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Long-term consequences of disrupting adenosine signaling during embryonic development

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Abstract

There is growing evidence that disruption in the prenatal environment can have long-lasting effects on an individual's health in adulthood. Research on the fetal programming of adult diseases, including cardiovascular disease, focuses on epi-mutations, which alter the normal pattern of epigenetic factors such as DNA methylation, miRNA expression, or chromatin modification, rather than traditional genetic alteration. Thus, understanding how *in utero* chemical exposures alter epigenetics and lead to adult disease is of considerable public health concern.

Few signaling molecules have the potential to influence the developing mammal as the nucleoside adenosine. Adenosine levels increase rapidly with tissue hypoxia and inflammation. Adenosine antagonists including the methylxanthines caffeine and theophylline are widely consumed during pregnancy. The receptors that transduce adenosine action are the A1, A2a, A2b, and A3 adenosine receptors (ARs). We examined the long-term effects of *in utero* disruption of adenosine signaling on cardiac gene expression, morphology, and function in adult offspring.

One substance that fetuses are frequently exposed to is caffeine, which is a non-selective adenosine receptor antagonist. Over the past several years, we examined the role of adenosine signaling during embryogenesis and cardiac development. We discovered that *in utero* alteration in adenosine action leads to adverse effects on embryonic and adult murine hearts. We find that cardiac A1ARs protect the embryo from *in utero* hypoxic stress, a condition that causes an increase in adenosine levels. After birth in mice, we observed that *in utero* caffeine exposure leads to abnormal cardiac function and morphology in adults, including an impaired response to β -adrenergic stimulation. Recently, we observed that *in utero* caffeine exposure induces transgenerational effects on cardiac morphology, function, and gene expression.

Our findings indicate that the effects of altered adenosine signaling are dependent on signaling through the A1ARs and timing of disruption. In addition, the long-term effects of altered adenosine signaling appear to be mediated by alterations in DNA methylation, an epigenetic process critical for normal development.

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1. Fetal Programming of Adult Disease

It is well recognized that disruption of the intrauterine environment by nutritional or chemical factors may influence the fetus, resulting in long-term adverse effects after birth and into adulthood^{1–4}. By disrupting normal prenatal development, environmental factors and chemical exposures lead to the fetal programming of adult disease, including cardiovascular disease^{5–7}. The timing of the *in utero* insult is important and can affect the outcomes in adulthood⁷.

Fetal programming of adult disease involves several potential mechanisms, including genetic and non-genetic events. Non-genetic factors include influences on cell division⁸, while genetic factors include epigenetic influences on gene activity and expression^{9–13}. Epigenetic changes not only affect the exposed embryo, but may also affect subsequent generations and contribute to the development of disease, as shown by endocrine disruptors vinclozolin and bisphenol A^{14, 15}. Research in our laboratory has demonstrated that *in utero* caffeine exposure disrupts adenosine action and leads to long-term adverse effects on cardiac function^{16–18}. Furthermore, depending on the timing of exposure, caffeine has transgenerational effects on cardiac function¹⁶.

2. Adenosine

Adenosine consists of an adenine group attached to a ribose moiety. Adenosine is present in all cells and is a component of nucleic acids and energy-carrying molecules^{19, 20}. Adenosine can be directly released from the cell or generated extracellularly²¹.

It is likely that several different humoral agents can transduce environmental effects on the embryo and developing fetus. However, adenosine is particularly attractive to study for several reasons. First, adenosine levels are dynamically regulated and increase markedly with tissue hypoxia and energy depletion²². Adenosine receptors may play an important role in sensing disruptions in the intrauterine environment, as it is present in all cells and is a component of nucleic acids and energy-carrying molecules^{19, 20}. For example, under basal conditions the interstitial adenosine levels are 1–50 nM, but these levels rapidly rise to more than 1 μ M with tissue ischemia, hypoxia, or inflammation^{20, 23}.

Adenosine and adenosine receptors influence a number of cellular processes, as well. For example, adenosine receptors activate transcription factors, e.g. NF- κ B that in turn activates pro-inflammatory molecules²⁴. Adenosine also plays a role in regulating cellular events by influencing the expression of the transcription factor Hypoxia Inducible Factor (HIF-1)²⁴.

3. Adenosine Receptors

Fluctuations in adenosine levels are sensed by transmembrane receptors that transduce adenosine's biological effects. There are two major classes of purine receptors – P1 and P2^{25, 26}. ATP and ADP bind to P2 purine receptors that include P2Y purine metabotropic receptors that couple with G-proteins²⁶. P2 receptors also include the P2X receptors that are ion channels²⁶.

Adenosine receptors (ARs) are P1 purine receptors^{25, 27, 28}. Similar to other G protein-coupled receptors (GPCRs), adenosine receptors contain seven putative transmembrane (TM) spanning domains^{25, 27, 28}. Adenosine receptors were initially cloned as orphan receptors²⁹. The identities of the genes encoding the A2a, A1, A2b, and A3 adenosine receptors were subsequently established in sequential order^{30–35}.

A1 and A3ARs activate G_{i/o} protein which inhibits adenylyl cyclase and leads to decreased levels of cAMP²⁷. Alternatively, A2a and A2bARs activate G_s protein which stimulates adenylyl cyclase and leads to increased levels of cAMP²⁷. In addition, A1ARs activate phospholipase C, and open ion channels such as calcium channels²⁷.

Each adenosine receptor subtype has a different pattern of tissue expression and ligand binding properties. In cell-based systems, A1ARs have the highest affinity for adenosine (K_i 10 nM)^{25, 27, 28}. The K_i values for adenosine for the A2a, A2b and A3 adenosine receptors are 200, 2000, and 10,000 nM, respectively, for the human receptors^{25, 27, 28}. A3ARs are also activated by the adenosine metabolite inosine (K_i 2300 nM)^{25, 27, 28}. Methylxanthines, including caffeine and theophylline, are nonselective adenosine receptor antagonists found in commonly consumed beverages including coffee and tea³⁶.

Highest levels of A1AR gene expression are detected in adult brain, fat, and testis³⁴. Less prominent A1AR expression is seen in the heart and kidneys³⁴. A2aAR gene expression is seen in brain, heart, and lung³³. A2bAR mRNA expression is highest in colon and bladder³⁷. A2bARs expression is also high in retina³⁸. A3AR is expressed in testis, heart, and retina³⁵. For A1 and A2aARs, the levels of gene and binding site expression are proportional, but for A3ARs gene expression is much greater than binding site expression³⁹.

In the brain, A2aARs are expressed in several brain regions, and heavy expression is seen in the striatum on cells expressing D2 dopamine receptors, an observation that dates back two decades³³. A2bAR expression is localized to the pars tuberalis region of the hypophysis^{32, 37}. Functional studies have suggested the presence of A3ARs in the central nervous system⁴⁰. A1ARs are among the most widespread GPCRs in the brain. In comparison with the relatively discrete expression of other receptor subtypes, A1AR expression is at high level throughout the brain^{34, 41}.

In the heart, A1AR expression is present in atria and ventricles, and atrial A1AR expression is greater than that seen in the ventricles⁴². A2aARs are present in coronary vessels in endothelial cells, smooth muscle cells of blood vessels and on myocytes⁴³. A3ARs are present in myocardial tissue, although at low levels³⁵. A2bARs are present on endothelial

cells, smooth muscles cells and fibroblasts⁴⁴. Adenosine receptors are thus localized at sites to modulate cardiovascular system function.

4. A1ARs Protect the Embryo from Hypoxic Stress

A1ARs are the earliest expressed adenosine receptors in the fetal heart⁴², and we demonstrated that cardiac A1AR expression protects the embryo from hypoxic insults^{45, 46}. It is likely that other adenosine receptor subtypes play important and possibly protective roles during development, however we have focused on the role of A1ARs in this report.

Because adenosine and A1ARs mediate adverse effects of hypoxia on the developing postnatal mammalian brain and lung⁴⁷, we anticipated that blockade of adenosine action would protect embryos from hypoxia⁴⁷. To our surprise, we observed that adenosine acting through A1ARs exerts dramatic protective effects during mammalian embryogenesis in response to hypoxic insults^{45, 46}.

Using a global A1AR knockout mouse model, we observed normal embryogenesis under normoxic conditions⁴⁶. However, embryos lacking A1ARs were significantly more growth retarded under hypoxic conditions compared to embryos expressing A1ARs⁴⁶. These data show that adenosine acting via A1ARs play an important role in protecting the embryo from hypoxia.

After E10.0 in mice, the embryo is dependent on the fetal heart for adequate nutrient delivery²³. Many developmental processes occur in the heart before this critical stage. The first evident assembly of the myocardial plate on either side of the neural tube begins at embryonic (E) day 7.0 in the mouse⁴⁸. The two primitive heart fields on either side of the neural tube then migrate towards the midline ventrally at E8.0 and fuse to form the single heart tube⁴⁸. The tubular heart begins to contract at E8.5 followed shortly thereafter by looping to the right⁴⁸. Cardiac cushion formation, which is the first step valve development, begins at E9.5⁴⁸. Valve formation and septation into the four chambered heart is completed by E12.5 and further development is primarily concerned with increasing the size of the heart⁴⁸.

Thus, to test if adenosine confers embryo protective effects by acting at the heart, mice that lack A1ARs only in the heart were developed⁴⁵. Remarkably, we observed that embryos lacking cardiac A1ARs had reduced survival and more severe growth retardation in hypoxia compared with littermates expressing A1ARs⁴⁵.

These observations show that adenosine plays a key role in protecting the embryo against intrauterine stress, and adenosine exerts protective effects through A1ARs expressed in the heart. It is likely that adenosine action on embryonic cardiac function plays a major role in embryonic responses to intrauterine stress.

5. Caffeine

One of the most common chemicals that fetuses are exposed to is caffeine, which is a non-selective adenosine receptor antagonist^{49, 50}. Cola drinks contain 35–60 mg of caffeine per

12 oz. can and a cup of regular coffee contains 100–150 mg of caffeine⁵¹. Caffeine is also present in chocolate, chocolate milk, gum, beef jerky, energy drinks, as well as other prepared foods such as coffee-flavored yogurts⁵². Average caffeine intake is between 150 and 200 mg per day among women of child bearing age⁵³.

Caffeine consumption during the first month of pregnancy is reported by 60% of women, many of whom are at a stage when they do not know they are pregnant⁵³. Caffeine consumption is associated with low birth weight and increased rates of spontaneous abortions^{54–62}. A recent Norwegian Mother and Child Cohort Study (MoBa) involving 59,123 pregnant women in Norway strongly supports the notion that caffeine consumption is associated with decreased birth weight, even at levels below recommended limits⁶¹. The idea that prenatal caffeine exposure is associated with low birth weight and leads to other adverse effects is not accepted by all^{53, 63}. However, prenatal caffeine exposure is concerning enough that Nordic countries and the United States recommend that pregnant women limit their caffeine intake to less than 200 mg per day^{64, 65}.

Caffeine readily crosses the placenta to reach the fetus, where the half-life of caffeine is longer (12–24 hrs) than adults (2–4 hrs) due to the absence of the metabolic enzyme CYP1A2 in placenta and fetus⁵⁰. In the embryo, caffeine influences cardiac gene expression and cardiac function^{66–68}.

In addition, caffeine exerts many cellular effects, including influences on intracellular calcium levels and inhibition of phosphodiesterase³⁶. Yet, with serum concentrations observed with human consumption, the physiological effects of caffeine are due to antagonism of adenosine receptors through competitive inhibition^{49, 50}.

6. Influences of Epigenetics on Embryonic Development

Epigenetic processes cause heritable changes in gene expression without altering the DNA sequence⁶⁹. Epigenetic processes, including histone modification, DNA methylation, and microRNA (miRNA), play important roles in embryogenesis and disease susceptibility⁶⁹. Increasing evidence demonstrates that epigenetic mechanisms are linked to gene activation, gene silencing and chromosomal instability^{9, 10, 70–72}. Physiological epigenetic processes act in cell-specific and temporally regulated manners to influence normal development, tissue formation, differentiation, and aging^{10, 12, 13}. Epigenetics also plays a critical role in the fetal programming of adult disease^{3, 69, 73, 74}.

DNA methylation is the process of adding a methyl group to a cytosine base in DNA to form 5-methylcytosine (5mC), and in mammals this event occurs at symmetric CG sites⁷⁵. DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs)⁷⁵. DNMT1 is associated with maintaining methylation patterns, as it primarily methylates hemimethylated DNA⁷⁵. DNMT3a and 3b primarily methylate unmethylated DNA, which is consistent with their role in *de novo* DNA methylation^{75, 76}. DNA methylation inhibits gene expression through suppressing transcription factor binding or recruiting histone deacetylases that cause chromatin condensation⁷⁶. Importantly, altered DNA methylation patterns can be stably inherited during DNA replication and mediate

persistent toxicological consequences in subsequent cellular and whole animal generations⁷⁵.

Members of the ten-eleven translocation (TET) family of proteins are 5mC dioxygenases that catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC)⁷⁷. These enzymes regulate DNA demethylation, since 5hmC is more sensitive than 5mC to deamination and to base-excision repair^{77, 78}.

Alterations in DNA methylation is a recognized mechanism for transmitting *in utero* environmental stress into an increased risk for adult disease²⁷. After fertilization, genomic DNA in the inner cell mass undergoes a rapid wave of demethylation of the genomic DNA^{75, 79, 80}. DNA methylation patterns are re-established during embryogenesis from E3.5 to E9.5 in mice, and these patterns can be altered by nutritional and environmental factors resulting in long-lasting effects^{75, 81–83}. During cellular differentiation, lineage-specific DNA methylation patterns undergo further remodeling⁸⁴.

Primordial germ cells (PGCs) in mice also go through specific DNA methylation remodeling events during embryogenesis⁸⁵. PGCs begin to migrate along the developing hindgut at E8.5 to the genital ridges⁸⁵. During and after this migration (E10.5–12.5), PGC DNA actively undergoes a wave of demethylation. This erasure process is important for removing genomic imprints from the previous generation^{85, 86}. These normal epigenetic processes can be affected by the intrauterine environment, including chemicals that alter DNA methylation in primordial germ cells and are correlated with adult diseases^{14, 15, 87–91}. In a report that indicates that *in utero* caffeine exposure can have long-term effects on PGCs, it was observed in rats that maternal caffeine consumption during gestation affected the fertility of adult male offspring⁹².

Another epigenetic process that may be influenced by alterations in the intrauterine environment includes miRNA expression. miRNAs are small 22 nucleotides long non-coding RNAs that reduce gene expression either by promoting mRNA degradation or inhibiting translation of target genes⁹³. miRNAs influence development by regulating cell proliferation, differentiation, and apoptosis⁹³.

7. Effects of Caffeine on the Embryo

Because caffeine is widely consumed, potential effects of caffeine on the developing fetus have been examined in animals and humans⁵³.

In rats, teratogenic effects of caffeine on the fetal heart are observed at doses in excess of 50 mg/kg⁹⁴. The most common cardiovascular malformations are ventricular defects⁹⁵. Cardiac morphogenesis has been found to be impaired in embryos from mothers treated with both ethanol and caffeine⁹⁶, showing that caffeine can amplify effects of other toxins. Caffeine administered to pregnant CD-1 mice led to a decrease in embryonic crown-rump lengths at E18.5⁹⁷. Caffeine induced decreased embryonic growth was accompanied with decrease embryonic carotid artery flow and decreased expression of A2AR⁹⁷. In addition, caffeine inhibits the fetal cardiac output recovery following maternal hypoxia exposure, again indicated that caffeine and amplify other environmental factors⁹⁷.

Considering the above, we tested if caffeine exerts effects on the embryo similar to that seen when A1ARs are deleted¹⁷. Pregnant mice in room air or hypoxia were treated with a single dose of caffeine at E8.5 resulting in circulating concentrations in the dam equivalent to those seen with two cups of coffee⁵⁰. The time of exposure was equivalent to 20–30 days of human gestation, a time when many women are not aware that they are pregnant.

Caffeine exposure was associated with reduced fetal viability¹⁷. When embryo size was assessed, the caffeine treated embryos were smaller compared to vehicle-treated embryos¹⁷. When cardiac histology was examined, caffeine resulted in reduced ventricular myocardial area and reduced HIF-1 α protein expression in hypoxia¹⁷.

Further analysis of caffeine effects on embryonic hearts revealed that caffeine had no effect on heart rate at E9.5, but induced increased heart rates in E12.5 hearts⁶⁷. These caffeine effects on embryonic heart rates are similar to those seen with specific antagonism of A1ARs by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX)⁶⁸. In addition, caffeine was able to abolish (E9.5) or blunt (E12.5) hypoxia-induced bradycardia in embryonic mice hearts⁶⁷. These data indicate that caffeine can directly influence embryonic heart function.

Caffeine also influences gene expression in the developing heart. We examined the effects of *in utero* caffeine exposure on cardiac gene expression. After treatment from E6.5–10.5, total RNA was isolated from E10.5 embryonic ventricles and used for qPCR analysis. Significant differences in gene expression were observed in ventricular tissue following caffeine treatment, including structural cardiac genes (*Myh6*, *Myh7*, *Myh7b*, *Tnni3*), transcription factors (*Nfatc1*, *Mef2c,d*, *Gata4*), and miRNAs (miR208a, miR208b, miR499; Fig. 1)⁶⁶. Because we observed decreased levels of DNA methylation in adult hearts after *in utero* caffeine exposure¹⁶, we examined the effects of caffeine exposure on the expression of important DNA methylation enzymes in the embryonic heart. Caffeine exposure (20 mg/kg) from E6.5–10.5 coincides with the remethylation of embryonic DNA that follows a massive wave of demethylation that occurs after fertilization⁸⁵. *In utero* exposure to caffeine during this developmental window leads to reduced expression of several genes that are involved in regulating DNA methylation levels, including DNA methyltransferases (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) and Tet genes (*Tet1*, *Tet2*, and *Tet3*; Fig. 1)⁶⁶.

In addition, a broader examination of the effects of *in utero* caffeine exposure on embryonic cardiac gene expression, through the use of RNAseq, revealed over 900 genes had altered expression after caffeine exposure⁶⁶. Pathway analysis of these genes revealed that caffeine alters the expression of many genes involved with cardiac hypertrophy and heart development⁶⁶. RNA-seq data also revealed the DNA methylation, histone modification, and alternative gene splicing pathways were also compromised by caffeine treatment⁶⁶. These data indicate that caffeine exposure influences epigenetic pathways during development that may explain the long-term effects that we observe with *in utero* exposure.

In contrast to animal studies, major teratogenic effects of caffeine have not been found in humans⁹⁴. Few studies, though, have evaluated effects of caffeine consumption during early embryogenesis^{53, 94}. Caffeine consumption is associated with low birth weight and increased rates of spontaneous abortions^{54–62}. While some of these effects on birth weight may be

minimal and not lead to gross birth defects, we demonstrated that similar concentrations of *in utero* caffeine exposure have long-term effects on adult offspring of treated mice^{16, 17}.

8. Long-Term Effects of In Utero Caffeine Exposure

Because *in utero* caffeine exposure had significant effects on embryonic heart function and gene expression, we examined the long-term effect of *in utero* caffeine exposure on adult heart function and gene expression. In addition, we examined epigenetic factors that may mediate long-term effects on the heart, including transgenerational effects.

To test for long-term effects of prenatal caffeine exposure, we examined cardiac function and morphology of adult male offspring. In the first set of experiments, pregnant dams from an inbred strain (C57Bl/6) of mice were treated with a single dose of caffeine (20 mg/kg) at E8.5¹⁷. Dams were allowed to give birth, and offspring were examined at 10–12 weeks of age. Using echocardiography, we observed a decrease in % fractional shortening (%FS) and an increase in left ventricular internal diameter (LVID) in caffeine-treated hearts¹⁷.

In a second set of long-term studies, we treated pregnant A1AR global knockout mice with 20 mg/kg of caffeine at E8.5 and examined offspring at 10–12 weeks. The groups of male mice examined included caffeine- or vehicle-treated and three genotypes (A1AR^{+/+}, A1AR^{+/-}, A1AR^{-/-}). Of these groups, the A1AR^{+/+} caffeine-treated mice had abnormal cardiac function and morphology¹⁶. Caffeine-treated adult A1AR^{+/+} hearts had an increase in left ventricle (LV) mass, an increase in left ventricular posterior wall (LVPW) thickness, and a decrease in cardiac output¹⁶.

In addition, we observed an altered pattern of DNA methylation in adult hearts exposed to caffeine *in utero* and an overall decrease in the percentage of methylated DNA¹⁶. Over 7,000 differentially methylated regions in the genome were identified when comparing caffeine vs. vehicle in A1AR^{+/+} mice¹⁶. Further analysis mapped these regions to genes that are associated with cardiac hypertrophy and heart disease pathways¹⁶.

In a third set of experiments, we examined the effects of *in utero* caffeine exposure from E6.5–E9.5 or E10.5–13.5 (20 mg/kg per day) on cardiac function and morphology in CD-1 mice, an outbred strain. This strain of mice has been demonstrated to have more severe transgenerational effects on health in adulthood than inbred strains when exposed to endocrine disruptors *in utero*¹⁵. We chose these exposure times because they coincide with two important DNA methylation transition events during embryogenesis, including a large scale DNA remethylation of the genome that occurs from E3.5–E9.5, and genome-wide demethylation that occurs during primordial germ cell development from E10.5–E12.5⁷⁵. In these sets of experiments, we examined the F1 generation (embryos exposed *in utero*), F2 generation (exposed gametes within the F1 generation embryos) and F3 generation (naïve to caffeine exposure).

At 12 weeks of age, mice treated from E6.5–9.5 did not have altered cardiac function or morphology¹⁸. However, at 22 weeks after birth, F1 generation offspring of dams treated from E6.5–9.5 with caffeine had altered cardiac morphology, including increased

interventricular septum (IVS) thickness during both systole and diastole, and increased left ventricle (LV) mass compared to vehicle controls¹⁸.

At one year of age, F1 generation mice exposed to caffeine *in utero* from E6.5–9.5 showed altered cardiac morphology, as well as altered cardiac function that was characteristic of dilated cardiomyopathy (Table 1)¹⁸. Hearts from the caffeine-treated group displayed systolic dysfunction including a decrease in the left ventricular posterior wall thickness during systole (LVPW;s) and an increase in left ventricle internal diameter during systole (LVID;s), which led to an increase in LV volume during systole (Table 1)¹⁸. These effects on cardiac morphology lead to reduced cardiac function, including a decrease in % fractional shortening (%FS) and a decrease in % ejection fraction (%EF; Table 1)¹⁸.

Indicating that the timing of caffeine exposure is critical for the observed effects on cardiac function and morphology, F1 adult offspring of dams exposed to caffeine from E10.5–13.5 did not display adverse effects on cardiac morphology or function in adulthood at 22 weeks or at 1 year of age¹⁸.

Although caffeine exposure from E10.5–13.5 has no effect on adult hearts in the F1 generation at 22 weeks or 1 year, we did observe changes in the F2 generation¹⁸. At 1 year of age, the F2 caffeine-treated group exhibited significant changes in cardiac morphology including decreased LVID, decreased LV volume and increased LVPW thickness during both systole and diastole (Table 1)¹⁸. These changes in morphology were associated with changes in cardiac function including an increase in % FS and an increase in % EF (Table 1)¹⁸. These changes in the caffeine-treated group are similar to those seen in hypertrophic cardiomyopathy with hyperdynamic function in humans^{98, 99}.

We also examined the F3 generation offspring, which were never directly exposed to caffeine. At 22 weeks, the F3 caffeine group displayed effects on morphology including increased LVPW thickness during systole and diastole compared to controls and an increase in LV mass¹⁸. At 1 year of age, we observed that hearts of the F3 generation caffeine group had an increase in LV mass compared to controls that did not coincide with an increase in body weight¹⁸.

In addition to altered cardiac function and morphology, *in utero* caffeine exposure alters gene expression levels in the F1, F2, and F3 generations of mice¹⁸. The most interesting difference in gene expression observed was with the *Myh7* gene. MYH7 is generally an embryonic and prenatal form of cardiac myosin that is greatly reduced in adult murine hearts, but *Myh7* expression is activated in adult hearts during cardiac stress and heart failure in mice^{100, 101}. In F1 generation mice exposed to caffeine from E6.5–9.5, we observed an increase in *Myh7* expression¹⁸. The increase in *Myh7* expression is indicative of the phenotype induced by caffeine as increased *Myh7* expression is associated with cardiomyopathy and heart failure^{101, 102}.

In contrast, we observed that F2 generation mice exposed to *in utero* caffeine from E10.5–13.5 had reduced expression of *Myh7*. This opposite effect on *Myh7* gene expression compared to the F1 generation mice exposed to caffeine from E6.5–9.5 correlates with the opposite effect that caffeine has on adult heart function in the F2 generation. These data

indicate that *Myh7* expression is sensitive to caffeine exposure and may be predictive of cardiac dysfunction in adult hearts (Fig. 2).

These data on the long-term effects of *in utero* caffeine exposure indicate strongly that the timing of exposure is critical to both the effects on the heart in adulthood and on which subsequent generation will be affected (Fig. 2). If exposed to caffeine early in development (E6.5–9.5) DNA methylation is altered and it leads to an increase in *Myh7* expression and decreased % fractional shortening in the adult F1 generation heart, which leads to a dilated cardiomyopathy phenotype (Fig. 2). On the other hand, when exposed to caffeine later in development (E10.5–13.5) no effects are observed in the F1 generation but the F2 generation is affected. F2 hearts display a decreased expression of *Myh7* and an increase in % fractional shortening, which leads to a hypertrophic cardiomyopathy phenotype in adults (Fig. 2).

9. Conclusion

These data indicate that *in utero* caffeine exposure has lasting effects on cardiac function and morphology in adult mice and that these effects are transmitted to subsequent generations. We demonstrate that the timing of caffeine exposure influences the generation affected and the phenotype observed. In addition, these data indicate that caffeine exposure during early embryogenesis inhibits expression of key DNA methylation enzymes and leads to long-term changes in cardiac DNA methylation patterns. These observations identify a potential mechanism by which fetal caffeine exposure mediates long-term effects on cardiac function.

Going forward, additional clinical investigation is needed to better address caffeine safety during pregnancy. Studies are needed to match known prenatal caffeine intake with long-term postnatal outcomes to determine if caffeine contributes to the programming of adult disease. As such, determining if prenatal caffeine exposure exerts epigenetic effects is needed too. Studies that better define the cellular targets of caffeine and adenosine action will also better define fundamental mechanisms that play adaptive roles in responses to hypoxia and other environmental insults.

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Figure 1. Altered gene and miRNA expression in embryonic hearts exposed to in utero caffeine
Caffeine treatment of pregnant dams from E6.5–10.5 altered the expression of important cardiac genes in embryonic hearts at E10.5. In utero caffeine treatment altered expression of cardiac transcription factors, structural genes, DNA methylation enzymes, and miRNAs. Bars $\pm$ SEM represent fold changes normalized to β -actin or snRNA RNU6-2 and relative to individual controls (n=5–7, ** P<0.01, *** P<0.001 vs. vehicle).

Figure 2. Timing of in utero caffeine exposure leads to different cardiac phenotypes in adult offspring

The timing of *in utero* caffeine treatment leads to differences in adult cardiac function, gene expression, and phenotype. Exposure to caffeine from E6.5–9.5 leads the F₁ generation to develop dilated cardiomyopathy with decrease % FS and increased *Myh7* expression. *In utero* caffeine exposure from E10.5–13.5 leads to a hypertrophic cardiomyopathy in the F₂ generation along with increased % FS and decreased *Myh7* expression.

Table 1

Echocardiography analysis of adult F1 generation mice exposed to caffeine or vehicle as embryos *in utero* from E6.5–9.5¹⁸.

Treatment	Generation	Age	IVS;d	LVID;d	LVPW;d	LV Vol;d	IVS;s	LVID;s	LVPW;s	LV Vol;s	% EF	% FS	LV Mass	Heart Rate	Weight	N
E6.5–9.5																
Vehicle	F1	1 year	0.95	4.68	1.07	102.53	1.33	3.30	1.49	44.87	56.47	29.66	169.77	495.54	62.13	4
Caffeine	F1	1 year	0.96	4.94	1.06	115.91	1.30	3.70	1.32	59.77	48.89	25.28	183.34	469.46	59.36	4
P-value			0.45	0.11	0.42	0.12	0.35	0.04	0.03	0.04	0.02	0.02	0.26	0.18	0.34	
E10.5–13.5																
Vehicle	F2	1 year	0.92	5.00	1.00	118.72	1.29	3.66	1.34	57.24	52.09	26.89	173.76	523.84	60.10	5
Caffeine	F2	1 year	0.89	4.75	1.11	105.32	1.25	3.32	1.50	45.54	57.01	30.07	169.31	526.52	59.62	7
P-value			0.22	0.03	0.00	0.03	0.24	0.02	0.00	0.02	0.04	0.04	0.33	0.44	0.47	

IVS, interventricular septum; LVID, left ventricle internal diameter; LVPW, left ventricular posterior wall; LV, left ventricle; FS, fraction shortening; Vol, volume; EF, ejection fraction; d, diastole; s, systole. Units: IVS, LVID, LVPW are measured in millimeters (mm); LV vol is measured in milliliters (ml); LV mass is measured in milligrams (mg). P-values were calculated by Student's t-test.