Cellular stress, excessive apoptosis and the effect of metformin in a mouse model of type 2 diabetic embryopathy

Yanqing Wu\textsuperscript{1}, Fang Wang\textsuperscript{1}, Mao Fu\textsuperscript{2}, Cheng Wang\textsuperscript{4}, Michael J. Quon\textsuperscript{2}, Peixin Yang\textsuperscript{1, 3}

\textbf{Author Affiliations:}
\begin{itemize}
\item \textsuperscript{1}Department of Obstetrics, Gynecology & Reproductive Sciences
\item \textsuperscript{2}Department of Medicine
\item \textsuperscript{3}Department of Biochemistry and Molecular Biology
\item University of Maryland School of Medicine
\item Baltimore, MD 21201
\item Olson Center for Women's Health
\item \textsuperscript{4}Department of Obstetrics and Gynecology
\item University of Nebraska Medical Center
\item Omaha, Nebraska 68198
\end{itemize}

First author’s surname and short title: Wu, A model of type 2 diabetic embryopathy

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\textbf{Address Correspondence to:}
Peixin Yang, PhD
University of Maryland School of Medicine
Department of Obstetrics, Gynecology & Reproductive Sciences
BRB11-039, 655 W. Baltimore Street
Baltimore, MD 21201
Email: pyang@upi.umaryland.edu
Tel: 410-706-8402
Fax: 410-706-5747

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ABSTRACT

Increasing prevalence of type 2 diabetes in women of childbearing age has led to higher incidence of diabetes-associated birth defects. Here, we established a model of type 2 diabetic embryopathy by feeding four-week old female mice with a high-fat diet (HFD, 60% fat). After 15 weeks on HFD, these female mice showed characteristics of type 2 diabetes mellitus (DM) and were mated with lean male mice. During pregnancy, control dams fed with a normal diet (10% fat) were maintained on either normal diet or on HFD, serving as a control group with elevated circulating free fatty acids. DM dams produced offspring with a rate of neural tube defects (NTDs) of 11.3%, whereas no embryos in the control groups developed NTDs. Elevated markers of oxidative stress, endoplasmic reticulum stress, caspase activation, and neuroepithelial cell apoptosis (causal events in type 1 diabetic embryopathy) were observed in embryos of DM dams. DM dams treated with 200 mg/kg metformin in drinking water ameliorated fasting hyperglycemia, glucose intolerance, and insulin resistance with consequent reduction of cellular stress, apoptosis and NTDs in their embryos. We conclude that cellular stress and apoptosis occur and metformin effectively reduces type 2 diabetic embryopathy in a useful rodent model.
Globally, nearly 60 million women of reproductive age (18-44 years old) have diabetes, and this number is expected to double by 2030 (1; 2). Pregestational maternal type 1 and 2 diabetes is strongly associated with high rates of severe structural birth defects including neural tube defects (NTDs) and congenital heart defects (3-6). The use of insulin during pregnancy in both animal models and humans greatly reduces the incidence of diabetes-induced embryonic malformations (7; 8). However, euglycemia is difficult to achieve in and maintain in women with pregestational Type 1 or Type 2 diabetes. Thus, offspring of women with diabetes still have significantly higher rates of birth defects than those of mothers without diabetes (5). Diabetic embryopathy remains a significant health problem for both women with diabetes and their children and additional effective therapeutic options are needed.

Previous studies in a type 1/insulin deficient diabetic embryopathy model demonstrate that oxidative stress, endoplasmic reticulum (ER) stress, and cellular stress-induced cell apoptosis (8-16) are causative events in induction of NTD formation. We and others have observed that maternal diabetes induces oxidative stress by suppressing endogenous expression of antioxidant enzymes while simultaneously increasing production of cellular reactive oxygen species (ROS) (17-20). Maternal diabetes triggers a spectrum of ER stress markers (12). Treatment of embryos in \textit{vitro} with 4-Phenylobutyric acid, an ER stress inhibitor, ameliorates NTD formation induced by hyperglycemia (12). Excess apoptosis is observed in the neuroepithelium of embryos exposed to maternal diabetes, and deletion of proapoptotic kinase genes reduces NTD incidence in these rodent embryos (9; 11; 12; 21). In addition, maternal diabetes-induced apoptosis is caspase 8 dependent (9; 11; 12; 21).
Prior research to delineate mechanisms underlying diabetic embryopathy has almost exclusively been performed using mouse models of type 1 diabetic/insulin deficient animals (9; 15; 16; 22; 23). Thus, the question remains whether similar mechanisms underlie type 2 diabetic embryopathy, a health problem of increasing importance. Hyperglycemia, a prominent feature of both type 1 and type 2 diabetes is a major contributing factor to teratogenicity of maternal diabetes (19; 24; 25). Nevertheless, many aspects of the pathophysiology of Type 1 and Type 2 diabetes are quite different. In particular, insulin resistance plays a prominent role in the pathophysiology of Type 2 diabetes while insulin deficiency is the primary defect in Type 1 diabetes. Thus, effects of Type 2 diabetes on developing embryos may be distinct from effects of Type 1 diabetes. As the number of women with type 2 diabetes continues to increase (1), a type 2 diabetic embryopathy model is urgently needed to help develop safe and effective therapeutic interventions to complement current therapies that are inadequate to oppose the rising incidence and prevalence of diabetes-induced embryopathy.

To date, animal models of type 2 diabetic embryopathy have not been well described or studied. In C57BL/6J mice, use of high fat diet (HFD) to cause diet induced obesity (DIO) recapitulates many features of the natural history of human obesity, metabolic syndrome, and frank type 2 diabetes (26; 27). This obese type 2 diabetic model manifests the characteristics of human type 2 diabetes (26; 27). After 15 weeks on HFD, female mice exhibit high fasting glucose levels, hyperinsulinemia, glucose intolerance and insulin resistance (26; 27). In the present study, we use the DIO mouse model to characterize study type 2 diabetic embryopathy and evaluate effects of metformin therapy.
Research Design and Methods

Mice and high-fat diet treatment

The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Four-week old female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animals were maintained in a temperature-controlled room on a 12-h light-dark cycle. After arrival, mice were divided into two groups and fed either a high-fat diet (HFD) (Research Diets, New Brunswick, NJ) or a normal diet (Research Diets, Harlan, US) for 15 weeks. The high-fat diet contained 20% protein, 20% carbohydrate and 60% fat. The normal diet contained 20% protein, 70% carbohydrate and 10% fat. Body weight was recorded weekly. After 15 weeks, the animals’ BMI was determined using the Lee’s Index. HFD mice and mice fed with the normal diet were mated with lean male mice. During pregnancy, mice in the normal diet group were either maintained on the normal diet (control group 1) or subsequently fed with HFD to serve as the high fatty acid control group (control group 2). Day 0.5 (E0.5) of pregnancy was established at noon on the day when a vaginal plug was observed. Fasting and random blood glucose levels were determined after 15 weeks on either HFD or normal diet, and at embryonic day 8.5 (E8.5). Blood was collected from orbital veins and sera was extracted by centrifugation and reserved to determine circulating free fatty acid and insulin concentrations. For long term storage, sera were kept at -80°C.

Blood glucose measurement, glucose tolerance test (GTT) and insulin tolerance test (ITT)

Blood glucose was measured using a handheld glucometer with appropriate test strips (FreeStyle Lite). For GTT, mice were fasted overnight and then injected intraperitoneally with
glucose at a dose of 2 g/kg body weight. Blood glucose levels were measured prior to injection, and at 15, 30, 60, 90, and 120 min after glucose injection. The trapezoidal rule was used to determine the area under the curve for GTT (AUC). For ITT, mice were fasted for 5 h and then injected intraperitoneally with insulin at a dose of 0.75 U/kg body weight. Blood glucose levels were measured prior to injection, and at 15, 30, 60, 90, and 120 min after injection.

**Serum free fatty acid and insulin measurements**

Free fatty acid (FFA) levels were determined using the FFA quantification kit (Sigma-Aldrich) according to the manufacturers’ instructions. Palmitic acid standards and 6 µl of serum per sample were incubated with reaction agents. Absorbance was measured at 570 nm in each sample to determine concentrations of FFA. The rat/mouse Insulin ELISA kit (Millipore, Cat.#EZRMI-13K) was used to assess the level of plasma insulin. Insulin standards and serum samples were reacted with assay buffer. Absorbance was read at 450 nm and 590 nm for each sample using a 96-well plate reader within 5 min. Quantitative insulin sensitivity check index (QUICKI) was calculated using the following formula: $1/\left[\log(\text{fasting insulin, } \mu\text{U/ml})+\log(\text{fasting glucose, } \text{mg/dl})\right]$.

**Dihydroethidium (DHE) Staining**

DHE staining was used to detect superoxide. DHE reacts with superoxide that is bound to cellular components including protein and DNA and exhibits bright red fluorescence. E8.5 embryos were fixed in 4% paraformaldehyde (PFA) for 30 min, washed three times with PBS (5 min per wash), and then embedded in OCT. 10-µm frozen embryonic sections were incubated with 1.5 µM DHE for 5 min at room temperature, and then washed three times with PBS for 5 min per
wash. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO).

**Lipid hydroperoxide quantification**

The degree of lipid peroxidation, an index of oxidative stress, was quantitatively assessed with an LPO assay as described by others (28) using the Calbiochem Lipid Hydroperoxide Assay Kit (Milliprole, Bedford, MA). Briefly, E8.5 embryos were homogenized in HPLC-grade water. Lipid hydroperoxides were extracted from embryos by deoxygenated chloroform, and then measurements of absorbance at 500 nm were taken after reaction with chromogen. Results were expressed as µM lipid hydroperoxides per microgram protein. Protein concentrations were determined with the BioRad DC protein assay kit (BioRad, Hercules, CA).

**Real-time PCR (RT-PCR)**

Using the Rneasy Mini kit (Qiagen), mRNA was isolated from E8.75 embryos, and then reversed transcribed using the high-capacity cDNA archive kit (Applied Biosystem, Grand Island, NY). RT-PCR for Calnexin, GRP94, PDIA, BiP, IRE1α, CHOP and β-actin were performed using the Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, IL) in the StepOnePlus system (Applied Biosystem). Primer sequences used are listed in Supplementary Table 1.

**Immunoblotting**

Immunoblotting was performed as previously described (29; 30). Briefly, embryos from
different experimental groups were sonicated in lysis buffer containing a protease inhibitor cocktail (Sigma, St Louis, MO). Equal amounts of protein and the Precision Plus Protein Standards (Bio-Rad) were resolved by SDS-PAGE electrophoresis and transferred onto Immunobilon-P membranes (Millipore, Billerica, MA). Membranes were incubated in 5% nonfat milk for 45 min and then incubated for 18 h at 4 °C with the primary antibodies. To help determine whether equivalent amounts of protein were loaded among all samples, membranes were stripped and incubated with a mouse antibody against β-actin (Abcam) to generate a signal used as a loading control. Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). The sources and dilutions of antibodies used in each experiment are listed in Supplementary Table 2.

**TUNEL assay**

The TUNEL assay was performed using the ApopTag Fluorescein in Situ Apoptosis Detection kit (Chemicon) as previously described (9; 11; 12; 21). Briefly, 10-μm frozen embryonic sections were fixed with 4% PFA in PBS and incubated with TUNEL reaction agents. Three embryos from three different dams (n = 3) per group were used, and two sections per embryo were examined. TUNEL-positive cells in an area (about 200 cells) of neuroepithelium were counted. The percentage of TUNEL-positive cells was calculated as a fraction of the total cell number, multiplied by 100, and values were averaged within the sections of one embryo.

**Metformin treatment**

Dams were given metformin (Sigma, St. Louis, MO) in their drinking water from E0.5 to
E10.5. The water consumption per mouse per day (24 h) was pre-determined and monitored daily during the course of metformin treatment. Because daily water consumption was different between lean control dams and obese diabetic dams, metformin concentrations in drinking water were adjusted accordingly to achieve comparable dosing. The purpose of giving metformin in drinking water was to help maintain a constant level of metformin. Metformin is typically given to patients in an extended release form so that effects of metformin can be sustained over long durations (31). The final metformin intake per mouse per day was approximately 200 mg/kg body weight, a dose previously described as safe and effective in mice (31-33). At E8.5, fasting glucose levels were determined, and GTT and ITT were performed, as described above. At E10.5, embryonic NTD formation was examined in metformin treated mice.

**Statistical analyses**

Data are presented as mean ± SE. Each set of experiments were repeated independently at least three times with comparable results, and embryonic samples from each replicate were taken from different dams. Statistical differences were evaluated using one-way analysis of variance (ANOVA) with SigmaStat 3.5 software. For one-way ANOVA analyses a *Tukey* test was used to estimate the significance of the results with $P < 0.05$ indicative of statistical significance. The *Chi square* test was used to estimate the significance of differences in NTD rates among experimental and control groups.
RESULTS

Induction of obese type 2 diabetes in female mice

Four-week old female mice were fed a high-fat (HFD) or normal (control) diet for 15 weeks. Mice in the HFD group continuously gained weight from 2 weeks onward and after 15 weeks. The mean body weight of mice in the HFD group was significantly greater than the control group (35.02 ± 0.81 versus 22.96 ± 0.49, \( P < 0.05 \)) (Fig.1a). The body mass index (BMI) of mice in the HFD group was also significantly higher than that of mice in the control group (Fig.1b).

Fasting glucose levels and random glucose levels of mice in the HFD group were significantly elevated when compared with mice in the normal diet group (Fig.1c, d). Plasma insulin levels in the HFD group were approximately two-fold higher than those of mice in the normal diet group (Fig.1e), indicating hyperinsulinemia in the HFD group. Quantitative insulin sensitivity check index (QUICKI) was used to assess insulin resistance. Mean QUICKI values of mice in the HFD group were significantly less than those of mice in the normal diet group (Fig.1f). Additionally, high FFA levels were observed in the HFD group (Fig.1g).

Glucose intolerance is the defining characteristic of type 2 diabetes. GTT was performed after 15 weeks feeding with different diets. After injecting mice in both the HFD and the normal diet group with glucose, blood glucose levels in all mice were increased during early time points of the GTT test (Fig.1h). At 30 min, the peak mean glucose levels of mice in the HFD group was much higher and glucose disappearance thereafter was much slower when compared with the mice with normal diet (Fig.1h). Indeed, the mean area under the curve (AUC) for GTT in mice on HFD was significantly higher than that of mice on normal diet (Fig. 1h). Thus, HFD induced significant and substantial glucose intolerance in mice after 15 weeks.
Insulin tolerance test was employed to evaluate insulin resistance in the mice. After insulin administration, blood glucose levels declined as expected in both groups; however, this decrease was significantly less in mice on HFD when compared with mice on normal diet (Fig.1i). During ITT, the glucose levels of mice in HFD group were higher at every time point when compared with mice in the normal diet group. This clearly demonstrates insulin resistance in the HFD group. Taken together, these findings are consistent with diet-induced obesity from HFD causing insulin resistance and key characteristics of type 2 diabetes. Therefore, the HFD group is referred to as the diabetes mellitus (DM) group hereafter.

Metabolic indices of obese type 2 diabetic dams

The mice in the normal diet group were randomized into two control groups: control group 1 (Ctrl 1)-normal diet the whole time including pregnancy, and the control group 2 (Ctrl 2), in which mice were switched to HFD after achieving pregnancy to serve as the high FFA control group. The metabolic indices of the DM group and the two control groups were determined at E8.5, an important time point for embryonic neural tube formation. The fasting and random glucose levels from mice in the DM group were significantly increased when compared with those from mice in the two control groups (Table 1). The fasting insulin level of mice in the DM group was more than two-fold greater than mice in either of the two control groups (Fig.1j). Furthermore, the QUICKI in mice from the DM group was significantly less than in mice from either of the two control groups (Fig.1k). The FFA level in mice from the DM group was significant higher than that of mice in the Ctrl1 group, but was comparable to that of mice in the Ctrl2 group (Fig.1l). DM dams retained glucose intolerance and insulin resistance observed before pregnancy (Fig. 1m, n).
Maternal type 2 diabetes induces NTD formation

To assess whether embryos from DM dams exhibit increased NTD formation, we examined NTD formation at E10.5. As shown in Table 1, 12 out of 106 embryos (11.3%) from DM dams had NTD, whereas no NTD was detected in embryos from either Ctrl 1 or Ctrl 2 dams. Histological examination of embryo sections of NTD embryos confirmed the presence of open neural tube structures in the DM group (Fig. 2a). In addition, 26 out of 132 embryos from diabetic dams (19.7%) were resorbed, whereas the two control groups had lower or similar resorption rates, respectively (Table 1). Blood glucose levels in the DM group with NTD embryos were significantly higher than those in the two control groups but were only slightly higher than those in the overall DM group (Fig. 2b).

Maternal type 2 diabetes triggers oxidative stress, ER stress, caspase activation and apoptosis in the developing embryo

Previous studies show that oxidative stress and ER stress are involved in induction of maternal type 1 diabetic embryopathy. In the present study, we examined whether maternal type 2 diabetes also induces oxidative stress and ER stress. The abundance of superoxide was determined by DHE staining. DHE-positive signals in the neuroepithelia of embryos from DM dams were robust (Fig. 2c). By contrast, minimal to no DHE signals were observed in either of the two control groups (Fig.2c). In addition, lipid peroxidation levels in embryos of DM dams were significantly higher than those in embryos from the two control groups (Fig.2d).

To determine the level of ER stress in embryos, we examined a number of ER stress markers. Protein levels of p-PERK, p-eIF2α, p-IRE1α and CHOP were significantly up-regulated in the
embryos from DM dams when compared with those in embryos from control dams (Fig.3a, b, c, d). Furthermore, maternal diabetes significantly increased mRNA levels of Calnexin, GRP94, PDIA, BiP and CHOP (Fig.3e). Only the mRNA level of IRE1α did not differ among the three groups (Fig.3e).

To test whether excessive cell apoptosis is involved in type 2 diabetic embryopathy, we used a TUNEL assay. The number of apoptotic cells in the neuroepithelia of embryos from DM dams was much greater than in embryos from the two control groups (Fig.4a). Cleaved caspase8 (an initiator caspase) and cleaved caspase3 (an executive apoptosis molecule) levels in embryos of DM dams were significantly increased when compared with embryos from either of the two control groups (Fig.4b).

**Metformin treatment partially normalizes the adverse metabolic phenotypes of type 2 diabetic dams**

Metformin is an anti-diabetic drug effective that increases insulin sensitivity and peripheral glucose uptake while inhibiting hepatic glucose production in type 2 diabetes (34). We assessed the effect of metformin treatment on adverse metabolic phenotypes of type 2 dams. Fasting and random glucose levels (Table 2), plasma insulin levels and QUICKI (Fig. 5a, b) were comparable in the two control groups treated with metformin as well as in the DM group treated with metformin. DM dams treated with metformin had demonstrated an amelioration of diabetic phenotype when compared with DM dams with no metformin treatment (Table 2, Fig. 5a, b). GTT and ITT were performed to evaluate effects of metformin therapy to ameliorate glucose intolerance and insulin resistance. Metformin treatment partially improved glucose intolerance and completely
reversed insulin resistance in DM dams (Fig.5c, d).

**Metformin treatment alleviates maternal type 2 diabetes-induced NTD formation**

To determine whether metformin treatment reduced type 2 diabetes-induced NTD formation, embryonic NTD were examined in the absence and presence of metformin therapy. Similar to a previous study reporting no adverse effects of metformin treatment on neurulation-stage (35), metformin treatment in our study did not induce any NTD in embryos of the control dams (Table 2). The NTD rate in embryos from DM dams treated with metformin was only 1.5%, which was significantly lower than that in embryos from DM dams without metformin treatment (11.9%) (Table 2). Metformin treatment of DM dams reduced the NTD rate to that observed in either of the control groups (Table 2). Metformin treatment reduced the resorption rate in DM dams but did not completely prevent maternal diabetes-induced resorption (Table 2). Moreover, metformin treatment alleviated oxidative stress, ER stress and apoptosis by reducing levels of lipid hydroperoxide, phosphorylation of PERK, eIF2α, IRE1α, up-regulation of CHOP and cleavage of caspase 3 and 8 (Fig. 6a-g). These data support the hypothesis that metformin is effective in treating type 2 diabetes and associated diabetic embryopathy.
DISCUSSION

Prior animal studies performed to elucidate mechanisms underlying diabetic embryopathy have been almost exclusively performed using a type 1/insulin deficient animal model (9; 15; 16; 22). With the increasing epidemic of type 2 diabetes in women of reproductive age, a useful animal model of type 2 diabetic embryopathy will be essential for understanding mechanisms and potential therapies for diabetic embryopathy from Type 2 diabetic women. In the present study, we developed and characterized a model of type 2 diabetes embryopathy using DIO in C57BL/6J mice on HFD. Although type 2 diabetes is a complex metabolic disorder, hyperglycemia with resulting glucotoxicity is a major mediator of diabetes teratogenicity. The modest hyperglycemia (an average blood glucose level: 9.56 ± 0.23 mmol/L) present in our type 2 diabetes animal model causes significant birth defects, specifically NTDs, when compared with normal mice even on a HFD after pregnancy to achieve comparable FFA levels and lipotoxicity. Our model of Type 2 diabetes pregnancy generates lower rates of NTD than models of type 1 diabetes, which have an average blood glucose level of 21.3 ± 1.2 mmol/L. These findings are consistent with observations in a human study that the increase of hyperglycemia linearly increases the incidence of birth defects (24).

In this study, treatment with metformin, a standard therapy for pregnant women with type 2 diabetes, inhibits gluconeogenesis in the liver (34), effectively normalized hyperglycemia in our type 2 diabetes animal model. We found that metformin improved glucose metabolism, reduced hyperglycemia and significantly ameliorated NTD formation. However, metformin treatment only partially prevented glucose intolerance. The incomplete correction of defective glucose metabolism by metformin may still allow transient hyperglycemia, which we may not have
detected in our study. Transient increases in maternal glucose may explain why we observed a higher NTD rate (1.5%) in diabetic dams treated with metformin compared with the rate in nondiabetic dams. Indeed, in vitro embryo culture studies have demonstrated that transient exposure to high glucose induces NTD formation (36).

The type 2 diabetic embryopathy model we employed displays hyperinsulinemia and high free fatty acid levels. Hyperinsulinemia associated with type 2 diabetes is unlikely to cause NTD formation because maternal insulin does not cross the blood/placental barrier (37). In addition, NTDs are not caused by embryonic pancreas-derived insulin, as pancreatic β-cells do not produce insulin until E11, which is after the neural tube closes (38). Although high fatty acid levels may still affect embryonic development, our findings demonstrate that high fatty acid levels did not contribute to NTD formation in our type 2 diabetic embryopathy model; lean mice fed a high-fat diet during pregnancy did not produce embryos with NTDs.

Mechanistic studies performed in type 1 diabetic embryopathy models reveal that oxidative stress, ER stress and caspase-dependent neuroepithelial cell apoptosis are causal events leading to NTD formation (8-16; 39-43). In the present study, we found that superoxide production and apoptosis was induced only in the developing neuroepithelia of embryos exposed to maternal type 2 diabetes. We also observed that maternal type 2 diabetes triggered the UPR and ER stress in the developing embryo. Additionally, we found that the cellular stress and apoptotic signaling pathways in embryos of type 2 diabetic dams mirrored that of type 1 diabetic embryopathy, suggesting that both type 1 and type 2 diabetic embryopathy share common mechanisms underlying NTD formation.

We used the C57BL/6J background in our study. A previous report showed no
significant increase of NTDs in the C57BL6J strain; however, that study is inconclusive because embryos from nondiabetic C57BL/6J dams have more than 12% NTDs (normal incidence 0-1% NTDs), and the study sample size is very small (n = 4 dams) (44). That study is contradictory to a report in 2001, which demonstrated that intravenous alloxan-induced diabetes in wild-type C57BL/6J significantly increased the occurrence of NTDs (45). Recent studies from us (9; 12; 21) and others (46; 47) have demonstrated that STZ-induced diabetes produces more than 22% NTDs in the C57BL/6J background. Thus, the C57BL/6J strain responds well to maternal diabetes. The 11.3% NTD rate in embryos from type 2 diabetic dams are significantly higher than or many fold higher than the 0% NTD rate from the nondiabetic group. In humans, maternal diabetes induces 2-6 fold higher NTD rates than those from the general population.

In summary, we employed obese type 2 diabetic mice to examine embryopathy caused by type 2 diabetes. We found that type 2 diabetes induces oxidative stress and ER stress in the developing neuroepithelium leading to NTD formation. Metformin treatment significantly reduced NTD formation through partial normalization of the metabolic defects in the type 2 diabetic embryopathy model. Hyperglycemia rather than lipotoxicity seems to be the predominant pathogenic feature inducing embryopathy. The type 2 diabetic embryopathy model is a unique and useful model that contains key aspects of the metabolic pathophysiology present in women with type 2 diabetes. Thus, our new model may be valuable in elucidating underlying causes of embryopathy in Type 2 diabetes that may lead to novel therapeutic interventions for diabetes-induced birth defects.
Author Contributions

Y. W. researched data and wrote the paper. F. W. researched data. M.J.Q participated in data analyses and writing the manuscript. M. F. and C. W participated in data analyses. P. Y. conceived the project, designed the experiments, and wrote the manuscript. All authors have approved the final version of the paper.

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Figure Legends

Figure 1. Induction of obese type 2 diabetes in female mice and metabolic indices of obese type 2 diabetic dams. a, Body weight measurements weekly during feeding with normal control diet (10% fat) (n = 15) or high-fat diet (HFD) (60% fat) (n = 15). b, Body mass index in normal diet group and high-fat diet group (n = 8) after 15 weeks feeding with different diet. c, Fasting glucose level (n = 12), d, Random glucose level (n = 16), e, plasma insulin level (n = 5), f, quantitative insulin sensitivity check index (QUICKI) (n = 5) and g, free fatty acid level (n = 7) after 15 weeks diet treatment. h, Blood glucose levels and AUC\textsubscript{glucose} level during GTT (n = 13) after 15 weeks feeding with different diets. i, Blood glucose levels during ITT (n = 6) after 15 weeks feeding with different diets. * indicate significant differences compared to the other group. j, Plasma insulin levels, k, QUICKI and l, free fatty acid levels on the two control groups (Ctrl1 and Ctrl2) and the obese type 2 diabetes mellitus (DM) group at E8.5. n = 10. m, Blood glucose levels and AUC\textsubscript{glucose} levels during GTT at E8.5 (n = 6). n, Blood glucose levels during ITT at E8.5 (n = 7). * indicate significant differences compared to the other two groups. Ctrl1: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy. Ctrl2: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy. The control group 2 served as the controls of high FFA observed in the DM group. DM: the obese type diabetes mellitus group.

Figure 2. Maternal type 2 diabetes induces NTD formation and oxidative stress in the developing embryo. a, closed and open neural tube structures of E10.5 embryos from control dams and diabetic dams (DM). b, Random blood glucose levels. n = 10 in the DM-NTD group.
Sample sizes for the other three groups are shown in Table 1. c, representative images of DHE staining. Red signals of DHE staining were observed in the V-shape neuroepithelium at E8.5. All cell nuclei were stained with DAPI (Blue). Bars = 30 µm. d, levels of lipid hydroperoxide (LPO) in E8.5 embryos. Experiments were performed using three embryos from three different dams (n = 3) per group. * indicate significant differences compared to the other groups. Ctrl1: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy. Ctrl2: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy. DM-NTD: the obese type 2 diabetes mellitus group with neural tube defects.

**Figure 3. Maternal type 2 diabetes triggers ER stress in the developing embryo.** Levels of p-PERK (a), p-eIF2α (b), p-IRE1α (c), and CHOP (d) in E8.5 embryos. e, mRNA level of Calnexin, GRP94, PDIA, BiP, IRE1α and CHOP. Experiments were performed using three embryos from three different dams (n = 3) per group. * indicate significant differences compared to the other two groups. Ctrl1: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy. Ctrl2: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy. DM: the obese type 2 diabetes mellitus group.

**Figure 4. Maternal type 2 diabetes activates caspase and induces neuroepithelial cell apoptosis in the developing embryo.** a, Representative images of the TUNEL assay showing apoptotic cells (Red signal). Cell nuclei were stained with DAPI (Blue). The bar graph showed the quantification of TUNEL positive cells. Three embryos from three different dams (n = 3) per group,
and two serial sections per embryo were analyzed. Bars = 30 µm. b, protein levels of cleaved caspase 3 and caspase 8 in E8.5 embryos. Experiments were performed using three embryos from three different dams (n = 3) per group. * indicate significant differences compared to the other two groups. Ctrl1: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy. Ctrl2: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy. DM: the obese type 2 diabetes mellitus group.

Figure 5. Metformin treatment normalizes some of the adverse metabolic phenotypes in type 2 diabetic dams. Plasma insulin levels (a) and QUICKI (b) after metformin treatments from E0.5 to the timing of assessment (E8.5). In order to reach a sustained effect, metformin was given through the drinking water. Daily water consumption and body weight were monitored, and metformin concentrations in the drinking water were adjusted accordingly. Dams were housed individually. The final amount of metformin given to one dam was 200 mg/kg body weight per 24 hours. Ctrl1: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy. Ctrl2: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy. DM: the obese type 2 diabetes mellitus group. Met: metformin. In a and b, there were four experimental groups: Ctrl1+Met (Metformin), Ctrl2+Met, DM+Met and the DM group without metformin treatment. c, Blood glucose levels and AUC_glucose levels during GTT at E8.5 after treating with metformin. d, Blood glucose levels during ITT at E8.5 after treating with metformin. During GTT, the number dams per group were the Ctrl1+Met group (n = 8), the DM+Met group(n = 6), Ctrl2+ Met (n = 8) and the DM group (n = 4). During ITT, the number dams per group were the Ctrl1+Met group (n = 3), the DM+Met group (n = 9), the
Ctrl2+Met group (n = 5) and the DM group (n = 6). *: indicate that the DM group was significantly different compared to the DM+Met, Ctrl1+Met and Ctrl2+Met groups. **: indicate that the DM group and the DM+Met group were not significantly different, and the DM group was significantly different compared to the Ctrl 1+Met and Ctrl 2+Met group. #: indicated that the DM and DM+Met groups were not significantly different, but they were significantly different compared to the Ctrl1+Met and Ctrl2+Met groups.

**Figure 6. Metformin treatment alleviates maternal type 2 diabetic induced-cellular stress and excessive apoptosis in the developing embryo.** (a), levels of lipid hydroperoxide (LPO) in E8.5 embryos. Protein levels of p-PERK (b), p-eIF2α (c), p-IRE1α (d), CHOP (e), cleaved caspase 8 (f) and caspase 3 (g) in E8.5 embryos. Experiments were performed using three embryos from three different dams (n = 3) per group. * indicate significant differences compared to the other three groups. Ctrl1 + Met: the control chow group in which mice were continuously fed with chow for 15 weeks and during pregnancy with metformin (Met) treatment. Ctrl2 + Met: the control group 2 in which mice were fed with chow for 15 weeks but fed with the HFD during pregnancy with metformin treatment. DM: the obese type 2 diabetes mellitus group.
References

19. Yang P, Li H: Epigallocatechin-3-gallate ameliorates hyperglycemia-induced embryonic vasculopathy and
malformation by inhibition of Foxo3a activation. American journal of obstetrics and gynecology 2010;203:75 e71-76
Table 1. Maternal type 2 diabetes induces NTD formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Fasting glucose (mmol/L)</th>
<th>Random glucose (mmol/L)</th>
<th>Number of embryos</th>
<th>Resorption rate (%)</th>
<th>NTD rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl 1 (n=15)</td>
<td>5.08±0.54</td>
<td>7.06±0.35</td>
<td>119</td>
<td>3(2.5%)</td>
<td>0(0.0)</td>
</tr>
<tr>
<td>DM (n=16)</td>
<td>7.92±0.51*</td>
<td>9.56±0.23*</td>
<td>106</td>
<td>26(19.7%)*</td>
<td>12(11.3%)*</td>
</tr>
<tr>
<td>Ctrl 2 (n=15)</td>
<td>5.76±0.54</td>
<td>7.38±0.26</td>
<td>107</td>
<td>4(3.6%)</td>
<td>0(0.0)</td>
</tr>
</tbody>
</table>

Ctrl 1: normal control diet group; DM: high fat diet group; Ctrl 2: 60% high fat diet during pregnancy. * indicate significant differences compared with the other two groups analyzed by the Tukey test or Chi square test.
Table 2. Metformin treatment alleviates maternal type 2 diabetes-induced NTD formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Fasting glucose (mmol/L)</th>
<th>Random glucose (mmol/L)</th>
<th>Number of embryos</th>
<th>Resorption rate (%)</th>
<th>NTD rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl 1+Met (n=6)</td>
<td>5.20±0.25</td>
<td>7.09±0.31</td>
<td>50</td>
<td>2(3.8%)</td>
<td>0(0.0%)</td>
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<tr>
<td>Ctrl 2+Met (n=8)</td>
<td>5.38±0.22</td>
<td>7.67±0.34</td>
<td>62</td>
<td>3(4.6%)</td>
<td>0(0.0%)</td>
</tr>
<tr>
<td>DM (n=10)</td>
<td>8.34±0.55*</td>
<td>9.46±0.27*</td>
<td>67</td>
<td>15(18.3%)*</td>
<td>8(11.9%)*</td>
</tr>
<tr>
<td>DM+Met (n=10)</td>
<td>6.05±0.33</td>
<td>8.03±0.25</td>
<td>64</td>
<td>9(12.3%)</td>
<td>1(1.5%)</td>
</tr>
</tbody>
</table>

Ctrl 1+Met: normal control diet group treating with metformin; Ctrl 2+Met: 60% fat diet during pregnancy treating with metformin; DM: high fat diet group; DM+Met: high fat diet group treating with metformin. * indicate that the DM group is significantly different compared with the DM+Met, the Ctrl 1+Met and the Ctrl 2+Met groups. **: indicate that the DM group was significantly different compared with the Ctrl 1+Met and the Ctrl 2+Met group but was not significantly different compared with the DM+Met group. Statistical differences were analyzed by the Tukey test or Chi square test.
Figure 1

**a**

Body weight (g) over time (week) for normal diet and HFD groups.

**b**

Body mass index comparison between normal diet and HFD groups.

**c**

Fasting glucose level comparison between normal diet and HFD groups.

**d**

Random glucose level comparison between normal diet and HFD groups.

**e**

Plasma insulin levels for normal diet and HFD groups.

**f**

QUICKI comparison between normal diet and HFD groups.

**g**

Free fatty acid (nmoL/µL) comparison between normal diet and HFD groups.

**h**

Blood glucose level (mmol/L) over time (min) for normal diet and HFD groups.

**i**

AUC glucose (h.nmol/L) comparison between normal diet and HFD groups.

**j**

Plasma insulin levels for Ctrl 1, DM, and Ctrl 2 groups.

**k**

QUICKI comparison for Ctrl 1, DM, and Ctrl 2 groups.

**l**

Free fatty acid (nmoL/µL) comparison for Ctrl 1, DM, and Ctrl 2 groups.

**m**

Blood glucose level (mmol/L) over time (min) for Ctrl 1, DM, and Ctrl 2 groups.

**n**

AUC glucose (h.mmol/L) comparison for Ctrl 1, DM, and Ctrl 2 groups.
Figure 4

(a) Diagram showing the expression levels of Caspase 8 and Caspase 3 in Ctrl 1, DM, and Ctrl 2 groups. The expression levels are normalized to β-actin.

(b) Western blot analysis of Caspase 8 and Caspase 3 cleavage in Ctrl 1, DM, and Ctrl 2 groups. The cleaved forms of Caspase 8 and Caspase 3 are marked with an asterisk (*) and quantified using densitometry.
Figure 5

(a) Plasma insulin level (ng/mL)

(b) QUICKI

(c) Blood glucose level (mmol/L)

(d) Blood glucose level (mmol/L)
### Supplementary Table 1. The sequences of primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sources</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calnexin-F</td>
<td>Primerbank ID: 6671664a1</td>
<td>ATGGAAGGGAAGTGGTTACTGT</td>
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<tr>
<td>Calnexin-R</td>
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<td>GCTTTGTAGGTGACCTTTGGAG</td>
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<tr>
<td>GRP94-F</td>
<td>Primerbank ID: 6755863a1</td>
<td>TCGTCAGAGCTGATGATGAAAGT</td>
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<tr>
<td>GRP94-R</td>
<td></td>
<td>GCGTTAACCCATCCAACTGAAT</td>
</tr>
<tr>
<td>PDIA3-F</td>
<td>Primerbank ID: 6679687a1</td>
<td>CGCCTCGATGTGTTGGA</td>
</tr>
<tr>
<td>PDIA3-R</td>
<td></td>
<td>CAGTGCAATCCACTTTGCTAA</td>
</tr>
<tr>
<td>BiP-F</td>
<td>Primerbank ID: 31981722a1</td>
<td>ACTTGGGGGACCACCTATTCT</td>
</tr>
<tr>
<td>BiP-R</td>
<td></td>
<td>ATCGCCAATCGACGCTCC</td>
</tr>
<tr>
<td>IRE1α-F</td>
<td>Primerbank ID: 13249351a1</td>
<td>ACACCGACCACGTATCTCA</td>
</tr>
<tr>
<td>IRE1α-R</td>
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<td>CTCAGGATAATGGTAGCCATGTC</td>
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<tr>
<td>CHOP-F</td>
<td>Own design</td>
<td>CGGAACCTGAGGAGAGAGTG</td>
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<tr>
<td>CHOP-R</td>
<td></td>
<td>CTGTCAGCCAAGGCTAGGGAC</td>
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</table>

F: forward; R: reverse.
**Supplementary Table 2.** The dilutions and sources of antibodies in immunoblotting.

<table>
<thead>
<tr>
<th>Antibodies name</th>
<th>Antibody sources</th>
<th>Dilution method</th>
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<tr>
<td>p-IRE1α</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>p-PERK</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>PERK</td>
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<tr>
<td>p-eIF2α</td>
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<tr>
<td>eIF2α</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
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<tr>
<td>CHOP</td>
<td>Cell Signaling technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>Caspase8</td>
<td>ENZO life sciences</td>
<td>1:1000</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>EMD Millipore</td>
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<tr>
<td>β-actin</td>
<td>Abcam</td>
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