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Reduced fetal cerebral blood flow predicts perinatal mortality in a mouse model of prenatal alcohol and cannabinoid exposure

Siara Kate Rouzer[1], Anirudh Sreeram[1] and Rajesh C. Miranda[1*]

Abstract

Background Children exposed prenatally to alcohol or cannabinoids individually can exhibit growth deficits and increased risk for adverse birth outcomes. However, these drugs are often co-consumed and their combined effects on early brain development are virtually unknown. The blood vessels of the fetal brain emerge and mature during the neurogenic period to support nutritional needs of the rapidly growing brain, and teratogenic exposure during this gestational window may therefore impair fetal cerebrovascular development.

Study Design To determine whether prenatal polysubstance exposure confers additional risk for impaired fetal-directed blood flow, we performed high resolution in vivo ultrasound imaging in C57Bl/6J pregnant mice. After pregnancy confirmation, dams were randomly assigned to one of four groups: drug-free control, alcohol-exposed, cannabinoid-exposed or alcohol-and-cannabinoid-exposed. Drug exposure occurred daily between Gestational Days 12–15, equivalent to the transition between the first and second trimesters in humans. Dams first received an intraperitoneal injection of either cannabinoid agonist CP-55,940 (750 µg/kg) or volume-equivalent vehicle. Then, dams were placed in vapor chambers for 30 min of inhalation of either ethanol or room air. Dams underwent ultrasound imaging on three days of pregnancy: Gestational Day 11 (pre-exposure), Gestational Day 13.5 (peri-exposure) and Gestational Day 16 (post-exposure).

Results All drug exposures decreased fetal cranial blood flow 24-hours after the final exposure episode, though combined alcohol and cannabinoid co-exposure reduced internal carotid artery blood flow relative to all other exposures. Umbilical artery metrics were not affected by drug exposure, indicating a specific vulnerability of fetal cranial circulation. Cannabinoid exposure significantly reduced cerebroplacental ratios, mirroring prior findings in cannabis-exposed human fetuses. Post-exposure cerebroplacental ratios significantly predicted subsequent perinatal mortality ($p = 0.019$, area under the curve, 0.772; sensitivity, 81%; specificity, 85.70%) and retroactively diagnosed prior drug exposure ($p = 0.005$; AUC, 0.861; sensitivity, 86.40%; specificity, 66.7%).

Conclusions Fetal cerebrovasculature is significantly impaired by exposure to alcohol or cannabinoids, and co-exposure confers additional risk for adverse birth outcomes. Considering the rising potency and global availability of cannabis products, there is an imperative for research to explore translational models of prenatal drug exposure,

*Correspondence:
Rajesh C. Miranda
rmiranda@tamu.edu

Full list of author information is available at the end of the article

including polysubstance models, to inform appropriate strategies for treatment and care in pregnancies affected by drug exposure.

Keywords Prenatal drug exposure, Polysubstance, Alcohol, Cannabinoid, Ultrasound, Blood flow, Fetal mortality, Maternal health

Introduction

Co-consumption of alcohol with cannabis is a rapidly emerging public health problem, partly due to widespread cannabis legalization and increased availability of potent synthetic cannabinoids [1]. Approximately one-third of young adults in the United States report using either drug within the past month [2]. Conservative estimates suggest that ~8–12% of infants are prenatally exposed to ethanol [3–5] and ~2–7% are prenatally exposed to cannabis throughout pregnancy [6]. However, others have reported much higher levels of exposure, with an estimated ~32–64% of infants experiencing alcohol exposure and ~20–41% experiencing cannabis exposure [7]. Notably, for both substances, self-reported levels of exposure were higher during the first two trimesters compared to the final trimester. Young adults who use both substances are known to preferentially engage in the practice of simultaneous alcohol and cannabinoid (SAC) consumption [8], which amplifies each drug's psychological effects [9]. However, the consequences of SAC for pregnancy health and fetal development are currently poorly understood.

Individually, prenatal exposures to either alcohol or cannabinoids have similar physical and developmental consequences for exposed offspring, each resulting in growth restriction and reduced birth weights [10–12], increased rates of preterm delivery [10, 13–15], and increased health risks associated with delivery [13, 16]. Cannabis exposure, in particular, has been tied to increased need for neonatal intensive care services [17] and greater risk of fetal demise [16]. It should be noted that some investigations have not reported adverse birth outcomes in humans due to prenatal cannabis exposure by itself, after controlling for intervening variables [18]. Other studies have only shown cannabis-associated impairments associated with polysubstance use, in which polysubstance exposure involving cannabis significantly increased the risk for birthweight deficits and preterm birth [19, 20]. Similarly, some studies in rodent models have also not documented cannabinoid effects on fetal growth, but have reported changes in sex ratios and neurobehavioral and physiological deficits in exposed offspring [21, 22]. These variable reports emphasize the need to understand the impact of intervening variables, particularly polysubstance use, on exposure-associated fetal growth outcomes. It is known that both ethanol and cannabinoids cross the placental barrier [23, 24], and consequently, have direct effects on the developing fetus. While little is known about the specific effects of co-use, two recent papers in mouse [25] and zebrafish [26] models indicate that teratogenic effects of prenatal alcohol exposure are amplified by concurrent cannabinoid exposure. Therefore, SAC is likely to result in increased deficits compared to either prenatal alcohol or cannabinoid exposures alone.

Here, we assessed the vulnerability of fetal brain vasculature following maternal SAC. Studies in children with fetal alcohol spectrum disorders, as well as translational animal models, have shown that prenatal alcohol exposure results in structural and functional changes in fetal brain circulation and arterial vasodilation, leading to both acute and persistent loss of cerebral blood flow to the developing brain (for review, see [27]). To date, few studies have explored the effects of *in utero* cannabinoid exposure on fetal cerebrovasculature. Ultrasound imaging in pregnant women during the second trimester has revealed that daily cannabis use is associated with higher umbilical artery systolic:diastolic ratios when compared to controls [28]. This anomaly persisted into the third trimester, with cannabis-exposed fetuses demonstrating a higher incidence of growth restriction, and some exhibiting absent or reversed end-diastolic blood flow in the umbilical artery, an outcome that predicts fetal distress [29]. Moreover, cannabis-exposed fetuses with growth restriction exhibit abnormally low cerebroplacental ratios (CPR), the ratio of middle cerebral artery to umbilical artery blood flow [30]. Low fetal CPR was also associated with low birth weight and an increased need for neonatal intensive care. Rapid reduction in fetal cerebral vessel diameter, length fraction, and area density have been further observed in murine models following acute maternal exposure to synthetic cannabinoid CP-55,940 [31].

To our knowledge, no studies have specifically addressed the impact of SAC on fetal vascular function. Therefore, we investigated the effects of singular or simultaneous exposure to alcohol and cannabinoids during a critical period of cerebral cortical plate neurogenesis and angiogenesis [32, 33] on fetal blood flow and subsequent viability at delivery. Our results indicate that both forms of prenatal drug exposure contribute to persistent reductions in fetal-directed cerebral blood flow, with SAC augmenting certain deficits further. Moreover, SAC was associated with low CPR and the highest rates of perinatal mortality among offspring compared to all other exposure conditions.

Methods

Breeding paradigm All procedures were performed with Texas A&M University's Institutional Animal Care Committee oversight. One male and two female C57Bl/6J female mice (Harlan laboratories, Houston, TX) were temporarily co-housed overnight for mating. Pregnancies were confirmed the next morning (Gestational Day [G]1) by the presence of a sperm plug. Dams were weighed on G1 and G10 to verify weight gain consistent with pregnancy, and subsequently on G15 and G18, to determine effects of drug exposure on gestational weight gain. After pregnancy confirmation, dams were randomly assigned to one of four groups: drug-free controls (CON), only alcohol-exposed (ALC), only cannabinoid-exposed (CB) or alcohol-and-cannabinoid-exposed (ALC+CB).

Prenatal drug exposure paradigm Exposures occurred daily between G12-15 (Fig. 1B). First, dams received an intraperitoneal injection of either cannabinoid agonist CP-55,940 (Tocris Bioscience, Bristol, UK; 750 µg/kg dissolved in 10% DMSO/saline), or vehicle (volume-equivalent 10% DMSO/saline). Next, dams were placed in vapor chambers (passive e-vape system, La Jolla Alcohol Research Inc., La Jolla, CA) for 30 min of inhalation of either ethanol (95% ethanol) or room air, as previously described [34]. Following exposure, dams were assessed for exposure-induced loss of righting reflex (LORR) and tail blood was collected (20µL) in heparinized capillary tubes to assess blood alcohol concentrations by gas chromatography, as previously described [35–38].

Ultrasonography Each pregnant dam underwent ultrasound imaging at G11 (pre-drug exposure), G13.5 (peri-drug exposure) and G16 (post-drug exposure) (Fig. 1A&B), as we previously described [39, 40]. Briefly, dams were anesthetized with isoflurane (2–3%), and maintained supine on a temperature-controlled sensor platform to monitor maternal electrocardiogram, respiration and core body temperature (Visualsonics, Toronto, Canada). For each pregnant dam, a single fetus located at the base of the uterine horn was selected for repeated imaging. Doppler measurements for umbilical arteries and fetal internal carotid/middle cerebral arteries were obtained using a high-frequency VEVO2100 ultrasound imaging machine coupled to a MS550D Microscan™ transducer with a center frequency of 40 MHz (Visualsonics, Canada). Following drug exposure, dams were monitored daily by researchers to assess maternal health outcomes and to verify date of parturition.

Data analysis and statistics Ultrasound subjects were assigned random identification codes and recordings were analyzed by an experimenter blinded to the exposure condition. Ultrasound imaging data were analyzed using the VEVO2100 measurement and analysis software (Visualsonics, Ca, Fig. 1C) to assess Velocity-Time Integral (VTI, in mm^3/sec, a measure of cardiac stroke volume through a specific blood vessel), peak systolic velocity (PSV, the peak quantity of blood flow during a systolic pulse) and Acceleration (Acc, in mm/Sect. ^{2}, a measure of arterial resistance) [39, 41, 42]. Each data point reflects measurements from one fetus, from one pregnant dam. Based on preliminary data, a power analysis (G*Power 3.1.9.4) for a two-way (gestational day x exposure) analysis of variance (ANOVA) and an alpha of 0.05 suggested a sample size of 7 pregnant dams per group. Results are presented as mean±SEM for each group.

Gestational weight gain and blood flow metrics were analyzed using a mixed-effects ANOVA, with independent variables of gestational day (within-subjects, three days: G11, G13.5 and G16) and drug exposure (between subjects, four groups: CON, ALC, CB and ALC+CB). In the event of a significant effect of exposure, or an interaction of gestational day x exposure, post-hoc Tukey tests were performed comparing all exposure groups within-day. To determine if drug exposure produced significant, within-animal changes in blood flow growth metrics, one-sample t-tests were performed within each exposure group. One-way ANOVAs were also used to compare drug-induced changes between exposure groups, and Pearson's r correlations were performed between fetal blood flow metrics and maternal/fetal mortality. To generate Kaplan-Meier Survival curves, viable (1) and non-viable (0) births were listed for each pregnancy according to exposure group, and analyzed using a Log-rank (Mantel-Cox) test to calculate overall changes in survival probability following drug exposure. Post-hoc pairwise comparisons were then employed to determine differences in survival between control pregnancies and each drug-exposed pregnancy. Receiver operating characteristic (ROC) analyses were performed to assess the accuracy of blood flow metrics in predicting either future fetal mortality or prior drug exposure. Gestational blood alcohol concentrations were assessed using a nested t-test of blood samples collected during G12-15. All data were assessed for outliers using the ROUT method of regression with a false-discovery rate of 1%, and identified outliers were removed from statistical analyses. Group differences were considered significant at $p \leq 0.05$. All statistics were performed using GraphPad Prism 9, except for ROC analyses (SPSS v28, IBM).

Results

SAC increased blood alcohol concentrations and LORR, and reduced gestational weight gain

A total of 40 dams were exposed in our paradigm (CON: 9, ALC: 9, CB: 10, ALC+CB: 12). Blood alcohol concentrations achieved in alcohol-exposed pregnancies were significantly lower ($\sim$ 25%) than concentrations achieved following combined exposure to alcohol and CP-55,940

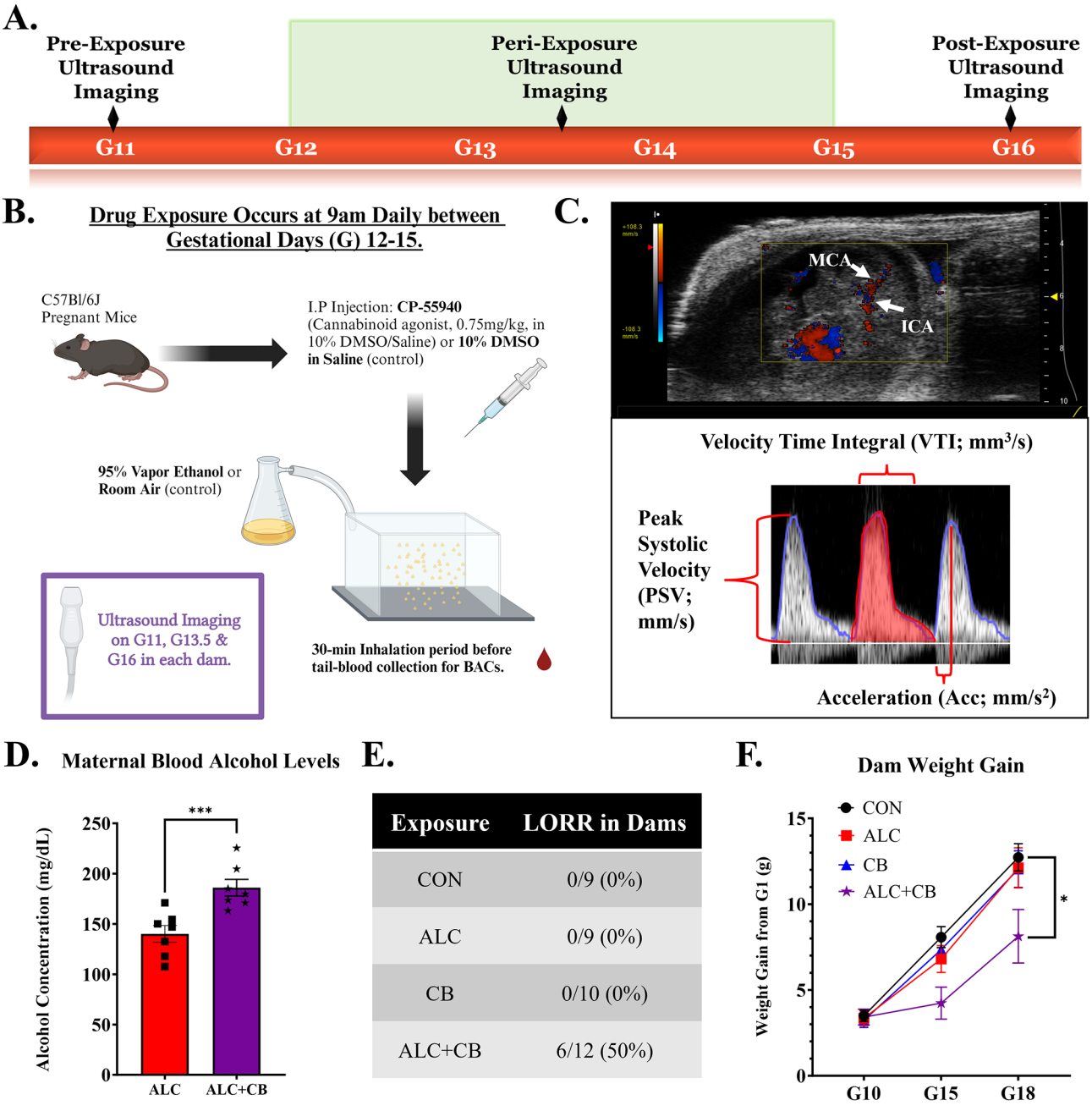


Fig. 1 Experimental outline and polysubstance exposure characterization. **A)** Experimental timeline for prenatal drug exposure and ultrasound imaging data collection. **B)** Summary figure of the daily prenatal drug exposure procedure. **C)** Example fetus, G13.5, imaged using VEVO2100, with example waveform analysis Fig. **D)** Average blood alcohol concentrations among alcohol-exposed subjects. **E)** Rates of loss of righting reflex (LORR) in drug-exposed dams immediately following drug exposure. **F)** Gestational weight gain across drug-exposed dams. *Abbreviations* G = Gestational Day, BAC = Blood Alcohol Concentration, MCA = Middle Cerebral Artery, ICA = Internal Carotid Artery, LORR = Loss of Righting Reflex, CON = Control, ALC = Alcohol, CB = Cannabinoid, ALC + CB = Alcohol + Cannabinoid. **Symbols**: * indicates $p < 0.05$, *** indicates $p < 0.001$

[Fig. 1D; $t(35) = 3.867$, $p < 0.001$], despite identical alcohol exposure conditions. Moreover, only ALC + CB dams experienced motor impairment, measured by LORR (Fig. 1E). All pregnant animals experienced gestational weight gain (significant main effect of gestational day [$F(2, 50) = 238.4$, $p < 0.001$]), with no group differences in weight gain on G10, prior to exposure (Supplementary Table 1). However, there was a significant interaction between gestation day x exposure [$F = (6, 64) = 5.146$, $p < 0.001$], due to decreased weight gain in ALC + CB dams at the end of the exposure period (G15, $p = 0.014$ compared to CON dams, Fig. 1F). Importantly, exposure-specific changes in maternal weight gain did not

correspond with changes in maternal food consumption (Supplementary Fig. 1).

From each exposure group, 7–8 dams were selected to undergo ultrasound imaging prior to (G11), during (G13.5) and after (G16) the drug exposure period. Indices for VTI, Acc and PSV were computed for umbilical and fetal middle cerebral and internal carotid arteries. Blood flow metric effect sizes are listed in Table 1. Importantly, no exposure condition significantly changed blood flow metrics in the umbilical artery (Supplementary Fig. 2).

All drug exposures significantly and persistently reduced middle cerebral artery blood flow metrics

All drug exposures - ALC, CB & ALC+CB - reduced fetal middle cerebral artery VTI relative to controls, but only after the exposure period, i.e., at G16 [significant interaction between gestational day and exposure: $F(6, 50) = 4.853$, $p < 0.001$, Fig. 2A&B, Supplementary Table 2). VTI significantly increased in CON fetuses between G11 and G16 ($p = 0.002$). This age-related increase was partly attenuated in ALC fetuses ($p = 0.049$) and completely abolished in CB and ALC+CB fetuses (all p's > 0.357; Fig. 2C; Supplementary Tables 3&4). As VTI measurements can reflect changes in arterial resistance and/or the quantity of blood flow, we also assessed middle cerebral artery Acc and PSV (Supplementary Fig. 3). Like VTI, all forms of prenatal drug exposure significantly reduced Acc (all p's < 0.005; Supplementary Table 5) and PSV (all p's < 0.005; Supplementary Table 6) relative to controls on G16, though drug-exposed groups did not differ statistically from each other.

Table 1 Effects of prenatal drug exposure on fetal blood flow metrics & associated effect sizes. Table of blood flow metric comparisons & associated effect sizes (Hedge's *g*, reported as Effect Size [95% CI upper, lower limits]). Bold text indicates accompanying *p*-value < 0.05. Italicized text indicates accompanying *p*-value < 0.1

VTI		**Main effect: exposure**	**Interaction: exposure x day**	**Hedge's *g*:**	**Hedge's *g*:**	**Hedge's *g*:**
		p-value	***p-value***	**CON vs. ALC**	**CON vs. CB**	**CON vs. ALC + CB**
MCA	G11	**0.0207**	**0.0006**	0.078 [-1.715, 1.871]	0.628 [-0.411, 1.667]	0.49 [-0.54, 1.519]
	G13.5			1.195 [0.094, 2.295]	1.279 [0.166, 2.391]	0.717 [-0.33, 1.763]
	G16			**1.374 [0.247, 2.502]**	**1.267 [0.156, 2.378]**	**1.735 [0.546, 2.924]**
ICA	G11	**0.0022**	**0.0306**	0.448 [-0.579, 1.475]	0.065 [-0.95, 1.079]	0.176 [-0.84, 1.192]
	G13.5			1.209 [0.106, 2.311]	0.22 [-0.797, 1.237]	0.69 [-0.354, 1.734]
	G16			0.84 [-0.218, 1.897]	0.692 [-0.352, 1.736]	**1.919 [0.694, 3.144]**
UA	G11	0.6007	0.7432	0.121 [-0.894, 1.136]	0.22 [-0.797, 1.238]	0.186 [-0.83, 1.203]
	G13.5			0.897 [-0.167, 1.961]	0.812 [-0.243, 1.867]	0.829 [-0.227, 1.886]
	G16			0.514 [-0.516, 1.545]	0.23 [-0.788, 1.248]	0.592 [-0.444, 1.629]
Acceleration		**Main effect: exposure**	**Interaction: exposure x day**	**Hedge's*g*:**	**Hedge's*g*:**	**Hedge's*g*:**
		p-value	***p-value***	**CON vs. ALC**	**CON vs. CB**	**CON vs. ALC + CB**
MCA	G11	**0.0011**	*0.0994*	0.267 [-0.441, 0.975]	0.193 [-0.51, 0.895]	0.006 [-0.701, 0.714]
	G13.5			1.114 [0.024, 2.204]	0.961 [0.239, 1.682]	1.615 [0.853, 2.377]
	G16			**1.402 [0.271, 2.534]**	**1.447 [0.733, 2.162]**	**1.319 [0.612, 2.026]**
ICA	G11	*0.0874*	0.3412	0.577 [0.408, 0.746]	0.179 [-0.521, 0.878]	0.337 [-0.375, 1.048]
	G13.5			0.629 [0.431, 0.828]	0.452 [-0.257, 1.161]	0.427 [-0.276, 1.129]
	G16			0.347 [-0.353, 1.047]	0.713 [0.012, 1.415]	**1.134 [0.432, 1.836]**
UA	G11	0.5798	0.8334	0.205 [-0.542, 0.951]	0.101 [-0.914, 1.116]	0.148 [-0.868, 1.164]
	G13.5			0.968 [0.21, 1.727]	0.415 [-0.61, 1.44]	1.756 [0.563, 2.949]
	G16			0.033 [-0.715, 0.78]	0.384 [-0.639, 1.408]	0.355 [-0.667, 1.377]
Peak Systolic Velocity		**Main effect: exposure**	**Interaction: exposure x day**	**Hedge's*g*:**	**Hedge's*g*:**	**Hedge's*g*: CON vs. ALC + CB**
		p-value	***p-value***	**CON vs. ALC**	**CON vs. CB**	
MCA	G11	**0.0437**	**0.011**	0.044 [-0.879, 0.967]	0.454 [-0.423, 1.331]	0.431 [-0.442, 1.304]
	G13.5			0.837 [-0.221, 1.895]	0.779 [-0.252, 1.81]	0.302 [-0.529, 1.132]
	G16			**1.189 [0.089, 2.289]**	**1.247 [0.339, 2.155]**	**1.277 [0.421, 2.132]**
ICA	G11	**0.0263**	**0.0326**	0.103 [-0.737, 0.942]	0.823 [-0.057, 1.704]	0.513 [-0.458, 1.483]
	G13.5			1.209 [0.23, 2.188]	0.487 [-0.627, 1.601]	0.615 [-0.24, 1.469]
	G16			0.766 [-0.07, 1.602]	0.652 [-0.132, 1.437]	**1.601 [0.751, 2.451]**
UA	G11	0.5708	0.5999	0.232 [-0.603, 1.067]	0.294 [-0.726, 1.313]	0.403 [-0.621, 1.428]
	G13.5			0.888 [-0.074, 1.85]	0.7 [-0.345, 1.745]	1.158 [0.062, 2.253]
	G16			0.445 [-0.401, 1.29]	0.342 [-0.679, 1.364]	0.58 [-0.455, 1.616]

Abbreviations CON=Control, ALC=Alcohol, CB=Cannabinoid, ALC+CB=Alcohol+Cannabinoid, VTI=Velocity-Time Integral, MCA=Middle Cerebral Artery, ICA=Internal Carotid Artery, UA=Umbilical Artery

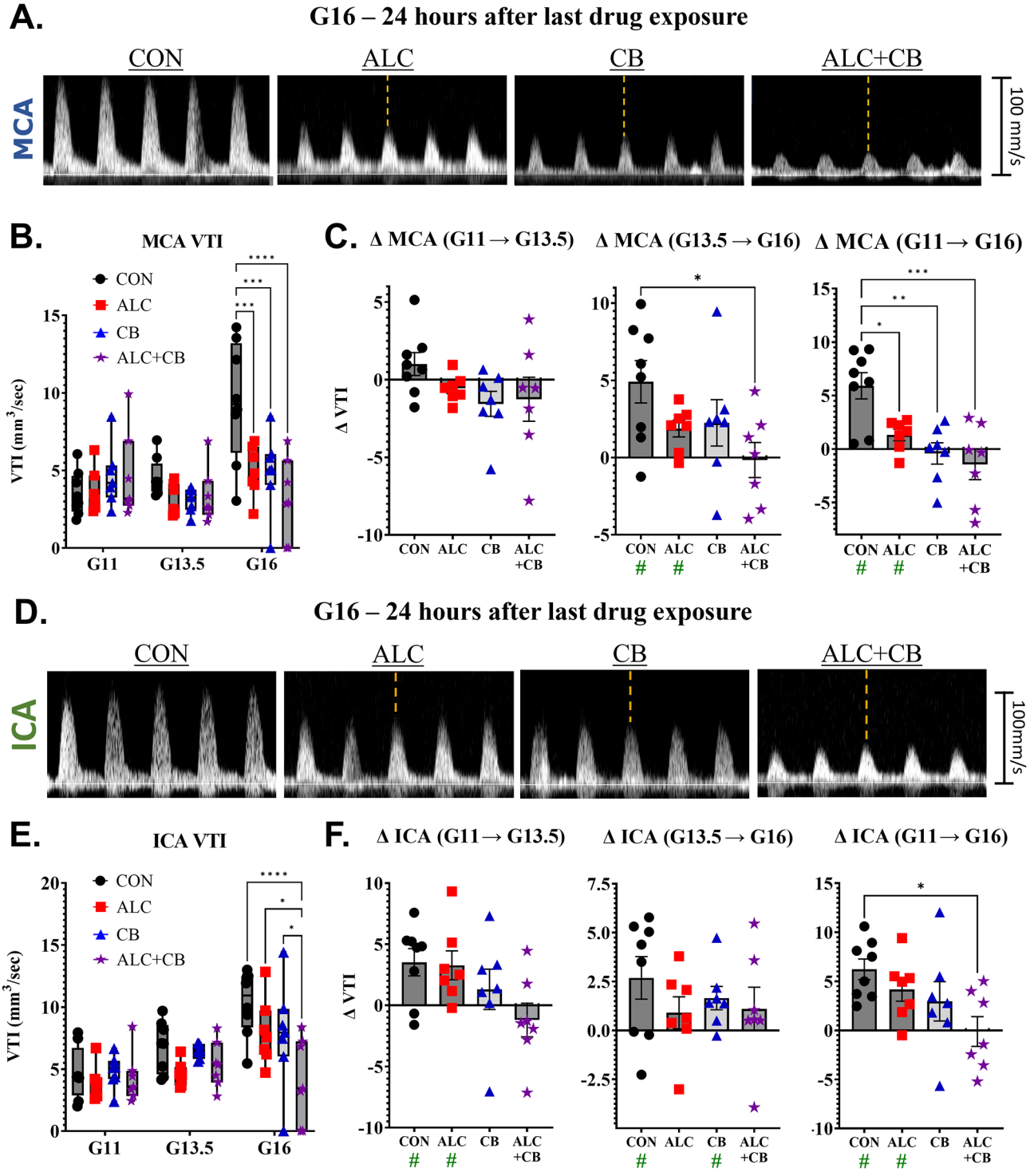


Fig. 2 Drug-associated changes in fetal blood flow in the middle cerebral and internal carotid arteries. **A)** Representative examples of systolic pulse waveforms from the fetal middle cerebral artery across drug groups on Gestational Day 16. **B)** Velocity-Time Integral measurements from the fetal middle cerebral artery across drug groups and assessment days. **C)** Within-fetus changes in middle cerebral artery Velocity-Time Integral measurements across drug groups and assessment days. **D)** Representative examples of systolic pulse waveforms from the fetal internal carotid artery across drug groups on Gestational Day 16. **E)** Velocity-Time Integral measurements from the fetal internal carotid artery across drug groups and assessment days. **F)** Within-fetus changes in internal carotid artery Velocity-Time Integral measurements across drug groups and assessment days. *Abbreviations*: G = Gestational Day, MCA = Middle Cerebral Artery, ICA = Internal Carotid Artery, VTI = Velocity-Time Integral, CON = Control, ALC = Alcohol, CB = Cannabinoid, ALC + CB = Alcohol + Cannabinoid. **Symbols**: Green # indicates a statistically significant change from 0. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$

SAC distinctly reduced internal carotid artery metrics on G16 compared to single-drug exposure groups

As with the middle cerebral artery, drug exposures reduced VTI in the fetal internal carotid artery only after the exposure period [significant interaction between gestational day and exposure: F (6, 75)=2.478, p=0.031]. However, post-hoc analyses revealed that this effect was driven by the ALC+CB group, which significantly reduced VTI relative to all other exposures (CON, ALC & CB: all p's<0.027). Neither ALC nor CB fetuses differed from controls (p's>0.150; Fig. 2D&E, Supplementary Table 7). While VTI increased in CON fetuses from G11 to G16 (p<0.001), this age-related increase was attenuated in ALC (p=0.032) and CB (p=0.032) fetuses (Fig. 2F), and abolished in ALC+CB fetuses (p=0.939). This VTI growth significantly differed between CON and ALC+CB fetuses exclusively (p=0.022; Supplementary Table 8). ALC+CB fetuses were also the only group to show significant reductions in internal carotid artery Acc on G16 (p=0.015; Supplementary Fig. 4A) relative to controls (Supplementary Table ;9). Furthermore, on G16, ALC+CB fetuses demonstrated reduced PSV compared to all other exposure groups (CON, ALC & CB: all p's<0.059; Supplementary Fig. 4B), with no other group differences observed (Supplementary Table 10).

Prenatal cannabinoid exposure diverts fetal blood away from the brain

Prior investigations in human fetuses link cannabis exposure to reduced CPR [30]. Here, assessments of CPR for mouse fetuses, as a ratio-percent of VTI in the middle cerebral artery relative to the umbilical artery, revealed a significant interaction between gestational day and exposure [F (6, 48)=3.082, p=0.012]. Post-hoc comparisons (Fig. 3A) showed that, relative to controls, CPR declined on G16 in both cannabinoid-exposed groups: CB (p=0.009) and ALC+CB (p=0.001) (Supplementary Table 11). Similarly, within-animal, only CB and ALC+CB dams demonstrated reduced CPR following the drug exposure period (Fig. 3B, Supplementary Table 12). Reduced CPR in prior investigations of cannabis-exposed human fetuses was also accompanied, in some cases, by reversed end-diastolic blood flow [28], an index of fetal distress [29]. Similar outcomes were observed in a few growth-restricted, cannabinoid-exposed fetal mice (1 CB fetus, 2 ALC+CB fetuses) assessed on G16, including near loss of blood flow in the middle cerebral artery (Fig. 3C, with an age-matched CON fetus for comparison; Supplementary Videos 1&2) and flow-reversal in umbilical circulation (Fig. 3D; Supplementary Videos 3&4).

Fetal blood flow metrics predict drug-exposure-associated perinatal fetal mortality

Kaplan-Meier analysis showed that drug-exposed groups, but not controls, experienced compromised fetal survival [Fig. 4A; $X^2(3, N=40)=15.33$, p=0.002]. Log-Rank (Mantel-Cox) analyses revealed that CON and ALC fetuses did not differ in survival rates (p=0.171). Compared to CON fetuses, both CB (p=0.041) and ALC+CB fetuses (p<0.001) were significantly less likely to be born alive. Likelihood of survival for ALC+CB offspring was significantly worse than ALC offspring (p=0.004), but not CB offspring (p=0.115). Maternal survival in the peri-partum period was also marginally compromised in CB and ALC+CB dams [$X^2(3, N=41)=6.385$, p=0.094; Supplementary Fig. 5]. In contrast, non-pregnant dams who underwent CB and ALC+CB exposures (n=6, data not shown) did not experience subsequent mortality over a 7-month observational period, consistent with prior research showing that CP-55,940 is not toxic or lethal to non-pregnant mice at concentrations equal to or greater than those included in our experimental design [43–45].

Lower VTI CPR on G16 was associated with increased fetal mortality during the peri-partum period (Fig. 4B). ROC analysis also showed that ΔVTI CPR (from G11-G16) predicted subsequent perinatal mortality (p=0.019, area under the curve (AUC), 0.772; sensitivity, 81%; specificity, 85.70%). Δ VTI CPR was also significantly diagnostic of prior exposure to any drug (p=0.005; AUC, 0.861; sensitivity, 86.40%; specificity, 66.7%), particularly cannabinoids (Fig. 4C). Finally, gestational exposure to a cannabinoid significantly predicted survival in the perinatal period (p=0.044; AUC, 0.733; sensitivity, 66.70%; specificity, 80%). All AUC analyses are detailed in Fig. 4C.

Discussion

Alcohol is often co-consumed with other psychoactive drugs, but the added risk for pregnancy health and infant birth outcomes due to co-consumption is seldom investigated. SAC in particular requires additional investigation, as existing literature on adult alcohol use disorders indicates that alcohol's effects may be potentiated by endocannabinoid signaling (reviewed in [46]). Moreover, two recently published papers in preclinical models indicate that SAC increases teratology [25, 26] over alcohol or cannabinoid exposures alone. Our study shows that SAC, but not alcohol or cannabinoids alone, decreased gestational weight gain. SAC also augmented maternal blood alcohol concentrations compared to alcohol-exposure alone, a phenomenon that has been observed acutely in human co-users [47], as well as rodent offspring with early postnatal exposure [48, 49]. As co-use in humans is also associated with higher cannabinoid levels in the bloodstream [50], as well as slower cannabinoid metabolism [51], it is likely that alcohol and cannabinoids

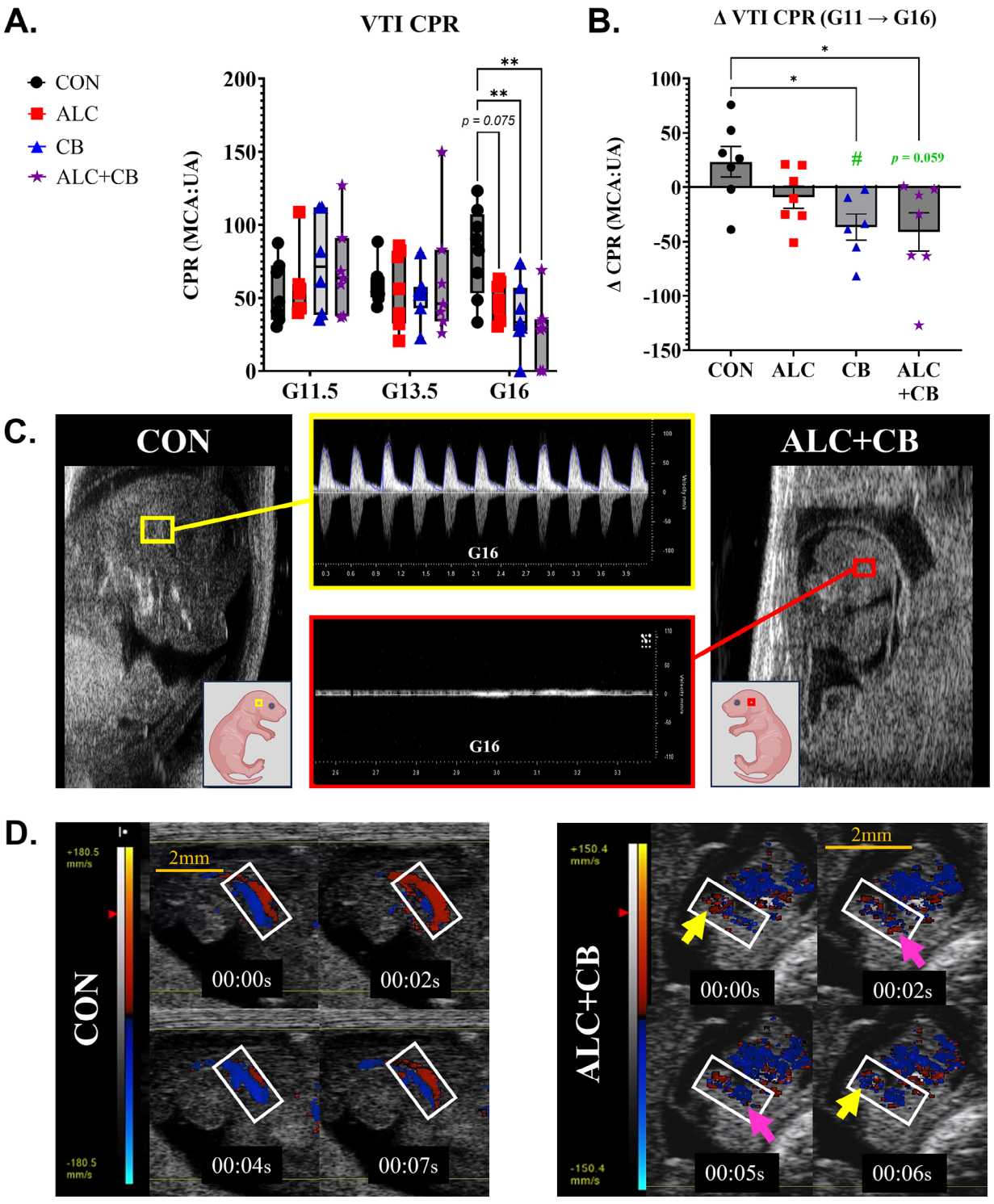
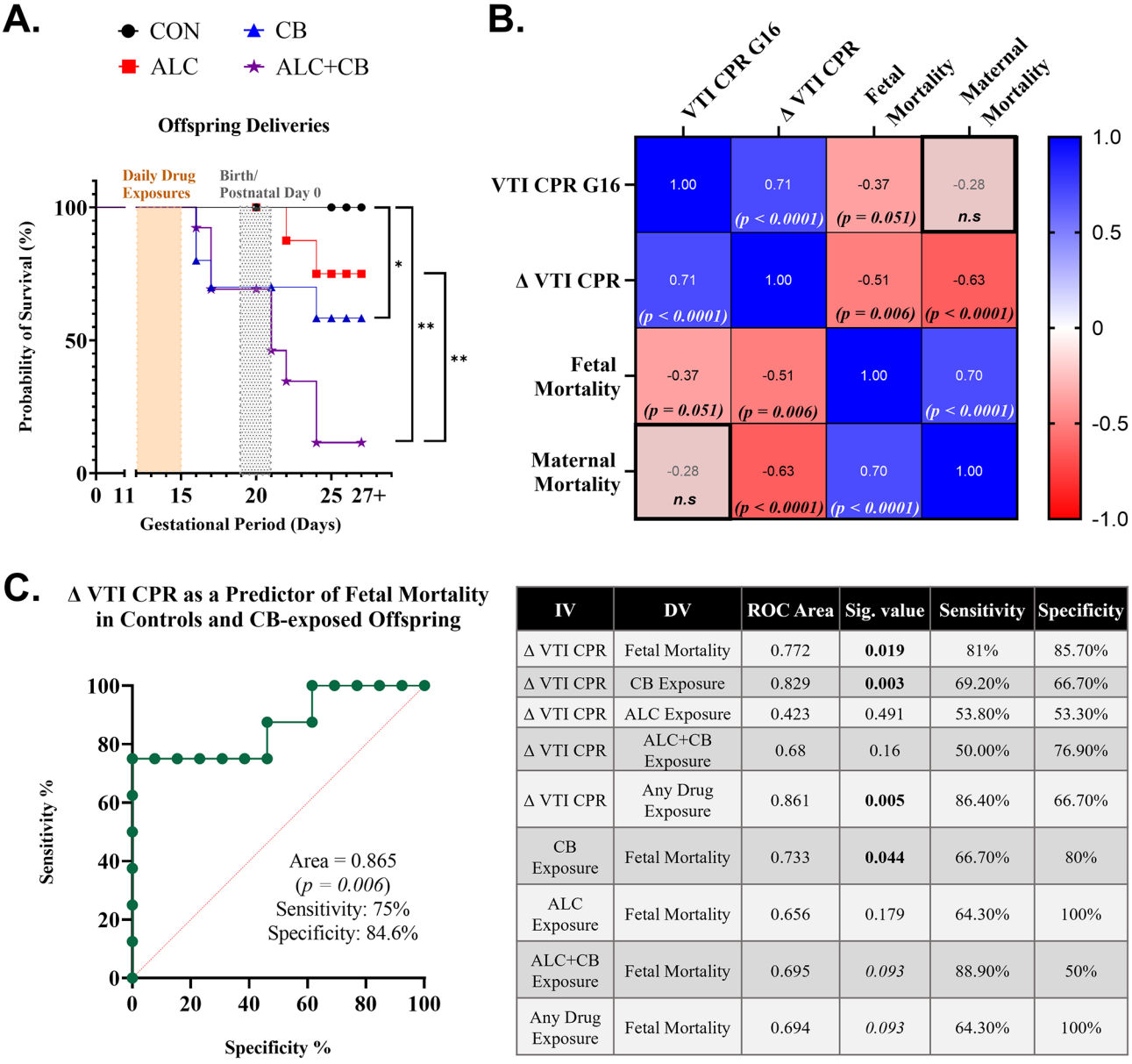


Fig. 3 Changes in fetal cerebroplacental ratios following prenatal drug exposure. **A)** Cerebroplacental ratios calculated within-animal across drug groups and assessment days. CPR = [MCA VTI/ UA VTI] * 100. **B)** Change within-animal in cerebroplacental ratios prior to drug exposure (Gestational Day 11) and following drug exposure (Gestational Day 16). **C)** Gestational Day 16 images of a fetus with no drug exposure (left, yellow) and a growth-restricted fetus with alcohol + cannabinoid exposure (right, red). Waveform images demonstrate a lack of directional blood flow in alcohol + cannabinoid fetus. **D)** Series of Color doppler images of a drug-free control fetus (left) and a growth-restricted alcohol + cannabinoid exposed fetus (right). In the control fetus, the umbilical artery and vein run parallel to each other without overlapping. In contrast, within the alcohol + cannabinoid exposed fetus, red and blue colors overlay within the same regions (arrows), indicating a loss of directional blood flow in the umbilical artery. *Abbreviations*: G = Gestational Day, VTI = Velocity-Time Integral, CPR = Cerebroplacental Ratio, CON = Control, ALC = Alcohol, CB = Cannabinoid, ALC + CB = Alcohol + Cannabinoid. **Symbols**: Green # indicates a statistically significant change from 0. * indicates $p < 0.05$, ** indicates $p < 0.01$



IV	DV	ROC Area	Sig. value	Sensitivity	Specificity
Δ VTI CPR	Fetal Mortality	0.772	**0.019**	81%	85.70%
Δ VTI CPR	CB Exposure	0.829	**0.003**	69.20%	66.70%
Δ VTI CPR	ALC Exposure	0.423	0.491	53.80%	53.30%
Δ VTI CPR	ALC+CB Exposure	0.68	0.16	50.00%	76.90%
Δ VTI CPR	Any Drug Exposure	0.861	**0.005**	86.40%	66.70%
CB Exposure	Fetal Mortality	0.733	**0.044**	66.70%	80%
ALC Exposure	Fetal Mortality	0.656	0.179	64.30%	100%
ALC+CB Exposure	Fetal Mortality	0.695	*0.093*	88.90%	50%
Any Drug Exposure	Fetal Mortality	0.694	*0.093*	64.30%	100%

Fig. 4 Fetal blood flow metrics as predictors of prior drug exposure and future perinatal mortality. **A)** Kaplan-Meier analysis of offspring survival rates across drug-exposed pregnancies. **B)** Pearson's *r* correlations between ultrasound measures of cerebroplacental ratios and perinatal mortality among offspring and dams. **C)** Predictive capacity of cerebroplacental ratios to prior drug exposure or subsequent perinatal offspring mortality. *Abbreviations*: G = Gestational Day, VTI = Velocity-Time Integral, CPR = Cerebroplacental Ratio, ALC = Alcohol, CB = Cannabinoid, ALC + CB = Alcohol + Cannabinoid, N.S = statistically non-significant. **Symbols**: * indicates $p < 0.05$, ** indicates $p < 0.01$

increase each other's bioavailability. In pregnant individuals, this may indicate that polysubstance use prolongs interactions between consumed substances and a developing fetus.

Whereas all drug exposures decreased fetal cranial blood flow, SAC also reduced internal carotid artery cardiac stroke volume, blood flow quantity, and arterial resistance relative to all other exposures. Moreover, the inhibitory effect of SAC on age-appropriate maturation of middle cerebral and internal carotid blood flow was more severe than following either alcohol or cannabinoid exposures alone. Individually, both alcohol and cannabinoids are known to inhibit fetal cerebral blood flow [31, 39, 52–54], and our data suggest that SAC may augment risk for decreased fetal cranial blood flow. Interestingly, no form of drug exposure inhibited umbilical arterial flow, suggesting a specific vulnerability of fetal cranial circulation.

Both cannabinoid exposure alone and SAC significantly and equivalently decreased fetal CPR, as previously documented for cannabis exposures in human pregnancy [30], and in growth-restricted fetuses, we

also observed end-diastolic flow similar to observations in human populations [28]. Reduced CPR suggests a diversion of resources, including nutrients derived from the mother, away from the developing fetal brain. In our study, reduced CPR at the end of the exposure period was a significant predictor of subsequent offspring mortality during the perinatal period. This outcome mirrors findings from one existing early study that reported synergistic increases in fetal reabsorption *in utero* following co-exposure to alcohol and delta-9-tetrahydrocannabinol in rodents [55]. While cannabinoid exposure by itself has been inconsistently associated with neonatal mortality in human populations [16, 56], its co-use with alcohol use may confer additional and unrecognized risk for adverse birth outcomes in human populations.

The mechanisms contributing to the synergy between alcohol and cannabinoids are not well understood, although prior research in zebrafish and mice indicates that cannabinoid receptor 1 (CNR1) may be uniquely impacted by SAC [57, 58], as polysubstance exposure augmented neurobehavioral outcomes and birth defects in exposed offspring in a CNR1-dependent manner. In human populations, the relationship between alcohol exposure and brain CNR1 levels is complex; acute exposure is associated with an increase in CNR1 availability, whereas chronic exposure results in decreased brain CNR1 [59]. Studies in animal models also generally associate chronic alcohol exposure with inhibition of the endocannabinoid system, including CNR1, though acute exposures produce more variable effects (reviewed in [46]). Endocannabinoid signaling plays a critical role in placental growth/viability [60] and the dilation [61] and growth [62] of microvessels. It is possible that the outcomes of SAC are mediated by a complex interplay of activation and inhibition of fetal endocannabinoid signaling across the placenta, blood vessels and brain, resulting in reduced fetal growth and viability. However, further research is required to identify underlying mechanisms, and importantly, to identify possible targets for intervention.

Limitations

To tightly control the dose and duration of episodic alcohol exposure, while maintaining consistent experimental procedures across all pregnancies, all dams were placed in vapor chambers daily from G12-15, receiving either vaporized ethanol or, as a control, room air. This model of forced drug exposure facilitates tight control of exposure conditions between pregnancies, but may also produce unintended stress during pregnancy. All dams also received intraperitoneal injections of either CP-55,940 or volume-equivalent controls. Although cannabinoids exhibit similar pharmacokinetics when administered intravenously or via inhalation, oral administration of both tetrahydrocannabinol (THC) and cannabidiol (CBD) produce notably lower plasma cannabinoid concentrations in exposed subjects, likely due to poor bioavailability and first-pass hepatic metabolism (see review: [63]). In rodents, the brain/plasma ratio of cannabinoid levels varies depending on not only the route of administration, but the specific phytocannabinoids administered, and the species and solvent used [64]. Although smoke inhalation remains the most common form of cannabinoid self-administration in humans, between-subject variability in absorption profiles is notably higher using this form of administration, and much more tightly regulated through intraperitoneal injection. However, this controlled administration may interact with cannabinoid-associated outcomes, particularly regarding pregnancy. In a recent study, pregnant rats receiving either CBD or Δ9-THC via intraperitoneal injection experienced more fetal resorptions than rats administered the same substances through inhalation [65]. Route of administration may not always alter fetal measurements, however, as a previous study determined that ethanol exposure via either intragastric gavage or intraperitoneal injection produced similar reductions in fetal arterial blood flow [39]. Thus, further research incorporating a breadth of administration procedures is needed to determine the influence of route of administration in observed offspring outcomes.

The cannabinoid used in this study, CP-55,940, exhibits greater potency and preference for CNR1 than THC, the main psychoactive component found in cannabis products. However, commercial and recreational cannabis products vary widely in their composition of over 100 naturally-occurring cannabinoids from the *cannabis sativa* plant [66], which poses challenges for mimicking translational models of exposure in the laboratory. Moreover, synthetic cannabinoids (e.g., "spice") are gaining popularity worldwide for reproducing the psychoactive effects of THC-containing products, specifically through ligand-binding preference for CNR1 [66, 67]. Synthetic cannabinoids are particularly favorable among young adults as a less expensive alternative to THC that is often undetectable through urine tests [68]. Notably, the vast majority of synthetic cannabinoid users are previous cannabis users, and use synthetic cannabinoids at levels comparable to [45] or exceeding cannabis, contributing to accidental overdose and synthetic cannabinoid toxicity [69, 70]. Thus, the potency and binding preference demonstrated by CP-55,940 serves as a viable model of synthetic cannabinoid use [45]. Although the dose chosen for this experiment falls within attainable levels in humans, mimicking high cannabis exposure [71, 72], further dose-response investigations comparing prenatal outcomes of synthetic cannabinoid vs. cannabis exposure

are warranted to improve translational modeling of prenatal cannabinoid exposure.

Finally, ultrasound imaging was performed under light isoflurane anesthesia, a necessary step to reduce pain and distress in experimental subjects, but one which complicates interpretation of the effects of drug exposure, although drug-control dams were similarly anesthetized. It is notable, however, that a parallel cohort of SAC-exposed pregnancies which did not include ultrasound imaging also experienced similar mortality during the perinatal period. The influence of anesthesia exposure on offspring neurodevelopmental outcomes is widely discussed [73, 74], and a notable collection of preclinical research has associated prenatal anesthesia exposure specifically with neurocognitive defects [75]. However, as most of these preclinical studies also involve invasive prenatal surgery, which our study did not, it is yet unclear whether anesthesia or surgery-associated stress influenced these observed offspring outcomes.

Conclusions

In this carefully controlled preclinical study, ultrasound imaging measures of fetal arterial blood flow were reduced by prenatal alcohol or synthetic cannabinoid exposure, which can disrupt normal embryonic growth and neural development, and polysubstance exposure augmented deficits in cerebral arterial blood flow. These intrauterine metrics subsequently predicted an increased risk of perinatal mortality in cannabinoid-exposed fetuses and their mothers, which may contribute, in part, to reports of greater health risks associated with delivery in cannabis-exposed pregnancies. Given the rising potency and accessibility of cannabis products, including synthetic cannabinoids worldwide, research investigating translational models of prenatal drug exposure, including polysubstance models, are urgently needed to develop informed treatment and care for drug-exposed pregnancies.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions

SKR and RCM contributed to the conception/design of this research, and SKR was responsible for the acquisition. Both SKR and AS contributed to the analysis of collected data, as well as the original manuscript preparation. Subsequently, SKR and RCM were involved in draft revisions and preparation for review. All authors read and approved the final version of the manuscript.

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Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Raw data files generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal procedures and experiments were approved by Texas A&M's Institutional Animal Care Committee. All animal experimental protocols were carried out in accordance with relevant guidelines and regulations. Experimental methods are described in this manuscript in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Neuroscience and Experimental Therapeutics, Texas A&M School of Medicine, 8447 Riverside Parkway, Bryan, TX 77807, USA

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