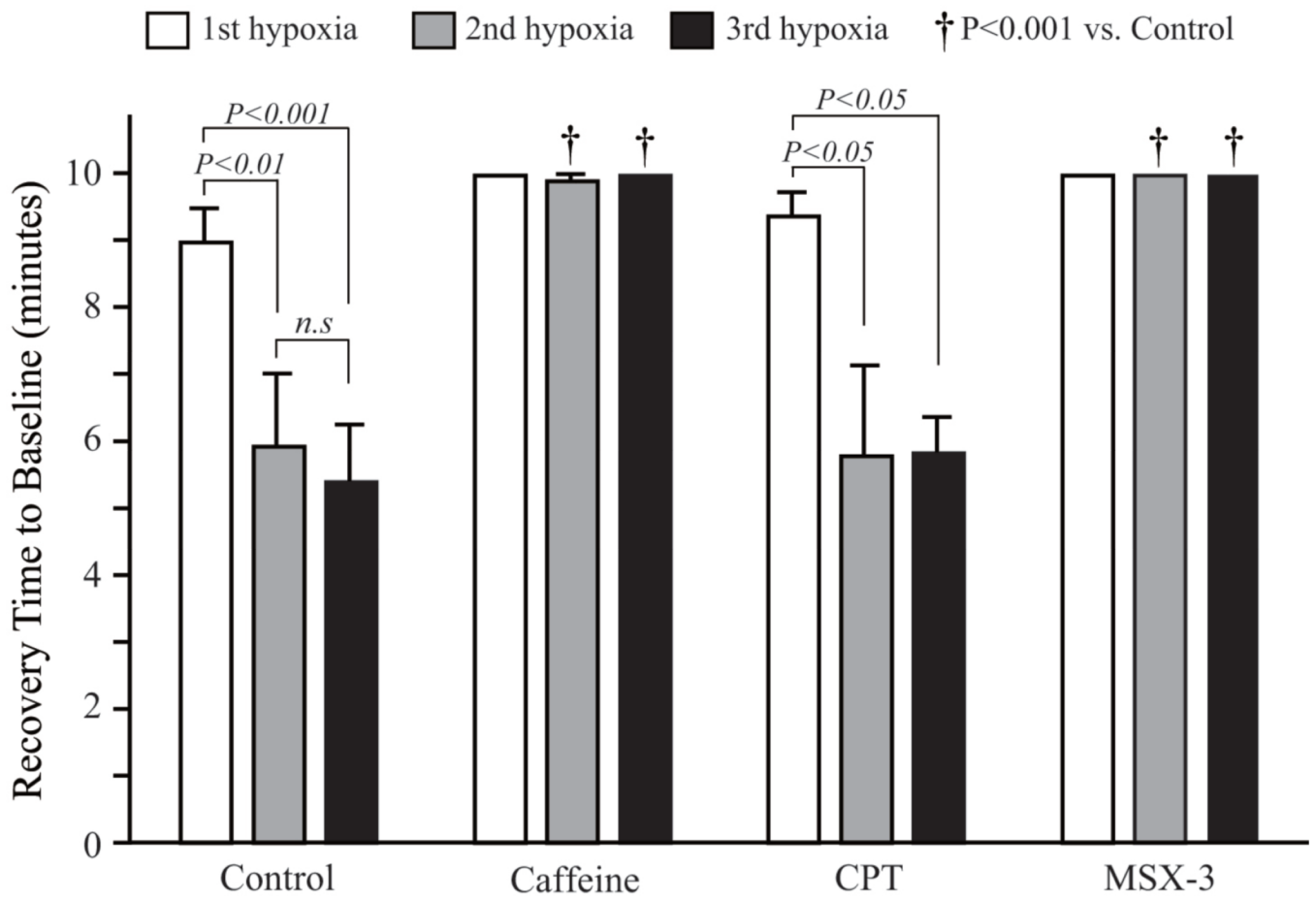


Figure 4. Fetal dorsal aortic flow recovery time to baseline following hypoxia, caffeine, and Adenosine receptor antagonist treatments
 Recovery time was defined as the time from the start of hypoxia to the point in time when fetal dorsal aortic flow recovered to the baseline. If no restoration to the baseline occurred, the recovery time was defined as 10 minutes.

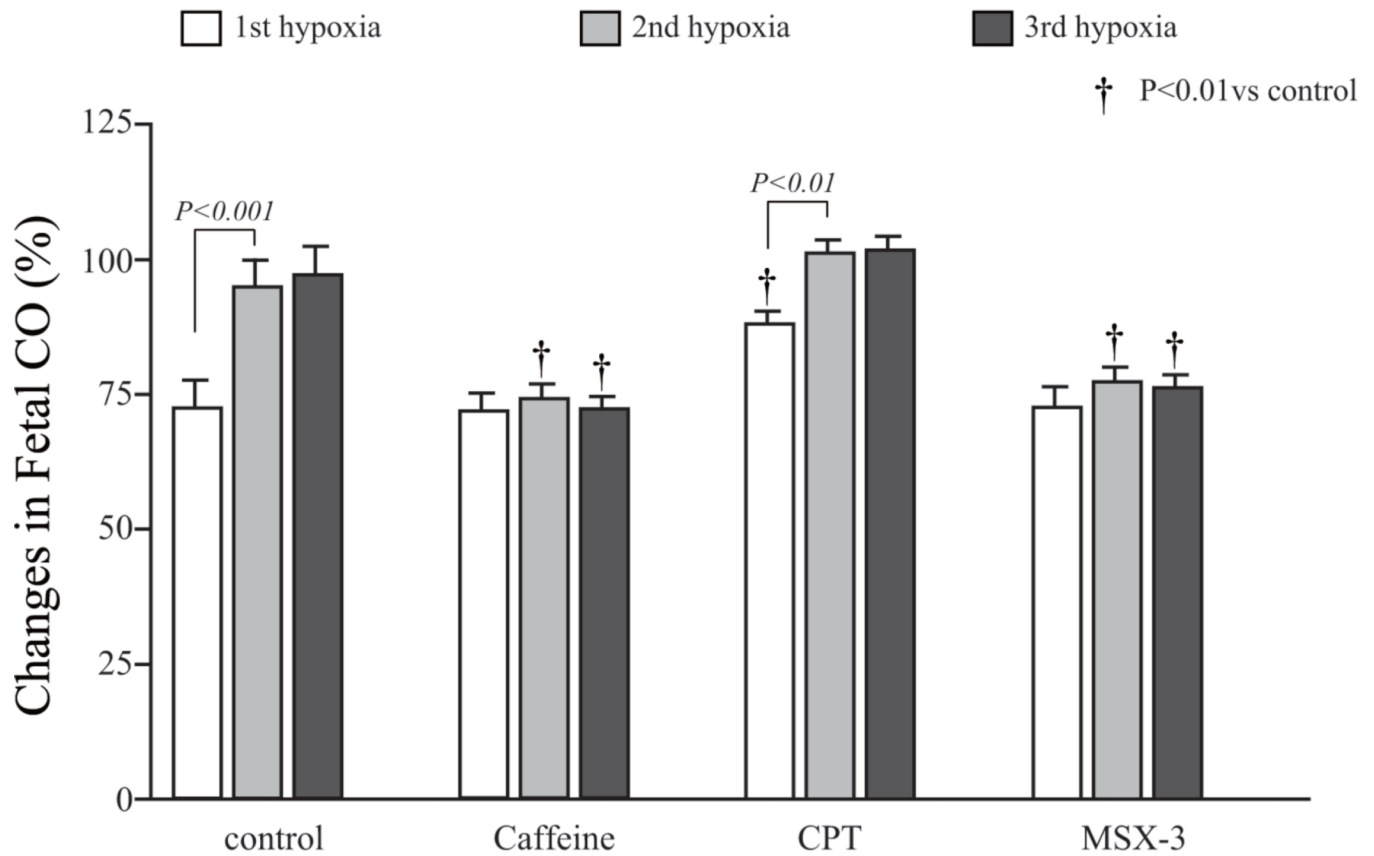
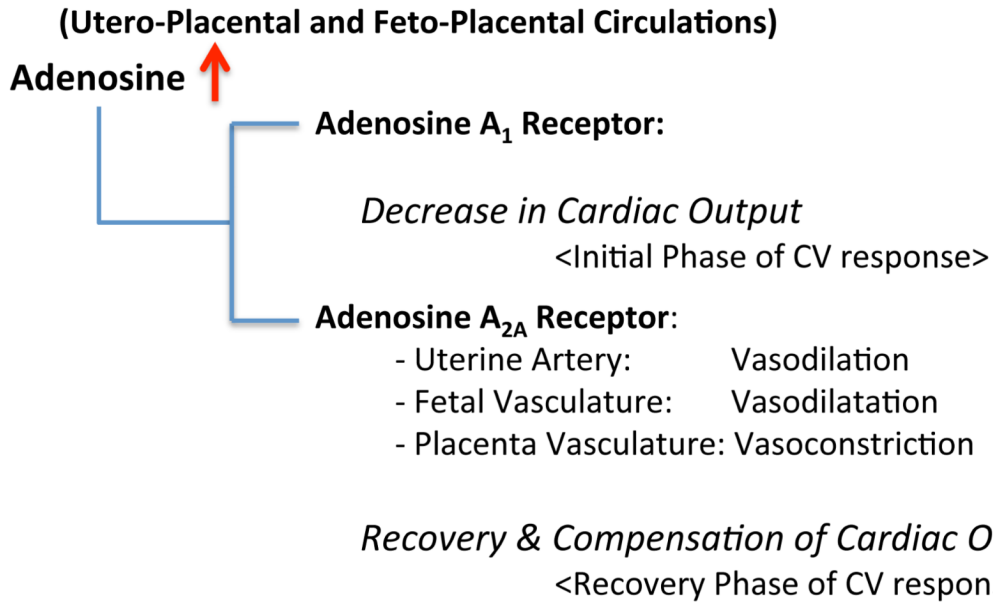


Figure 5. Mean percentage changes in fetal cardiac output following the onset of hypoxia
 The mean percentage change of the fetal cardiac output after the first hypoxia in the CPT group was higher than that in the control group. Control and CPT groups showed a similar trend of changes in fetal CO, while Caffeine and adenosine A2A antagonist, MSX-3, treated group did not recover the fetal CO to the baseline levels following 2nd and 3rd hypoxia exposure.

A. Hypoxia



B. Hypoxia + Caffeine

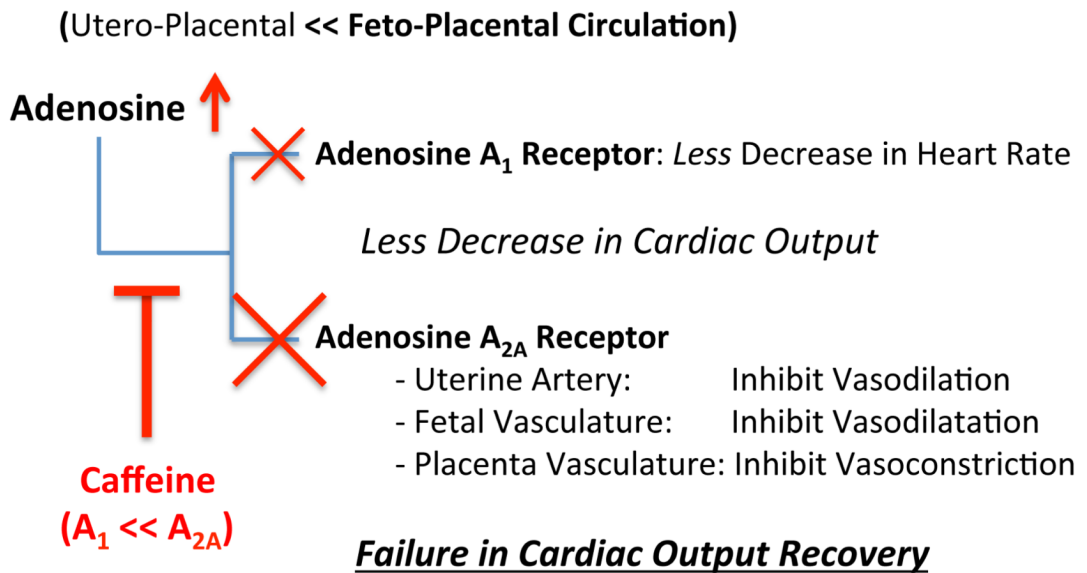


Figure 6. Schematic diagram of maternal hypoxia and caffeine exposure effects on utero-placental and fetoplacental circulations

Maternal hypoxia increases adenosine concentration within utero-placental (maternal) and fetoplacental (fetal) circulations in which both adenosine A₁ and A_{2A} receptors are activated (**panel A**). Adenosine A₁ receptor activation decreases maternal and fetal heart rate and cardiac output (initial hypoxia response), and A_{2A} receptor activation increases uterine and fetal arterial blood flow by vasodilation with relatively decreased placenta vascular flow (vasoconstriction) resulting in fetal cardiac output recovery (recovery phase of hypoxia). Caffeine, a non-selective adenosine antagonist, inhibits adenosine receptor activation. While initial decrease in cardiac output of utero- and feto-placental circulations by combined

hypoxia and caffeine exposure is less than hypoxia only, inhibition of adenosine A_{2A} receptor by caffeine leads to decrease in uterine and fetal arterial blood flow (inhibition of vasodilation) and blood flow steal to the placental vasculature (blood flow redistribution within fetoplacental circulation by placental vasodilation,) resulting in failure in cardiac output recovery of fetoplacental circulation (**panel B**).

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Table 1

Baseline hemodynamics

	Control	Caffeine	A ₁ antagonist (CPT)	A _{2A} antagonist (MSX-3)	p-value
Mother					
HR (/min)	517 ± 12.4	533 ± 19.6	500 ± 64.1	506 ± 13.8	n.s.
CO (ml/min)	27.7 ± 1.0	27.3 ± 1.4	25.2 ± 3.1	28.4 ± 1.5	n.s.
Fetus					
HR (/min)	183 ± 22.6	181 ± 7.5	209 ± 38.2	176 ± 11.0	n.s.
CO (µl/min)	83.1 ± 7.9	73.9 ± 5.9	81.7 ± 10.7	62.9 ± 7.6	n.s.
SV (µl)	0.47 ± 0.031	0.41 ± 0.031	0.41 ± 0.36	0.36 ± 0.035	n.s.

HR: heart rate, CO: cardiac output, SV: stroke volume