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Maternal Delta-9-Tetrahydrocannabinol Exposure Induces Abnormalities of the Developing Heart in Mice

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Abstract

Introduction: Cannabis is increasingly being consumed by pregnant women for recreational purposes as well as for its antiemetic and anxiolytic effects despite limited studies on its safety during pregnancy. Importantly, phytocannabinoids found in cannabis can pass through the placenta and enter the fetal circulation. Recent reports suggest gestational cannabis use is associated with negative fetal outcomes, including fetal growth restriction and perinatal intensive care, however, the effects of delta-9-tetrahydrocannabinol (THC) on fetal heart development remains to be elucidated.

Materials and Methods: We aimed to determine the outcomes of maternal THC exposure on fetal heart development in mice by administering 0, 5, or 10 mg/kg/day of THC orally to C57BL/6 dams starting at embryonic day (E)3.5. Offspring were collected at E12.5 for molecular analysis, at E17.5 to analyze cardiac morphology or at postnatal day (PND)21 to assess heart function.

Results: Maternal THC exposure in E17.5 fetuses resulted in an array of cardiac abnormalities with an incidence of 44% and 55% in the 5 and 10 mg/kg treatment groups, respectively. Maternal THC exposure in offspring resulted in ventricular septal defect, higher semilunar valve volume relative to orifice ratio, and higher myocardial wall thickness. Notably, cell proliferation within the ventricular myocardium was increased, and expression of multiple cardiac transcription factors was downregulated in THC-exposed E12.5 fetuses. Furthermore, heart function was compromised with lower left ventricular ejection fraction, fractional shortening, and cardiac output in PND21 pups exposed to THC compared to controls.

Discussion: The results show that maternal THC exposure during gestation induces myocardial hyperplasia and semilunar valve thickening in the fetal heart and postnatal cardiac dysfunction. Our study suggests that maternal cannabis consumption may induce abnormalities in the developing heart and cardiac dysfunction in postnatal life.

Keywords: tetrahydrocannabinol; cannabis; fetal heart development; congenital heart defects; teratogen

Introduction

Congenital heart defects (CHDs) are the most common structural birth defect, occurring in ~1–2% of all births.[1,2] Currently, an estimated 12 million people are living with congenital heart disease globally and the prevalence is rising.[3] Research into the etiology of CHDs to mitigate congenital heart disease has never been more critical.

Genetics are believed to only count for 15% of CHDs, whereas environmental, nongenetic factors are associated with most CHDs and could be prevented.[4] Due to the recent and ongoing cannabis legalization in many countries, a growing number of pregnant women are consuming cannabis or cannabis extracts for their anxiolytic and antiemetic effects.[5] Some studies have found that babies maternally exposed to cannabis are associated with an overall higher rate of CHDs,[6,7] smaller birth weight,[8–10] and a higher rate of morbidity.[11]

Delta-9-tetrahydrocannabinol (THC), the primary cannabinoid found within cannabis, has been shown

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to cross the placenta and enter fetal circulation.[12] Due to critical cardiogenic events occurring at the first several weeks of pregnancy, many women could consume cannabis and affect fetal development without even knowing that they are pregnant[13] or erroneously believe cannabis use to combat morning sickness is safe.[14]

Early studies have shown THC prevented ovulation,[15,16] induced fetal resorptions,[17] reduced fetal bodyweight, and caused cleft palate and skeletal anomalies.[17,18] More recent studies have reported altered development of multiple organs, including placental defects resulting in fetal growth restriction,[19,20] sex-specific pancreatic defects causing glucose insensitivity,[21] cognitive impairments with altered behavior,[22–25] and ocular defects.[26,27] Although many reports have shown that THC exposure can impair the development of the pancreas, brain, eye, and placenta, and THC analogs can impair cardiogenesis in chick embryos,[28] the effects of maternal THC exposure on mammalian heart development are not fully elucidated.

Due to the overall lack of adequate research on the effects of cannabis products on fetal heart development, this study was performed to confidently determine if maternal THC exposure, at relevant doses, results in abnormalities of the fetal heart. Since recent epidemiological studies show that cannabis use in pregnancies is associated with a higher rate of CHDs,[6,7] we hypothesized that maternal THC exposure impairs heart development and causes abnormalities of the fetal heart in mice. To test this hypothesis, two doses of THC at 5 and 10 mg/kg/day oral administration were used to demonstrate a dose-dependent effect. THC treatment started at embryonic day 3.5 (E3.5) until fetal harvest or parturition. These oral doses of THC were selected as they produce, in rodents, plasma THC levels similar to cannabis smokers in humans using cigarettes containing 3–6% THC.[29–31]

Materials and Methods

Animals

This study used C57BL/6J mice (Charles River Laboratories, Laval, Canada) in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care and was approved by the Animal Care Committee at Western University, Canada. An 8–10 weeks old female mouse was paired with a male mouse at 5:00 PM. Vaginal plugs present the next morning at 9:00 AM indicated successful breeding, the embryo was deemed to be E0.5. Once plugged, female mice were separated from male mice. Fetuses from pregnant mice were collected at timepoints E12.5 and E17.5 via caesarian section or pups were collected at postnatal day 21 (PND21) after parturition (Supplementary Fig. S1).

Dams and pups were anesthetized using an intramuscular ketamine solution injection (ketamine 25 mg/mL, xylazine 2.5 mg/mL, and atropine 30 μg/mL), required samples were collected after mice were unresponsive, and were then sacrificed by cervical dislocation. Fetus and heart weights were measured after drying on paper towels. Torsos from E17.5 fetuses were fixed and processed for histology. Whole E12.5 fetuses were fixed and processed for histology. E12.5 ventricles were flash frozen in liquid nitrogen.

THC treatment in pregnant dams

In brief, THC in ethanol (CAS No. 1972-08-3; Cayman Chemicals) was dissolved in cremophor and saline (1:1:18). Ethanol was evaporated using nitrogen gas to remove all ethanol from the final THC solution. Immediately before feeding, the THC solution was diluted to a final concentration of 1:36 cremophor and saline. Starting at E3.5, dams were administered an oral daily dose of THC or vehicle control. Pregnant dams were fed THC at doses of 0, 5, or 10 mg/kg/day via drench nozzle until collection or parturition (Supplementary Fig. S1). The doses of THC were selected because they produce plasma THC levels in rodents similar to cannabis smokers (cigarettes containing 3–6% THC) in humans.[29–31]

Histology

Fetuses collected at E17.5 or E12.5 were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μm thick transverse serial sections from the beginning of the aortic arch to the end of the apex of the heart.[32,33] Sections were stained with Mayer's hematoxylin (Thermo Fisher Scientific, Waltham, MA) and counterstained with eosin (Thermo Fisher Scientific). Hematoxylin and eosin (H&E) stained slides were used to analyze heart morphology using an inverted light microscope (Zeiss Observer D1, Germany). Images were taken using Zen 2.0 software (Zeiss) and were analyzed either in Zen, QuPath, or AMIRA.

Immunohistochemistry

Slides with E12.5 fetal heart sections were microwaved (BP-111; Microwave Research & Applications, Carol Stream, IL) in citric acid buffer (0.01 M, pH 6) for

12 min at 94°C for antigen retrieval.[32,33] Following blocking with 1% goat serum, sections were immunostained using an anti-phospho-histone H3 on serine-10 (pHH3, 1:500 in TBST, Ab47297; Abcam, Toronto, Canada) antibody to quantify cell proliferation since pHH3 is a specific marker for cell mitosis.[34] Sections were subsequently incubated in biotinylated goat anti-rabbit IgG (1:300 in TBST, BA-1000-1.5; Vector Laboratories, Burlington, Canada) and incubated in ABC reagent (1:100 in PBS; Vector Laboratories) followed by 3-3′ di-aminobenzidine tetrahydrochloride (Sigma-Aldrich, Toronto, Canada) and H_2O_2 for visualization of the signal. Sections were counterstained with Mayer's hematoxylin.

AMIRA 3 dimensional reconstructions

AMIRA software (Thermo Fisher) was used to reconstruct 3 dimensional (3D) models of semilunar valves of E17.5 hearts using H&E-stained serial sections. AMIRA calculated absolute volumes of labeled components and these values were utilized to create ratios of various parameters for normalization. Representative images of reconstructions were smoothed utilizing the unconstrained smoothing function within AMIRA to create a smooth anatomical representation of the 3D structure without altering the quantitative data.[35]

QuPath analysis

QuPath v0.2.0 was used for quantitative analysis of cell number and stained area for the ventricular myocardium and semilunar valves.[36] Images of the heart and semilunar valves were taken and imported into QuPath. The percentage of area that was stained was determined by exporting selected regions to ImageJ within QuPath. The total cell number was determined using the cell detection function on the region of interest with parameters optimized to accurately identify cell nuclei. Any red blood cells erroneously identified as nuclei were deleted. The cell density of semilunar valves and compact myocardium was determined by dividing the number of identified nuclei by the total area. The cell size of ventricular myocardial walls was determined by dividing the stained area by the number of identified nuclei.

Real-time polymerase chain reaction analysis

Ventricular tissues were isolated from E12.5 control and THC-exposed fetuses and flash frozen in liquid nitrogen. Samples were stored in −80°C until RNA isolation and reverse transcription was done. Total RNA was extracted using the TRIzol reagent method and 200 ng of RNA was reverse transcribed using Maloney murine leukemia virus reverse transcriptase (Invitrogen, Burlington, Canada) and random primers. Evergreen qPCR MasterMix (Applied Biological Systems, Vancouver, Canada) was used for quantitative polymerase chain reaction (qPCR) on cDNA. Primers were designed using Primer3 software v4.1.0 (Supplementary Table S1). An Eppendorf RealPlex machine (Hamburg, Germany) was used to amplify cDNA for 35 cycles. mRNA levels were extrapolated using C_t method by normalizing to the housekeeper, 28S-Ribosomal RNA.[37]

Echocardiography

At PND21, pup heart function was measured using a Vevo 2100 ultrasound imaging system with an MS700 transducer (30–70 MHz; VisualSonics, Toronto, Canada). Mice were anaesthetized using 2–4% isoflurane and then were fastened to the heating dock in the supine position. The ultrasound probe was positioned to obtain a parasternal short-axis view. The end diastolic left ventricular internal diameter and end systolic left ventricular internal diameter were measured, and ejection fraction and fractional shortening were calculated. Cine-loop videos of heart contractions were recorded.[38,39]

Statistical analysis

Data are presented as means ± standard error of the mean and analyzed using GraphPad Prism (La Jolla, CA). An unpaired Student's *t*-test was used for comparisons between two groups and a one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test was used for analysis for three and more groups. A chi-square test was used to analyze the incidence of cardiac abnormalities. p-Values < 0.05 were considered statistically significant.

Results

Maternal THC exposure causes growth restriction

The percentage of successful pregnancies was determined by dividing the number of pregnant mice, as determined by caesarian section and parturition, by the number of vaginal plugs identified, indicating copulation. Percentage of successful pregnancies was significantly lower in both THC groups ($p < 0.01$, Fig. 1A).

At the time of collection, the number of fetuses, and the weight of each fetus and placenta were recorded.

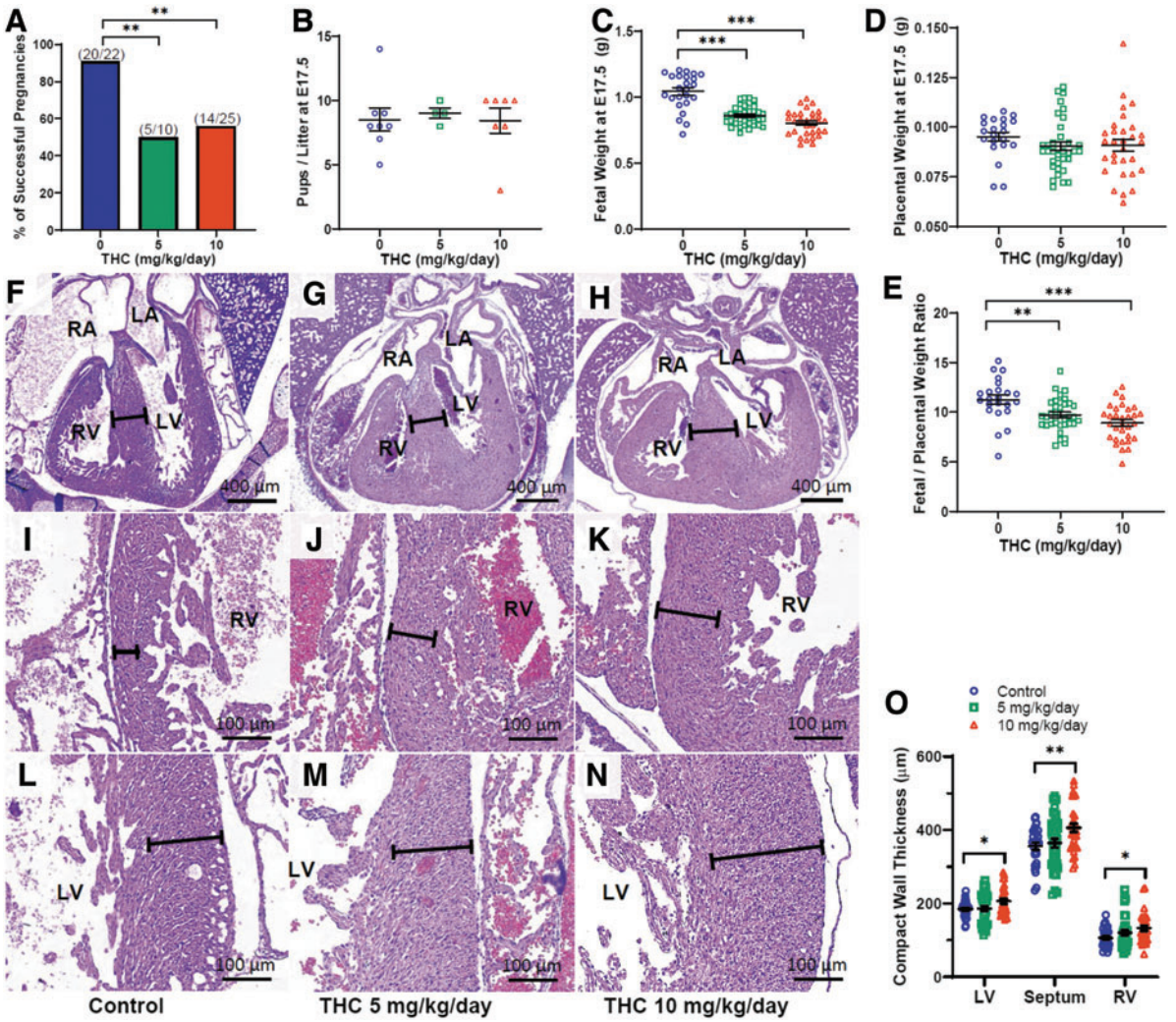

FIG. 1. Effects of maternal THC exposure on pregnancy, fetal growth, and fetal myocardial wall thickness at E17.5. **(A)** The percentage of successful pregnancies. **(B)** Average number of pups per litter. **(C–E)** Fetal weight, placental weight, and fetal to placental weight ratio. **(F–H)** Images of the four-chambered view of the heart. **(I–K)** RV and (**L–N**) LV myocardial sections of E17.5 fetuses. Black lines indicate measurements made for thickness. **(O)** LV, Se, and RV compact myocardium thickness measurements. Data are mean ± SEM and analyzed with a one-way ANOVA followed by a Bonferroni's *post hoc* test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control. ANOVA, analysis of variance; E, embryonic day; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; Se, septum; SEM, standard error of the mean; THC, delta-9-tetrahydrocannabinol.

The number of pups per litter in both THC groups was not significantly different than those of the control (Fig. 1B). Fetal body weight at E17.5 in both THC groups were significantly lower than those of the control ($p < 0.001$, Fig. 1C); however, placental weight did not differ (Fig. 1D). Fetal body weight to placental weight ratio was significantly lower in both THC groups compared to those of the controls ($p < 0.01$, Fig. 1E), indicating fetal growth restriction.

Maternal THC exposure induces cardiac abnormalities

Hearts from E17.5 fetuses exposed to 0, 5, or 10 mg/kg/day of THC gestationally were analyzed histologically

for morphological abnormalities. Fetuses that had parameters that were two standard deviations away from the mean of the control were classified as having cardiac abnormalities. The incidence of heart abnormalities in E17.5 fetuses exposed to 0, 5, and 10 mg/kg/day of THC were 0%, 44%, and 55%, respectively (Table 1).

In the low dose of THC, significantly higher rates of pulmonary valve thickening (17%, $p < 0.05$), right ventricle (RV) thickening (19%, $p < 0.01$), and left ventricle (LV) thickening (14%, $p < 0.05$) were seen (Table 1). Other defects and abnormalities observed but not significantly different compared to the control included aortic narrowing, aortic valve thickening, and septal thickening (Table 1).

In the high dose of THC, multiple heart abnormalities had significantly higher incidence rates compared to those of the controls (Table 1). These included pulmonary valve thickening (26%, $p < 0.01$), LV thickening (26%, $p < 0.01$), RV thickening (29%, $p < 0.001$), and septal thickening (23%, $p < 0.01$). Other defects seen included pulmonary artery narrowing and aortic valve thickening (Table 1).

Interestingly, one heart had a VSD (Table 1 and Supplementary Fig. S2), which came from a litter exposed gestationally to 5 mg/kg/day of THC. No other fetuses in the 5 mg/kg/day THC group or any fetuses in the control or 10 mg/kg/day THC groups had a VSD.

Table 1. Rate of Cardiac Abnormalities in Delta-9-Tetrahydrocannabinol-Exposed Fetuses at E17.5

	Control 35 fetuses (4 litters)		THC 5 mg/kg 36 fetuses (4 litters)		THC 10 mg/kg 31 fetuses (4 litters)	
	n	%	*n*	%	*n*	%
Normal	35	100	20***	56***	14***	45***
Abnormal	0	0	16***	44***	17***	55***
VSD	0	0	1	3	0	0
PV thickening	0	0	6*	17*	8**	26**
PA narrowing	0	0	0	0	2	6
AoV thickening	0	0	0	0	2	6
Ao narrowing	0	0	1	3	0	0
LV thickening	0	0	5*	14*	8**	26**
Se thickening	0	0	3	8	7**	23**
RV thickening	0	0	7**	19**	9***	29***

Data were analyzed using a chi-square test.

*$p < 0.05$, **$p < 0.0.01$, ***$p < 0.0.001$ compared to the control. Although the overall incidence of abnormalities was higher in THC 10 mg/kg group, there was no statistical difference between 5 and 10 mg/kg THC dose groups (p = n.s.).

Ao, aorta; AoV, aortic valve; LV, left ventricle; PA, pulmonary artery; PV, pulmonary valve; RV, right ventricle; Se, septal; THC, delta-9-tetrahydrocannabinol; VSD, ventricular septal defect.

Effects of maternal THC exposure on myocardial wall development

Measurement of the thickness of the ventricular compact myocardium in each of the LV, RV, and septum in the 4-chamber view in E17.5 fetuses was performed in duplicate in the LV and RV at the side and bottom of the chamber and singularly for the septum in the middle of the myocardial wall (Fig. 1F–N). Compared to the controls, the compact myocardium in the high dose of THC group was significantly thicker in the LV, RV, and septum ($p < 0.05$ and $p < 0.01$, Fig. 1O).

Using E17.5 fetuses with diagnosed myocardial wall thickening, the total area of the compact myocardium in the LV, RV, and septum was measured using QuPath analysis and found significantly larger when exposed to the high dose of THC compared to those of the controls (five fetuses per treatment group, $p < 0.05$, Fig. 2A–C). Further, THC-exposed fetuses diagnosed with myocardial wall thickening had a significantly higher number of nuclei within each compartment ($p < 0.05$, Fig. 2D), whereas cardiomyocyte cell size did not differ between THC exposure and controls (Fig. 2E).

To determine if the resulting larger cell number in E17.5 fetal hearts was due to proliferation, E12.5 fetuses exposed to either 0 or 10 mg/kg/day of THC starting at E3.5 were collected, and corresponding heart sections were stained for pHH3 (Fig. 2F–I). The number of pHH3 positive cells normalized to area was significantly higher in the THC group compared to that of the controls for the LV, RV, and septum ($p < 0.05$, Fig. 2J). The percentage of cell proliferation was also significantly higher in the THC group compared to that of the controls for the LV, RV, and septum ($p < 0.05$, Fig. 2K).

Effects of maternal THC exposure on semilunar valve development

The aortic valves were assessed using histological sections to determine the effect of maternal THC exposure on the development of the aortic valve (Fig. 3A–C). Fetuses exposed gestationally to 10 mg/kg/day of THC had higher total aortic valve to orifice area ratios ($p < 0.05$, Fig. 3P). Notably, aortic orifice area was dose-dependently lower in the low-dose ($p < 0.05$) and high-dose ($p < 0.001$) THC groups compared to those of the controls (Fig. 3Q). Interestingly, only the ratio of right coronary cusp (RCC) area to orifice area was found to be significantly larger in the high dose of THC compared to that of the controls ($p < 0.001$, Fig. 3R).

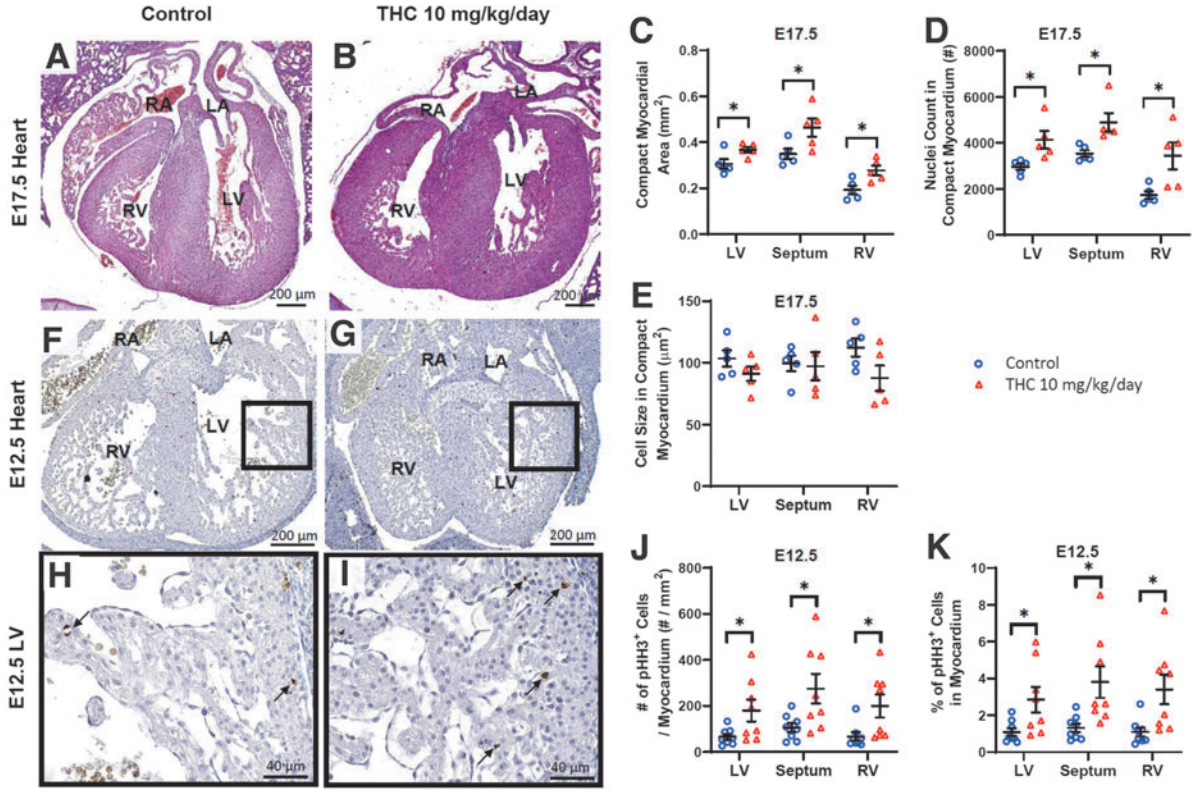

FIG. 2. Effects of maternal THC exposure on cell size and proliferation of the fetal heart. **(A, B)** Sections of E17.5 hearts were stained with H&E and total compact area and nuclear density was measured. **(C)** Compact myocardium area within LV, Se, and RV. **(D)** Nuclear density within the LV, Se, and RV. **(E)** Cell size in compact myocardium within LV, Se, and RV. **(F, G)** Sections of E12.5 hearts stained with pHH3, phosphohistone H3 to identify proliferating cells. **(H, I)** Closeup of LV stained with pHH3. **(J)** Number of positively stained pHH3 cells normalized to area of the compact myocardium for LV, Se, and RV. **(K)** Percentage of positively stained pHH3 cells out of total nuclei for LV, Se, and RV, respectively. Data are mean ± SEM and were analyzed by unpaired Student's *t*-test for each parameter. *$p < 0.05$ versus control. H&E, hematoxylin and eosin.

Subsequently, 3D reconstructions analysis showed that the aortic valve to orifice ratio was significantly higher in the high dose of THC group compared to that of the controls ($p < 0.001$, Fig. 3J–L, V). Each of the left coronary cusp (LCC, $p < 0.001$), noncoronary cusp (NCC, $p < 0.01$), and RCC ($p < 0.01$) volume to orifice ratio was also found to be significantly higher in the high dose of THC group compared to those of the controls (Fig. 3W).

Pulmonary valves were also assessed to determine the effect of maternal THC exposure on the development of the pulmonary valve (Fig. 3D–I). Fetuses exposed gestationally to both 5 and 10 mg/kg/day of THC showed a dose-dependent smaller pulmonary trunk diameters compared to those of the control ($p < 0.01$ and $p < 0.001$, respectively, Fig. 3S). Total pulmonary leaflet thickness, sum of the left cusp (LC) and right cusp (RC) thickness, relative to the orifice diameter was found to be significantly bigger in both the low dose ($p < 0.01$) and high dose THC groups ($p < 0.001$, Fig. 3T) compared to that of the controls.

Not only were both the LC and RC thickness to orifice diameter ratios significantly higher in high dose of the THC group ($p < 0.01$ for LC, $p < 0.05$ for RC), but the RC thickness to orifice diameter ratio was also significantly higher in the low dose of THC group compared to those of the controls ($p < 0.01$, Fig. 3U). 3D reconstructions of the pulmonary valve (Fig. 3M–O) displayed a similar trend with total leaflet volume to orifice ratio being significantly higher in the high-dose

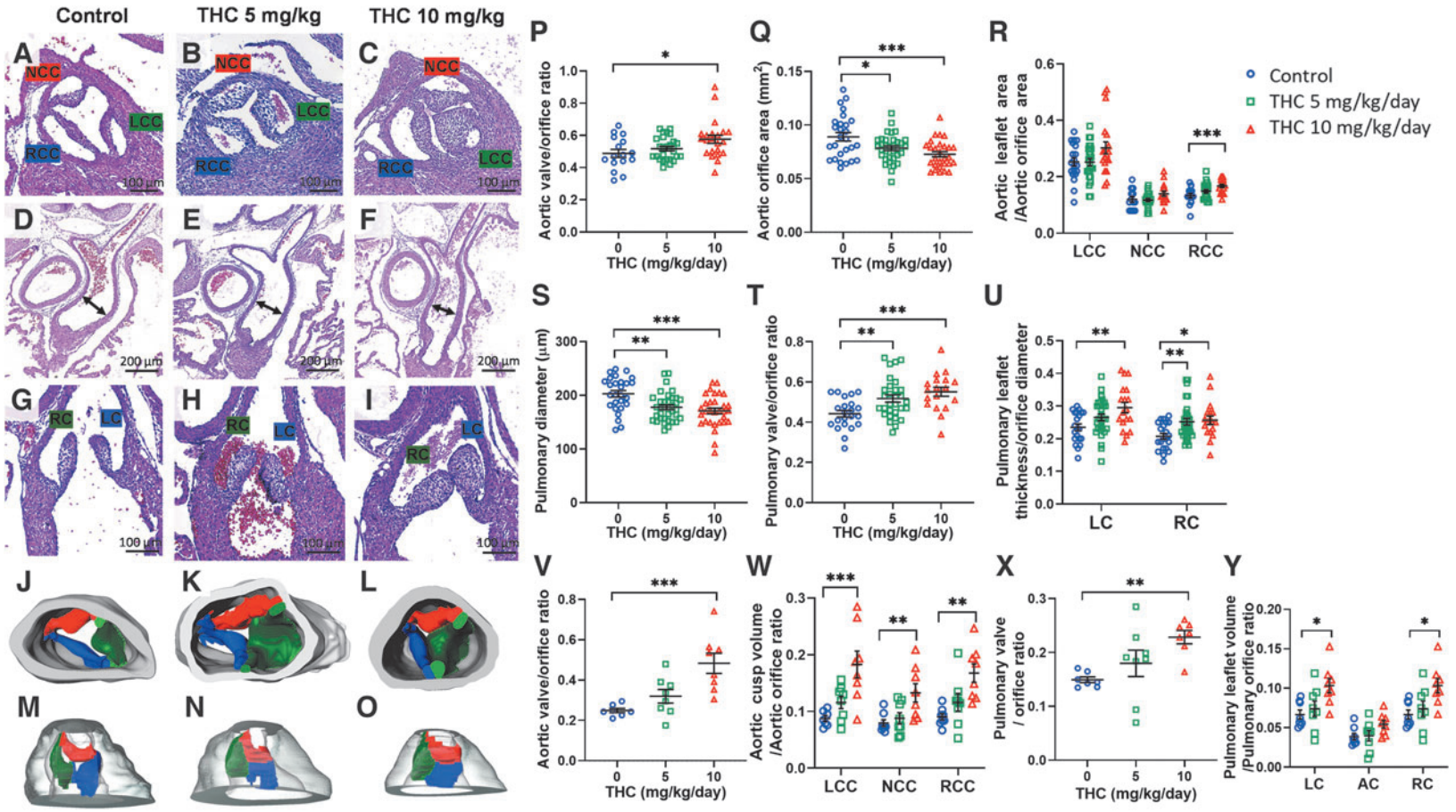

FIG. 3. Effects of maternal THC exposure on aortic and PVs development in E17.5 fetuses. **(A–C)** H&E-stained sections of AoVs, **(D–F)** pulmonary trunk, and **(G–I)** PVs. 3D reconstructions were performed on H&E-stained sections for **(J, K)** AoVs and (**M–O**) PVs. **(P)** AoV to orifice area ratio, **(Q)** aortic orifice area, **(R)** aortic leaflet area to aortic orifice area, **(S)** pulmonary trunk diameter, **(T)** PV to orifice ratio, **(U)** pulmonary leaflet thickness to orifice diameter ratio, **(V)** AoV to orifice ratio, **(W)** aortic cusp to orifice ratio, **(X)** PV to orifice ratio, and **(Y)** pulmonary leaflet to orifice ratio. Data are mean ± SEM and were analyzed with a one-way ANOVA followed by a Bonferroni's *post hoc* test for each independent variable. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control. Four litters per treatment group. 3D, 3 dimensional; AC, anterior cusp; AoV, aortic valve; LC, left cusp; LCC, left coronary cusp; NCC, noncoronary cusp; PV, pulmonary valve; RC, right cusp; RCC, right coronary cusp.

THC group ($p < 0.01$, Fig. 3X), and both the LC and RC leaflet volume to orifice ratio being significantly larger in the high-dose THC group ($p < 0.05$, Fig. 3Y) compared to that of the controls, whereas no significant differences were found in the anterior cusp of pulmonary valve (Fig. 3Y).

To determine cell proliferation in the leaflet thickness in the PV and aortic valves, using QuPath, cell nuclei density was calculated on closeups of the aortic (Fig. 4A, B) and pulmonary valves (Fig. 4C, D). The aortic valve cell nuclei density was significantly higher in the LCC ($p < 0.01$), NCC ($p < 0.05$), and RCC ($p < 0.001$) in the high dose of THC group compared to those of the controls (Fig. 4E). For the pulmonary valve, cell nuclei density was significantly higher in the LC and the RC than those of the controls ($p < 0.001$, Fig. 4F).

Maternal THC lowers expression of crucial cardiac genes in E12.5 hearts

Total RNAs were isolated from E12.5 hearts and mRNA levels of important developmental transcriptional regulators, and signaling molecules implicated in cell proliferation and differentiation were quantified using qPCR analysis. Cell proliferation inhibitors *p21* and *p27* were significantly downregulated ($p < 0.01$ and $p < 0.05$, respectively, Fig. 5A, B) while *CDK4* was significantly upregulated ($p < 0.01$, Fig. 5C) in the THC group compared to those of the controls. Furthermore, important valve development genes, *NFATc1* and *NFATc2*, were downregulated in the THC group compared to those of the controls ($p < 0.05$ and $p < 0.01$, respectively, Fig. 5E, F). However, expression of *Cyclin D1*, *NFATc3*, *NFATc4* (Fig. 5D, G, H), *Nkx2.5*, and *Gata4* (Supplementary Fig. S3A, B) were not significantly altered.

Postnatal survival, bodyweight, heart weight, and cardiac function

The animal survival from birth to PND21 was monitored by checking the number of pups alive every day following parturition. No differences were seen between the control and 10 mg/kg/day of THC group (Fig. 6A). PND21 pup bodyweight was significantly

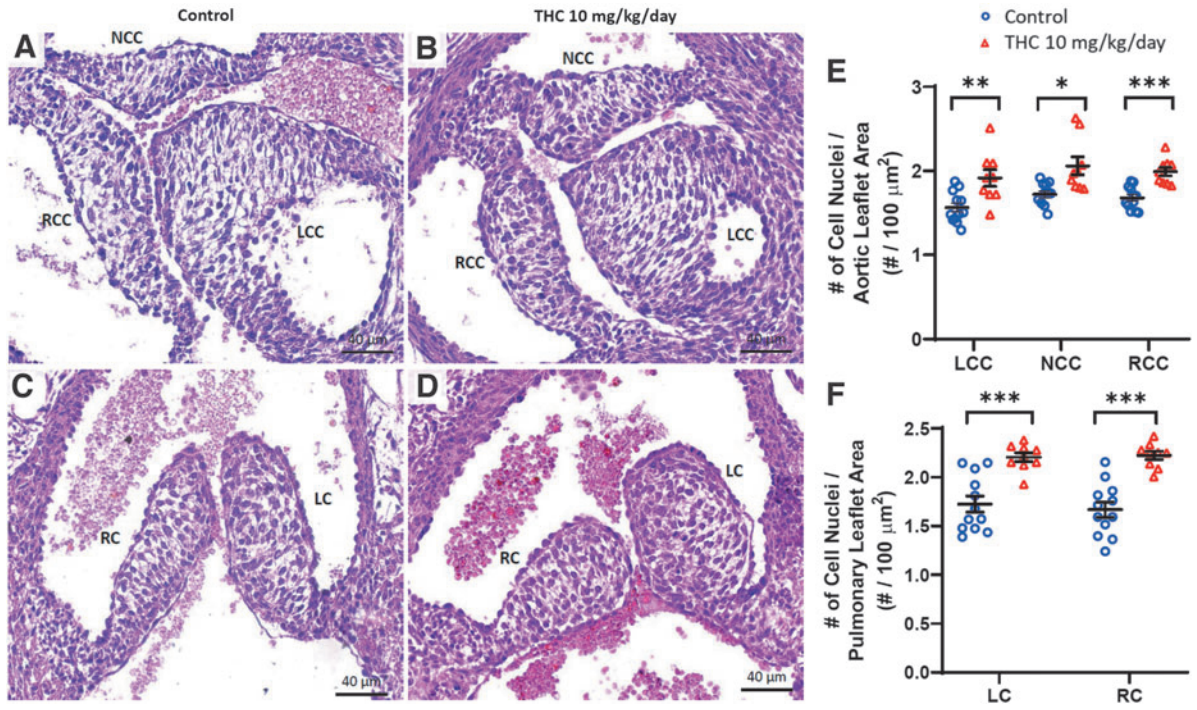

FIG. 4. Effects of maternal THC exposure on semilunar valve cell density in E17.5 hearts. H&E-stained sections of AoVs **(A, B)** and PVs **(C, D)**. Nuclei were counted based upon hematoxylin staining and normalized to leaflet area in AoVs **(E)** and PVs **(F)** using QuPath. Data are mean ± SEM and were analyzed by unpaired Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control. Three litters per group.

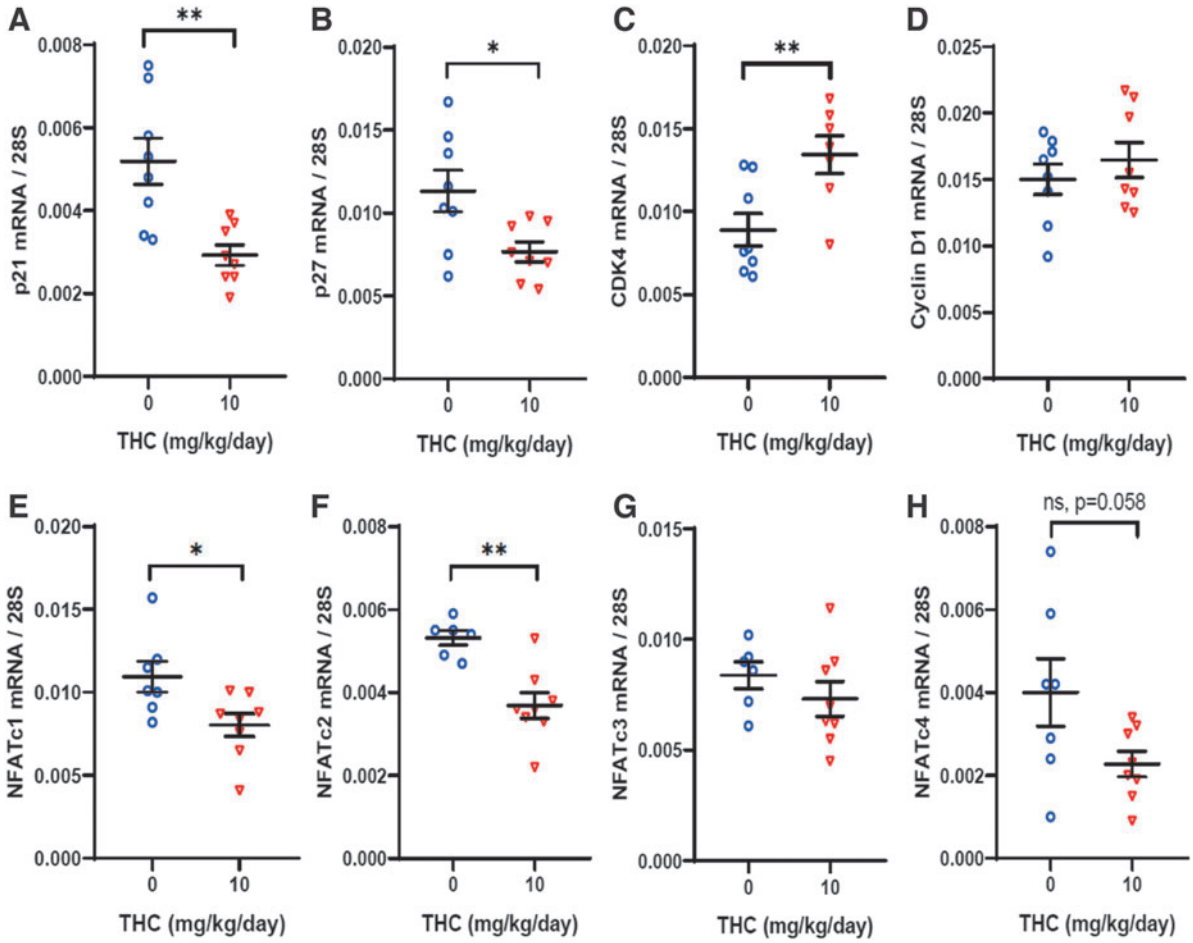

FIG. 5. Effects of maternal THC exposure on the expression of transcription and growth factors in E12.5 fetal hearts. mRNA levels were analyzed by qPCR for the expression of p21 **(A)**, p27 **(B)**, CDK4 **(C)**, Cyclin D1 **(D)**, NFATc1 **(E)**, NFATc2 **(F)**, NFATc3 **(G)**, and NFATc4 **(H)**. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus control by unpaired Student's *t*-test. CDK4, cyclin-dependent kinase 4.

lower in pups exposed to 10 mg/kg/day of THC compared to those of the controls ($p < 0.001$, Fig. 6B). Heart weight was significantly lower in PND21 pups exposed to maternal THC compared to the control ($p < 0.01$, Fig. 6C). Heart weight to body weight ratio was calculated and pups exposed to maternal THC had significantly higher heart weight to body weight ratios compared to those of the controls ($p < 0.01$, Fig. 6D).

Cardiac function was assessed using echocardiography on pups at PND21 (Table 2). Both LV fractional shortening and ejection fraction were significantly lower in the THC group compared to the control group ($p < 0.01$). Heart rate ($p < 0.05$), stroke volume ($p < 0.01$), and cardiac output ($p < 0.001$) were also found to be significantly lower in the THC group compared to the control group. LV volume in systole or diastole, and LV mass were not significantly different between the groups (Table 2).

Discussion

This study established a model of oral THC use during pregnancy to evaluate the effects of maternal THC exposure on fetal heart development, postnatal cardiac function, and the potential mechanisms inducing such phenotypes. The two doses of THC were chosen because they produce plasma THC concentrations commonly seen in humans after consuming cannabis containing 3–6% THC.[29–31] The results demonstrated that maternal THC exposure induced fetal heart

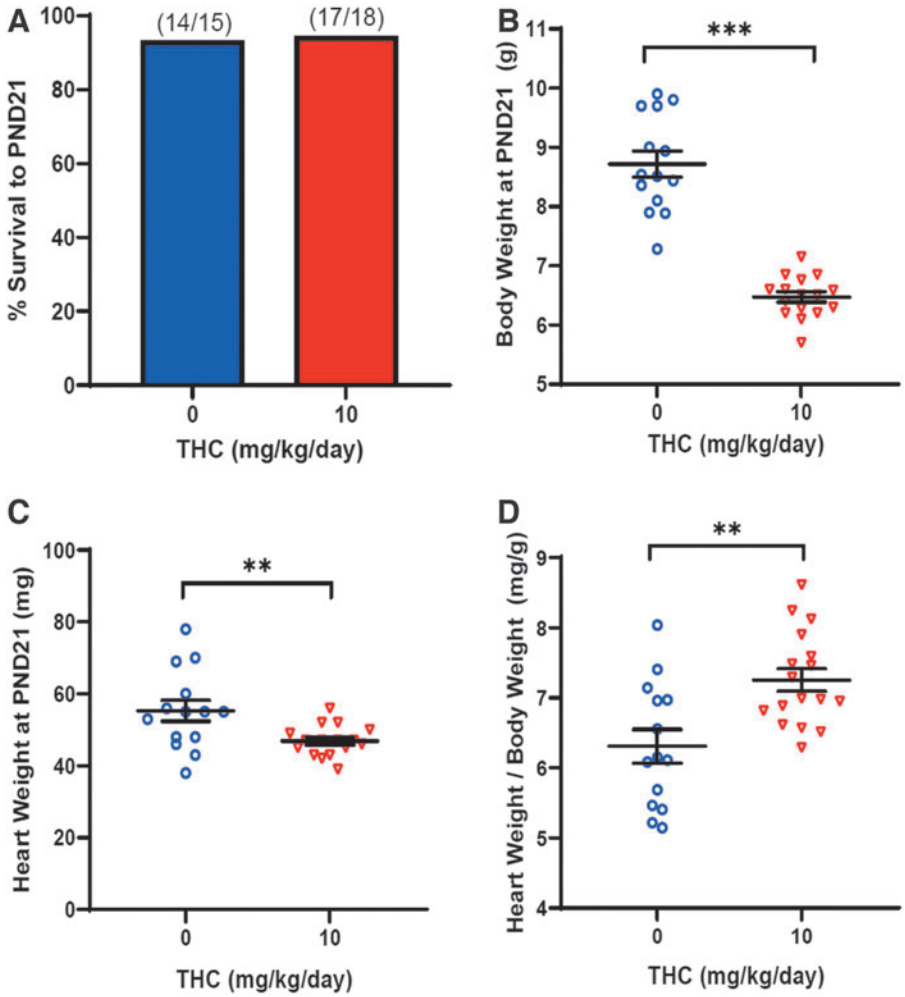

FIG. 6. Effects of maternal THC exposure on postnatal survival, bodyweight, and heart weight at PND21. Mice were gestationally exposed to 0 or 10 mg/kg/day of THC starting at E3.5 until parturition. **(A)** Percent survival of pups from birth to PND21. **(B)** Bodyweight of pups at PND21. **(C)** Heart weight of pups at PND21. **(D)** Heart weight to bodyweight ratio of pups at PND21. Data were analyzed using **(A)** chi-square test or **(B–D)** unpaired Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. PND21, postnatal day 21.

abnormalities in mice with an apparent dose-dependent relationship. Two main phenotypes were fetal myocardial hyperplasia and semilunar valve thickening. In addition, we also observed one case of VSD in a fetus of maternal THC exposure. Furthermore, postnatal heart function was compromised in PND21 pups.

E17.5 fetuses exposed to maternal THC were found to have significantly thicker LV, RV, and septal compact myocardium compared to those of the controls. Further histological analysis showed the number of nuclei found in the ventricular myocardium to be significantly higher in THC-exposed fetuses, but the average cardiomyocyte cell size was unaffected. The data suggest that the increase in thickness is due to hyperplasia instead of cellular hypertrophy. Furthermore, cell mitosis and proliferation in E12.5 fetal hearts, measured by pHH3 staining,[34] were significantly increased, and gene analysis at E12.5 supported hyperplasia as many cell regulators were altered. Although *cyclin D1*, a nuclear protein whose levels oscillate in a cell cycle-dependent manner, was not affected, *p21* and *p27* were downregulated, and *CDK4* was upregulated. CDK4 and cyclin D1 form a complex to become activated and control G_1/S cell cycle progression; however, p21 and p27 inhibit CDK-Cyclin complex activity.[40]

Table 2. Effects of Gestational Delta-9-Tetrahydrocannabinol Exposure on Postnatal Cardiac Function at Postnatal Day 21

	Control 9 pups (2 litters)	THC 10 mg/kg 11 pups (2 litters)
LV volume, systole (μL)	17.9 ± 2.2	19.0 ± 1.5
LV volume, diastole (μL)	39.8 ± 3.3	34.5 ± 2.2
LV mass AW corrected (mg)	32.4 ± 2.5	28.7 ± 2.4
LV mass AW (mg)	40.6 ± 3.1	35.9 ± 3.1
LV fractional shortening (%)	28.4 ± 1.6	22.0 ± 1.3**
LV ejection fraction (%)	56.0 ± 2.7	45.8 ± 2.3**
Heart rate (BPM)	414.8 ± 9.5	383.5 ± 6.3*
Stroke volume (μL)	22.0 ± 1.7	15.5 ± 1.1**
Cardiac output (μL)	9.0 ± 0.6	5.9 ± 0.4***

Echocardiography was performed using Vevo 2100 ultrasound imaging system with an MS700 transducer (30–70 MHz) on PND21 pups exposed to 0 or 10 mg/kg/day of THC gestationally starting at E3.5 until parturition. All data are mean ± SD and analyzed using an unpaired Student's *t*-test.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control.

AW, anterior wall; BPM, beats per minute; LV, left ventricle; SD, standard deviation.

Lowered *p21* and *p27* gene expression indicates an increased CDK-Cyclin complex activity, and an increased *CDK4* expression would lead to increased complexes and therefore increased cell proliferation. Due to the previously discovered role of NFATc3/4 in embryonic ventricular development, *NFATc3/4* transcript levels were also measured via qPCR. It was hypothesized that THC would bind to a receptor and induce calcium influx in cardiomyocytes, which would increase activation of the calcineurin/NFAT pathway. Specifically, NFATc3/4 have been found to be crucial in ventricular development and the regulation of cell proliferation and mitochondrial morphology.[41] However, neither *NFATc3* nor *NFATc4* was found to be upregulated, suggesting that the NFAT pathway is not significantly affected in the fetal heart by maternal THC exposure. Alternatively, other potential mechanisms could increase cell proliferation in the fetal heart. THC could induce Rho kinase through GPR55 agonism.[42]

Increased Rho kinase activity would lead to altered Rho GTPase activity causing increased cardiomyocyte proliferation.[43] Whether these mechanisms are involved in cardiomyocyte proliferation induced by maternal THC exposure merits further investigation.

Although some cases of cardiac hypertrophy in infants can spontaneously resolve within the first year of life,[44] neonatal cardiac hypertrophy is associated with a higher prevalence of morbid cardiovascular events.[45] Cardiac hypertrophy increases the risk of heart failure, stroke, coronary artery disease, and sudden cardiac death. Although we found no immediate risk of mortality within 21 days following parturition in the present study, heart function was impaired, including reduced LV ejection fraction, fractional shortening, and cardiac output after maternal THC exposure. Offspring with compromised cardiac function could be more susceptible to cardiovascular stressors.

THC exposure during gestation was found to decrease the size of both the aortic orifice size and pulmonary trunk opening as well as increase the ratio of the leaflet to orifice of each semilunar valve in E17.5 fetuses. Cellular analysis of each leaflet within the semilunar valves found the cell density to be higher, suggesting an increase in cell proliferation or decrease in remodeling of the valves. The later phases of valve development are characterized by transition of undifferentiated mesenchyme into specialized valvular interstitial cells, and remodeling and stratification of the extracellular matrix that continues after birth.[46] NFAT signaling has been shown to have multiple important roles in valvulogenesis.[47] NFATc1 is crucial for proper suppression of endocardial mesenchymal transition (EndMT) and promotion of endocardial cell proliferation in the developing valve while defining the endocardial-neural crest mesenchyme boundary.

Downregulation of *NFATc1* results in a shifted boundary and overpopulated endocardial-derived mesenchyme resulting from increased EndMT.[48] Furthermore, NFATc1 promotes RANKL-cathepsin K signaling, which regulates extracellular matrix remodeling.[49] Importantly, *NFATc1* was found to be downregulated in hearts collected from E12.5 fetuses exposed to THC. In combination with an increased cellular density within the valves and decreased *NFATc1* mRNA expression, decreased NFAT signaling is likely the cause of semilunar valve thickening in this model.

In humans, cardiac valve malformations including narrowing or stenosis of the aortic and pulmonary valves are the most common type of CHDs and are present in approximately half of all cases of CHDs.[50] Prognosis of the disease is a progressively worsening condition that results in ventricular hypertrophy and heart failure if there is no intervention.[51] During fetal heart development, the myocardium adapts to increases in functional demands by cell proliferation.[52,53] In the present study, semilunar valve thickening and narrowing of aorta and pulmonary trunk in THC-exposed fetuses may increase ventricular pressures (preload and afterload) of the heart. The hemodynamic

stress may serve as a potential mechanism underlying a higher cardiomyocyte proliferation in the fetal heart by maternal THC exposure.

In conclusion, maternal THC exposure during pregnancy, with doses equivalent to 3–6% THC containing cannabis smoking in humans, induces abnormalities of the developing heart, including semilunar valve thickening, myocardial hyperplasia, and postnatal cardiac dysfunction in mice. With the rising popularity of the high potency cannabis strains containing 17–28% of THC,[54] maternal exposure to these strains during gestation may produce further deleterious effects to the fetal heart. Our study suggests that maternal cannabis consumption may have a negative impact on fetal heart development during pregnancy and should be avoided.

Authors' Contributions

G.I.R., X.L., and Q.F conceived and designed the experiment; G.I.R., F.Y., and X.L. performed experiments; G.I.R. and X.L. performed data analysis; S.R.L. contributed to the study design and the preparation and dosing of THC. All authors reviewed the article.

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Author Disclosure Statement

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Table S1

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Abbreviations Used

3D = 3 dimensional
AC = anterior cusp
ANOVA = analysis of variance
Ao = aorta
AoV = aortic valve
CDK4 = cyclin-dependent kinase 4
E = embryonic day
EndMT = endocardial mesenchymal transition
H&E = hematoxylin and eosin
LA = left atrium
LC = left cusp
LCC = left coronary cusp
LV = left ventricle
NCC = noncoronary cusp
NFAT = nuclear factor of activated T-cells
PA = pulmonary artery
pHH3 = phospho-histone H3
PND = postnatal day
PND21 = postnatal day 21
PV = pulmonary valve
qPCR = quantitative polymerase chain reaction
RA = right atrium
RC = right cusp
RCC = right coronary cusp
RV = right ventricle
Se = septum
SEM = standard error of the mean
THC = delta-9-tetrahydrocannabinol