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THE EFFECTS OF METHAMPHETAMINE EXPOSURE ON CARDIOVASCULAR DEVELOPMENT IN COMBINATION WITH HYPOXIA IN DANIO RERIO

Sarah Donaldson
University of Akron, sed42@zips.uakron.edu

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Sarah Donaldson

Department of Biology

Honors Research Project

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_____ Reader (printed)	_____ Honors Faculty Advisor (printed)
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_____ Reader (printed)	

Abstract

The purpose of this study was to determine the effects of methamphetamine combined with hypoxia on the development and function of the cardiovascular system in *Danio rerio* embryos. It was hypothesized that the combined effect of the drug and decreased oxygen concentration would result in decreased cardiac parameters due to underdevelopment of the ventricle and vessels resulting from cardiomyopathy and lack of blood flow to tissues. It was found that methamphetamine exposure correlated to an increase in stroke volume, caudal artery and vein diameters and RBC velocities alone, while having a multiplicative effect of increasing arterial RBC velocity when combined with hypoxia. Methamphetamine exposure alone did not have a significant effect on heart rate and cardiac output. This experiment shows that the negative effects of prenatal abuse of methamphetamine could be amplified by hypoxia induced by various pathologies.

Introduction

Methamphetamine is a well-known, popularly abused central nervous system stimulant known to modify and possibly damage the cardiovascular system, increasing heart rate, blood pressure and blood glucose (Logan 2002). Long-term methamphetamine abuse has been linked to psychotic behavior (including hallucination and delusions similar to schizophrenia), motor and memory impairment (Nagai and Yamada 2010). Methamphetamine acts at the level of neuron synapse by increasing the release of neurotransmitters such as dopamine and serotonin into the synapse, while also blocking the reuptake of these neurotransmitters thus increasing activity in the brain (Won et. al 2013). Methamphetamine similarly acts on the heart to increase contractility and blood pressure by activating α- and β-adrenergic receptors in the cardiac muscle, as well as other cardiac pathologies including arrhythmias, sudden cardiac death and cardiomyopathy (Logan 2002, Won et. al 2013).

While methamphetamine has been studied in detail in adults, little research has been done on its effect on fetal development (American College of Obstetricians and Gynecologists

2011). Several studies have shown children who have been prenatally exposed to methamphetamines have had significantly lower birth weight, smaller gestational age and increased possibility of defects in the cardiovascular, nervous and gastrointestinal systems (American College of Obstetricians and Gynecologists 2011). However, these studies were not corrected for the possibility of the mothers abusing other substances while pregnant. Prenatal methamphetamine exposure is also known to decrease uterine and placental blood flow, which is thought to be the cause of hypoxemia and hypertension in the fetus (Khoradmehr et. al 2015). The methamphetamine-induced hypoxia to the fetus could then lead to decreased cardiovascular function due to many factors such as ventricle dilation, myocardial thinning, or septum defects (Patterson and Zhang 2010).

This study was conducted to determine to what extent methamphetamine exposure combined with hypoxia exposure effects cardiovascular development. While methamphetamine exposure has the potential to increase the heart rate, potential significant underdevelopment of the heart resulting from a hypoxic environment would result in a decreased stroke volume, which in turn would cause decreased cardiac output, and RBC velocity. When exposed to methamphetamine or hypoxia alone, significant underdevelopment of the heart provides evidence that when combined, this effect would be amplified and cause a decrease in vessel diameter. This research will expand on that already performed on the effects of methamphetamine exposure during pregnancy on fetal growth, cognition, and behavior in humans and rats (Smith et al 2003, Vorhees et al. 1994). The zebrafish embryonic development is characteristic of most vertebrates, therefore any consequences observed in the experimental fish could be extended to the possible birth defects of children from mothers who abused methamphetamines while pregnant with the child and have lung/heart disease (Bakkers 2011). This research could be beneficial to providing insight into the possible physiological mechanisms of methamphetamines during embryogenesis and fetal development, and it effects on the human fetal cardiac morphology and physiology.

Methods

Animals

Adult, long-finned zebrafish (*Danio rerio*) were used from breeding stock at the University of Akron Research Vivarium and bred to obtain embryos for exposure. The zebrafish model was chosen due to the similarities in cardiac development to other vertebrates, including humans, as well as the relative ease of breeding and experimentation, as zebrafish are characteristically transparent as embryos making them excellent models for microscopy (Bakkers 2011). The adults were housed in 75L acrylic tanks at 26°C (±1°C) with a 14:10 hour light: dark cycle. Breeding boxes were introduced into the tanks just before the start of the dark cycle, breeding occurred just after the end of the dark cycle. The eggs were collected within one hour post fertilization (hpf) and immediately placed in a treatment flask (200mL) and incubated until 72 hpf.

Chemicals

Methamphetamine hydrochloride was obtained from Sigma Aldrich (lot number: SLBG3762V, item number: M8750) and the procedures for schedule II substances were followed. Exposures of 3.0mg/L intended for exposure were mixed the morning of egg collection. The solutions (control and experimental) were mixed with dechlorinated tap water.

Treatment

Once the methamphetamine dilutions were created and separated into 200mL flasks, hypoxia was induced by bubbling the water with N_2 gas (2.5 ± 0.5 mg O_2/L) and normoxia was maintained via compressed air bubbles (7.0 ± 0.5 mg O_2/L). The flasks were sealed with corks to minimize change in O_2 concentrations. Once collected, 25 fertilized eggs were placed in the flasks in one of four treatments: normoxic conditions alone, normoxic conditions with methamphetamine (3.0 mg/L), hypoxia alone, or hypoxia with methamphetamine (3.0 mg/L). Two flasks were used for each treatment. After 24 hours, the embryos were temporarily removed from the flasks so that the dead embryos could be removed and the water could be changed and replaced with identical treatment dilution solutions and oxygen concentrations.

The embryos were placed back in the same treatment flask. At 72 hpf, the water was changed a second time, but replaced instead with dechlorinated tap water and normoxic conditions in all flasks. At this time, the majority of the larvae had hatched and data collection began.

Measurement

For data collection, the larvae were placed in 1mL dechlorinated wells and placed under an inverted light microscope (Leica DMIRB) with a temperature-controlled stage (Harvard Apparatus). 5-second videos were recorded using a high-speed camera (Red Lake MASD, 125 frames/second) of both the heart and of the trunk vessels of the tail of each larva. Image Pro Software (version 4.5) was used to measure cardiac parameters, including heart rate, stroke volume, RBC velocity and trunk vessel diameter using the digital motion technique, as described by Schwerte and Pelster (2000).

For each embryo, using approximately 800 frames of the trunk vessel video, a cast was taken using the digital motion technique to measure the arterial and venous diameters by drawing five lines across the trunk artery and vein, a technique derived from Bagatto (2005). The frames were used to measure arterial and venous RBC velocity by taking the differential of the frames and measuring the length of seven blood cells at peak flow, which is equivalent to the length each cell moved per 1/125 of a second.

The heart rate was measured with a five second video of the heart, where the number of frames between each contraction was counted for three beats. The area and greatest width of the ventricle was measured using Image Pro software at systole and diastole for three heartbeats. These values were used to calculate ventricular volume using the following equation described by Bagatto and Burggren (2006):

$$\textit{Ventricular volume} = (8A^2)/(3\pi L)$$

Where A is equivalent to the ventricular area at either systole or diastole, and L is the length of the ventricle. This equation determined end systolic volume (ESV) and end diastolic volume (EDV), which were used to calculate stroke volume (SV) by subtracting ESV from EDV. The stroke volume was multiplied by heart rate (HR) to calculate overall cardiac output (Q).

Statistics

First, a one-way nested analysis of variance (ANOVA) was performed to test for variance between treatment flasks. No significant variance from flask effects was measured. Therefore, the cardiovascular parameters (vessel diameter, RBC velocity, SV, heart rate, and Q) were analyzed using a crossed two-way ANOVA to test the main effects of oxygen concentration and methamphetamine. (Appendix A, Table 1-2). When significance in any of the main effects was found with the ANOVA, a *post hoc* Fisher's LSMeans Difference multiple comparison test was conducted to determine the source of significance (Appendix A, Table 3-4). Statistics were performed using JMP Pro 13 (SAS institute) with alpha set at $p < 0.05$.

Results

Stroke Volume

Stroke volume (SV) was measured by subtracting ESV from EDV at 72hpf for all treatment groups. Oxygen concentration had a slight effect on ESV (F ratio=3.26, p=0.0764), and methamphetamine exposure had a slight effect on EDV (F ratio=1.98, p=0.0759). Methamphetamine exposure had a significant effect on stroke volume (F ratio=5.36, p=0.024), shown Figure 1A-C. The *post hoc* analysis showed a significant increase in both EDV and SV with methamphetamine and hypoxia co-exposure compared to the normoxic group not exposed to methamphetamine.

Heart Rate

The heart rate (HR), shown in Figure 1D, measured in beats per minute (bpm), was measured at 72 hpf for all treatment groups. While no significance was found with methamphetamine exposure (F ratio=1.75, p=0.1911), oxygen concentration had a significant effect on heart rate (F ratio=4.64, p=0.0355). The *post hoc* analysis showed a significant increase in heart rate in the hypoxia group not exposed to the drug compared to the normoxia methamphetamine co-exposed group.

Cardiac Output

No significant effect of oxygen concentration or methamphetamine exposure was determined with the ANOVA (F ratio=1.45, p=0.2329 and F ratio=3.70, p=0.0595, respectively).

However, the *post hoc* analysis showed a significant increase in cardiac output between the hypoxia and methamphetamine co-exposed group compared to the normoxia group not exposed to the drug.

Artery Diameter

Exposure to methamphetamine had a significant effect on caudal artery diameter (F ratio=4.10, p=0.0476), while the oxygen concentration showed no significant change in diameter (F ratio=0.18, p=0.6690). No significance was found in the *post hoc* analysis for the source of the effect.

Vein Diameter

Vein diameter was significantly affected by methamphetamine exposure alone (F ratio=5.06, p=0.0285). Interestingly, the *post hoc* LSMeans Difference test showed a slight increase in diameter with co-exposure to hypoxia and methamphetamine (p=0.0797), leading to a 16.2% increase in vein diameter of the hypoxic, methamphetamine-exposed fish compared to the hypoxia group not exposed to the drug, shown in Figure 2B.

Arterial RBC Velocity

The red blood cell velocity was measured in the caudal vessels for all treatment groups at 72 hpf. While no effect was measured between the oxygen concentration treatments (F ratio=1.52, p=0.2216), methamphetamine exposure had a significant effect on RBC velocity in the caudal artery (F ratio=15.1, p=0.0003). A significant interaction was also found between the oxygen concentration and methamphetamine co-exposure (F ratio=5.35, p=0.0244). The *post hoc* LSMeans Difference test showed an increase in arterial RBC velocity with hypoxia and methamphetamine co-exposure compared to the normoxic group not exposed to methamphetamine and as well as an increase with co-exposure compared to the hypoxia group not exposed to the drug (see Figure 2C).

Venous RBC Velocity

Oxygen concentration had no effect on RBC velocity in the caudal vein (F ratio=0.027). However, exposure to methamphetamine had a significant effect on venous RBC velocity (F ratio=9.02, p=0.0040). While the ANOVA did not show a significant interaction (F ratio=0.2979, p=0.5874), the *post hoc* analysis showed a significant increase in RBC velocity in the vein

between the normoxia and methamphetamine co-exposed group compared to the hypoxia group not exposed to the drug and from the normoxia group not exposed to the drug.

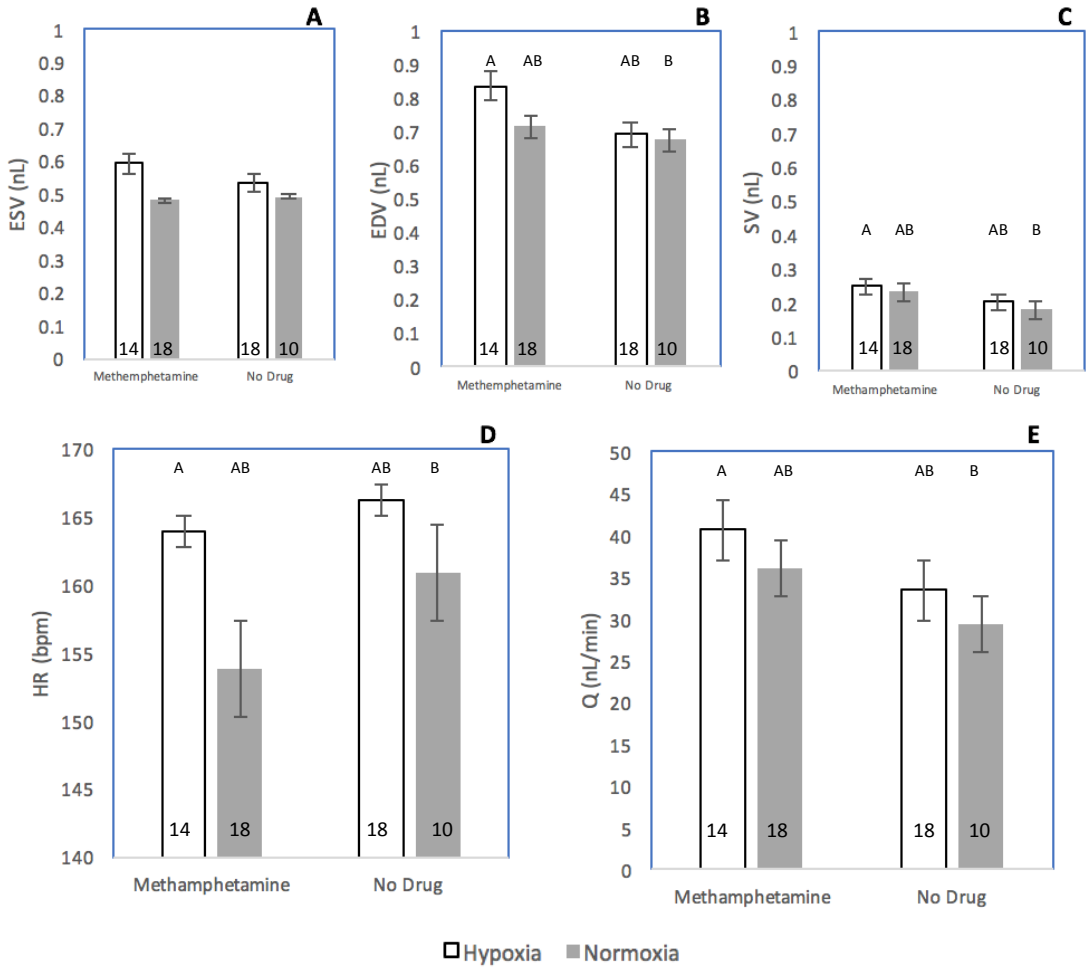

Figure 1. End Diastolic Volume (EDV), End Systolic Volume (ESV), Stroke Volume, Heart Rate and Cardiac Output (Q) for all treatment groups, recorded at 72 hpf. Sample sizes are noted on the bars, and error bars represent standard error. Bars not connected by the same letter are significantly different (significance determined by *post hoc* Fisher's LSD test). (A) EDV and (B) ESV were both unaffected by either treatment. (C) Stroke Volume only slightly increased from the normoxia control when meth and hypoxia were combined. (D) Heart rate was unaffected by methamphetamine, but significantly increased with hypoxia compared to the normoxia control. (E) Q was unaffected by either exposure.

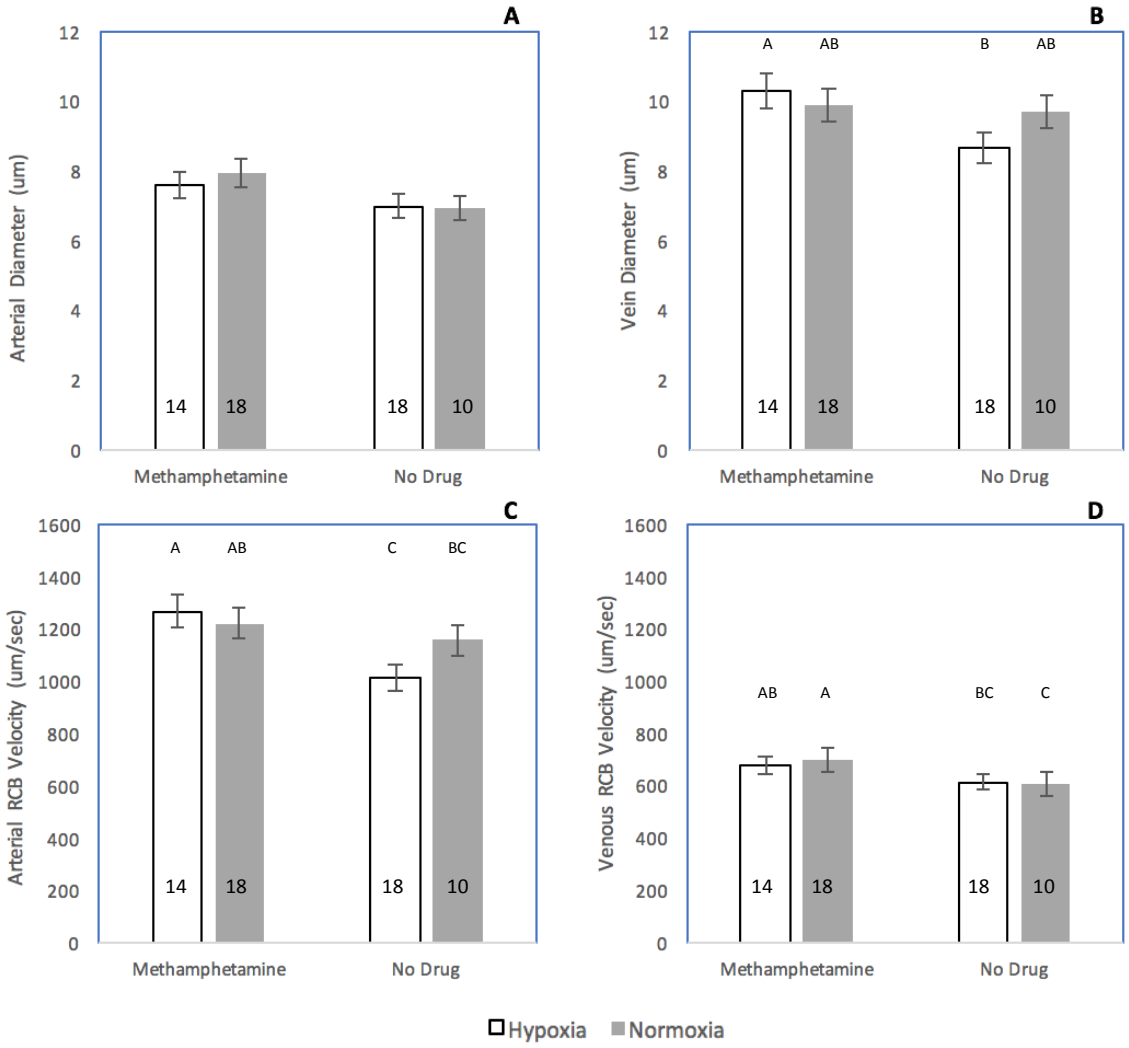

Figure 2. Caudal vessel diameters (A-B) and caudal vessel RBC velocities (C-D) of all treatment groups recorded at 72 hpf. Sample sizes are noted on the bars, error bars represent standard error. Bars not connected by the same letter are significantly different (significance determined by *post hoc* Fisher's LSD test). (A) Artery diameter increased with methamphetamine exposure compared to the unexposed embryos. (B) Vein diameter was unaffected by methamphetamine exposure but did increase with hypoxia when compared to the normoxia control. (C). Arterial RBC velocity significantly increased when methamphetamine and hypoxia were combined compared to the normoxia control. (D) Venous RBC velocity was unaffected by exposure to hypoxia, but did increase significantly compared to the unexposed treatment groups when exposed to methamphetamine.

Discussion

The cardiovascular system function was shown to be affected by embryonic methamphetamine exposure until 48 hpf. While drug exposure showed no significant effects on heart rate or cardiac output, there were significant effects on stroke volume, artery and vein diameter, and arterial and venous RBC velocity. The results contradicted the hypothesis.

In the human fetus, methamphetamine is known to act on the heart, resulting in ventricular myopathy and cardiac underdevelopment such as myocardial thinning, septum defects and smaller ventricular volume (Patterson and Zhang 2010). It was hypothesized that methamphetamine exposure would significantly decrease the stroke volume and cardiac output when combined with hypoxia (Patterson and Zhang 2010, Smith et al 2003, Vorhees et al. 1994). However, stroke volume significantly increased (Figure 1C) along with a slight increase in cardiac output (Figure 1E). End diastolic volume increased slightly, possibly due to increased ventricular compliance or increased preload, which can increase the volume of blood entering the ventricle thus increasing the volume pumped out (Klabunde 2012). As methamphetamine exposure could result in ventricular thinning (Patterson and Zhang 2010), compliance resulting from a thinner myocardium in the ventricle could have increased resulting in a greater volume of blood capable of entering the ventricle to be pumped out. Preload on the ventricle could have been increased by a higher aortic blood pressure resulting from the methamphetamines acting on the vessel α-adrenergic receptors stimulating a “flight or fight” response (Logan 2002). The increase in stroke volume with methamphetamine exposure is a contributor to the slight increase in cardiac output, while a second contributor is also the increased heart rate with hypoxia exposure (Figure 1D). This effect of hypoxia on heart rate was surprising, as research indicates that cardiac contractility would decrease in response to hypoxia and result in bradycardia (Patterson and Zhang 2010, Klabunde 2012). These results could be suggestive of a relatively small sample size, as a power analysis shows that doubling the sample size will nearly double the power of the analysis (see Appendix A, Table 5).

Methamphetamine exposure had significant effects on artery and vein diameters, as well as on RBC velocity in the caudal artery and vein. While it was hypothesized that the vasoconstrictive properties of methamphetamine would override the vasodilatory effects of

hypoxia and result in a decrease in vessel diameter, the opposite was discovered (Figure 2A-B) (Logan 2002, Klabunde 2012). Hypoxia results in vasodilation of the tissue vessels to increase flow and perfusion to the tissues in response to a decrease in oxygen concentration via release of nitric oxide, endothelial-derived hyperpolarizing factor (EDHF) and prostacyclin, all of which inhibit smooth muscle contraction resulting in vasodilation (Klabunde 2012). Conversely, methamphetamine acts on the α-adrenergic receptors of smooth muscle, causing the release of endothelin-1 (ET-1), which is a powerful vasoconstrictor (Logan 2002, Won et. al 2013, Klabunde 2012). Due to the significantly larger arterial and venous vessel diameters, it could be hypothesized that the vasoconstrictive effects of methamphetamine were outcompeted by the vasodilatory effects of hypoxia. However, this mechanism would need further study, as Schwerte et. al (2006) found that α- and β-adrenergic receptors in fish larvae are present but inactive until approximately 4 days post-fertilization. This means that the exact mechanism of action methamphetamine has on the heart of 72 hpf larvae could be more complex than originally thought, as the receptors are not active and would therefore be insensitive to methamphetamine to alter vasoactive motion of the vessels.

Methamphetamine exposure had a significant effect on arterial and venous RBC velocity (Figure 2C-D). The RBC velocity in the caudal artery was also significantly increased when methamphetamine exposure was combined with hypoxia exposure, suggesting a multiplicative interaction. Klabunde (2012) explains Poiseuille's equation as blood flow (or velocity) being proportional to the radius of the vessel to the power of 4 ($F \propto r^4$). The vascular response to hypoxia results in vasodilation, which would exponentially increase flow and velocity in accordance to Poiseuille's equation. However, methamphetamine's vasoconstrictive properties via α-adrenergic receptor activation could result in a decrease in flow, opposing the vasodilatory effects of hypoxia. Rather than the opposing forces cancelling out, the vasoconstriction effect could have been surpassed by the increase in vessel diameter, which has a more significant effect on velocity than pressure alone (Klabunde 2012), or due to the inactive α-adrenergic receptors in the vessels giving no vasoconstrictive response to methamphetamine (Schwerte et. al 2006). The significant interaction of hypoxia and methamphetamine co-exposure on arterial RBC velocity could be caused by a combination of the vasodilatory

response to hypoxia and the increase in blood pressure and cardiac output in response to methamphetamine.

The functionality of the cardiac system is key to whole body development in any organism, as its purpose is to generate pressure to perfuse tissues with oxygen and nutrients (Klabunde 2012). With impeded flow, the tissue may fail to develop properly. In human infants, prenatal methamphetamine exposure resulted in smaller head circumference, reduced brain volume and lower birth weight, all possibly resulting in poorly developed tissue due to variations in cardiac function and blood flow (American College of Obstetricians and Gynecologists 2011, Klabunde 2012). Methamphetamine exposure in adult rats resulted in accelerated glucose and fatty acid catabolism, and could also be shown to act similarly on the systemic metabolism of other mammals with further study (Zheng et. al 2014). Therefore, if the metabolic demand of embryos exposed to methamphetamines increases, the flow of blood to the metabolizing tissues must increase as well to supply the tissue with nutrients needed to support the metabolic demand. The underlying response to hypoxia and methamphetamine exposure would then be to increase cardiac parameters such as stroke volume, heart rate, vessel diameter and RBC velocity to maintain the delivery of oxygen and nutrients to the metabolizing tissue while in a low oxygen environment.

Conclusions

As the results have shown, methamphetamine exposure has significant effects on cardiovascular function, and in several cases a multiplicative effect when combined with hypoxia. Oxygen concentration significantly affected heart rate, while methamphetamine exposure had an effect on stroke volume, caudal artery and vein diameter, and arterial and venous RBC velocity. EDV, ESV, and Cardiac output were unaffected by either exposure. A multiplicative effect of co-exposure to hypoxia and methamphetamine was found in arterial RBC velocity. These results show that while several cardiac parameters were altered due to the individual effects of hypoxia or methamphetamine, combined exposure can synergistically act on the cardiovascular system.

This study could have been improved by increasing the sample sizes (shown with a power analysis of the results where the p-value was between 0.07 and 0.059, see Appendix A, Table 4) of the treatment groups, thus increasing the accuracy of the results depicting changes in the cardiovascular system in response to co-exposure as well as the reliability of the results without the action of confounding effects. Exposure of the embryos could have been altered by lengthening methamphetamine exposure to 72 hpf, or by performing multiple water changes daily to ensure constant methamphetamine exposure as well as oxygen concentrations. The dosage of methamphetamine could also be increased to determine if increased methamphetamine concentration could further effect the cardiac development. This area of research could also benefit from simultaneous exposure of methamphetamine combined with other popularly abused drugs, such as nicotine or heroine, and their effects on cardiac parameters, as it better reflects the reality of pregnant women abusing multiple drugs while pregnant, as it is becoming a more common occurrence (American College of Obstetricians and Gynecologists 2011).

In Ohio alone, the availability of methamphetamine is a 10 on a 0-10 scale (0 being not available and 10 being extremely easy to get), and the use of the drug is on the rise according to the Ohio Department of Mental Health and Addiction Services (2016). This poses a large risk, as 53% of the methamphetamine users were female, and of those more than 50% were of child-bearing age (Ohio Department of Mental Health and Addiction Services 2016). There is a risk of these women abusing methamphetamines while pregnant, which would pose a serious risk to the cardiovascular functionality of the fetus. As the results suggested, if any of these women were at risk for or diagnosed with COPD, anemia, or other heart disease resulting in hypoxemia it could amplify the effects of methamphetamine on their unborn child and intensify the negative side effects. Further research is needed to fully understand and map out the exact mechanism of methamphetamine in the embryo to fully understand its effects.

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Appendix A

Main Effects ANOVA results (Parameter Estimates):

A. ESV

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	2.351e-5	0.0764	1.02018e-7	3.2594	0.0764
DRUG [C]	2.351e-5	0.5153	1.34202e-8	0.4288	0.5153
OXYGEN[H]*DRUG[C]	2.351e-5	0.8216	1.60717e-9	0.0513	0.8216

B. EDV

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	2.87e-5	0.1644	9.25465e-8	1.9847	0.1644
DRUG [C]	2.87e-5	0.0759	1.52551e-7	3.2716	0.0759
OXYGEN[H]*DRUG[C]	2.87e-5	0.6032	1.27439e-8	0.2733	0.6032

C. Stroke Volume

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	0.010643	0.3584	0.00549962	0.8574	0.3584
DRUG [C]	0.010643	0.0243*	0.03437140	5.3586	0.0243*
OXYGEN[H]*DRUG[C]	0.010643	0.8372	0.00027349	0.0426	0.8372

D. Heart Rate

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	1.775878	0.0355*	828.97948	4.6417	0.0355*
DRUG [C]	1.775878	0.1911	312.73833	1.7511	0.1911
OXYGEN[H]*DRUG[C]	1.775878	0.5067	79.76813	0.4466	0.5067

E. Cardiac Output

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	1.795862	0.2329	265.63382	1.4544	0.2329
DRUG [C]	1.795862	0.0595	676.01809	3.7015	0.0595
OXYGEN[H]*DRUG[C]	1.795862	0.9321	1.33842	0.0073	0.9321

Table 1. The results of the main effects ANOVA on cardiac parameters, showing both the parameter estimates and the effects tests. The F ratio values and p-values for main effects were taken from the effects tests. (A) End Systolic Volume, (B) End Diastolic Volume, (C) Stroke Volume, (D) Heart Rate, (E) Cardiac Output.

A. Arterial Diameter

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	0.20044	0.6690	0.4201818	0.1847	0.6690
DRUG [C]	0.20044	0.0476*	9.3326088	4.1020	0.0476*
OXYGEN[H]*DRUG[C]	0.20044	0.6080	0.6055101	0.2661	0.6080

B. Venous Diameter

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	0.206693	0.4440	1.437627	0.5942	0.4440
DRUG [C]	0.206693	0.0285*	12.238816	5.0588	0.0285*
OXYGEN[H]*DRUG[C]	0.206693	0.0797	7.707494	3.1858	0.0797

C. Arterial RBC Velocity

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	20.17003	0.2216	35195.80	1.5277	0.2216
DRUG [C]	20.17003	0.0003*	346773.72	15.0519	0.0003*
OXYGEN[H]*DRUG[C]	20.17003	0.0244*	123363.00	5.3547	0.0244*

D. Venous RBC Velocity

TERM	STD ERROR	PROB>|T|	SUM OF SQUARES	F RATIO	PROB>F
OXYGEN [H]	35195.80	1.5277	259.975	0.0270	0.8701
DRUG [C]	346773.72	15.0519	86944.134	9.0210	0.0040*
OXYGEN[H]*DRUG[C]	123363.00	5.3547	2871.381	0.2979	0.5874

Table 2. The results of the main effects ANOVA on vascular parameters, showing both the parameter estimates and the effects tests. The F ratio values and p-values were taken from the effects tests. (A) Caudal Artery Diameter, (B) Caudal Vein Diameter, (C) Arterial RBC Velocity, (D) Venous RBC Velocity.

Post hoc Analysis results where significance was found in co-exposure:

A. ESV

Level		Least Sq Mean
H,M	A	0.00057592
H,C	A	0.00053447
N,M	A	0.00048037
N,C	A	0.00046024

B. EDV

Level			Least Sq Mean
H,M	A		0.00082459
N,M	A	B	0.00071373
H,C	A	B	0.00069078
N,C		B	0.00063993

C. SV

Level			Least Sq Mean
H,M	A		0.24867199
N,M	A	B	0.23335769
H,C	A	B	0.20379428
N,C		B	0.17968955

D. HR

Level			Least Sq Mean
H,C	A		166.24876
H,M	A	B	163.92242
N,C	A	B	160.97033
N,M		B	153.89661

E. Q

Level			Least Sq Mean
H,M	A		40.725223
N,M	A	B	36.086120
H,C	A	B	33.507578
N,C		B	29.483420

Table 3. The *post hoc* LSMeans Difference test values for (A) End Systolic Volume, (B) End Diastolic Volume, (C) Stroke Volume, (D) Heart Rate, and (E) Cardiac Output where a significant effect was measured between the main effects. Levels not connected by the same letter are significantly different. (where H is hypoxia, N is normoxia, M is methamphetamine and C is control [no methamphetamine exposure]).

A. Artery Diameter

Level		Least Sq Mean
N,M	A	7.9588980
H,M	A	7.5798111
H,C	A	6.9747043
N,C	A	6.9401722

B. Vein Diameter

Level			Least Sq Mean
H,M	A		10.313681
N,M	A	B	9.894498
N,C	A	B	9.702567
H,C		B	8.646056

C. Arterial RBC Velocity

Level				Least Sq Mean
H,M	A			1265.2108
N,M	A	B		1221.7238
N,C		B		1158.5645
H,C			C	1015.3568

D. Venous RBC Velocity

Level				Least Sq Mean
N,M	A			695.75411
H,M	A	B		677.22738
H,C		B	C	613.10247
N,C			C	603.14623

Table 4. The *post hoc* LSMeans Difference test values for (A) Artery Diameter, (B) Vein Diameter, (C) Arterial RBC Velocity, and (D) Venous RBC Velocity where a significant effect was measured between the main effects. Levels not connected by the same letter are significantly different. (where H is hypoxia, N is normoxia, M is methamphetamine and C is control [no methamphetamine exposure]).

Power Analysis Tables where 0.080< p >0.0595:

A. ESV

α	σ	δ	Number	Power
0.0500	0.000177	4.123e-5	60	0.4265
0.0500	0.000177	4.123e-5	120	0.7164

B. EDV

α	σ	δ	Number	Power
0.0500	0.000216	5.042e-5	60	0.4278
0.0500	0.000216	5.042e-5	120	0.7180

C. Cardiac Output

α	σ	δ	Number	Power
0.0500	13.51429	3.35663	60	0.4725
0.0500	13.51429	3.35663	120	0.7698

D. Venous Diameter

α	σ	δ	Number	Power
0.0500	1.555417	0.358411	60	0.4186
0.0500	1.555417	0.358411	120	0.7065

Table 5. The power analysis values for all measurements where the ANVOA showed a p value between 0.070 and 0.059. The sample size was increased from n=60 to n=120 and the power analyzed for improvement in data to reject or confirm the hypothesis. An increase in power is considered improvement. (A) End Systolic Volume with respect to oxygen concentration, (B) End Diastolic Volume with respect to methamphetamine exposure, (C) Cardiac Output with respect to co-exposure to hypoxia and methamphetamine, (D) Caudal Vein Diameter with respect to the co-exposure to hypoxia and methamphetamine.