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The green tea polyphenol EGCG alleviates maternal diabetes–induced neural tube defects by inhibiting DNA hypermethylation

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Abstract

BACKGROUND—Maternal diabetes increases the risk of neural tube defects in offspring. Our previous study demonstrated that the green tea polyphenol, Epigallocatechin gallate, inhibits high glucose-induced neural tube defects in cultured embryos. However, the therapeutic effect of Epigallocatechin gallate on maternal diabetes–induced neural tube defects is still unclear.

OBJECTIVE—We aimed to examine whether Epigallocatechin gallate treatment can reduce maternal diabetes–induced DNA methylation and neural tube defects.

STUDY DESIGN—Nondiabetic and diabetic pregnant mice at embryonic day 5.5 were given drinking water with or without 1 or 10 μ M Epigallocatechin gallate. At embryonic day 8.75, embryos were dissected from the visceral yolk sac for the measurement of the levels and activity of DNA methyltransferases, the levels of global DNA methylation, and methylation in the CpG islands of neural tube closure essential gene promoters. embryonic day 10.5 embryos were examined for neural tube defect incidence.

RESULTS—Epigallocatechin gallate treatment did not affect embryonic development because embryos from nondiabetic dams treated with Epigallocatechin gallate did not exhibit any neural tube defects. Treatment with 1 μ M Epigallocatechin gallate did not reduce maternal diabetes–induced neural tube defects significantly. Embryos from diabetic dams treated with 10 μ M Epigallocatechin gallate had a significantly lower neural tube defect incidence compared with that of embryos without Epigallocatechin gallate treatment. Epigallocatechin gallate reduced neural tube defect rates from 29.5% to 2%, an incidence that is comparable with that of embryos from nondiabetic dams. Ten micromoles of Epigallocatechin gallate treatment blocked maternal diabetes–increased DNA methyltransferases 3a and 3b expression and their activities, leading to the suppression of global DNA hypermethylation. Additionally, 10 μ M Epigallocatechin gallate abrogated maternal diabetes–increased DNA methylation in the CpG islands of neural tube closure essential genes, including *Grhl3*, *Pax3*, and *Tulp3*.

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The authors have nothing to report.

CONCLUSION—Epigallocatechin gallate reduces maternal diabetes–induced neural tube defects formation and blocks the enhanced expression and activity of DNA methyltransferases, leading to the suppression of DNA hypermethylation and the restoration of neural tube closure essential gene expression. These observations suggest that Epigallocatechin gallate supplements could mitigate the teratogenic effects of hyperglycemia on the developing embryo and prevent diabetes–induced neural tube defects.

Keywords

DNA methyltransferases; Epigallocatechin gallate; essential gene; green tea polyphenol; hypermethylation; maternal diabetes; neural tube closure; neural tube defects

Currently nearly 60 million women of reproductive age (18–44 years old) worldwide have diabetes, and this number has been estimated to double by 2030.^{1–4} Clinical studies and animal model investigations have revealed that maternal diabetes increases the risk of neural tube defects in offspring and that hyperglycemia is a teratogen.^{1–3,5–10}

Although strict glycemic control by lifestyle and pharmacological treatment can decrease the incidence of hyperglycemia-induced embryonic malformations in pregnancies affected by preexisting maternal diabetes,^{1–3,6,7} euglycemia is difficult to achieve and maintain, and even transient exposure to high glucose could lead to abnormal embryonic development.^{1–3,11–13} Thus, diabetes-induced birth defects are significant public health problems, and there is an urgent need for new therapeutic approaches against diabetic embryopathy.

Neural tube defects are common complex congenital malformations of the central nervous system that form during embryogenesis.¹⁴ There are approximately 5 times more neural tube defects in offspring from diabetic mothers than in those from nondiabetic mothers, despite modern preconception care.¹⁵ Studies from our group^{1,5,6,16–25} and others²⁶ have demonstrated that maternal diabetes induces cellular stress, including oxidative stress and endoplasmic reticulum stress, and that those cellular stresses cause apoptosis in the embryonic neural tissue, leading to neural tube defect formation. Recently several studies have suggested that altered DNA methylation disrupts the folate metabolic pathway and causes neural tube defects.^{27–29} Therefore, we hypothesize that altered DNA methylation is involved in neural tube defect formation in diabetic pregnancies.

Our previous studies have revealed that naturally occurring polyphenols exert protective effects against high glucose–induced neural tube defects in vitro.^{30,31} Epigallocatechin gallate is the major polyphenol in green tea (*Camellia sinensis*) and makes up to approximately 30% of the solids in green tea.³²

Epigallocatechin gallate is the subject of increasing research interest because it has demonstrated beneficial effects in studies of diabetes, Parkinson's disease, Alzheimer's disease, stroke, and obesity.³³ The cancer-preventive effects of Epigallocatechin gallate have been widely reported in epidemiological, cell culture, animal, and clinical studies.³⁴ One of the mechanisms by which Epigallocatechin gallate exerts effects on cancer cells is through the inhibition of DNA methyltransferases and reactivation of DNA methylation–silenced

gene expression.³⁵ Thus, in the present study, we investigated whether Epigallocatechin gallate could reduce or prevent neural tube defect formation in embryos from diabetic dams and inhibit maternal diabetes–increased DNA methylation.

Methods and Materials

Animals and reagents

All animal procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar harbor, ME). Streptozotocin from Sigma (St Louis, MO) was dissolved in 0.1 M citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, Canada).

The mouse model of diabetic embryopathy

Our mouse model of diabetic embryopathy was described previously.^{21,36} Briefly, female mice were intravenously injected daily with 75 mg/kg streptozotocin over 2 days to induce diabetes. Nondiabetic wild-type mice with vehicle injection served as controls. Diabetes was defined as 12 hour fasting blood glucose levels of ≥ 250 mg/dL, which normally occurred at 3–5 days after streptozotocin injections. Once the level of hyperglycemia indicative of diabetes (≥ 250 mg/dL) was achieved, insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. The mice were then mated with wild-type male mice at 3:00 PM.

We designated that the morning when a vaginal plug was present as embryonic day 0.5. On embryonic day 5.5, insulin pellets were removed to permit frank hyperglycemia (>250 mg/dL glucose level), so the developing conceptuses would be exposed to hyperglycemic conditions. Wild-type, nondiabetic female mice with vehicle injections and sham operation of insulin pellet implants served as nondiabetic controls. On embryonic day 8.75, the mice were euthanized, and conceptuses were dissected out of the uteri for analysis. Embryos were harvested at embryonic day 8.75 for analysis and at embryonic day 10.5 for neural tube defect examination.

At embryonic day 10.5, embryos were examined under a Leica MZ16F stereo-microscope (Leica, Wetzlar, Germany) to identify neural tube defects. Images of embryos were captured by a DFC420 5-megapixel digital camera with software (Leica). Normal embryos were classified as having completely closed neural tube and no evidence of other malformations. Malformed embryos were classified as showing evidence of failed closure of the anterior neural tubes, resulting in exencephaly, a major type of neural tube defect.

Epigallocatechin gallate treatment

Epigallocatechin gallate treatment was performed as described previously.³⁷ Concentrations of either 1 or 10 μ M Epigallocatechin gallate (Sigma) were given to wild-type nondiabetic and diabetic pregnant mice at embryonic day 5.5 in drinking water.

Real-time polymerase chain reaction

Using the Trizol (Invitrogen, Carlsbad, CA), messenger RNA was isolated from embryonic day 8.5 embryos and then reversed transcribed using the high-capacity complementary DNA archive kit (Applied Biosystems, Grand Island, NY). Real-time polymerase chain reaction for *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Grhl3* (grainyhead-like-3), *Pax3* (paired box gene 3), *Tulp3* (tubby-like-3), and β -actin was performed using the Maxima SYBR Green/ROX quantitative polymerase chain reaction master mix assay (Thermo Scientific, Rockford, IL) in the StepOnePlus system (Applied Biosystems, Grand Island, NY). Primer sequences are listed in Table 1.

Western blotting

Western blotting was performed as described previously.²¹ To extract proteins, embryos were sonicated in ice-cold lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitor cocktail (Sigma). Equal amounts of protein from different experimental groups and the Precision Plus protein standards (Bio-Rad Laboratories, Hercules, CA) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and then immunoblotted by primary antibodies at 1:1000 dilutions in 5% nonfat milk. Antibodies to protein DNA methyltransferase-1, DNA methyltransferase-3a, and DNA methyltransferase-3b were purchased from Cell Signaling Technology.

Horseradish peroxidase-conjugated goat antirabbit, goat antimouse (Jackson ImmunoResearch Laboratories, West Grove, PA) or goat antirat (Chemicon, Temecula, CA) secondary antibodies at 1:1000 were used. The intensity of the target protein bands were identified by densitometry and normalized by the densities of β -actin (Abcam, Cambridge, United Kingdom). Signals were detected by SuperSignal West Femto maximum sensitivity substrate kit (Thermo Scientific), and chemiluminescence emitted from bands was captured by a UVP Bioimage EC3 system (Upland, CA). All experiments were repeated 3 times with the use of independently prepared tissue lysates.

Measurement of DNA methyltransferase activities

DNA methyltransferase activities were measured by an EpiQuik DNA methyltransferase activity/inhibition assay kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions. Briefly, we first extracted nuclear from embryonic day 8.5 embryos and then incubated nuclear with substrate and assay buffer for 1 hour, later added capture antibody for wash, after the wash added detection antibody, and finally added fluorodeveloping solution for fluorescence development and measurement.

Detection of global DNA methylation level

The global DNA methylation level was detected by a MethylFlash methylated DNA quantification kit (Colorimetric; Epigentek, Farmingdale, NY) according to the manufacturer's instructions. In brief, we extracted genomic DNA from embryonic day 8.5 embryos and then bound DNA to the assay well, washed the well and added the capture antibody, washed the well again and added detection antibody and enhancer solution, and finally added color developing solution for color development and measurement.

Methylation-specific PCR

DNA methylation patterns in the CpG islands of *Grhl3*, *Pax3*, and *Tulp3* genes were determined by methylation-specific polymerase chain reaction.³⁸ Methylation-specific polymerase chain reaction distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated cytosines to uracil, and subsequent polymerase chain reaction using primers designed for either methylated or unmethylated DNA. Polymerase chain reaction was performed with 3.0 μ L of bisulfite-modified DNA template in a 25 μ L reaction. The CpG islands were identified on the Li Lab website (www.urogene.org). Primer sequences are listed in Table 2.

Statistical analysis

Sample sizes were preestimated based on our previous studies¹⁷ before experiments were performed. Data on neural tube defect rates of each experimental group were analyzed by Fisher exact test or a χ^2 test. Data on protein and messenger RNA expression were presented as means $\pm$ SE. A one-way analysis of variance was performed using the SigmaStat 3.5 software (Systat Software Inc, San Jose, CA) followed by a Tukey test to estimate the significance of results ($P < .05$).

Results

Epigallocatechin gallate ameliorates maternal diabetes–induced neural tube defects

Mouse embryonic neurulation occurs during embryonic day 8.5–10.5.³⁹ To determine whether Epigallocatechin gallate treatment could ameliorate maternal diabetes–induced neural tube defects, concentrations of either 1 μ M or 10 μ M Epigallocatechin gallate were given to wild-type nondiabetic and diabetic pregnant mice at embryonic day 5.5 in drinking water.

The neural tube defect rate in embryos from diabetic dams was significantly higher than that in embryos from nondiabetic dams, with or without Epigallocatechin gallate treatment (Table 3). Treatment with 10 μ M Epigallocatechin gallate dramatically decreased neural tube defect formation in embryos from diabetic dams, compared with untreated diabetic dams (Table 3). Treatment with 1 μ M Epigallocatechin gallate did not reduce maternal diabetes–induced neural tube defects significantly. Therefore, 10 μ M Epigallocatechin gallate was used in subsequent experiments.

Epigallocatechin gallate inhibits maternal diabetes–increased DNA methyltransferase expression

To assess whether Epigallocatechin gallate treatment prevents diabetes–induced neural tube defects by regulating embryonic DNA methylation level, we first examined the DNA methyltransferase expression level in embryos from diabetic dams, with or without Epigallocatechin gallate treatment. Messenger RNA expression of *Dnmt1* and *Dnmt3a* did not differ in embryos from both the nondiabetic and diabetic groups, regardless of Epigallocatechin gallate treatment (Figure 1A). However, *Dnmt3b* messenger RNA expression was significantly higher in embryos from the diabetic group than in embryos

from the nondiabetic group, with or without Epigallocatechin gallate treatment. We also observed that Epigallocatechin gallate treatment blocked maternal diabetes–induced *Dnmt3b* expression (Figure 1A).

We next examined the DNA methyltransferase protein expression by immunoblotting. The levels of all 3 DNA methyltransferases, DNA methyltransferase-1, DNA methyltransferase-3a, and DNA methyltransferase-3b, were significantly higher in embryos from the diabetic group than that in embryos from the nondiabetic group. Treatment with 10 μ M Epigallocatechin gallate suppressed maternal diabetes–increased DNA methyltransferase protein expression, whereas Epigallocatechin gallate treatment did not further suppress DNA methyltransferase protein expression in embryos from the nondiabetic group (Figure 1B). These data suggested that Epigallocatechin gallate treatment inhibits maternal diabetes–increased DNA methyltransferase expression.

Epigallocatechin gallate reduces maternal diabetes–increased DNA methyltransferase activity and global methylation levels

Total DNA methyltransferase activity was determined in embryos from diabetic dams vs nondiabetic dams. DNA methyltransferase activity was dramatically increased in embryos from diabetic dams compared with those in embryos from nondiabetic dams, with or without Epigallocatechin gallate treatment. However, the DNA methyltransferase activity in embryos from diabetic dams treated with 10 μ M Epigallocatechin gallate was similar to those seen in embryos from nondiabetic dams, with or without Epigallocatechin gallate treatment, and was significantly lower than that in the diabetic group without Epigallocatechin gallate treatment (Figure 2A).

We tested whether Epigallocatechin gallate treatment could affect global DNA methylation levels in embryos from diabetic dams. Consistent with our observation of DNA methyltransferase activity, maternal diabetes increased global DNA methylation levels in embryos from the diabetic group, compared with the nondiabetic group, and Epigallocatechin gallate treatment blocked maternal diabetes–increased global DNA methylation levels (Figure 2B).

Epigallocatechin gallate decreases methylation in the CpG islands of neural tube closure essential genes

Hypermethylation of CpG islands in the promoter regions of genes involved in embryogenesis is an important mechanism to silence gene expression.³⁵ To determine whether maternal diabetes induces hypermethylation of CpG islands in the promoters of neural tube closure essential genes, we measured DNA methylation in the CpG islands of several neural tube closure essential genes, including *Grhl3*, *Pax3*, and *Tulp3*. CpG islands were identified the promoter regions of the *Grhl3*, *Pax3*, and *Tulp3* gene (Figure 3A). We defined a CpG island using the following criteria: size >200 bp, guanine-cytosine percentage >50%, CpG observed/CpG expected >0.6.⁴⁰

Through methylation-specific polymerase chain reaction, we observed that DNA methylation levels in the CpG islands of *Grhl3*, *Pax3*, and *Tulp3* were increased in embryos

from diabetic dams (Figure 3B). Epigallocatechin gallate treatment abrogated maternal diabetes–increased methylation in CpG islands of these 3 genes (Figure 3B).

In addition, we measured the messenger RNA levels of *Grhl3*, *Pax3*, and *Tulp3*. Consistent with increased DNA methylation in the promoters of these 3 genes, the messenger RNA levels of these genes were significantly down-regulated by maternal diabetes, and treatment with 10 μ M Epigallocatechin gallate reversed maternal diabetes–suppressed *Grhl3*, *Pax3*, and *Tulp3* expression (Figure 3C).

Comment

Although it is well documented that maternal diabetes induces neural tube defects in offspring^{1–3,6} and it has been reported that altered DNA methylation is involved in neural tube defect formation,^{27–29,35} the relationship between maternal diabetes–induced neural tube defects and DNA methylation remains unclear. Here we demonstrated that neural tube defects were reduced in association with reduced DNA methylation.

Yet the causal relationship between Epigallocatechin gallate–suppressed DNA methylation and Epigallocatechin gallate–reduced neural tube defects was not established. Future studies on a dose-response effect of Epigallocatechin gallate on both DNA methylation of the NT closure genes and neural tube defect formation is essential for the establishment of this causal relationship.

Epigallocatechin gallate is the major polyphenol in green tea and has beneficial effects in preventing the negative effects of cancer, diabetes, and Parkinson's disease, among other conditions.³³ Previous studies have indicated that Epigallocatechin gallate inhibits DNA methyltransferase activity, causes CpG island hypomethylation, and reactivates hypermethylation-silenced genes.³⁵

Here we revealed that Epigallocatechin gallate treatment blocks hypermethylation in promoters of neural tube closure–essential genes during embryogenesis and ameliorates diabetes-induced neural tube defects. DNA hypermethylation is a critical epigenetic mechanism for the silencing of many genes, including those essential for neural tube closure.^{28,41,42} DNA hypermethylation of CpG islands in the promoters of active genes is a mechanism for locking the chromatin in a repressed state.^{43,44}

In the present study, we showed that DNA hypermethylation of the CpG islands of several neural tube closure–essential genes, including *Grhl3*, *Pax3*, and *Tulp3*, occurs in response to maternal diabetes. *Grhl3* is required for neural tube closure, and its null mutants exhibit neural tube defects similar to those observed in diabetic embryopathy.⁴⁵ Loss of *Pax3* also results in neural tube defects by having a negative impact on the folate metabolic pathway.^{46,47} *Tulp3*-null embryos are manifested in neural tube defects through the induction of excessive neuroepithelial cellapoptosis.⁴⁸

The expression of these 3 genes is inhibited by maternal diabetes in neurulation-stage embryos; however, the underlying mechanism of the inhibition of these 3 gene is unknown.

The present study demonstrates that DNA hypermethylation in the CpG islands of the promoters of these 3 genes is responsible for their down-regulation in diabetic embryopathy.

Because Epigallocatechin gallate is a DNA methylation inhibitor and reduces high glucose–induced neural tube defect formation in vitro,⁴⁹ we tested 1 and 10 μ M Epigallocatechin gallate to determine which dose might have a protective effect against maternal diabetes–induced neural tube defect formation. Both doses of Epigallocatechin gallate reduced neural tube defect incidence in embryos from diabetic dams; however, only 10 μ M Epigallocatechin gallate significantly decreased the risk of neural tube defects.

In the present study, we demonstrated that Epigallocatechin gallate treatment blocks maternal diabetes–induced neural tube defects through the inhibition of DNA hypermethylation of the promoters of neural tube closure–essential genes. Future studies that explore Epigallocatechin gallate as a therapeutic intervention against maternal diabetes–induced neural tube defects will need to carefully examine any potential toxicities high-dose Epigallocatechin gallate may have to the developing embryo. However, we did not observe any Epigallocatechin gallate toxicities to the developing embryo.

Epigallocatechin gallate has been used as a dietary supplement for humans. As a stand-alone supplement, Epigallocatechin gallate is inexpensive and comes in a capsule form that can be taken orally.⁵⁰ Epigallocatechin gallate capsules (200 mg) taken daily for 12 weeks in patients with human papilloma virus–infected cervical lesions are safe and effective.⁵¹ Epigallocatechin gallate has also been shown to be safe and effective in other human diseases.³³ Human studies have demonstrated that high Epigallocatechin gallate doses, equivalent to the doses we used, exert beneficial effects.⁵² The Epigallocatechin gallate dosing typically available in current pharmacopoeia⁵² are in a close range of the doses used in the current study and forms the basis for future human clinical trials.

In our previous and present studies, we have demonstrated that maternal diabetes–increased DNA methylation and that maternal diabetes–induced oxidative stress are involved in neural tube defect formation.^{1–3,6,53} Understanding how to diminish DNA hypermethylation and oxidative stress in embryos from diabetic dams may be critical to reducing the risk of neural tube defects in the offspring of diabetic mothers.

DNA hypermethylation is involved in many pathological processes during embryogenesis, including adverse fetal programming, systemic sclerosis, systemic inflammatory syndrome, autism spectrum disorders, gestational diabetes, offspring obesity, fetal telomere length, and stem cell epigenetics.^{54–62} Similarly, oxidative stress is the pathogenesis of a variety of adverse pregnancy outcomes including preeclampsia, fetal alcohol syndrome, fetal brain inflammation, and maternal inflammation–induced offspring cerebral injury,^{61,63–69} and the effects of antioxidant treatments have been the subjective of intensive investigations.^{70–72}

Recent studies have demonstrated that alterations of epigenetic modifications, including DNA methylation and histone acetylation, are involved in the pathogenesis of an array of adverse pregnancy outcomes.^{41,55,58–62,73} Thus, our finding that Epigallocatechin gallate prevents diabetic embryopathy by inhibiting DNA hypermethylation has a significantly high

clinical value. Epigallocatechin gallate may be effective in preventing adverse pregnancy outcomes associated with epigenetic modifications.^{41,55,58–62,73}

In addition to neural tube defects, Epigallocatechin gallate may also be effective in correcting epigenetic alterations associated in other structural birth defects such as congenital heart defects.^{1,8,56} Although the efficacy of general antioxidants, including folic acid, in reducing adverse pregnancy outcomes is controversial,^{74–76} antioxidants with specific inhibitory effects on DNA methylation, such as Epigallocatechin gallate, may be better therapeutics.

Consistent with our current observation of Epigallocatechin gallate treatment preventing neural tube defects, recent studies have revealed the effectiveness of a group of natural compounds in preventing diabetic embryopathy in animal models.^{30,31,37,49,77} However, the effectiveness of these natural compounds needs be tested in human diabetic pregnancies.

In addition to birth defects, maternal diabetes causes a variety of adverse pregnancy outcomes including preterm birth, small for gestational age, fetal hypertrophic cardiomyopathy, preeclampsia, stillbirth and perinatal deaths.^{78–84} Future studies may test the effectiveness of Epigallocatechin gallate on these adverse pregnancy outcomes induced by maternal diabetes.

Previous studies have demonstrated that neural tube defects may result from diabetes as a consequence of oxidative stress, endoplasmic reticulum stress, or apoptosis.^{17,85} Neural tube closure—essential genes, which are repressed by DNA methylation, are required for cell survival and cellular homeostasis.⁸⁶ Therefore, future studies will aim to reveal the relationship of these factors with Epigallocatechin gallate treatment.

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References

1. Wang F, Wu Y, Quon MJ, Li X, Yang P. ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development. *Am J Physiol Endocrinol Metab.* 2015; 309:E487–99. [PubMed: 26173459]
2. Gabbay-Benziv R, Reece EA, Wang F, Yang P. Birth defects in pregestational diabetes: defect range, glycemic threshold and pathogenesis. *World J Diabetes.* 2015; 6:481–8. [PubMed: 25897357]
3. Yang P, Reece EA, Wang F, Gabbay-Benziv R. Decoding the oxidative stress hypothesis in diabetic embryopathy through proapoptotic kinase signaling. *Am J Obstet Gynecol.* 2015; 212:569–79. [PubMed: 25434839]
4. Wang F, Reece EA, Yang P. Advances in revealing the molecular targets downstream of oxidative stress-induced proapoptotic kinase signaling in diabetic embryopathy. *Am J Obstet Gynecol.* 2015; 213:125–34. [PubMed: 25595581]

5. Gu H, Yu J, Dong D, et al. High glucose-repressed CITED2 expression through miR-200b triggers the unfolded protein response and endoplasmic reticulum stress. *Diabetes*. 2016; 65:149–63. [PubMed: 26450995]
6. Dong D, Reece EA, Lin X, Wu Y, Arias-Vilella N, Yang P. New development of the yolk sac theory in diabetic embryopathy: molecular mechanism and link to structural birth defects. *Am J Obstet Gynecol*. 2016; 214:192–202. [PubMed: 26432466]
7. Yang P, Zhao Z, Reece EA. Activation of oxidative stress signaling that is implicated in apoptosis with a mouse model of diabetic embryopathy. *Am J Obstet Gynecol*. 2008; 198:130.E1–7. [PubMed: 18166327]
8. Wang F, Fisher SA, Zhong J, Wu Y, Yang P. Superoxide Dismutase 1 in vivo ameliorates maternal diabetes mellitus-induced apoptosis and heart defects through restoration of impaired Wnt signaling. *Circ Cardiovasc Genet*. 2015; 8:665–76. [PubMed: 26232087]
9. Wang F, Weng H, Quon MJ, et al. Dominant negative FADD dissipates the proapoptotic signalosome of the unfolded protein response in diabetic embryopathy. *Am J Physiol Endocrinol Metab*. 2015; 309:E861–73. [PubMed: 26419589]
10. Becerra JE, Khoury MJ, Cordero JF, Erickson JD. Diabetes mellitus during pregnancy and the risks for specific birth defects: a population-based case-control study. *Pediatrics*. 1990; 85:1–9. [PubMed: 2404255]
11. Reece EA, Wiznitzer A, Homko CJ, Hagay Z, Wu YK. Synchronization of the factors critical for diabetic teratogenesis: an in vitro model. *Am J Obstet Gynecol*. 1996; 174:1284–8. [PubMed: 8623857]
12. Oakley GP Jr. Failing to prevent birth defects caused by maternal diabetes mellitus. *Am J Obstet Gynecol*. 2012; 206:179–80. [PubMed: 22381596]
13. Correa A, Gilboa SM, Botto LD, et al. Lack of periconceptional vitamins or supplements that contain folic acid and diabetes mellitus-associated birth defects. *Am J Obstet Gynecol*. 2012; 206:218.e1–13. [PubMed: 22284962]
14. Au KS, Ashley-Koch A, Northrup H. Epidemiologic and genetic aspects of spina bifida and other neural tube defects. *Dev Disabil Res Rev*. 2010; 16:6–15. [PubMed: 20419766]
15. Correa A, Gilboa SM, Besser LM, et al. Diabetes mellitus and birth defects. *Am J Obstet Gynecol*. 2008; 199:237.e1–9. [PubMed: 18674752]
16. Li X, Weng H, Reece EA, Yang P. SOD1 overexpression in vivo blocks hyperglycemia-induced specific PKC isoforms: substrate activation and consequent lipid peroxidation in diabetic embryopathy. *Am J Obstet Gynecol*. 2011; 205:84.e1–6. [PubMed: 21529760]
17. Yang PX, Li XZ, Xu C, et al. Maternal hyperglycemia activates an ASK1-FoxO3a-caspase 8 pathway that leads to embryonic neural tube defects. *Sci Signal*. 2013 Aug 27;6:ra74. [PubMed: 23982205]
18. Weng H, Li X, Reece EA, Yang P. SOD1 suppresses maternal hyperglycemia-increased iNOS expression and consequent nitrosative stress in diabetic embryopathy. *Am J Obstet Gynecol*. 2012; 206:448.e1–7.
19. Yang P, Cao Y, Li H. Hyperglycemia induces inducible nitric oxide synthase gene expression and consequent nitrosative stress via c-Jun N-terminal kinase activation. *Am J Obstet Gynecol*. 2010; 203:185.e5–11.
20. Yang P, Zhao Z, Reece EA. Blockade of c-Jun N-terminal kinase activation abrogates hyperglycemia-induced yolk sac vasculopathy in vitro. *Am J Obstet Gynecol*. 2008; 198:321.e1–7. [PubMed: 18177823]
21. Yang P, Li X, Xu C, et al. Maternal hyperglycemia activates an ASK1-FoxO3a-caspase 8 pathway that leads to embryonic neural tube defects. *Sci Signal*. 2013; 6:ra74. [PubMed: 23982205]
22. Li XZ, Xu C, Yang PX. c-Jun NH2-terminal kinase 1/2 and endoplasmic reticulum stress as interdependent and reciprocal causation in diabetic embryopathy. *Diabetes*. 2013; 62:599–608. [PubMed: 22961085]
23. Wu Y, Wang F, Fu M, Wang C, Quon MJ, Yang P. Cellular stress, excessive apoptosis, and the effect of metformin in a mouse model of type 2 diabetic embryopathy. *Diabetes*. 2015; 64:2526–36. [PubMed: 25720389]

24. Wang F, Reece EA, Yang P. Oxidative stress is responsible for maternal diabetes-impaired transforming growth factor beta signaling in the developing mouse heart. *Am J Obstet Gynecol.* 2015; 212:650.e1–11. [PubMed: 25595579]
25. Gu H, Yu J, Dong D, Zhou Q, Wang JY, Yang P. The miR-322-TRAF3 circuit mediates the pro-apoptotic effect of high glucose on neural stem cells. *Toxicol Sci e.* 2015; 144:186–96.
26. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res.* 2010; 107:1058–70. [PubMed: 21030723]
27. Dunlevy LPE, Burren KA, Mills K, Chitty LS, Copp AJ, Greene NDE. Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Defects Res A.* 2006; 76:544–52.
28. Imbard A, Benoist JF, Blom HJ. Neural tube defects, folic acid and methylation. *Int J Environ Res Public Health.* 2013; 10:4352–89. [PubMed: 24048206]
29. Afman LA, Blom HJ, Driittij MJ, Brouns MR, van Straaten HW. Inhibition of transmethylation disturbs neurulation in chick embryos. *Brain Res Dev Brain Res.* 2005; 158:59–65. [PubMed: 15996755]
30. Wu Y, Wang F, Reece EA, Yang P. Curcumin ameliorates high glucose-induced neural tube defects by suppressing cellular stress and apoptosis. *Am J Obstet Gynecol.* 2015; 212:802.e1–8. [PubMed: 25595578]
31. Zhong J, Reece EA, Yang P. Punicagin exerts protective effect against high glucose-induced cellular stress and neural tube defects. *Biochem Biophys Res Commun.* 2015; 467:179–84. [PubMed: 26453010]
32. Yang CS, Wang X, Lu G, Picinich SC. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer.* 2009; 9:429–39. [PubMed: 19472429]
33. Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem Pharmacol.* 2011; 82:1807–21. [PubMed: 21827739]
34. Thawonsuan J, Kiron V, Satoh S, Panigrahi A, Verlhac V. Epigallocatechin-3-gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol Biochem.* 2010; 36:687–97. [PubMed: 19680766]
35. Fang MZ, Wang Y, Ai N, et al. Tea polyphenol (–)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* 2003; 63:7563–70.
36. Li XZ, Weng HB, Xu C, Reece EA, Yang PX. Oxidative Stress-induced JNK1/2 activation triggers proapoptotic signaling and apoptosis that leads to diabetic embryopathy. *Diabetes.* 2012; 61:2084–92. [PubMed: 22688338]
37. Xu C, Li XZ, Wang F, Weng HB, Yang PX. Trehalose prevents neural tube defects by correcting maternal diabetes-suppressed autophagy and neurogenesis. *Am J Physiol Endocr Metab.* 2013; 305:E667–78.
38. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA.* 1996; 93:9821–6. [PubMed: 8790415]
39. Sakai Y. Neurulation in the mouse: manner and timing of neural tube closure. *Anat Record.* 1989; 223:194–203. [PubMed: 2712345]
40. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proce Natl Acad Sci USA.* 2006; 103:1412–7.
41. Januar V, Desoye G, Novakovic B, Cvitic S, Saffery R. Epigenetic regulation of human placental function and pregnancy outcome: considerations for causal inference. *Am J Obstet Gynecol.* 2015; 213:S182–96. [PubMed: 26428498]
42. Shih Ie M, Chen L, Wang CC, et al. Distinct DNA methylation profiles in ovarian serous neoplasms and their implications in ovarian carcinogenesis. *Am J Obstet Gynecol.* 2010; 203:584.e1–22. [PubMed: 20965493]
43. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene.* 2002; 21:5427–40. [PubMed: 12154405]

44. Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene*. 2002; 21:5380–7. [PubMed: 12154400]
45. Ting SB, Wilanowski T, Auden A, et al. Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor Grhl-3. *Nat Med*. 2003; 9:1513–9. [PubMed: 14608380]
46. Trembath D, Sherbondy AL, Vandyke DC, et al. Analysis of select folate pathway genes, PAX3, and human T in a Midwestern neural tube defect population. *Teratology*. 1999; 59:331–41. [PubMed: 10332959]
47. Burren KA, Savery D, Massa V, et al. Gene-environment interactions in the causation of neural tube defects: folate deficiency increases susceptibility conferred by loss of Pax3 function. *Hum Mol Genet*. 2008; 17:3675–85. [PubMed: 18753144]
48. Ikeda A, Ikeda S, Gridley T, Nishina PM, Naggert JK. Neural tube defects and neuroepithelial cell death in Tulp3 knockout mice. *Hum Mol Genet*. 2001; 10:1325–34. [PubMed: 11406614]
49. Yang P, Li H. Epigallocatechin-3-gallate ameliorates hyperglycemia-induced embryonic vasculopathy and malformation by inhibition of Foxo3a activation. *Am J Obstet Gynecol*. 2010; 203:75.e1–6.
50. Sartippour MR, Shao ZM, Heber D, et al. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr*. 2002; 132:2307–11. [PubMed: 12163680]
51. Ahn WS, Yoo J, Huh SW, et al. Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions. *Eur J Cancer Prev*. 2003; 12:383–90.
52. Thielecke F, Boschmann M. The potential role of green tea catechins in the prevention of the metabolic syndrome—a review. *Phytochemistry*. 2009; 70:11–24. [PubMed: 19147161]
53. Dong D, Yu J, Wu Y, Fu N, Villela NA, Yang P. Maternal diabetes triggers DNA damage and DNA damage response in neurulation stage embryos through oxidative stress. *Biochem Biophys Res Commun*. 2015; 467:407–12. [PubMed: 26427872]
54. Hartley JD, Ferguson BJ, Moffett A. The role of shed placental DNA in the systemic inflammatory syndrome of preeclampsia. *Am J Obstet Gynecol*. 2015; 213:268–77. [PubMed: 25794631]
55. Behnia F, Parets SE, Kechichian T, et al. Fetal DNA methylation of autism spectrum disorders candidate genes: association with spontaneous preterm birth. *Am J Obstet Gynecol*. 2015; 212:533.e1–9. [PubMed: 25687563]
56. Karachaliou M, Georgiou V, Roumeliotaki T, et al. Association of trimester-specific gestational weight gain with fetal growth, offspring obesity, and cardiometabolic traits in early childhood. *Am J Obstet Gynecol*. 2015; 212:502.e1–14. [PubMed: 25557209]
57. Salihu HM, Pradhan A, King L, et al. Impact of intrauterine tobacco exposure on fetal telomere length. *Am J Obstet Gynecol*. 2015; 212:205.e1–8. [PubMed: 25173189]
58. Almgren M, Schlinzig T, Gomez-Cabrero D, et al. Cesarean delivery and hematopoietic stem cell epigenetics in the newborn infant: implications for future health? *Am J Obstet Gynecol*. 2014; 211:502.e1–8. [PubMed: 24996659]
59. Lesseur C, Armstrong DA, Paquette AG, Li Z, Padbury JF, Marsit CJ. Maternal obesity and gestational diabetes are associated with placental leptin DNA methylation. *Am J Obstet Gynecol*. 2014; 211:654.e1–9. [PubMed: 24954653]
60. Suter MA, Ma J, Vuguin PM, et al. In utero exposure to a maternal high-fat diet alters the epigenetic histone code in a murine model. *Am J Obstet Gynecol*. 2014; 210:463.e1–11. [PubMed: 24793723]
61. Donzelli G, Carnesecchi G, Amador C, et al. Fetal programming and systemic sclerosis. *Am J Obstet Gynecol*. 2015; 213:839.e1–8. [PubMed: 26232509]
62. Liu Y, Hoyo C, Murphy S, et al. DNA methylation at imprint regulatory regions in pre-term birth and infection. *Am J Obstet Gynecol*. 2013; 208:395.e1–7. [PubMed: 23477525]
63. Hamel MS, Hughes BL, Rouse DJ. Whither oxygen for intrauterine resuscitation? *Am J Obstet Gynecol*. 2015; 212:461–2.e1. [PubMed: 25659471]
64. Lim R, Acharya R, Delpachitra P, et al. Activin and NADPH-oxidase in preeclampsia: insights from in vitro and murine studies. *Am J Obstet Gynecol*. 2015; 212:86.e1–12. [PubMed: 25046804]
65. Hill AJ, Drever N, Yin H, Tamayo E, Saade G, Bytautiene E. The role of NADPH oxidase in a mouse model of fetal alcohol syndrome. *Am J Obstet Gynecol*. 2014; 210:466.e1–5. [PubMed: 24334207]

66. Macones GA. Applying for fellowships in the United States: an expensive and risky proposition. *Am J Obstet Gynecol.* 2014; 210:500. [PubMed: 24878203]
67. Simoes RV, Cruz-Lemini M, Bargallo N, Gratacos E, Sanz-Cortes M. Brain metabolite differences in one-year-old infants born small at term and association with neurodevelopmental outcome. *Am J Obstet Gynecol.* 2015; 213:210.e1–11. [PubMed: 25891998]
68. Kase BA, Northrup H, Au KS. Novel single nucleotide polymorphisms in the superoxide dismutase 1 and 2 genes among children with myelomeningocele. *Am J Obstet Gynecol.* 2013; 209:388.e1–7. [PubMed: 23792044]
69. Beloosesky R, Ginsberg Y, Khatib N, et al. Prophylactic maternal N-acetylcysteine in rats prevents maternal inflammation-induced offspring cerebral injury shown on magnetic resonance imaging. *Am J Obstet Gynecol.* 2013; 208:213.e1–6. [PubMed: 23433325]
70. Kalanderian A, Abate N, Patrikeev I, et al. Pioglitazone therapy in mouse offspring exposed to maternal obesity. *Am J Obstet Gynecol.* 2013; 208:308.e1–7. [PubMed: 23313309]
71. Grigsby PL, Novy MJ, Sadowsky DW, et al. Maternal azithromycin therapy for *Ureaplasma* intraamniotic infection delays pre-term delivery and reduces fetal lung injury in a primate model. *Am J Obstet Gynecol.* 2012; 207:475.e1–14. [PubMed: 23111115]
72. Wakimoto T, Uchida K, Mimura K, et al. Hydroxylated fullerene: a potential antiinflammatory and antioxidant agent for preventing mouse preterm birth. *Am J Obstet Gynecol.* 2015; 213:708.e1–9. [PubMed: 26196453]
73. Monk D. Genomic imprinting in the human placenta. *Am J Obstet Gynecol.* 2015; 213:S152–62. [PubMed: 26428495]
74. Bateman BT, Huybrechts KF, Fischer MA, et al. Chronic hypertension in pregnancy and the risk of congenital malformations: a cohort study. *Am J Obstet Gynecol.* 2015; 212:337.e1–14. [PubMed: 25265405]
75. Agopian AJ, Lupo PJ, Canfield MA, Mitchell LE. National Birth Defects Prevention Study. Swimming pool use and birth defect risk. *Am J Obstet Gynecol.* 2013; 209:219.e1–9. [PubMed: 23628264]
76. Hernandez RK, Werler MM, Romitti P, Sun L, Anderka M. National Birth Defects Prevention Study. Nonsteroidal antiinflammatory drug use among women and the risk of birth defects. *Am J Obstet Gynecol.* 2012; 206:228.e1–8. [PubMed: 22196851]
77. Dong D, Reece EA, Yang P. The Nrf2 activator vinylsulfone reduces high glucose-induced neural tube defects by suppressing cellular stress and apoptosis. *Reprod Sci.* Jan 21. pii: 1933719115625846. [Epub ahead of print].
78. Hauth JC, Clifton RG, Roberts JM, et al. Maternal insulin resistance and preeclampsia. *Am J Obstet Gynecol.* 2011; 204:327.e1–6. [PubMed: 21458622]
79. Gonzalez AB, Young L, Doll JA, Morgan GM, Crawford SE, Plunkett BA. Elevated neonatal insulin-like growth factor I is associated with fetal hypertrophic cardiomyopathy in diabetic women. *Am J Obstet Gynecol.* 2014; 211:290.e1–7. [PubMed: 24813596]
80. Boghossian NS, Yeung E, Albert PS, et al. Changes in diabetes status between pregnancies and impact on subsequent newborn outcomes. *Am J Obstet Gynecol.* 2014; 210:431.e1–14. [PubMed: 24361790]
81. Yee LM, Cheng YW, Inturrisi M, Caughey AB. Effect of gestational weight gain on perinatal outcomes in women with type 2 diabetes mellitus using the 2009 Institute of Medicine guidelines. *Am J Obstet Gynecol.* 2011; 205:257.e1–6. [PubMed: 22071055]
82. Peterson C, Grosse SD, Li R, et al. Preventable health and cost burden of adverse birth outcomes associated with pregestational diabetes in the United States. *Am J Obstet Gynecol.* 2015; 212:74.e1–9. [PubMed: 25439811]
83. Yanit KE, Snowden JM, Cheng YW, Caughey AB. The impact of chronic hypertension and pregestational diabetes on pregnancy outcomes. *Am J Obstet Gynecol.* 2012; 207:333.e1–6. [PubMed: 22892187]
84. Desai N, Roman A, Rochelson B, et al. Maternal metformin treatment decreases fetal inflammation in a rat model of obesity and metabolic syndrome. *Am J Obstet Gynecol.* 2013; 209:136.e1–9. [PubMed: 23659985]

85. Wang F, Wu Y, Gu H, et al. Ask1 gene deletion blocks maternal diabetes-induced endoplasmic reticulum stress in the developing embryo by disrupting the unfolded protein response signalosome. *Diabetes*. 2015; 64:973–88. [PubMed: 25249581]
86. Harris MJ, Juriloff DM. An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res Part A Clin Mol Teratol*. 2010; 88:653–69. [PubMed: 20740593]

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FIGURE 1.

Epigallocatechin gallate treatment blocks maternal diabetes–increased DNA methyltransferase expression

A, Messenger RNA levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. **B**, Protein levels of DNA methyltransferase-1, DNA methyltransferase-3a, and DNA methyltransferase-3b in E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. Experiments were performed using 3 embryos from 3 different dams per group ($n = 3$). *Asterisk* indicates significant difference compared with the other groups ($P < .05$).

DM, diabetic mellitus; *E*, embryonic day; *EGCG*, Epigallocatechin gallate; *NC*, nondiabetic control; *NC-EGCG*, nondiabetic control–Epigallocatechin gallate; *DM-EGCG*, diabetic mellitus–Epigallocatechin gallate.

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FIGURE 2.

EGCG inhibits diabetes–increased DNA methyltransferase activity and global DNA methylation

A, Total DNA methyltransferase activity was tested in E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. **B**, Global DNA methylation levels were determined in E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. Experiments were performed using 3 embryos from 3 different dams per group. *Asterisk* indicates significant differences compared with the other groups ($P < .05$).

DM, diabetic mellitus; *E*, embryonic day; *EGCG*, Epigallocatechin gallate; *NC*, nondiabetic control; *NC-EGCG*, nondiabetic control–Epigallocatechin gallate; *DM-EGCG*, diabetic mellitus–Epigallocatechin gallate.

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FIGURE 3.

EGCG reduces DNA hypermethylation in gene promoter CpG islands

A, CpG islands (blue) in the promoter regions of neural tube closure–essential genes, including *Grhl3*, *Pax3*, and *Tulp3*. **B**, Methylation levels in CpG islands of *Grhl3*, *Pax3*, and *Tulp3* were detected in the E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. **C**, Messenger RNA levels of *Grhl3*, *Pax3*, and *Tulp3* were determined in the E8.75 embryos from nondiabetic and diabetic dams with or without Epigallocatechin gallate treatment. Experiments were performed using 3 embryos from 3 different dams per group. Asterisk indicates significant differences compared with the other groups ($P < .05$).

DM, diabetic mellitus; DM-EGCG, diabetic mellitus-Epigallocatechin gallate; E, embryonic day; EGCG, Epigallocatechin gallate; MSP, methylation-specific primer; NC, nondiabetic control; NC-EGCG, nondiabetic control-Epigallocatechin gallate; NSP, nonmethylation-specific primer.

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TABLE 1

Primers used for RT-PCR

Primer name	Primer sequences
<i>Dnmt1</i>	Forward primer, 5'- AAGAATGGTGTGTCTACCGAC -3'
	Reverse primer, 5'- CATCCAGGTTGCTCCCCTTG -3'
<i>Dnmt3a</i>	Forward primer, 5'- GATGAGCCTGAGTATGAGGATGG -3'
	Reverse primer, 5'- CAAGACACAATTCGGCCTGG -3'
<i>Dnmt3b</i>	Forward primer, 5'- CGTTAATGGGAACCTCAGTGACC -3'
	Reverse primer, 5'- GGGAGCATCCTTCGTGTCTG -3'
<i>Grhl3</i>	Forward primer, 5'- CCCGGCAAGACCAATACCG -3'
	Reverse primer, 5'- AACCCCATGAATGCTCTCAAAT -3'
<i>Pax3</i>	Forward primer, 5'- TTTCACCTCAGGTAATGGGACT -3'
	Reverse primer, 5'- GAACGTCCAAGGCTTACTTTGT -3'
<i>Tulp3</i>	Forward primer, 5'- CCAAAAACACGGCATCTTGAG -3'
	Reverse primer, 5'- GGGCTATACGCAAAGTCCTCTAA -3'
<i>β-Actin</i>	Forward primer, 5'- GTGACGTTGACATCCGTAAAGA -3'
	Reverse primer, 5'- GCCGGACTCATCGTACTCC -3'

RT-PCR, Real-time polymerase chain reaction.Zhong et al. The green tea polyphenol EGCG prevents diabetic embryopathy. *Am J Obstet Gynecol* 2016.

TABLE 2

Primers used for MSP

Primers name	Primer sequences
<i>Grhl3</i>	Left M primer, 5'- TTAAAGCGTAACGTAGAGTAAACGT -3'
	Right M primer, 5'- ACCTCGATATACTAAAAAACCGAA -3'
	Left U primer, 5'- TTAAAGTGTAATGTAGAGTAAATGT -3'
	Right U primer, 5'- ACCTCAATATACTAAAAAACCAAA -3'
<i>Pax3</i>	Left M primer, 5'- GTATTGTGTTTCGTTTTTCGTTTC -3'
	Right M primer, 5'- GCTACGTAAATAATTCTACCCCGA -3'
	Left U primer, 5'- TTGTGTTTGTTTTTTGTGTTT -3'
	Right U primer, 5'- ACTACATAAATAATTCTACCCCAAAC -3'
<i>Tulp3</i>	Left M primer, 5'- TTTTCGATTTTTTTATTTGTAATGC -3'
	Right M primer, 5'- CAACTCAATTCTAATCCTACTCGTA -3'
	Left U primer, 5'- TTTGATTTTTTTATTTGTAATGTGT -3'
	Right U primer, 5'- CCAACTCAATTCTAATCCTACTCATA -3'

M, methylated; *MSP*, methylation-specific polymerase chain reaction; *U*, unmethylated.

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TABLE 3

EGCG treatment ameliorates maternal diabetes-induced neural tube defects

Group	Total embryos	Embryos with NTD	Blood glucose levels	NTD rate, %
Nondiabetic, no EGCG (n = 12)	85	0	167.71 ± 7.1	0
Nondiabetic, 1 μ M EGCG (n = 4)	27	0	157.3 ± 8.2	0
Nondiabetic, 10 μ M EGCG (n = 4)	24	0	152.5 ± 11.3	0
Diabetic, no EGCG (n = 12)	78	23	442.2 ± 14.7	29.5 ^a
Diabetic, 1 μ M EGCG (n = 7)	46	11	417.5 ± 18.4	23.9 ^a
Diabetic, 10 μ M EGCG (n = 8)	51	1	406.0 ± 20.1	2.0

EGCG, Epigallocatechin gallate; NTD, neural tube defect.

^aSignificant difference compared with the other groups in χ^2 test ($P < .05$). The diabetic no Epigallocatechin gallate group is significantly different when compared with the nondiabetic no Epigallocatechin gallate, nondiabetic 1 μ M Epigallocatechin gallate, nondiabetic 10 μ M Epigallocatechin gallate, and diabetic 10 μ M Epigallocatechin gallate groups. The diabetic 1 μ M Epigallocatechin gallate group and the diabetic no Epigallocatechin gallate group are not significantly different, and the diabetic 1 μ M Epigallocatechin gallate group is significantly different when compared with the nondiabetic no Epigallocatechin gallate, nondiabetic 1 μ M Epigallocatechin gallate, nondiabetic 10 μ M Epigallocatechin gallate, and diabetic 10 μ M Epigallocatechin gallate groups.

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