CALL FOR PAPERS | Cardiovascular Mitochondria and Redox Control in Health and Disease

Maternal high-fat diet impairs cardiac function in offspring of diabetic pregnancy through metabolic stress and mitochondrial dysfunction

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Maternal high-fat diet impairs cardiac function in offspring of diabetic pregnancy through metabolic stress and mitochondrial dysfunction. Am J Physiol Heart Circ Physiol 310: H681–H692, 2016. First published January 22, 2016; doi:10.1152/ajpheart.00795.2015.—Offspring of diabetic pregnancies are at risk of cardiovascular disease at birth and throughout life, purportedly through fuel-mediated influences on the developing heart. Preventative measures focus on glycemic control, but the contribution of additional offenders, including lipids, is not understood. Cellular bioenergetics can be influenced by both diabetes and hyperlipidemia and play a pivotal role in the pathophysiology of adult cardiovascular disease. This study investigated whether a maternal high-fat diet, independently or additively with diabetes, could impair fuel metabolism, mitochondrial function, and cardiac physiology in the developing offspring’s heart. Sprague-Dawley rats fed a control or high-fat diet were administered placebo or streptozotocin to induce diabetes during pregnancy and then delivered offspring from four groups: control, diabetes exposed, diet exposed, and combination exposed. Cardiac function, cellular bioenergetics (mitochondrial stress test, glycolytic stress test, and palmitate oxidation assay), lipid peroxidation, mitochondrial histology, and copy number were determined. Diabetes-exposed offspring had impaired glycolytic and respiratory capacity and a reduced proton leak. High-fat diet-exposed offspring had increased mitochondrial copy number, increased lipid peroxidation, and evidence of mitochondrial dysfunction. Combination-exposed pups were most severely affected and demonstrated cardiac lipid droplet accumulation and diastolic/systolic cardiac dysfunction that mimics that of adult diabetic cardiomyopathy. This study is the first to demonstrate that a maternal high-fat diet impairs cardiac function in offspring of diabetic pregnancies through metabolic stress and serves as a critical step in understanding the role of cellular bioenergetics in developmentally programmed cardiac disease.

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glycemic control is good (1, 34, 51, 64), also suggesting that lipids play an underrecognized role (9, 30).

The proposed pathogenic mechanism of adult diabetic cardiomyopathy involves abnormal cardiac metabolism followed by oxidative stress, mitochondrial dysfunction, and mitophagy (9, 16, 21, 25, 26). It is increasingly recognized that cellular bioenergetics play a pivotal role in the maintenance of health and pathophysiology of disease (62), especially in the heart (12). Because of its high energy demands, the heart requires a constant fuel source for continued contractile activity, making it prone to failure from metabolic abnormalities and mitochondrial dysfunction (58). Fatty acids are a major source of energy for the normal, resting adult heart (26, 28, 41, 42). Despite this preference, the heart has a remarkable ability to utilize various substrates depending on supply and demand. This fuel flexibility allows ongoing energy production under various metabolic conditions including ischemia (oxygen supply), starvation (fuel supply), and exercise (demand) and even during developmental maturation (28, 31, 58). Impaired fuel flexibility from excess circulating fuels and insulin resistance makes the heart prone to failure (12, 26). We hypothesized that a similar pathogenesis could affect offspring of diabetic pregnancies.

The objective of this study was to determine whether a maternal high-fat (HF) diet and diabetes, either independently or additively, impair metabolic fuel flexibility, mitochondrial function, and cardiac physiology in the developing offspring’s heart. Utilizing a rat model of partially treated diabetes developing during later gestation in combination with control (CD) or HF diet, we simulated the adverse metabolic and cardiac consequences experienced by infants of diabetic mothers. To test our hypothesis, we measured cardiac function, cellular bioenergetics (mitochondrial stress test, glycolytic stress test, and palmitate oxidation assay), lipid peroxidation, mitochondrial dysfunction (fuel supply), and exercise (demand) and even during later gestation while avoiding glucose-mediated teratogenic influences on early organogenesis (3). This is because diabetes during early pregnancy is associated with a higher risk of structural heart defects (11, 38) that could confound cardiovascular outcomes in exposed offspring. After STZ injection, twice-daily glucose concentrations were measured by tail nick sampling with a One Touch Ultra meter (LifeScan, Milpitas, CA). Maternal β-hydroxybutyrate levels were measured concurrently each morning with a Precision Xtra Ketone Meter (Abbott Diabetes Care, Alameda, CA). Sliding-scale subcutaneous Humulin R (100 U/ml; Eli Lilly, Indianapolis, IN) was administered in the morning and Lantus recombinant insulin glargine (100 U/ml; Sanofi Aventis, Bridgewater, NJ) in the evening to keep glucose levels in a target range of 200 – 400 mg/dl and ketosis to a minimum. Dams that received STZ but did not manifest significant hyperglycemia (nonfasting blood glucose ≥200 mg/dl) were excluded. Dams delivered spontaneously (on gestational day 22) to yield newborn offspring in four distinct groups: CD-CB (control), CD-STZ (diabetes exposed), HF-CB (HF diet exposed), and HF-STZ (combination exposed).

Plasma Analyses

Blood was collected by venipuncture at baseline, postdiet, gestational day 14, and postpartum time points in dams and immediately after humane euthanasia by cervical dislocation in newborn pups. Aliquots of whole blood and plasma fractions were stored at –80°C until analyses. Plasma triglyceride levels were measured with a Triglyceride Colorimetric Assay Kit (Thermo Fisher Scientific, Waltham, MA), and nonesterified fatty acid levels were measured with a Wako HR Series NEFA-HR (2) Colorimetric Kit (Wako Diagnostics, Richmond, VA). Both were quantitated with a SpectraMax Plus plate reader ( Molecular Devices, Sunnyvale, CA). Plasma leptin, insulin, and C-peptide concentrations were measured with the Milliplex MAP Rat Metabolic Magnetic Bead Panel and analyzed with free access to water and chow. Female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) received CD (TD2018 Teklad; Harlan Laboratories, Madison, WI) or HF (TD95217 custom diet Teklad; Harlan Laboratories) diet for at least 28 days prior to breeding to simulate a dietary “lifestyle.” Diets were selected to equate commonly attainable low-fat (18% of calories as fat) or HF (40% of calories as fat) diet with more saturated and monounsaturated fat content. Dietary differences are detailed in Tables 1 and 2.

Females were bred with normal, CD-fed males. Gestational day 0 was designated by a positive swab for spermatozoa. On gestational day 14, dams received either 0.09 M citrate buffer (CB) placebo or 65 mg/kg of intraperitoneal streptozotocin (STZ) (Sigma Life Sciences, St. Louis, MO) in CB to induce diabetes. The timing of diabetes induction was selected to study the effects of developing diabetes during later gestation while avoiding glucose-mediated teratogenic influences on early organogenesis (3). This is because diabetes during early pregnancy is associated with a higher risk of structural heart defects (11, 38) that could confound cardiovascular outcomes in exposed offspring. After STZ injection, twice-daily glucose concentrations were measured by tail nick sampling with a One Touch Ultra meter (LifeScan, Milpitas, CA). Maternal β-hydroxybutyrate levels were measured concurrently each morning with a Precision Xtra Ketone Meter (Abbott Diabetes Care, Alameda, CA). Sliding-scale subcutaneous Humulin R (100 U/ml; Eli Lilly, Indianapolis, IN) was administered in the morning and Lantus recombinant insulin glargine (100 U/ml; Sanofi Aventis, Bridgewater, NJ) in the evening to keep glucose levels in a target range of 200 – 400 mg/dl and ketosis to a minimum. Dams that received STZ but did not manifest significant hyperglycemia (nonfasting blood glucose ≥200 mg/dl) were excluded. Dams delivered spontaneously (on gestational day 22) to yield newborn offspring in four distinct groups: CD-CB (control), CD-STZ (diabetes exposed), HF-CB (HF diet exposed), and HF-STZ (combination exposed).

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**Table 1. Caloric content of maternal control and high-fat diets**

<table>
<thead>
<tr>
<th>Diet</th>
<th>kcal/g</th>
<th>%kcal Fat</th>
<th>%kcal Protein</th>
<th>%kcal Carbohydrate</th>
<th>Fat Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>3.1</td>
<td>18</td>
<td>24</td>
<td>58</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>HF</td>
<td>4.3</td>
<td>40</td>
<td>19</td>
<td>41</td>
<td>Vegetable shortening, milk fat, soybean oil</td>
</tr>
</tbody>
</table>

Dietary content of maternal control (CD) and high-fat (HF) diets is detailed. Dams are placed on specified diets for 28 days prior to breeding and throughout pregnancy.

**Table 2. Fatty acid composition of maternal diets**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Lauric 12:0</th>
<th>Myristic 14:0</th>
<th>Palmitic 16:0</th>
<th>Stearic 18:0</th>
<th>Oleic 18:1</th>
<th>LA 18:2n6</th>
<th>ALA 18:3n3</th>
<th>ARA 20:4n6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>0.32</td>
<td>0.07</td>
<td>6.8</td>
<td>1.6</td>
<td>12</td>
<td>29</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HF</td>
<td>1.24</td>
<td>5</td>
<td>32</td>
<td>18</td>
<td>81</td>
<td>35</td>
<td>4</td>
<td>0.04</td>
</tr>
<tr>
<td>Difference</td>
<td>4.7-fold</td>
<td>11.3-fold</td>
<td>6.8-fold</td>
<td>ω−3:6 ratio 9.67 vs. 8.73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fatty acid composition (wt:wt, %) of maternal CD and HF diets is detailed. Dams are placed on specified diets for 28 days prior to breeding and throughout pregnancy. LA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid.
with the Luminex 200 Milliplex Analyzer according to the manufacturer’s directions.

**Echocardiography**

Structural and functional cardiac physiology was evaluated by echocardiography with the Vvo2100 high-frequency imaging system (VisualSonics, SomoSite, Toronto, ON, Canada) equipped with heated stage. Echocardiography was done under light isoflurane anesthesia with temperature and EKG monitoring to ensure physiological stability. Images were captured in B mode (brightness mode), M mode (motion mode) and pulsed-wave (PW) Doppler mode with parasternal long axis (PLAX), parasternal short axis (PSAX), and the apical four chamber views and analyzed with the Vvo2100 Imaging System Software to assess ventricular size and systolic and diastolic function. Reported ventricular measurements and systolic function are from left ventricular trace in PLAX views. Diastolic function is reported from PSAX analyses.

**Cardiac Lipid Droplet Analysis**

After harvest, newborn rat hearts were weighed, snap frozen in liquid nitrogen, and stored at −80°C prior to batch staining for lipid droplet analyses. Frozen sections (10 μm) were fixed in 40% formaldehyde, stained in Oil Red O for 10 min followed by background staining with hematoxylin containing acetic acid, and blued in ammonia water. Sections were mounted with aqueous mounting medium. With a uniform approach for all samples, four regions of each left ventricle (anterior wall, septum, posterior wall, and outer wall) were imaged systematically at ×60 using a Nikon 90i microscope with a programmable motorized stage (Nikon Instruments, Melville, NY). Images were captured with a 25-μm grid overlay using NIS-Elements software. Lipid droplets were counted with a point counting method. The average number of lipid droplets touching the grid in each of the four ventricular sections was determined for each heart.

**Neonatal Cardiomyocyte Isolation**

Hearts were harvested and neonatal cardiomyocytes were isolated from three or four littermates as previously described (48, 55). In summary, hearts were digested in 0.1% trypsin and 0.02% DNase (in 0.15 M NaCl) and filtered into bovine serum. After pelleting, cells were resuspended in DMEM-1 [supplemented with 10% bovine serum albumin (BSA) and 1% penicillin-streptomycin] with 0.0002% DNase and then incubated for 1 h in 5% CO2 at 37°C. Cardiomyocytes were detached, resuspended in DMEM-1, counted, plated at a seeding density of 150,000 cells/well in 0.1% gelatin-coated Seahorse V7-PS microplates, and then incubated overnight at 5% CO2 at 37°C with DMEM-2 (DMEM-1 supplemented with 100 μM 5-bromo-2'-deoxyuridine) medium.

**Extracellular Flux Analyses**

Mitochondrial stress test, glycolytic stress test, and palmitate oxidation assay were completed on primary neonatal cardiomyocytes from each litter by real-time extracellular flux analyses with a Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA) as previously described (17, 47, 53a). Specifically, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured under the following specified conditions.

**Mitochondrial stress test. OCR** was measured on 150,000 plated cells in XF assay medium (Seahorse Bioscience no. 100965-000) supplemented with 10 mM glucose and 1 mM pyruvate. Measurements were taken at baseline and after timed injections of 2 μM oligomycin (ATP synthase inhibitor), 0.3 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, oxidative phosphorylation uncoupler), and a mixture of 2 μM rotenone (respiratory complex I inhibitor) and 4 μM Antimycin A (respiratory complex III inhibitor). Real-time OCR was averaged and recorded three times during each conditional cycle.

**Glycolytic stress test. ECAR** was measured on 150,000 plated cells in XF base medium (Seahorse Bioscience no. 102353-100) at baseline and after timed injections of 10 mM glucose (to test glycolytic response to exogenous glucose), 2 μM oligomycin (to stimulate anaerobic glycolysis), and 100 mM 2-deoxyglucose (glycolysis inhibition). The contribution of both lactate (from anaerobic glycolysis) and CO2 production (from cellular respiration) was determined by calculating the proton production rate (PPR) as outlined by Mookerjee et al. (43).

**Palmitate oxidation assay.** BSA-conjugated palmitate was prepared as previously described (50). Palmitate oxidation was interrogated by measuring OCR at baseline and after injection of 0.15 mM palmitate-BSA and 40 μM etomoxir to inhibit carnitine-palmitoyl transport and mitochondrial respiration via fatty acid oxidation. Etomoxir injection was repeated to ensure that maximal inhibition was obtained.

**Extracellular flux validation.** Experimental optimization was conducted in compliance with the manufacturer’s recommendations for Seahorse XF24 (53a). Experimental replicates were normalized to cell count. This was the most appropriate normalization method for this experiment because primary isolated cardiomyocytes are nondividing and require extracellular matrix protein (gelatin) coating of the XF plates for uniform adhesion, making normalization to protein concent-

<table>
<thead>
<tr>
<th>Table 3. Model characteristics</th>
<th>CD-CB</th>
<th>CD-STZ</th>
<th>HF-CB</th>
<th>HF-STZ</th>
<th>Diet</th>
<th>Diabetes</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal weight gain, g</td>
<td>28 ± 3.64</td>
<td>27 ± 4.24</td>
<td>63 ± 13.89</td>
<td>47 ± 8.75</td>
<td>≤0.001</td>
<td>0.29</td>
<td>0.35</td>
</tr>
<tr>
<td>Late-gestation blood glucose, mg/dl</td>
<td>91 ± 1.06</td>
<td>322 ± 11.1</td>
<td>93 ± 1.28</td>
<td>348 ± 12.5</td>
<td>0.10</td>
<td>≤0.001</td>
<td>0.15</td>
</tr>
<tr>
<td>Maternal postpartum triglyceride, mg/dl</td>
<td>0.25 ± 0.01</td>
<td>0.49 ± 0.05*</td>
<td>0.31 ± 0.02</td>
<td>0.81 ± 0.11*</td>
<td>0.04*</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Maternal postpartum NEFA, meq/l</td>
<td>0.20 ± 0.04</td>
<td>0.41 ± 0.15</td>
<td>0.42 ± 0.08</td>
<td>0.72 ± 0.10</td>
<td>≤0.001</td>
<td>0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Maternal postpartum leptin, ng/ml</td>
<td>1.40 ± 0.21</td>
<td>1.06 ± 0.17</td>
<td>2.90 ± 0.60</td>
<td>1.95 ± 0.27</td>
<td>≤0.001</td>
<td>0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Litter size, n</td>
<td>10.67 ± 1.12</td>
<td>10.85 ± 1.16</td>
<td>10.73 ± 1.23</td>
<td>11.09 ± 1.60</td>
<td>0.91</td>
<td>0.83</td>
<td>0.94</td>
</tr>
<tr>
<td>Offspring birth weight, g</td>
<td>6.17 ± 0.07</td>
<td>6.01 ± 0.08</td>
<td>6.00 ± 0.08</td>
<td>5.69 ± 0.10</td>
<td>≤0.001</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Offspring insulin, pmol/l</td>
<td>111 ± 14</td>
<td>468 ± 157</td>
<td>387 ± 116</td>
<td>1,224 ± 357</td>
<td>0.16</td>
<td>0.23</td>
<td>0.25</td>
</tr>
<tr>
<td>Offspring C-peptide, ng/ml</td>
<td>2.70 ± 0.22</td>
<td>3.29 ± 0.68</td>
<td>1.97 ± 0.19</td>
<td>7.47 ± 1.21*</td>
<td>≤0.001</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Offspring triglyceride, mg/dl</td>
<td>93 ± 4.29</td>
<td>102 ± 10.77</td>
<td>114 ± 15.49</td>
<td>91 ± 10.53</td>
<td>0.60</td>
<td>0.51</td>
<td>0.12</td>
</tr>
<tr>
<td>Offspring NEFA, meq/l</td>
<td>0.32 ± 0.02</td>
<td>0.36 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.25 ± 0.02*</td>
<td>≤0.001</td>
<td>0.05</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Values are means ± SE. CD-CB, control; CD-STZ, diabetes induced; HF-CB, high-fat diet fed; HF-STZ, high-fat fed and diabetes induced; NEFA, nonesterified fatty acid. Significant values in boldface. *Remains significant by 1-way ANOVA and Dunnnett’s posttest when interaction effect is significant by 2-way ANOVA.
Mitochondrial Histology

Isolated neonatal cardiomyocytes (150,000 cells) were plated in XF assay medium (Sea-
horse Bioscience no. 100965-000) supplemented with 10 mM glucose and 1 mM pyruvate and incubated at 37°C for 1 h before the assay was run to mimic the time point at which basal OCR was measured as described above.

Mitochondrial DNA Copy Number

Total DNA was extracted from newborn rat hearts with a standard phenol-chloroform extraction (60). In summary, isolated cardiomyocytes were resuspended in a lysis buffer (10 mM Tris HCl pH 8, 1 mM EDTA, and 0.1% SDS), homogenized, and incubated for 3 h with 20 mg/ml proteinase K and 100 mg/ml RNase A. DNA integrity and concentration were determined by spectrophotometry using an Epoch plate reader (BioTek) and stored at 4°C. Relative mitochondrial DNA copy number was determined by real-time PCR quantitation of the standard curve with MxPro software (Agilent).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Diet-, diabetes-, and interaction-related effects were interrogated by two-way ANOVA with Bonferroni post hoc test. When the interaction was significant, differences between controls (CD-CB) and each exposed group (CD-STZ, HF-CB, and HF-STZ) were interrogated via one-way ANOVA and Dunnett’s post hoc test. Changes in dam weight and triglyceride levels over time were analyzed by linear regression analysis. Group data were averaged (CD-CB, CD-STZ, HF-CB, and HF-STZ) and descriptively expressed as means ± SE. A value of P < 0.05 was considered significant in all cases.

RESULTS

Animal Model Characteristics: Maternal HF Diet with Diabetes Exacerbates Maternal Hyperlipidemia and Offspring Hyperinsulinemia

Dams and offspring from 48 litters (with 486 live-born pups) were used to characterize the features of our animal model. Group comparisons are described for each of the following experimental groups in Table 3: CD-CB (control, 12 litters), CD-STZ (diabetes exposed, 13 litters), HF-CB (diet exposed, 12 litters) and HF-STZ (combination exposed, 11 litters). Dams on a HF diet (n = 23) gained an average of 35.6 g more body weight than those fed CD (n = 25), and the trend persisted over time (P = 0.002). Diabetic dams had higher whole blood glucose levels, while dams receiving CB had nonfasting blood glucose levels <200 mg/dl regardless of their diet. Dams fed a HF diet had significantly higher leptin and circulating lipid levels, which rose drastically with diabetes induction. Compared with control dams, postpartum triglyceride levels were 2-fold higher in diabetic dams, 3-fold higher in HF-fed dams, and 15-fold higher in HF-STZ dams. Despite significant maternal dyslipidemia, newborn rat pups had no significant dif-

Table 4. Summarized echocardiographic left ventricular measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IVSd, mm</th>
<th>IVSs, mm</th>
<th>LVPWd, mm</th>
<th>LVPWs, mm</th>
<th>LVIDd, mm</th>
<th>LVIDs, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-CB</td>
<td>108</td>
<td>0.52 (0.02)</td>
<td>0.60 (0.02)</td>
<td>0.53 (0.01)</td>
<td>0.66 (0.06)</td>
<td>2.26 (0.03)</td>
<td>1.47 (0.03)</td>
</tr>
<tr>
<td>CD-STZ</td>
<td>122</td>
<td>0.48 (0.01)</td>
<td>0.57 (0.01)</td>
<td>0.51 (0.01)</td>
<td>0.60 (0.01)</td>
<td>2.19 (0.04)</td>
<td>1.40 (0.04)</td>
</tr>
<tr>
<td>HF-CB</td>
<td>98</td>
<td>0.48 (0.01)</td>
<td>0.58 (0.01)</td>
<td>0.50 (0.01)</td>
<td>0.57 (0.01)</td>
<td>2.21 (0.04)</td>
<td>1.42 (0.04)</td>
</tr>
<tr>
<td>HF-STZ</td>
<td>62</td>
<td>0.48 (0.01)</td>
<td>0.59 (0.02)</td>
<td>0.50 (0.02)</td>
<td>0.61 (0.01)</td>
<td>2.18 (0.05)</td>
<td>1.44 (0.05)</td>
</tr>
</tbody>
</table>

Values are means (SE). IVS, intraventricular septum; LVPW, left ventricular posterior wall; LVID, left ventricular internal diameter; d, during diastole; s, during systole.

Table 5. Summarized echocardiographic systolic measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HR, beats/min</th>
<th>SV, μl</th>
<th>EF, %</th>
<th>SF, %</th>
<th>CO, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-CB</td>
<td>108</td>
<td>270 (2.98)</td>
<td>11.34 (0.49)</td>
<td>61.01 (1.21)</td>
<td>24.33 (0.98)</td>
<td>3.11 (0.16)</td>
</tr>
<tr>
<td>CD-STZ</td>
<td>122</td>
<td>268 (3.27)</td>
<td>10.79 (0.42)</td>
<td>63.23 (1.32)</td>
<td>21.11 (0.97)</td>
<td>3.03 (0.15)</td>
</tr>
<tr>
<td>HF-CB</td>
<td>98</td>
<td>258 (3.59)*</td>
<td>10.70 (0.40)</td>
<td>62.95 (1.37)</td>
<td>24.78 (0.86)</td>
<td>2.85 (0.14)</td>
</tr>
<tr>
<td>HF-STZ</td>
<td>62</td>
<td>259 (5.45)*</td>
<td>10.08 (0.77)</td>
<td>58.90 (1.95)*</td>
<td>23.67 (1.19)*</td>
<td>2.73 (0.25)</td>
</tr>
</tbody>
</table>

Values are means (SE). HR, heart rate; SV, stroke volume; EF, ejection fraction; SF, shortening fraction [calculated as %SF = (LVDDd - LVDd)/LVDDd] × 100, where LVD is left ventricular diameter; CO, cardiac output. Significant differences: *dietary effect (P ≤ 0.05), †diabetes effect (P ≤ 0.05), interaction effect (P ≤ 0.05).
ference in circulating triglyceride levels. Pups exposed to both diabetes and HF diet had increased circulating insulin and C-peptide levels, but leptin levels were not different.

Echocardiography: Offspring Exposed to Maternal HF Diet and Diabetes Had Impaired Systolic and Diastolic Cardiac Function

Newborn offspring exposed to a maternal HF diet (P < 0.0001) had a larger mean heart-to-body weight ratio by morphometric measures [CD-CB 0.0071 ± 0.0002, n = 68; CD-STZ 0.0077 ± 0.0002, n = 77; HF-CB 0.0089 ± 0.0003, n = 89; HF-STZ 0.0094 ± 0.0003, n = 66]. However, left ventricular hypertrophy was not confirmed by echocardiography (Table 4). Systolic and diastolic function for each newborn rat is detailed in Tables 5 and 6, respectively. Together, combination-exposed offspring had the poorest systolic and diastolic function with a significantly lower mean heart rate, ejection/shortening fraction (a marker of systolic function), and E:A (a measure of ventricular filling/compliance and a marker of diastolic function).

Lipid Droplet Analysis: Lipid Droplets Accumulate in HF Diet-Exposed Newborn Hearts

As demonstrated in Fig. 1, newborn pups exposed to a maternal HF diet, especially in combination with diabetes, had an increased number and size of lipid droplets in ventricular sections. Diet accounted for a 173% increase in lipid droplets, while diabetes accounted for a 57% increase. The interaction was significant.

Mitochondrial Stress Test: Offspring Exposed to Maternal Diabetes and HF Diet Have Impaired Cardiac Mitochondrial Respiration

Real-time OCR during mitochondrial stress testing is illustrated for neonatal cardiomyocytes from each group in trace (Fig. 2A) and bar (Fig. 2B) formats in Figure 2. Diabetes-exposed cardiomyocytes had significantly lower basal, maximal, spare, and nonmitochondrial OCR consistent with mitochondrial dysfunction. ATP-linked and proton leak OCR were also lower. Additionally, HF diet-exposed neonatal cardiomyocytes had lower basal OCR. Combination-exposed (HF-STZ) neonatal cardiomyocytes demonstrated the poorest mitochondrial respiration.

Glycolytic Stress Test: Diabetes-Exposed Offspring Have Decreased Cardiac Glycolytic Capacity

Real-time ECAR, a marker of anaerobic glycolysis, is illustrated for neonatal cardiomyocytes from each group in trace (Fig. 3A) and bar (Fig. 3B) formats in Figure 3. Diabetes-exposed cardiomyocytes had significantly lower basal, glucose-stimulated, and oligomycin-stimulated glycolytic capac-

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Table 6. Summarized echocardiographic diastolic measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>E:A, mm/s (ratio)</th>
<th>IVRT, ms</th>
<th>IVCT, ms</th>
<th>MVET, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-CB</td>
<td>108</td>
<td>0.75 (0.02)</td>
<td>30.43 (0.89)</td>
<td>22.13 (0.96)</td>
<td>100 (3.01)</td>
</tr>
<tr>
<td>CD-STZ</td>
<td>122</td>
<td>0.69 (0.01)$^+$</td>
<td>33.09 (0.87)</td>
<td>25.20 (0.98)$^*$</td>
<td>103 (2.30)</td>
</tr>
<tr>
<td>HF-CB</td>
<td>98</td>
<td>0.70 (0.17)$^+$</td>
<td>33.04 (1.24)</td>
<td>23.00 (0.99)$^*$</td>
<td>103 (2.30)</td>
</tr>
<tr>
<td>HF-STZ</td>
<td>62</td>
<td>0.66 (0.02)$^+$</td>
<td>31.77 (1.16)</td>
<td>23.00 (0.99)$^*$</td>
<td>100 (2.82)</td>
</tr>
</tbody>
</table>

Values are means (SE). Significant differences: $^*$dietary effect (P ≤ 0.05), $^+$diabetes effect (P ≤ 0.05). E, early ventricular filling; A, ventricular filling from atrial contraction; IVRT, isovolumetric relaxation time; IVCT, isovolumetric contraction time; MVET, mitral valve ejection time.
ity. Although the combination-exposed group had the lowest glycolytic response, no significant interaction effect was found. Determining the PPR as described previously (43) allowed us to confirm that differences in ECAR were due to a decreased capability for anaerobic glycolysis (Fig. 4). Indeed, diabetes-exposed neonatal cardiomyocytes had a lower PPR under basal, glucose-stimulated, and oligomycin-stimulated conditions, suggesting a decreased glycolytic capacity. After glucose stimulation, the contribution of lactate from anaerobic glycolysis was significantly lower in diabetes-exposed neonatal cardiomyocytes. There was no difference in the contribution of Krebs cycle CO₂ (aerobic respiration) to ECAR with glucose stimulation. It was anticipated that oligomycin, an inhibitor of ATP production through cellular respiration, would stimulate anaerobic glycolysis in all cardiomyocytes. Unexpectedly, ECAR actually decreased in diabetes-exposed cardiomyocytes (Fig. 3). Upon further analyses, we found that this was due to a decreased contribution of CO₂ (aerobic respiration) to the total PPR after oligomycin injection (Fig. 4C). This substantiates that cardiac fuel flexibility is impaired in offspring of diabetic mothers because of a decreased ability to utilize glucose for ATP production even when mitochondrial respiration is inhibited.

**Palmitate Oxidation Assay: Combination-Exposed Offspring Have Lower Basal and Maximal OCR Despite Response to Exogenous Palmitate**

Real-time OCR in response to exogenous palmitate serves as a marker of mitochondrial fatty acid oxidation and is illustrated in trace format (Fig. 5A) and bar format for basal (Fig. 5B) and palmitate response (Fig. 5C) for each group in Figure 5. Neonatal cardiomyocytes from diabetes-exposed offspring had a lower response to exogenous palmitate. Combination-exposed (HF-STZ) neonatal cardiomyocytes had a very low basal OCR, so despite increased respiration to exogenous palmitate maximal OCR never increased to that of the other groups.

**MDA Assay: HF Diet-Exposed Offspring Have Evidence of Cardiac Lipid Peroxidation**

Exposure to a maternal HF diet (P ≤ 0.0001), but not diabetes (P = 0.53), was associated with increased MDA levels in isolated neonatal cardiomyocytes, which indicates oxidative stress through lipid peroxidation (Fig. 6).

**Mitochondrial Histology: Combination-Exposed Offspring Have Abnormal Cardiomyocyte Mitochondrial Structure and Membrane Potential**

Isolated neonatal cardiomyocytes stained with MitoTracker Green to identify mitochondria, TMRE to identify a charged mitochondrial membrane potential (necessary for ATP production), and Hoechst to identify DNA (both nuclear and mitochondrial) are depicted in Fig. 7. Normal mitochondria are typically long, thin, and abundant in cardiac tissue (40% of cellular components); they are dynamic and constantly undergo fusion and fission (21). When they become adynamic (57) or depolarized (loss of membrane potential) they undergo fragmentation and become susceptible to mitophagy (18). Using live-cell, confocal video imaging, we found long, thin, well-charged, and active mitochondria undergoing fusion and fission in isolated cardiomyocytes from control (CD-CB) offspring. Diabetes-exposed (CD-STZ) neonatal cardiomyocytes had no visibly discernible differences in fusion and fission by video observation, but they seemed to have fewer mitochondria overall. HF diet-exposed (HF-CB) neonatal cardiomyocytes
Fig. 3. Glycolytic stress test. Extracellular acidification (ECAR) trace (A) and bar graphs (B) illustrate the glycolytic response of newborn cardiomyocytes to glucose, oligomycin, and 2-deoxyglucose from CD-CB, CD-STZ, HF-CB, and HF-STZ offspring. Diabetes-exposed offspring had lower basal, glucose-stimulated, and oligomycin-stimulated ECAR regardless of diet exposure. Data are expressed as means ± SE; n = 5–7 litters/group. \( *P < 0.05 \) by 2-way ANOVA.

Fig. 4. Proton production rate (PPR). A: calculated (43) PPR for neonatal cardiomyocytes under basal, glucose-stimulated, oligomycin-stimulated, and 2-deoxyglucose-inhibited conditions is represented for CD-CB, CD-STZ, HF-CB, and HF-STZ offspring. B and C: the contributions of both lactate from anaerobic glycolysis and CO₂ production from cellular respiration during glucose stimulation (B) and oligomycin stimulation (C) confirm a decreased glycolytic capacity in diabetes-exposed neonatal cardiomyocytes. Diabetes exposure was associated with lower lactate production from anaerobic glycolysis under glucose stimulation and with lower CO₂ production from aerobic respiration under oligomycin stimulation consistent with impaired cardiac fuel flexibility. Data are expressed as means ± SE; n = 5–7 litters/group. \( *P < 0.05 \) by 2-way ANOVA.
had an increased number of shorter, more fragmented mitochondria, but they maintained their charge and some ability for fusion and fission by video observation. Combination-exposed (HF-STZ) neonatal cardiomyocytes had fragmented, depolarized mitochondria with very little to no movement on live-cell video imaging (see merged image, Fig. 7).

Mitochondrial DNA Copy Number: HF Diet-Exposed Cardiomyocytes Have Higher Mitochondrial Copy Number

To verify observed differences in mitochondrial number seen by histology, mitochondrial DNA copy number was determined by real-time PCR using two distinct primers. Gene expression analyses of the MT-CO1 (Fig. 8A) and D-loop (Fig. 8B) mitochondrial control region demonstrated similar group differences. Diabetes exposure alone (CD-STZ) was associated with a lower mitochondrial copy number than control, but HF diet exposure was associated with a higher copy number. Overall, there was a significant interaction effect.

DISCUSSION

The contribution of excess circulating fuels, including lipids, to the pathogenesis of developmentally programmed cardiac disease has been proposed (10, 20, 30) but remains poorly understood. It is well known that the triad of insulin resistance, hyperglycemia, and hyperlipidemia in adults causes diabetic cardiomyopathy (9, 25). Infants born to diabetic or obese mothers are at risk of similar heart disease at birth, even if maternal glycemic control is good (1, 34, 49, 61). Epidemiological studies demonstrate that circulating maternal triglyceride levels independently and temporally correlate with both fetal overgrowth and ventricular hypertrophy in offspring of diabetic mothers (1, 19, 33, 53, 56), which suggests a strong lipid-mediated influence. Although previous studies have reported individual effects of maternal diabetes or a HF diet on cardiovascular disease risk (7, 13, 14, 33, 35), our study adds evidence that a maternal HF diet in combination with diabetes is especially detrimental for the developing fetal heart.
To our knowledge, this is the first study to demonstrate that a maternal HF diet further impairs diastolic and systolic function in offspring of diabetic pregnancies through lipid droplet accumulation, metabolic abnormalities, oxidative stress, and mitochondrial dysfunction. Using a rat model to identify independent and additive effects, we found that diabetes-exposed neonatal cardiomyocytes had lower mitochondrial respiratory capacity and glycolytic capacity and a decreased ability to utilize glucose for ATP production even when mitochondrial respiration was inhibited with oligomycin. We suspect that this impairment in cardiac fuel flexibility caused a compensatory decrease in proton leak necessary to maintain an adequate mitochondrial membrane potential, as demonstrated by a lower OCR for proton leak, thereby increasing the risk of damaging reactive oxygen species (ROS) production (8). HF diet-exposed neonatal cardiomyocytes had lipid droplet accumulation, an increased mitochondrial number, and evidence of increased oxidative stress (lipid peroxidation). Together, combination-exposed neonatal cardiomyocytes had significantly impaired metabolic fuel flexibility, histological evidence of mitochondrial dysfunction, and diastolic and systolic dysfunction on echocardiography.

How does mitochondrial dysfunction impair cardiac function? Mitochondria are the major site for ROS production (9). It is known that electrons can leak from the respiratory chain, leading to production of superoxide, which can be converted to hydrogen peroxide and transformed to very potent hydroxyl radicals that cause damage to macromolecules, including structural lipids (lipid peroxidation) (45). Oxidative stress can lead to further mitochondrial dysfunction, fragmentation, mitophagy, and eventually cell death (18, 56). Mature cardiomyocytes cannot proliferate, and as a result heart failure ensues. This is a proposed primary mechanism contributing to diabetic cardiomyopathy in adults (19, 23). Our findings suggest that a similar pathogenesis contributes to cardiac dysfunction in newborns born to diabetic mothers and that a maternal HF diet increases this risk. In our rat model, cardiomyocytes from combination-exposed (HF-STZ) pregnancies had fragmented and poorly charged mitochondria with little to no fission or fusion seen on live-cell imaging. These characteristics suggest significant injury that may mark cells for apoptosis (18, 57).

If metabolic abnormalities, oxidative stress, and mitochondrial dysfunction persist beyond the neonatal time point, this could also explain the increased risk of cardiac disease found throughout life in infants born to mothers with diabetes or obesity (22, 44, 54). However, caution should be used in making this inference because significant metabolic differences in cardiac metabolism between fetal and adult time points (39, 42) could exacerbate disease severity, progression, and long-term prognosis. The fetal heart relies predominantly on anaerobic glycolysis to meet ATP demands (40). However, shortly after birth, the placenta is no longer available to provide a continuous supply of nutrients, and the circulating oxygen supply rapidly increases, driving mitochondrial ATP production to sustain cardiac energetic demands with age (58). Indeed, fatty acid oxidation becomes the primary energy source for the normal resting adult heart (41). In the neonatal heart, a rapid increased reliance on aerobic metabolism, alongside a decreased proton leak (or very tightly coupled respiration) and lower spare respiratory capacity (found in HF-STZ neonatal cardiomyocytes), may add to increased oxidative injury. However, with normal metabolic maturation and the heart’s robust mitochondrial turnover (61), cardiomyocytes may recover over time. Indeed, this may be why ventricular hypertrophy found in infants of diabetic mothers often regresses in the first months of life. Therefore, future studies are necessary to determine whether there is a lasting consequence that may lead to cardiovascular disease later in life.

Study Limitations

There were several identifiable limitations to our animal model. First, a high perinatal mortality rate in HF diet-exposed offspring (44% in HF-CB and 43% in HF-STZ vs. 9% in CD-CB and 11% in CD-STZ litters) confounded data because only healthy pups could have echocardiography or viable hearts for primary cardiomyocyte isolation and metabolic studies. For this reason, we anticipate that the negative impact of a maternal HF diet is actually greater than reported in our results.

Second, it is well recognized that maternal hyperglycemia, particularly early in the pregnancy (during organogenesis), is associated with structural birth defects including cardiac anomalies (3, 6), which would impact cardiac function in newborn offspring. Gestational diabetes, which typically develops later in pregnancy, is less likely associated with structural heart defects (11). The cardiac hypertrophy and diastolic and systolic dysfunction found in infants of diabetic pregnancies typically develops during this later time frame (51, 63). For these reasons, we induced diabetes on gestational day 14 after organogenesis was complete. Also, both structure and function were assessed on echocardiography. Only one living newborn pup in the HF-CB group had a structural heart defect detected, and functional data for this pup were not included in the final analyses.

Third, diabetes induced by STZ is not a perfect model for either type 1 diabetes (immunologically mediated) or type 2 diabetes (insulin resistance). However, our model allows us to study the effects of maternal hyperglycemia, hyperlipidemia, and fetal hyperinsulinemia on the developing offspring late in gestation (see

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Table 3). This is important not only because it helps avoid diabetes-related effects on organogenesis but also because the timing coordinates with increased placental lipid transport and fetal pancreatic development/function (insulin secretion) in the latter part of pregnancy. Moreover, women with gestational diabetes are usually not diagnosed or treated until the last trimester, making this a translatable time point for dietary intervention.

Another limitation of our study is that mitochondria were not examined by electron microscopy or other analyses for evidence of mitophagy. Additionally, cardiomyocyte apoptosis was not evaluated. Given our observations on confocal imaging and live-cell video, this is planned in future studies. In addition, it is not yet known whether changes in cardiac metabolism are a cause or compensatory mechanism for heart failure, and while reactive species may be culprits in the decline of myocardial function under conditions of hyperglycemia/hyperlipidemia, the actual cause of dysfunction in myocardial fuel utilization remains unknown. These questions represent areas in desperate need of research.

Conclusions

In conclusion, using a rat model we found that maternal HF diet further impairs cardiac function in offspring of diabetic
pregnancy through metabolic abnormalities, oxidative stress, and mitochondrial dysfunction. Current measures to prevent the complications of diabetic pregnancy involve vigilant glycemic control (2). However, by itself, better glycemic control does not negate risks to the developing baby (4, 7, 36, 37, 64), including the risk of cardiovascular disease (1, 64). Our study is the first to specifically delineate the role of maternal dietary fat intake in cardiac metabolic health in offspring of diabetic pregnancies. Findings demonstrate a significant detrimental effect from this combination and uncover an underrecognized and targetable risk factor that, when addressed during pregnancy, could improve the lifelong heart health of at-risk infants. Our findings guide further investigation into the role of cellular bioenergetics in developmentally programmed cardiac disease and serve as a crucial step in discovering novel diagnostic, preventative, and therapeutic strategies to improve heart health in infants born after diabetic pregnancy.

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REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

CARDIAC BIOENERGETICS IN DIABETIC OFFSPRING


