OBSTETRICS

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

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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 μM 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 μM Epigallocatechin gallate did not reduce maternal diabetes—induced neural tube defects significantly. Embryos from diabetic dams treated with 10 μM 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 μM Epigallocatechin gallate abrogated maternal diabetes—increased DNA methylation in the CpG islands of neural tube closure essential genes, including *Grhl3*, *Pax3*, and *Tulp3*.

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.

Key words: 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 others[26] 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]

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TABLE 1
Primers used for RT-PCR

Primer name	Primer sequences
Dnmt1	Forward primer, 5'- AAGAATGGTGTTGTCTACCGAC -3'
	Reverse primer, 5'- CATCCAGGTTGCTCCCCTTG -3'
Dnmt3a	Forward primer, 5'- GATGAGCCTGAGTATGAGGATGG -3'
	Reverse primer, 5'- CAAGACACAATTCGGCCTGG -3'
Dnmt3b	Forward primer, 5'- CGTTAATGGGAACTTCAGTGACC -3'
	Reverse primer, 5'- GGGAGCATCCTTCGTGTCTG -3'
Grhl3	Forward primer, 5'- CCCGGCAAGACCAATACCG -3'
	Reverse primer, 5'- AACCCCATGAATGCTCTCAAAT -3'
Pax3	Forward primer, 5'- TTTCACCTCAGGTAATGGGACT -3'
	Reverse primer, 5'- GAACGTCCAAGGCTTACTTTGT -3'
Tulp3	Forward primer, 5'- CCAAAAACACGGCATCTTGAG -3'
	Reverse primer, 5'- GGGCTATACGCAAAGTCCTCTAA -3'
β-Actin	Forward primer, 5'- GTGACGTTGACATCCGTAAAGA -3'
	Reverse primer, 5'- GCCGGACTCATCGTACTCC -3'

RT-PCR, Real-time polymerase chain reaction.
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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 stereomicroscope (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 μM 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.