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Sapropterin reduces coronary artery malformation in offspring of pregestational diabetes mice

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) and oxidative stress are critical to embryonic coronary artery development. Maternal diabetes increases oxidative stress and reduces eNOS activity in the fetal heart. Sapropterin (Kuvan®) is an orally active, synthetic form of tetrahydrobiopterin (BH4) and a co-factor for eNOS with antioxidant properties. The aim of the present study was to examine the effects of sapropterin on fetal coronary artery development during pregestational diabetes in mice. Diabetes was induced by streptozotocin to adult female C57BL/6 mice. Sapropterin (10 mg/kg/day) was orally administered to pregnant mice from E0.5 to E18.5. Fetal hearts were collected at E18.5 for coronary artery morphological analysis. Sapropterin treatment to diabetic dams reduced the incidence of coronary artery malformation in offspring from 50.0% to 20.6%. Decreases in coronary artery luminal diameter, volume and abundance in fetal hearts from diabetic mothers, were prevented by sapropterin treatment. Maternal diabetes reduced epicardial epithelial-to-mesenchymal transition (EMT) and expression of transcription and growth factors critical to coronary artery development including hypoxia-inducible factor 1a (*Hif1a*), *Snail1*, *Slug*, *β-catenin*, retinaldehyde dehydrogenase 2 (*Aldh1a2*), basic fibroblast growth factor (*bFGF*) and vascular endothelial group factor receptor 2 (*Vegfr2*) in E12.5 hearts. Additionally, eNOS phosphorylation was lower while oxidative stress was higher in E12.5 hearts from maternal diabetes. Notably, these abnormalities were all restored to normal levels after sapropterin treatment. In conclusion, sapropterin treatment increases eNOS activity, lowers oxidative stress and reduces coronary artery malformation in offspring of pregestational diabetes. Sapropterin may have therapeutic potential in preventing coronary artery malformation in maternal diabetes.

1. Introduction

Diabetes is a global health concern. In 2013, approximately 21.4 million pregnancies worldwide were affected by diabetes, of which 16% were pregestational diabetes [1]. Women with pregestational diabetes are at an increased risk of having a child with a congenital heart defect (CHD) [2]. Hypoplastic coronary artery disease (HCAD) is a congenital abnormality characterized by a marked decrease in luminal diameter and length of one or more major branches of coronary arteries [3–6]. HCAD can be asymptomatic at birth but are often associated with myocardial infarction and sudden cardiac death under physical exertion later in life [7,8]. In the clinic, newborns are screened for many forms of CHDs, however, coronary artery branches are not reliably imaged using non-invasive tools perinatally [9]. HCAD is a rare form of congenital coronary artery malformations (CAMs) including anomalous origins of the left and right coronary arteries, single coronary artery, and coronary artery fistula, which collectively affect ~1% of the general population [10,11]. While the clinical etiology of congenital coronary artery anomalies is undefined, we have recently shown that pregestational diabetes results in CAMs in the fetal heart of offspring in mice [12].

The heart is the first functional organ to form during embryogenesis, and vascularization in the primitive heart initiates at E9.5 in the proepicardial organ [13,14]. Cells from this primitive structure migrate

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to cover the surface of myocardium, forming an epicardium, which is fully formed by E12.5. These cells then undergo coordinated epithelial-to-mesenchymal transition (EMT) and become epicardial-derived cells (EPDCs) [14]. EPDCs migrate to the subepicardial space and differentiate into vascular smooth muscle cells, endothelial cells and adventitial fibroblasts, all which are needed to form coronary vessels [14]. The endocardium and sinus venosus also contribute to this coronary plexus, and a complete coronary network is established by E15 [15,16]. A misstep in any of these processes including alterations in gene expression, cell proliferation and epicardial EMT may result in coronary artery malformations.

Hyperglycemia-induced reactive oxygen species (ROS) is not conducive to heart and coronary artery development as it oxidizes cardiogenic and angiogenic molecules. Endothelial nitric oxide synthase (eNOS) is critical to embryonic heart development as nitric oxide (NO) regulates transcription factor expression, progenitor cell growth and EMT [17]. In fact, eNOS$^{-/-}$ mice display hypoplastic coronary arteries and postnatal myocardial infarction [18]. Tetrahydrobiopterin (BH4) is a co-factor for eNOS, and an antioxidant. It stabilizes the enzyme dimer and allows arginine binding [19]. Under oxidative stress, BH4 levels decline, eNOS is uncoupled and unable to form NO, instead generating superoxide [20]. Although BH4 improves eNOS coupling and vascular endothelial function in diabetes [21], the ability of BH4 to regulate coronary artery development in the fetal heart is not known. The FDA-approved drug, sapropterin dihydrochloride (Kuvan®), is a stable, orally active, synthetic form of BH4. In the present study, we aim to determine the effects of sapropterin on coronary artery development under pregestational diabetes in mice. Our hypothesis was that sapropterin treatment improves epicardial EMT and reduces CAMs during pregestational diabetes.

2. Materials and methods

2.1. Animals

C57BL/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbour, Maine), and a breeding program was implemented to generate postnatal and fetal mice. All animals used in this study were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011). All procedures were approved by the Animal Care Committee at Western University, following the Canadian Council on Animal Care guidelines.

2.2. Induction of diabetes and sapropterin treatment

Diabetes was induced to six to eight-week old female C57BL/6 mice by streptozotocin (STZ, 50 mg/kg body weight, I.P.) injections for five consecutive days, as previously described [22,23]. STZ was dissolved in a sterile saline solution at the time of administration, with sterile saline as vehicle controls. Non-fasting blood glucose levels were measured one week following the last injection using a glucose meter (One Touch Ultra2, LifeScan Canada, Burnaby, BC). Mice with blood glucose levels above 11.0 mmol/L were considered to be diabetic and subsequently bred with adult wild-type male mice. A vaginal plug indicated embryonic day 0.5 (E0.5) and the pregnant female mice were placed in a cage with littermates. A subset of the diabetic dams received a 10 mg/kg per body weight oral dose of sapropterin dihydrochloride (Kuvan®, BioMarin Pharmaceutical Inc., Novato, CA, USA) each day of gestation. Sapropterin dihydrochloride was dissolved in water and combined with a small amount of peanut butter. Blood glucose levels, food and water intake were monitored throughout pregnancy.

2.3. Histological and immunohistochemical analysis

To analyze coronary artery development and resulting vasculature, embryos were harvested at E12.5 and E18.5 for morphological and immunohistochemical analyses. Briefly, the thoraces of embryos were isolated and fixed in 4% paraformaldehyde overnight. The samples were dehydrated, paraffin embedded and subsequently serial sectioned into 5-μm sections. Prior to immunostaining, antigen retrieval was conducted in citric acid buffer (0.01 mol/L, pH 6.0) for 12 min at 94 °C using a microwave (BP-111; Microwave Research & Applications, Inc., Carol Stream, IL). The following primary antibodies were applied and incubated overnight; anti-α-smooth muscle actin (1:3,000, mus monoclonal, Sigma-Aldrich A2547, Lot 032M4822), biotinylated lectin-1 (1:250, Bandeiraea Simplicifolia, Sigma-Aldrich L2380, Lot 054M4086V), anti-sex-determining region Y protein antibody (1:200, mus monoclonal, Santa Cruz SC-398567, Lot J1316), anti-Wt1 (1:300, rb polyclonal, Santa Cruz SC-192, Lot F0513), anti-E-cadherin (1:200, goat polyclonal, Santa Cruz SC-31020, Lot D2413), and anti-phosphohistone H3 (pHH3) (1:500, rb polyclonal, Abcam ab5176, Lot GR72823-1), followed by either biotinylated goat anti-rabbit IgG (1:500) or biotinylated donkey anti-goat IgG (1:500), for 1 h. Signals were amplified through the ABC reagent (Vector Laboratories), which enabled visualization by 3-3′ diaminobenzidine tetrahydrochloride (DAB, Sigma). Counterstaining was performed with Mayer's Hematoxylin (Thermo Scientific, Waltham, MA), and images were captured with a light microscope (Observer D1, Zeiss). To analyze coronary artery volume and branching, the AMIRA software (FEI Visualization Sciences Group) was used to create three-dimensional reconstructions of α-smooth muscle actin stained sections 25-μm apart. A ratio of coronary artery to myocardial volume was obtained using the AMIRA software. Counts of coronary arteries, capillaries, and coronary progenitor cells were taken from a minimum of three heart sections and normalized to the myocardium.

Table 1
Specific primer sequences for real-time PCR analysis.

Gene	Accession no.	Product Size	Primer Sequence
Hif1-α	NM_001313919	237	F: CAGCCTCACCAGACAGAGCA R: GTGCACAGTCACCTGGTTGC
Snail1	NM_011427.3	133	F: CACACGCTGCCTTGTGTCT R: GGTCAGCAAAAGCACGGTT
Slug	NM_011415.2	161	F: CAACGCCTCCAAGAAGCCCA R: GAGCTGCCGACGATGTCCAT
Aldh1a2	NM_009022.4	219	F: GGCAGCAATCGCTTCTCACA R: CAGCACTGGCCTTGGTTGAA
β-catenin	NM_001165902.1	178	F: CTTGGCTGAACCATCACAGAT R: AGCTTCCTTTTTGGAAAGCTG
bFgf	NM_008006.2	174	F: CAAGGGAGTGTGTGCCAACC R: TGCCCAGTTCGTTTCAGTGC
Vegfa	NM_001025257.3	194	F: GATTGAGACCCTGGTGGACAT R: TCTCCTATGTGCTGGCTTTGG
Vegfr2	NM_001363216.1	189	F: GGCGGTGGTGACAGTATCTT R: CTCGGTGATGTACACGATGC
Tbx18	NM_023814.4	199	F: GAGCAGCAACCCGTCTGTGA R: GGGACTGTGCAATCGGAAGG
28S	NR_003279.1	178	F: GGGCCACTTTTGGTAAGCAG R: TTGATTCGGCAGGTGAGTTG

F: forward primer; R: reverse primer.

2.4. Analysis of 4-hydroxynonenal and NO levels

To analyze lipid peroxidation, as an indication of oxidative stress, and NO levels in embryonic hearts, E12.5 ventricles from all four groups were harvested and embedded in FSC22 frozen section media (Leica). Samples were cryosectioned (CM1950, Leica) into 8 μm thick sections, and immunostained with 4-hydroxynonemal (4-HNE) antibody (1:300, 2 h, goat polyclonal, ABM Y072093, Lot AP1217), to indicate lipid peroxidation, or incubated with 4-amino-5-methylamino-2′,7′-difluororescein (DAF-FM) diacetate (5 mM, Invitrogen D23844, Lot 895268), to indicate NO levels. The 4-HNE immunostain was followed by CY3-conjugated anti-goat IgG secondary antibody (1:1000;

Table 2
Effects of sapropterin (BH4) on nonfasting maternal blood glucose levels and incidence of CAMs in E18.5 hearts during pregestational diabetes.

Litters (n)	Control	Diabetes	BH4	Diabetes + BH4	Diabetes + Insulin
	3	9	4	5	3
Blood glucose at E0.5 (mmol/L)	7.5 ± 1.8	15.9 ± 4.9*	8.0 ± 0.6	16.8 ± 4.2†	6.2 ± 0.8
Blood glucose at E18.5 (mmol/L)	7.5 ± 0.6	25.1 ± 4.4**	9.1 ± 1.0	26.9 ± 5.2††	7.6 ± 0.7
CAM/total fetuses (n)	0/14	19/38*	0/23	7/34†	0/20
Males: CAM/total fetuses (n)	0/14	9/38*	0/23	3/34	0/20
Females: CAM/total fetuses (n)	0/14	10/38*	0/23	4/34	0/20
CAMs (%)	0.0	50.0	0.0	20.6	0.0

Data are mean ± SEM. Blood glucose levels were analyzed by one-way ANOVA followed by Tukey's test. The incidence of CAM was analyzed by χ^2 test. CAM, coronary artery malformation. *$P < 0.05$, **$P < 0.01$ vs. respective controls. †$P < 0.05$, ††$P < 0.05$ vs. diabetes.

Jackson Immunoresearch). Probing with DAF-FM for 45 min was followed by a 30 min incubation in PBS. Signals were detected using fluorescence microscopy (Observer D1, Zeiss, Oberkochen, Germany). Using a fixed exposure time, a minimum of 4 images were captured per heart, and fluorescence intensity per area of myocardium were measured using AxioVision Software (Zeiss, Oberkochen, Germany).

2.5. Assessment of epicardial EMT using ex vivo heart explant culture

To assess epicardial EMT, ventricles of E12.5 embryonic hearts from non-diabetic dams were harvested and cultured on a collagen gel matrix for 96 h. To prepare the collagen matrix, 1 mg/mL type I rat tail collagen (BD Bioscience) was added to 2x M199 media (M5017; Sigma) containing 5 mM D-glucose or 30 mM D-glucose in a 24-well plate. The casted collagen wells were hydrated with 500 µL 1x M199 media plus 10% FBS and insulin-transferrin-selenium, with or without 0.1 mM BH4 (Sigma), and 5 mM or 30 mM D-glucose. E12.5 ventricles were isolated in sterile saline, placed centrally in a well containing the set collagen matrix and media, and incubated at 37 °C for 4 days. Images were captured with a phase contrast microscope (Zeiss, Oberkochen, Germany), and spindle-shaped cell outgrowth distance was quantified.

2.6. Western blotting for eNOS and Akt phosphorylation

Ventricular myocardial tissue from E14.5 hearts, isolated in PBS, was used to analyze eNOS and Akt activity. Briefly, 30 µg of protein from isolated ventricular tissue was separated via 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with antibodies against p-NOS3 (Ser1177; 1:1000, rb polyclonal, Cell Signaling 9571S, Lot 7), NOS3 (1:1000, rb polyclonal, Santa Cruz SC-654, Lot G2414), p-Akt (Ser473; 1:5000, rb polyclonal, Cell Signaling 9271, Lot 19), Akt (1:5000, rb polyclonal, Cell Signaling 9272, Lot 22), and α-actinin (1:5000, mus monoclonal, Sigma-Aldrich A7811, Lot 029K4844). Washed blots were probed with horseradish peroxidase–conjugated secondary antibodies (1:2500; Bio-Rad). The signal was detected using enhanced chemiluminescence and quantified by densitometry.

2.7. Real-time RT-PCR analysis

Total RNA was extracted from E12.5 hearts using TRIzol reagent (Invitrogen). 200 ng of RNA was reverse transcribed using the Maloney murine leukemia virus reverse transcriptase and random primers. Evergreen qPCR MasterMix (Applied Biological Systems, Vancouver, BC) was used to conduct Real-time PCR on cDNA. Primers were designed for HIF-1α, Aldh1a2, β-catenin, bFGF, Vegfa, Vegfr2, Slug, Snail1, and Tbx18 using the Primer3 software v4.1.0 (Table 1). Samples were amplified for 35 cycles using the Eppendorf Realplex (Hamburg, Germany). mRNA levels were extrapolated using a comparative C_T method by normalizing to 28S-Ribosomal RNA.

2.8. Statistical analysis

Data are shown as mean ± SEM. A two-way analysis of variance (ANOVA) was used for multiple group comparisons between diabetic and control dams with and without sapropterin treatment, and their interactions, followed by the Bonferroni post-hoc test (GraphPad Software, Version 5, La Jolla, CA, USA). The incidence of coronary artery malformations was assessed with Chi-square test. Differences were regarded significant with $P < 0.05$.

3. Results

3.1. Sapropterin reduces coronary artery malformations in offspring of diabetic mice without altering blood glucose levels

The present study was conducted using the same model of pregestational diabetes previously employed [12,22,23]. A week following STZ administration, diabetes was confirmed by measuring non-fasting blood glucose levels. Female mice with > 11 mmol/L blood glucose were considered diabetic and bred with normal males. The glycemic state of the diabetic dam steadily increased from 15.9 ± 4.9 mmol/L at E0.5 to 25.1 ± 4.4 mmol/L by E18.5 (Table 2). The diabetic dams fed with sapropterin during gestation displayed a similar increase in hyperglycemia, gradually reaching 26.9 ± 5.2 mmol/L at the end of pregnancy. Of note, treatment with sapropterin did not affect blood glucose levels in either the control or diabetic groups. A daily dose of insulin was administered to a cohort of diabetic dams throughout gestation, which normalized their elevated blood glucose levels (Table 2).

Half of all fetuses from untreated diabetic dams examined at E18.5 displayed CAM. This ratio was significantly reduced to 20.6% with sapropterin treatment (Table 2). Furthermore, 36.8% of fetal hearts presented with both CHD and CAM. Sapropterin effectively prevented the dual defect occurrence, as no hearts from the offspring of sapropterin-treated diabetic dams had both CHD and CAM. No CAMs or CHDs were found in either of the control groups or the insulin treatment groups, indicating that any vascular abnormalities seen were induced by diabetes and not a teratogenic effect of STZ. Finally, CAM incidence or the effectiveness of sapropterin treatment did not show any bias towards the male or female sex (Table 2).

3.2. Sapropterin diminishes diabetes-induced fetal hypoplastic coronary arteries and restores capillary density

Hearts with hypoplastic coronary arteries have marked decreases in vessel number and luminal diameter [12]. Immunostaining with α-smooth muscle actin and biotinylated lectin-1 was used to identify hypoplastic coronary arteries and capillaries, respectively in E18.5 hearts. The left coronary artery originating from the aortic orifice in fetuses from diabetic dams was significantly smaller in luminal diameter than control ($P < 0.01$, Fig. 1A and E). Similarly, the diameter of the right ventricular coronary artery in offspring from diabetic dams

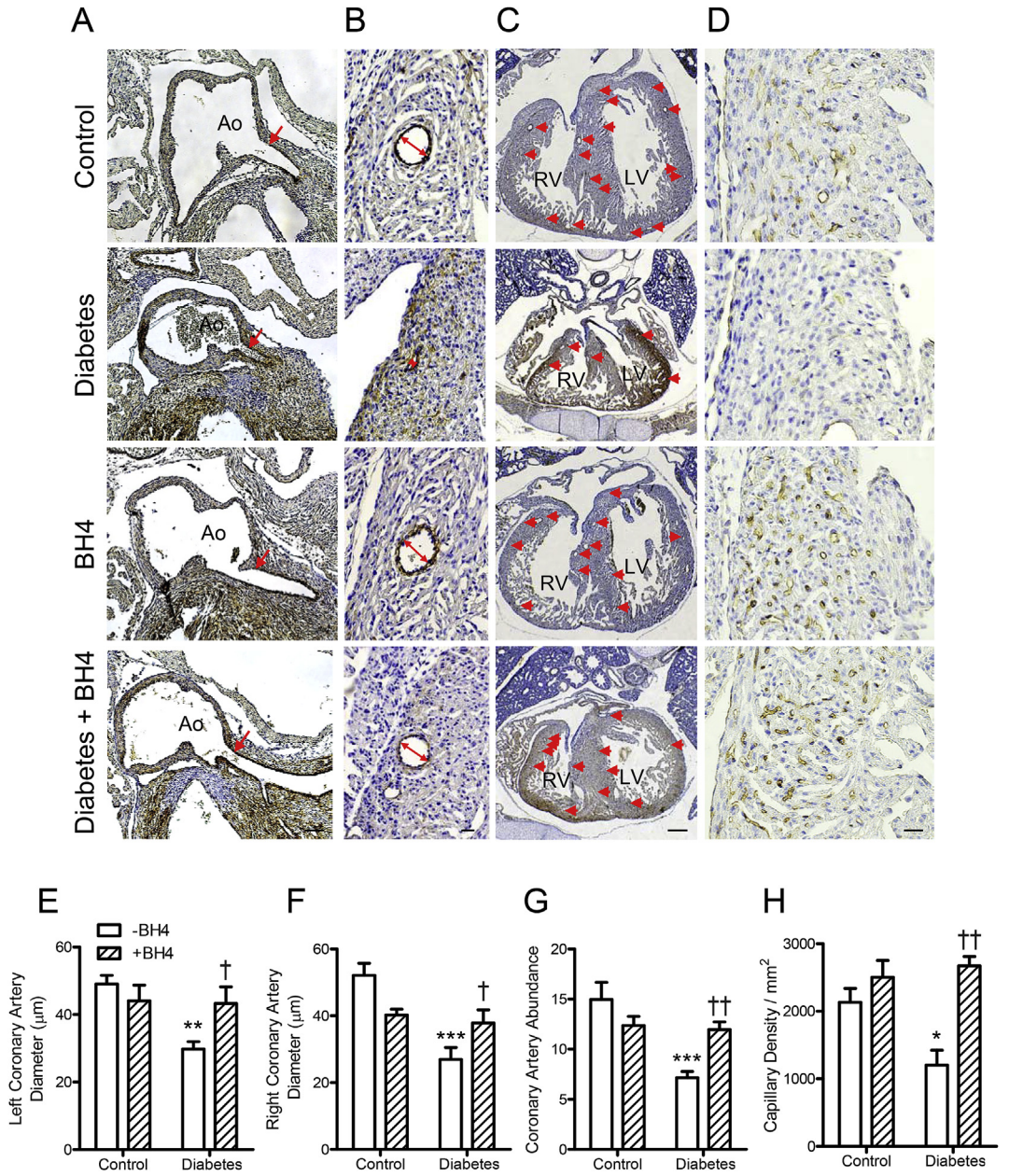

Fig. 1. Effects of sapropterin (BH4) on coronary artery malformations induced by pregestational diabetes. Representative histological sections of E18.5 hearts from offspring of control and diabetic dams with and without BH4 treatment. α-smooth muscle actin staining of vascular smooth muscle cells specifying the left (**A**) and right (**B**) coronary artery, with arrows indicating diameter of the artery lumen measurements. (**C**) α-smooth muscle actin staining for total number of coronary arteries throughout the ventricular and septal myocardium. (**D**) Biotinylated lectin-1 immunostaining marking endothelial cells forming myocardial capillaries in the right ventricle. **(E–H)** Analysis of left **(E)** and right **(F)** coronary artery diameter, coronary artery abundance **(G)** and capillary density **(H)**. n = 7–9 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. untreated control, †$P < 0.05$ and ††$P < 0.01$ vs. untreated diabetes. Scale bars represent 50, 20, 200 and 20 μm in A, B, C and D, respectively.

was also smaller than control ($P < 0.001$, Fig. 1B and F). Sapropterin treatment prevented this diabetes-induced reduction in luminal diameter of the left and right coronary arteries ($P < 0.05$, Fig. 1A, B, E and F). Sapropterin treatment also rescued a maternal diabetes-induced decrease in coronary artery abundance within the ventricular myocardium ($P < 0.01$, Fig. 1C and G). Interestingly, strong α-smooth muscle actin staining was noted in hearts from diabetic dams throughout the myocardium at E18.5, suggesting a delayed maturation of cardiomyocytes. Finally, immunostaining of lectin-1 marking endothelial cells revealed a reduced capillary density in the ventricular myocardium of E18.5 hearts from diabetic dams comparted to controls, which was restored to normal with sapropterin treatment ($P < 0.05$, Fig. 1D and H). Coronary arteries were reconstructed in 3-dimensions to illustrate branching and extrapolate volume measurements. Fig. 2A shows a notable decrease of coronary artery arborisation in E18.5 hearts from diabetic dams. The typical branching patterns were restored in offspring from diabetic mothers treated with sapropterin, resulting in normalization of the coronary artery volume to myocardial volume ratio ($P < 0.01$, Fig. 2B). In addition, the volume of the ventricular myocardium was significantly lower in E18.5 hearts of offspring of diabetic dams, compared to control ($P < 0.01$, Fig. 2C). This decrease was restored to normal with sapropterin treatment ($P < 0.05$, Fig. 2C).

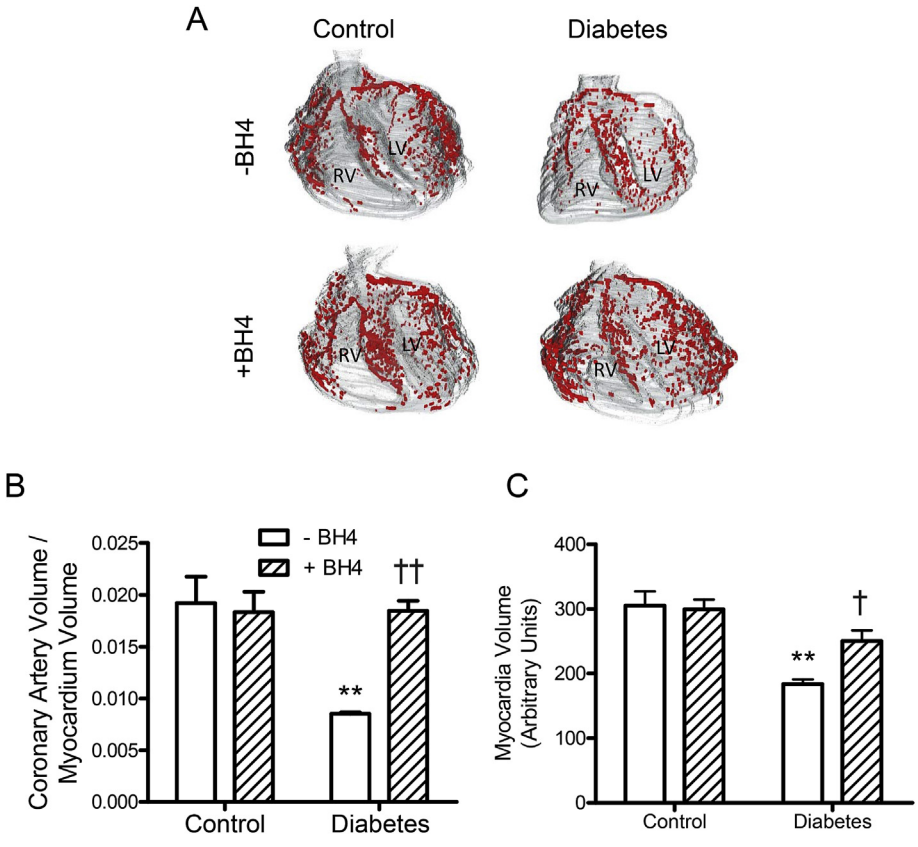

Fig. 2. Effects of sapropterin (BH4) on coronary artery volume at E18.5. (**A**) Frontal views of 3D reconstructions of the coronary arteries with superimposed myocardium. (**B**) Total coronary artery volume normalized to myocardial volume. n = 4 hearts per group. $^{**}P < 0.01$ vs. untreated control, $^{††}P < 0.01$ vs. untreated diabetes.

3.3. Sapropterin regulates coronary artery progenitor proliferation and EMT

To determine if diabetes-induced perturbations in coronary artery formation could give rise to the hypoplastic phenotype seen at E18.5, and whether sapropterin affected coronary artery precursors, epicardial proliferation and EMT were examined at E12.5. The epicardium is the source of coronary artery progenitors, which migrate from this epithelial layer via EMT into the myocardium [24]. Immunostaining for phosphorylated histone H3 (pHH3), marking cells undergoing division, revealed less proliferation, reduced cell density, a loosely attached epicardium in hearts from diabetic dams compared to controls ($P < 0.01$, Fig. 3A and D). Sapropterin treatment restored the number of $pHH3^+$ cells to almost control levels and rescued epicardial detachment (Fig. 3A). Concurrent with these changes in proliferation, a significantly greater number of $E\text{-cadherin}^+$ epicardial cells were found in embryonic hearts from diabetic dams, which was abrogated by sapropterin treatment ($P < 0.01$, Fig. 3B and E). To determine the number of cells actively going through the process of EMT, immunostaining for the transcription factor Wt1 was conducted and showed significantly fewer positive cells in the epicardium and subepicardium in E12.5 hearts from diabetic dams compared to controls ($P < 0.05$, Fig. 3D). These abnormalities were also prevented by sapropterin treatment ($P < 0.05$, Fig. 3D, F-G).

3.4. Tetrahydrobiopterin normalizes high glucose-impaired epicardial EMT ex vivo

To further investigate the ability of sapropterin to restore epicardial EMT and thereby prevent diabetes-induced CAMs, effects of BH4 on EPDC migration was studied *ex vivo* under high glucose conditions. The ventricular myocardium of E12.5 hearts from control dams was isolated and cultured on collagen gel in both high glucose (30 mmol/L) and normal glucose (5 mmol/L) conditions for 4 days (Fig. 4A). The outgrowth radius of spindle shaped cells from the explanted heart tissue was measured. The data shows that high glucose impaired EPDC migration through the collagen matrix with significantly shorter outgrowth distance compared to normal glucose ($P < 0.01$, Fig. 4A). The addition of 0.1 mmol/L BH4 restored the distance travelled by the spindle-shaped EPDCs to normal levels ($P < 0.05$, Fig. 4A and B).

3.5. Sapropterin restores expression of coronary vessel development and growth genes

Pregestational diabetes has been shown to alter gene expression in the developing heart [12,22,25]. To determine if the expression of key transcriptional regulators and signaling molecules responsible for epicardial EMT, angiogenesis, differentiation and growth were affected by maternal diabetes and sapropterin treatment, qPCR analysis was performed on E12.5 hearts. The mRNA levels of *Hif-1α*, *Snail1*, *Slug*, *Aldh1a2*, *β-catenin*, *bFGF*, *Vegfr2*, and *Tbx18* were significantly lower in hearts from diabetic dams compared to controls ($P < 0.05$, Fig. 5A–F, H-I). Treatment with sapropterin significantly improved the expression levels of *Hif-1α*, *Snail1*, *Aldh1a2*, *β-catenin*, *bFGF* and *Vegfr2* ($P < 0.05$, Fig. 5A–F and H).

3.6. Sapropterin inhibits oxidative stress and restores Akt/eNOS activity and NO levels

To assess levels of oxidative stress in the developing myocardium, immunofluorescent staining of 4-hydroxynonenal (4-HNE), a product of lipid peroxidation, was conducted on E12.5 hearts. Quantification of red fluorescence intensity indicated higher levels of myocardial 4-HNE in offspring from diabetic dams compared to controls ($P < 0.05$, Fig. 6A and B). Sapropterin administration to diabetic dams significantly reduced lipid peroxidation ($P < 0.05$, Fig. 6B). Next, we assessed the effects of pregestational diabetes on Akt and eNOS activity in E14.5 hearts. Western blotting was used to analyze the phosphorylated and total amounts of Akt and eNOS (Fig. 6C). Fetal hearts from

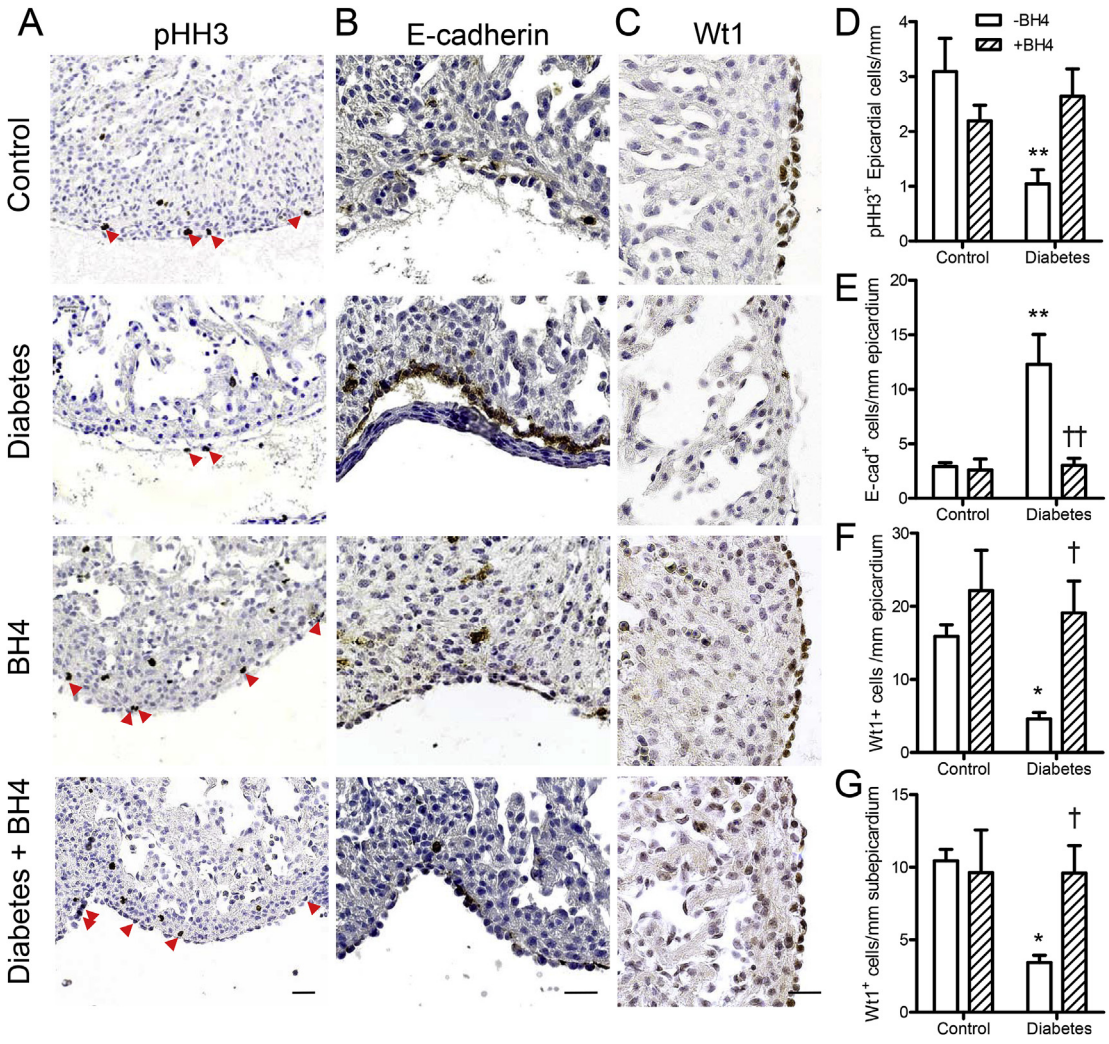

Fig. 3. Effects of sapropterin (BH4) on epicardial cell proliferation and markers of EMT in E12.5 hearts. (**A**) Representative images of immunostaining for phosphorylated histone H3 marking proliferating cells (red arrows), (**B**) E-cadherin (brown staining) representing cell-to-cell adhesion, and (**C**) $Wt1^+$ cells (brown staining) indicating EMT. Quantification of $pHH3^+$ cells (**D**), E-cadherin$^+$ cells (**E**) in the epicardium, and $Wt1^+$ cells in the epicardium **(F)** and subepicardium **(G)**. n = 3–6 hearts per group, $^*P < 0.05$, $^{**}P < 0.01$ vs. untreated control, †$P < 0.05$, ††$P < 0.01$ vs. untreated diabetes. Scale bars are 20 μm.

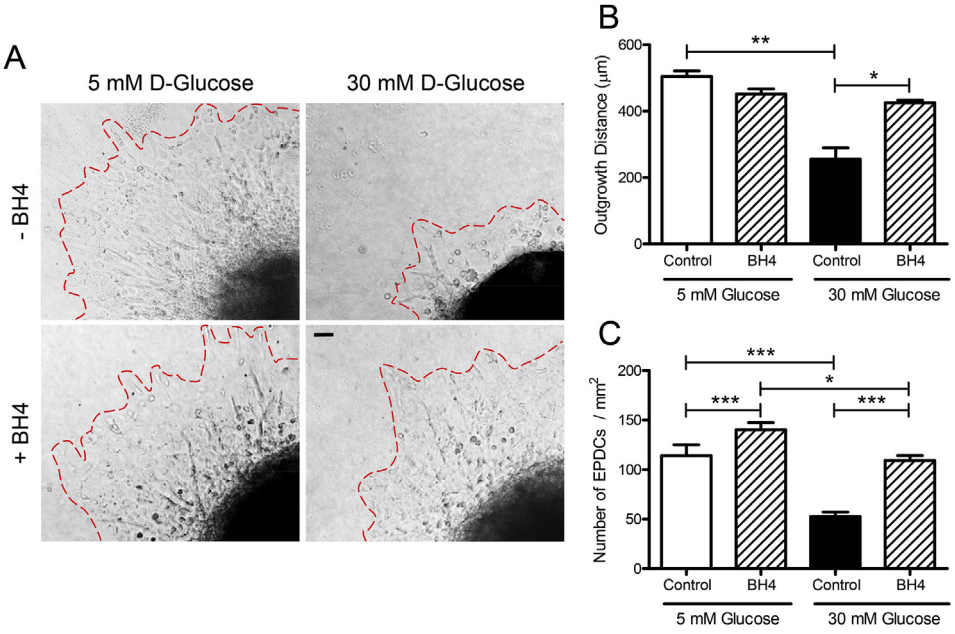

Fig. 4. Effects of BH4 on epicardial EMT *ex vivo*. (**A**) Representative images of epicardial cell outgrowth and EMT from E12.5 heart explants grown on a collagen matrix in normal (5 mM) and high (30 mM) glucose conditions with and without BH4 (0.1 mM). Dashed line indicated the border of the migrated cells. (**B**) Measurements of the average distance travelled by epicardial cells. (**C**) Quantification of spindle shaped cells (epicardial-derived cells, EPDCs) that have undergone EMT. $^*P < 0.05$, $^{**}P < 0.01$. n = 3–5 explants per group from five litters.

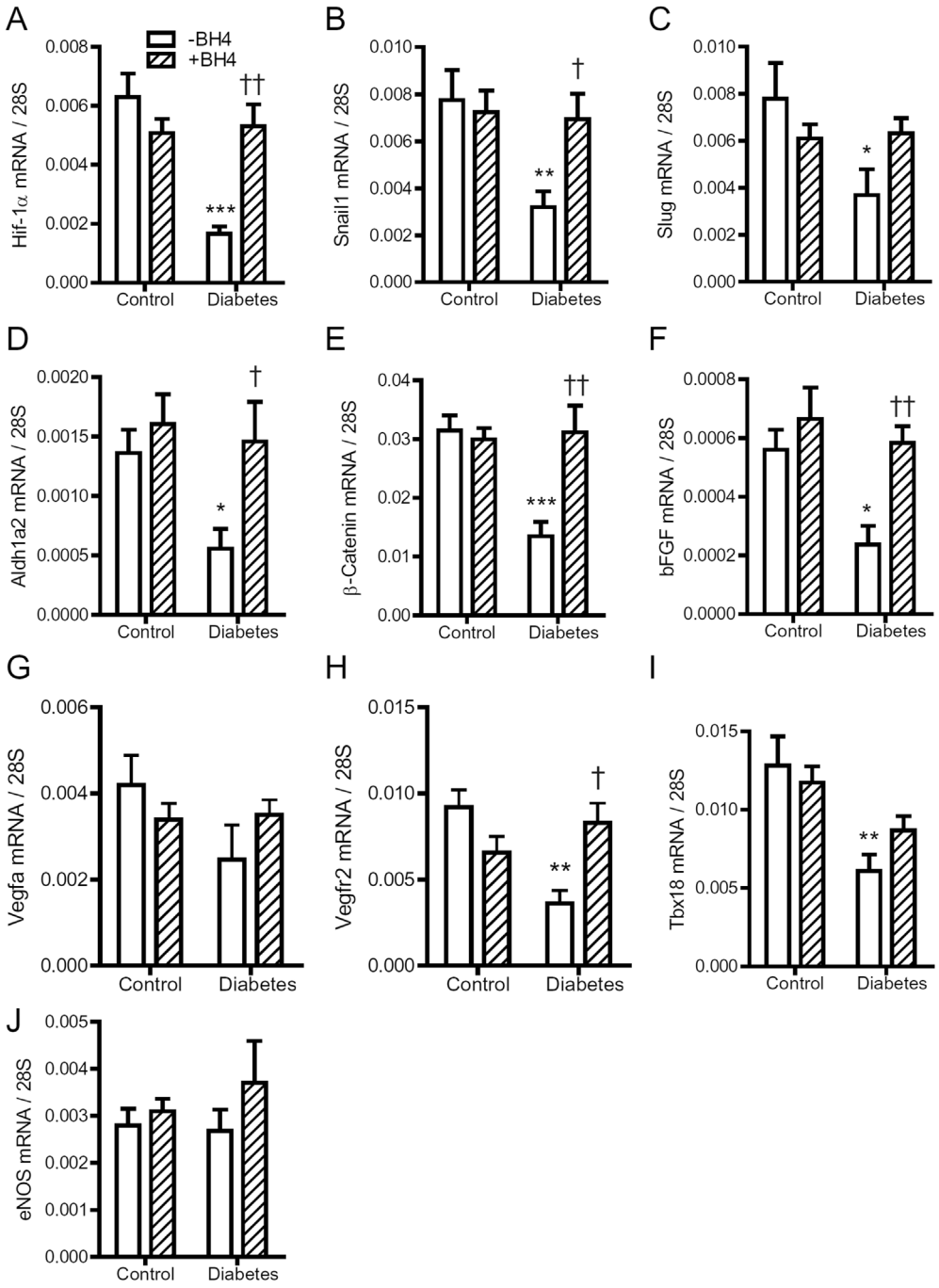

Fig. 5. Effects of sapropterin (BH4) on gene expression of transcription and growth factors critical to coronary artery development in E12.5 hearts of offspring from diabetic and control dams. mRNA levels were analyzed by qPCR. (**A-F, H and I**) The expression of *Hif-1α, Snail1, Slug, Aldh1a2, β-catenin, bFGF, Vegfr2* and *Tbx18* were significantly decreased under maternal diabetes. All (**A-F, H**) were restored to control levels by BH4 treatment except *Slug* and *Tbx18* (**C** and **I**). (**G and J**) *Vegfa* and *eNOS* expression was not significantly changed. n = 4–7 hearts per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated control, and $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs. untreated diabetes.

diabetic dams had significantly lower levels of p-Akt and p-eNOS compared to controls, without any major changes in total protein levels ($P < 0.001$, Fig. 6C–E). Phosphorylation of both enzymes was recovered to control levels with sapropterin treatment ($P < 0.05$, Fig. 6C–E). To assess NO levels in E12.5 hearts, a fluorescence probe DAF-FM diacetate was used. Quantification of green fluorescence intensity of DAF-FM indicated lower myocardial NO levels in the offspring of diabetic dams compared to control ($P < 0.01$, Fig. 7A and B). Sapropterin administration to diabetic dams significantly increased NO levels in the fetal heart ($P < 0.01$, Fig. 7B).

4. Discussion

The current study displays the efficacy of the FDA-approved drug sapropterin dihydrochloride (Kuvan®) in reducing congenital malformations of coronary arteries induced by a clinically relevant model of pregestational diabetes. In accordance with our previous studies [12], CAMs were observed in the offspring of diabetic female mice. These CAMs manifest as hypoplastic coronary arteries, with marked decreases in luminal diameter and abundance, which translates to an overall reduction in the volume of coronary vasculature. We show that daily sapropterin administration to diabetic dams prevents hypoplastic coronary arteries, restores myocardial coronary arborisation and increases capillary density. Additionally, sapropterin decreases oxidative stress, and increases Akt/eNOS activity, NO levels, and epicardial EMT, leading to normal coronary artery development.

Recently, we have shown the effectiveness of sapropterin in preventing CHDs in the offspring of diabetic mice [23]. Sapropterin treatment throughout gestation to diabetic dams decreased the incidence of CHDs in their offspring from 59.4% to 26.5%, despite dams

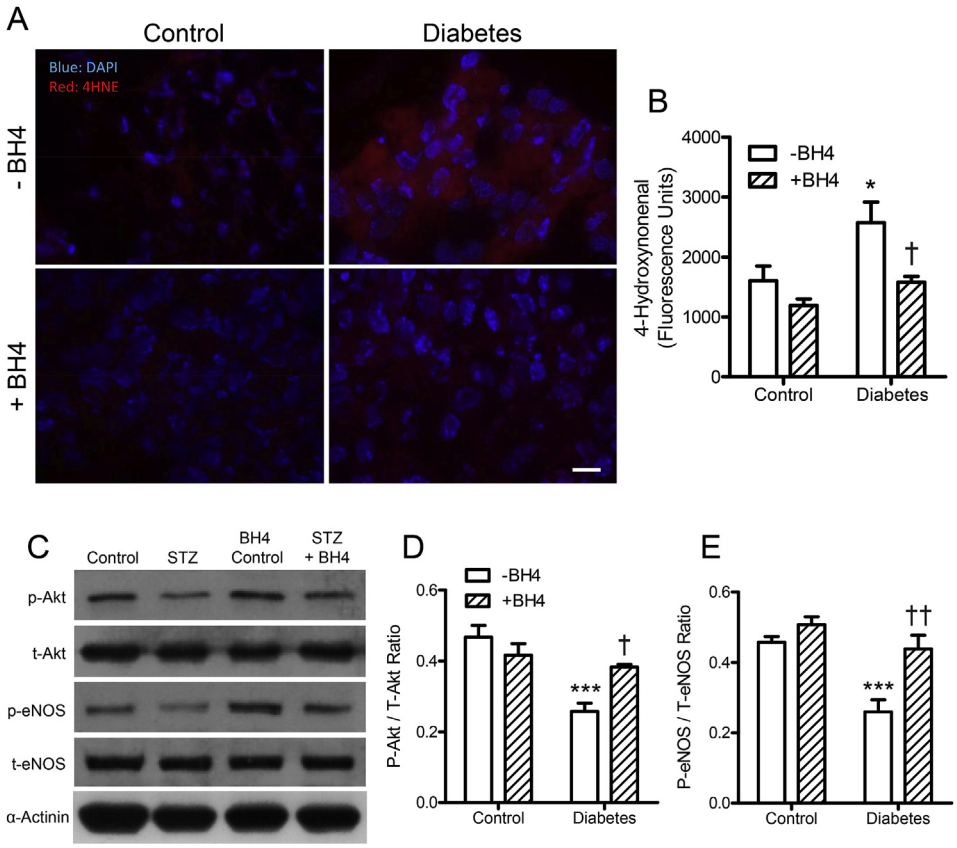

Fig. 6. Effects of sapropterin (BH4) on oxidative stress and Akt/eNOS phosphorylation in fetal hearts. (A) Representative immunofluorescence images of 4-hydroxynonenal staining for lipid peroxidation in the ventricular myocardium at E12.5. (**B**) Quantification of 4-HNE red immunofluorescence signal. (**C**) Representative Western blots for phosphorylated and total levels of Akt and eNOS with α-actinin as a loading control in E14.5 hearts. (**D and E**) Densitometric analysis of phosphorylated to total protein levels of Akt (**D**) and eNOS (**E**). n = 4 hearts per group. $^{***}P < 0.001$ vs. untreated control, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ vs. untreated diabetes.

having a comparable hyperglycemic status [23]. No major CHDs were reported in the treatment group, and insulin administration completely prevented the presence of malformations [23]. Correspondingly, our previous work show that N-acetylcysteine (NAC) treatment in the same model of pregestational diabetes, significantly reduced the incidence of CHDs and CAMs from 58.1% to 16.3% and from 46.6% to 6.0%, respectively [12,22]. In fact, only 3% of fetal hearts from diabetic dams treated with NAC showed dual congenital heart and coronary artery anomalies [12]. In the present study, sapropterin treatment completely prevented the presence of both a CHD and CAM within the same heart, and significantly reduced CAM incidence from 50.0% to 20.6%. Whether sapropterin and NAC have an additive or synergistic effect in preventing CHDs and CAMs remains to be determined in future studies.

STZ is commonly used in animal models of diabetes to induce congenital malformations [12,22,23,25]. Following STZ administration in our study, the glycemic levels of female mice steadily rose throughout pregnancy. Daily insulin treatment, the clinical gold-standard for controlling hyperglycemia in diabetes [26], to a cohort of these diabetic mice normalized blood glucose levels. Interestingly, no CAMs were seen with insulin treatment, indicating that STZ does not have teratogenic effects on coronary artery development, and the defects seen in non-insulin treated STZ cohort are due to hyperglycemia. Unlike insulin, sapropterin did not affect blood glucose levels of the diabetic dam, suggesting that its beneficial effects are independent of maternal glucose levels. Clinically, a male predominance of coronary artery anomalies has been reported [27,28]. In the present study, the incidence of pregestational diabetes-induced CAMs did not show a sex bias and sapropterin treatment was equally effective in both females and males. Of note, in dams with blood glucose exceeding 30 mmol/L, fetuses harvested at E18.5 were 69% females, suggesting that more males with CAMs may have succumbed to maternal diabetes, which may explain a lack of sex difference in the incidence of CAMs in our study.

The epicardium is a major source of coronary arteries during embryonic heart development. Between E11.5 and E12.5 many cells in the epicardium undergo EMT and delaminate, forming EPDCs [14,29]. These EPDCs migrate into the myocardium and differentiate into vascular smooth muscle cells, endothelial cells and perivascular fibroblasts, and form coronary arteries [13]. Wt1 is the master regulator of epicardial EMT, and transcriptionally controls the expression of many mediators of EMT such as Snail1, Aldh1a2 and E-cadherin [30,31]. In mice deficient of Wt1, epicardial cells fail to undergo EMT, resulting in null coronary artery formation [32]. In the present study, consistent with our previous work [12], pregestational diabetes decreased epicardial and subepicardial expression of Wt1, and inhibited epicardial cell proliferation and EMT in the developing heart. We also showed lower mRNA levels of many drivers of EMT including, Snail1, Slug, and Aldh1a2, and increased expression of E-cadherin. Notably these changes were restored to control levels with sapropterin treatment. Interestingly, BH4 has been shown to increase cell proliferation in retinal microvascular endothelial cells, mesangial (smooth muscle) cells of the kidney, and human vascular endothelial cells (HUVECs), and promote cell migration and tubulogenesis [33–35]. Consistent with these studies, we showed a pro-angiogenic effect of sapropterin in the fetal heart of diabetic pregnancies in the present study.

Akt and eNOS are critical for proper heart development. In fact, Akt1/3 double knockout mice exhibit CHDs and early neonatal lethality [36]. We have previously shown that eNOS-deficient mice display major cardiac defects including ASD, VSD and hypoplastic coronary arteries [18,37,38]. Diabetes impairs eNOS function. For example, eNOS Ser1177 phosphorylation is decreased in the heart of diabetic patients [39] and eNOS is uncoupled with a lower dimer to monomer ratio in E12.5 hearts from diabetic dams, indicating decreased eNOS activity [23]. In the present study, maternal diabetes induces significant reductions in Akt and eNOS phosphorylation as well as NO levels in fetal hearts, which were restored by sapropterin treatment. eNOS-derived NO is a key signaling molecule involved in proliferation, differentiation, EMT and ROS handling [17]. High levels of 4-

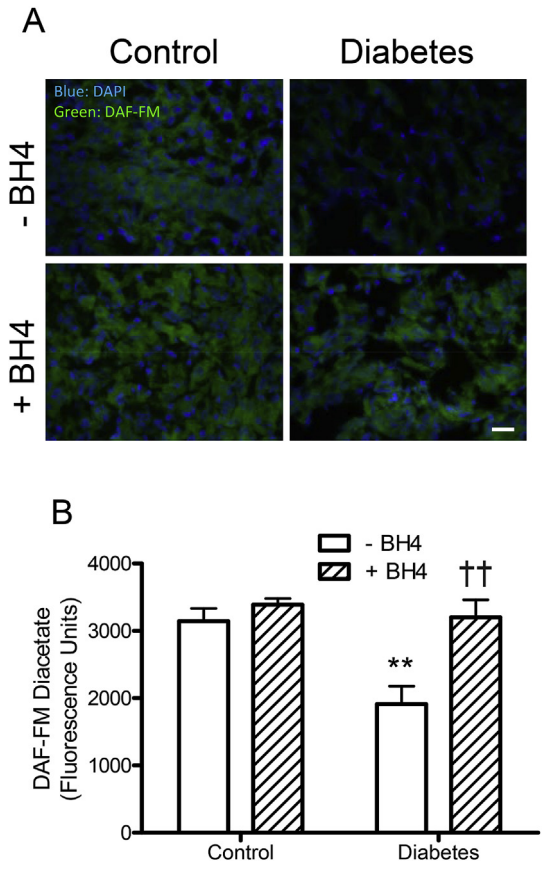

Fig. 7. Effects of sapropterin (BH4) on nitric oxide levels in E12.5 hearts. **(A)** Representative fluorescence images of DAF-FM probing for nitric oxide in the ventricular myocardium at E12.5. **(B)** Quantification of DAF-FM green fluorescence signals. N = 4–5 hearts per group. $^{**}P < 0.01$ vs. untreated control, $\dagger\dagger P < 0.01$ vs. untreated diabetes.

hydroxynonenal, a marker of lipid peroxidation, reduces eNOS phosphorylation in bovine aortic endothelial cells by decreasing cellular BH4 bioavailability [40]. In our study, sapropterin treatment in diabetic dams significantly reduced ROS and 4-hydroxynonenal levels in fetal hearts. These findings suggest that sapropterin prevents hyperglycemia-induced oxidative stress and maintains redox balance and eNOS function in the developing heart.

A notable observation from immunohistochemical analysis is α-SMA positive staining in cardiomyocytes in addition to coronary arteries in E18.5 hearts of offspring from diabetic mothers. During early cardiogenesis, immature cardiomyocytes express α-SMA and as cardiac development progresses, α-SMA is eventually replaced by cardiac actin isoforms [41,42]. The persistent α-SMA expression in cardiomyocytes and smaller heart size at E18.5 in offspring of pregestational diabetes suggest that maternal diabetes delays fetal heart maturation, which is also prevented by sapropterin treatment.

The present study is limited to simulate type 1 diabetes. In pregnant women, the prevalence of pregestational type 2 diabetes is rapidly increasing in recent years [43]. The effects of type 2 diabetes on CAMs remain to be investigated in future studies. Additionally, coronary arteries are formed from at least 3 sources of progenitors, the epicardium, endothelial cells of sinus venosus and the endocardium [14–16]. The present study examined the changes of epicardial progenitors, which is considered a major contributor to coronary arteries [14]. Whether the other two sources of coronary progenitors are affected by pregestational diabetes and/or sapropterin requires further investigation.

In conclusion, oral treatment with the FDA-approved drug sapropterin dihydrochloride (Kuvan®), a stable, synthetic form of BH4, reduces the incidence of CAMs induced by maternal diabetes in mice. Sapropterin increases Akt/eNOS phosphorylation and NO levels, decreases oxidative stress in the developing heart, and promotes cell proliferation, EMT and growth of coronary artery progenitors. Sapropterin did not negatively affect litter size, fetal weight or heart development in our studies [23], suggesting an excellent safety profile in normal mouse pregnancies. Currently sapropterin is prescribed as a phenylalanine hydroxylase activator for patients with phenylketonuria (PKU), a genetic disorder. The potential of sapropterin as a treatment to prevent CHDs and CAMs in the offspring of women with pregestational diabetes needs to be tested in clinical trials.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.10.002.

Author contributions

A.E, X.L., and Q.F. conceived the experiments. A.E, X.L., K·N., and Q.F. designed the experiments. A.E., Y.L., M.Y.K., and X.L. performed the experiments and data analyses. A.E. drafted the manuscript. A.E., K·N., and Q.F. revised the manuscript. All authors contributed to the interpretation of results and proofreading of the manuscript.

References

[1] L. Guariguata, U. Linnenkamp, J. Beagley, D.R. Whiting, N.H. Cho, Global estimates of the prevalence of hyperglycaemia in pregnancy, Diabetes Res. Clin. Pract. 103 (2014) 176–185.
[2] N. Oyen, L.J. Diaz, E. Leirgul, H.A. Boyd, J. Priest, E.R. Mathiesen, T. Quertermous, J. Wohlfahrt, M. Melbye, Prepregnancy diabetes and offspring risk of congenital heart disease: a nationwide cohort study, Circulation 133 (2016) 2243–2253.
[3] F.T. Zugibe, F.T. Zugibe Jr., J.T. Costello, M.K. Breithaupt, Hypoplastic coronary artery disease within the spectrum of sudden unexpected death in young and middle age adults, Am. J. Forensic Med. Pathol 14 (1993) 276–283.
[4] W.C. Roberts, B.N. Glick, Congenital hypoplasia of both right and left circumflex coronary arteries, Am. J. Cardiol. 70 (1992) 121–123.
[5] N. Amabile, A. Fraisse, J. Quilici, Hypoplastic coronary artery disease: report of one case, Heart 91 (2005) e12.
[6] C. McFarland, R.S. Swamy, A. Shah, Hypoplastic coronary artery disease: a rare cause of sudden cardiac death and its treatment with an implantable defibrillator, J. Cardiol. Cases 4 (2011) e148–e151.
[7] C. Basso, B.J. Maron, D. Corrado, G. Thiene, Clinical profile of congenital coronary artery anomalies with origin from the wrong aortic sinus leading to sudden death in young competitive athletes, J. Am. Coll. Cardiol. 35 (2000) 1493–1501.
[8] A.J. Taylor, K.M. Rogan, R. Virmani, Sudden cardiac death associated with isolated congenital coronary artery anomalies, J. Am. Coll. Cardiol. 20 (1992) 640–647.
[9] H.W. Goo, Coronary artery imaging in children, Korean J. Radiol. 16 (2015) 239–250.
[10] O. Yamanaka, R.E. Hobbs, Coronary artery anomalies in 126,595 patients undergoing coronary arteriography, Cathet. Cardiovasc. Diagn. 21 (1990) 28–40.
[11] G. Tuo, M. Marasini, C. Brunelli, L. Zannini, M. Balbi, Incidence and clinical relevance of primary congenital anomalies of the coronary arteries in children and adults, Cardiol. Young 23 (2013) 381–386.
[12] H. Moazzen, X. Lu, M. Liu, Q. Feng, Pregestational diabetes induces fetal coronary artery malformation via reactive oxygen species signaling, Diabetes 64 (2015) 1431–1443.
[13] D.E. Reese, T. Mikawa, D.M. Bader, Development of the coronary vessel system, Circ. Res. 91 (2002) 761–768.

[14] A.C. Gittenberger-de Groot, E.M. Winter, M.M. Bartelings, M.J. Goumans, M.C. DeRuiter, R.E. Poelmann, The arterial and cardiac epicardium in development, disease and repair, Differentiation 84 (2012) 41–53.
[15] B. Wu, Z. Zhang, W. Lui, X. Chen, Y. Wang, A.A. Chamberlain, R.A. Moreno-Rodriguez, R.R. Markwald, B.P. O'Rourke, D.J. Sharp, D. Zheng, J. Lenz, H.S. Baldwin, C.P. Chang, B. Zhou, Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling, Cell 151 (2012) 1083–1096.
[16] K. Red-Horse, H. Ueno, I.L. Weissman, M.A. Krasnow, Coronary arteries form by developmental reprogramming of venous cells, Nature 464 (2010) 549–553.
[17] Y. Liu, Q. Feng, NOing the heart: role of nitric oxide synthase-3 in heart development, Differentiation 84 (2012) 54–61.
[18] Y. Liu, X. Lu, F.L. Xiang, R.E. Poelmann, A.C. Gittenberger-de Groot, J. Robbins, Q. Feng, Nitric oxide synthase-3 deficiency results in hypoplastic coronary arteries and postnatal myocardial infarction, Eur. Heart J. 35 (2014) 920–931.
[19] E.R. Werner, N. Blau, B. Thony Tetrahydrobiopterin, Biochemistry and pathophysiology, Biochem. J. 438 (2011) 397–414.
[20] S. Cai, J. Khoo, S. Mussa, N.J. Alp, K.M. Channon, Endothelial nitric oxide synthase dysfunction in diabetic mice: importance of tetrahydrobiopterin in eNOS dimerisation, Diabetologia 48 (2005) 1933–1940.
[21] T. Heitzer, K. Krohn, S. Albers, T. Meinertz, Tetrahydrobiopterin improves endothelium-dependent vasodilation by increasing nitric oxide activity in patients with type II diabetes mellitus, Diabetologia 43 (2000) 1435–1438.
[22] H. Moazzen, X. Lu, N.L. Ma, T.J. Velenosi, B.L. Urquhart, L.J. Wisse, A.C. Gittenberger-de Groot, Q. Feng, N-Acetylcysteine prevents congenital heart defects induced by pregestational diabetes, Cardiovasc. Diabetol. 13 (2014) 46.
[23] A. Engineer, T. Saiyin, X. Lu, A.S. Kucey, B.L. Urquhart, T.A. Drysdale, K. Norozi, Q. Feng, Sapropterin treatment prevents congenital heart defects induced by pregestational diabetes mellitus in mice, J. Am. Heart Assoc. 7 (2018) e009624.
[24] A.C. Gittenberger-de Groot, E.M. Winter, R.E. Poelmann, Epicardium-derived cells (EPDCs) in development, cardiac disease and repair of ischemia, J. Cell Mol. Med. 14 (2010) 1056–1060.
[25] S.D. Kumar, S.T. Dheen, S.S. Tay, Maternal diabetes induces congenital heart defects in mice by altering the expression of genes involved in cardiovascular development, Cardiovasc. Diabetol. 6 (2007) 34.
[26] R. Starikov, J. Bohrer, W. Goh, M. Kuwahara, E.K. Chien, V. Lopes, D. Coustan, Hemoglobin A1c in pregestational diabetic gravidas and the risk of congenital heart disease in the fetus, Pediatr. Cardiol. 34 (2013) 1716–1722.
[27] A. Tongut, Z. Ozyedek, I. Cerezci, S. Erenturk, A.C. Hatemi, Prevalence of congenital coronary artery anomalies as shown by multi-slice computed tomography coronary angiography: a single-centre study from Turkey, J. Int. Med. Res. 44 (2016) 1492–1505.
[28] F. De Giorgio, A. Abbate, E. Stigliano, A. Capelli, V. Arena, Hypoplastic coronary artery disease causing sudden death. Report of two cases and review of the literature, Cardiovasc. Pathol. 19 (2010) e107–e111.
[29] C.L. Cai, J.C. Martin, Y. Sun, L. Cui, L. Wang, K. Ouyang, L. Yang, L. Bu, X. Liang, X. Zhang, W.B. Stallcup, C.P. Denton, A. McCulloch, J. Chen, S.M. Evans, A myocardial lineage derives from Tbx18 epicardial cells, Nature 454 (2008) 104–108.
[30] O.M. Martinez-Estrada, L.A. Lettice, A. Essafi, J.A. Guadix, J. Slight, V. Velecela, E. Hall, J. Reichmann, P.S. Devenney, P. Hohenstein, N. Hosen, R.E. Hill, R. Munoz-Chapuli, N.D. Hastie, Wt1 is required for cardiovascular progenitor cell formation through transcriptional control of Snail and E-cadherin, Nat. Genet. 42 (2010) 89–93.
[31] R. Vicente-Steijn, R.W. Scherptong, B.P. Kruithof, S.N. Duim, M.J. Goumans, L.J. Wisse, B. Zhou, W.T. Pu, R.E. Poelmann, M.J. Schalij, M.D. Tallquist, A.C. Gittenberger-de Groot, M.R. Jongbloed, Regional differences in WT-1 and Tcf21 expression during ventricular development: implications for myocardial compaction, PLoS One 10 (2015) e0136025.
[32] A. von Gise, B. Zhou, L.B. Honor, Q. Ma, A. Petryk, W.T. Pu, WT1 regulates epicardial epithelial to mesenchymal transition through beta-catenin and retinoic acid signaling pathways, Dev. Biol. 356 (2011) 421–431.
[33] K.S. Edgar, O.M. Galvin, A. Collins, Z.S. Katusic, D.M. McDonald, BH4-Mediated enhancement of endothelial nitric oxide synthase activity reduces hyperoxia-induced endothelial damage and preserves vascular integrity in the neonate, Investig. Ophthalmol. Vis. Sci. 58 (2017) 230–241.
[34] J. Wang, Q. Yang, Y. Nie, H. Guo, F. Zhang, X. Zhou, X. Yin, Tetrahydrobiopterin contributes to the proliferation of mesangial cells and accumulation of extracellular matrix in early-stage diabetic nephropathy, J. Pharm. Pharmacol. 69 (2017) 182–190.
[35] L. Chen, X. Zeng, J. Wang, S.S. Briggs, E. O'Neill, J. Li, R. Leek, D.J. Kerr, A.L. Harris, S. Cai, Roles of tetrahydrobiopterin in promoting tumor angiogenesis, Am. J. Pathol. 177 (2010) 2671–2680.
[36] Z.Z. Yang, O. Tschopp, N. Di-Poi, E. Bruder, A. Baudry, B. Dummler, W. Wahli, B.A. Hemmings, Dosage-dependent effects of Akt1/protein kinase Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice, Mol. Cell. Biol. 25 (2005) 10407–10418.
[37] Q. Feng, W. Song, X. Lu, J.A. Hamilton, M. Lei, T. Peng, S.P. Yee, Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase, Circulation 106 (2002) 873–879.
[38] Y. Liu, X. Lu, F.L. Xiang, M. Lu, Q. Feng, Nitric oxide synthase-3 promotes embryonic development of atrioventricular valves, PLoS One 8 (2013) e77611.
[39] U. Streit, H. Reuter, W. Bloch, T. Wahlers, R.H. Schwinger, K. Brixius, Phosphorylation of myocardial eNOS is altered in patients suffering from type 2 diabetes, J. Appl. Physiol. 114 (2013) 1366–1374.
[40] J. Whitsett, M.J. Picklo Sr, J. Vasquez-Vivar, 4-Hydroxy-2-nonenal increases superoxide anion radical in endothelial cells via stimulated GTP cyclohydrolase proteasomal degradation, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 2340–2347.
[41] J. Woodcock-Mitchell, J.J. Mitchell, R.B. Low, M. Kieny, P. Sengel, L. Rubbia, O. Skalli, B. Jackson, G. Gabbiani, Alpha-smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles, Differentiation 39 (1988) 161–166.
[42] I.D. Marinas, R. Marinas, I. Pirici, L. Mogoanta, Vascular and mesenchymal factors during heart development: a chronological study, Rom. J. Morphol. Embryol. 53 (2012) 135–142.
[43] S.J. Coton, I. Nazareth, I. Petersen, A cohort study of trends in the prevalence of pregestational diabetes in pregnancy recorded in UK general practice between 1995 and 2012, BMJ Open 6 (2016) e009494.