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Modulation of nuclear factor- κ B signaling and reduction of neural tube defects by quercetin-3-glucoside in embryos of diabetic mice

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Abstract

Background—Diabetes mellitus in early pregnancy increases the risk of birth defects in infants. Maternal hyperglycemia stimulates the expression of nitric oxide (NO) synthase 2 (NOS2), which can be regulated by transcription factors of the nuclear factor- κ B (NF- κ B) family. Increases in reactive nitrogen species (RNS) generate intracellular stress conditions, including nitrosative, oxidative, and endoplasmic reticulum (ER) stresses, and trigger programmed cell death (or apoptosis) in the neural folds, resulting in neural tube defects (NTDs) in the embryo. Inhibiting NOS2 can reduce NTDs; however, the underlying mechanisms require further delineation. Targeting NOS2 and associated nitrosative stress using naturally occurring phytochemicals is a potential approach to preventing birth defects in diabetic pregnancies.

Objectives—This study aims to investigate the effect of quercetin-3-glucoside (Q3G), a polyphenol flavonoid found in fruit, in reducing maternal diabetes-induced NTDs in an animal model, and to delineate the molecular mechanisms underlying Q3G action in regulating NOS2 expression.

Study Design—Female mice (C57BL/6) were induced to develop diabetes using streptozotocin before pregnancy. Diabetic pregnant mice were administered Q3G (100 mg/kg) daily via gavage feeding, introduction of drug to the stomach directly via a feeding needle, during neurulation from embryonic (E) day 6.5 to E9.5. After treatment, E10.5 embryos were collected and examined for

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Implications and Contributions:

- A. Diabetes mellitus in early pregnancy increases the risk of birth defects in infants. The underlying mechanisms and interventions for prevention remain to be explored.
- B. Oral treatment with naturally occurring quercetin-3-glucoside (Q3G) reduces neural tube defects in embryos of diabetic mice. Q3G suppresses the activation of nuclear factor κ B (NF- κ B) transcription factors, leading to decreases in nitric oxide synthase 2 expression and alleviation of nitrosative stress.
- C. Dietary supplementation of Q3G may be a promising approach to preventing birth defects induced by diabetes in pregnancy.

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the presence of NTDs and apoptosis in the neural tube. Expression of Nos2 and superoxide dismutase 1 (Sod1; an antioxidative enzyme) was quantified using Western blot assay. Nitrosative, oxidative, and endoplasmic reticulum (ER) stress conditions were assessed using specific biomarkers. Expression and posttranslational modification of factors in the NF- κ B system were investigated.

Results—Treatment with Q3G (suspended in water) significantly decreased NTD rate (24.7%) and apoptosis in the embryos of diabetic mice, compared with those in the water-treated diabetic group (3.1%; $p<0.001$). Q3G decreased the expression of Nos2 and nitrosative stress ($p<0.05$). It also increased the levels of Sod1 ($p<0.05$), further increasing the antioxidative capacity of the cells. Q3G treatment also alleviated of ER stress in the embryos of diabetic mice ($p<0.05$). Q3G reduced the levels of p65 (RelA; $p<0.05$), a member of the NF- κ B transcription factor family, but augmented the levels of the inhibitor of κ B α (I κ B α ; $p<0.05$), which suppresses p65 nuclear translocation. In association with these changes, the levels of I κ B kinase α (Ikka) and I κ B α phosphorylation were elevated ($p<0.05$).

Conclusion—Q3G reduces the NTD rate in the embryos of diabetic dams. Q3G suppresses Nos2 and increases Sod1 expression, leading to alleviation of nitrosative, oxidative, and ER stress conditions. Q3G may regulate the expression of Nos2 via modulating the NF- κ B transcription regulation system. Q3G, a naturally occurring polyphenol that has high bioavailability and low toxicity, is a promising candidate agent to prevent birth defects in diabetic pregnancies.

Keywords

Diabetic embryopathy; neural tube defects; quercetin-3-glucoside; nitric oxide synthase; superoxide dismutase; nitrosative stress; nuclear factor- κ B

Introduction

Diabetes mellitus in early pregnancy can cause birth defects in infants, a complication known as diabetic embryopathy^{1, 2}. Defects in the central nervous system, including anencephaly, exencephaly, and spina bifida, are the result of a failure in neural tube formation during early embryogenesis, collectively referred to as neural tube defects (NTDs).³ The development of NTDs is associated with increased programmed cell death (apoptosis) in the neural folds during neurulation.¹ Although the mechanisms underlying apoptosis induction are not fully understood, an inflammation-like condition with elevated nitric oxide (NO) levels has been observed in the embryos of diabetic animals.⁴⁻⁶ High levels of reactive nitrogen species (RNS), derived from NO, augment protein S-nitrosylation and nitration, manifesting as nitrosative stress.^{7, 8} The perturbation of protein activity affects the function of the mitochondria, leading to generation of reactive oxygen species (ROS), and the endoplasmic reticulum (ER), resulting in disruption of protein folding, to further exacerbating oxidative and ER stresses.⁹⁻¹¹ ROS can be scavenged by antioxidative enzymes, including superoxide dismutases (SODs) and catalase.¹² However, these enzymes in the embryo are suppressed by maternal hyperglycemia.¹³

NO is generated by NO synthases (NOS), including NOS1 (neuronal), NOS2 (inducible), and NOS3 (endothelial). The elevation of NO levels in the embryos of diabetic animals may

be ascribed to the dramatic upregulation of *Nos2*;⁵ because other NOS isoforms are downregulated.^{14, 15} The pivotal role of *Nos2* in diabetic embryopathy has been demonstrated in mouse models where deletion of the *Nos2* gene or inhibition of NOS2 leads to significant decreases in the rates of embryonic and fetal abnormalities under maternal hyperglycemic conditions.^{5, 16}

The expression of the *NOS2* gene is regulated by nuclear proteins, including nuclear factor (NF) κ B transcription factors.^{17, 18} NF- κ B proteins, including p50 and p65 (RelA), form dimers and are retained in the cytoplasm by inhibitor of κ B (I κ B).¹⁹ Phosphorylation of I κ B by I κ B kinase (IKK) triggers I κ B degradation and releases p50/p65 complex.²⁰ The NF- κ B dimer translocates into the nucleus to regulate target gene transcription.¹⁹

Targeting NOS2 may be an effective approach to reduction of embryonic malformations.^{5, 16} NOS2 inhibiting agents that have low toxicity and are potentially applicable in humans have been explored to prevent diabetes-induced birth defects.²¹ Among them, quercetin (QC), a naturally occurring flavonoid in fruits, has been shown to reduce *Nos2* expression in the embryos of diabetic mice.²¹

However, QC has low water-solubility and low rate of absorption in the gastrointestinal (GI) tract.^{22, 23} In comparison, its derivative, quercetin-3-glucoside (Q3G; isoquercetin), has higher water-solubility and higher absorption rates, due to the presence of the glucoside group.^{22, 24, 25} Q3G also possesses antioxidative and anti-inflammatory properties.^{26, 27} These characteristics make Q3G a promising candidate intervention to reduce birth defects in diabetic pregnancies.

Materials and Methods

Diabetic animal model

Use of animals was approved by the Institutional Animal Care and Use Committee of University of Maryland, Baltimore. Female mice (C57BL/6J) were injected intravenously with streptozotocin (Sigma-Aldrich, St. Louis, MO) in 0.1 M citrate buffer (pH 4.5; 65 mg/kg body weight) to eliminate insulin-producing β -cells in the pancreas. Diabetes mellitus (DM) was defined as blood glucose levels reaching 14 mM (250 mg/dl). Female mice injected with citrate buffer were used as non-diabetic (ND) controls. Euglycemia (~8 mM) was restored in the diabetic mice by subcutaneous implantation of insulin pellets (Linshin Canada, Scarborough, ON, Canada). Control mice were sham operated on? Female mice were paired with normal male mice of the same strain in the afternoon. The presence of the vaginal plug the next morning was designated as embryonic (E) day 0.5. At E5.5, insulin implants were removed to make the female mice hyperglycemic before the beginning of neurulation (E8.5).

Pregnant mice were fed Q3G (CAS No: 21637-25-2, Cayman Chemical, Ann Arbor, MI) suspended in water at 100 mg/kg body weight, or water (vehicle = VEH; 0.1 ml) via gavage feeding, injection of the solution into the stomach via a feeding needle, once a day from E6.5 to E9.5. Maternal blood glucose levels were monitored every day. Three groups were

included in this study: ND, DM+VEH, and DM+Q3G. At E10.5 (late stage of neurulation²⁹), embryos were collected for examination of NTDs.

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation in apoptotic cells.³⁰ E10.5 embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) at 4 °C for 24 hours and embedded in paraffin wax through dehydration with ethanol and xylene. Tissue sections in 5- μ m thickness were cut using a microtome. Tissue sections were dewaxed in xylene and rehydrated through a reverse ethanol concentration series to water.

After equilibration with PBS, the tissue sections were treated with proteinase K (20 μ g/ml) for 15 min at room temperature, incubated with TUNEL labeling mix containing dUTP-fluorescein (Roche, Indianapolis, IN) for 2 hours at 37°C, and counterstained with 4',6-diamidino-2-phenylindole (DAPI). The sections were observed under a fluorescent microscope (Zeiss, Thornwood, NY). Microscopic images were captured using a CCD camera connected to an image analysis station and arranged using Adobe Photoshop software program.

Western blot assay

The neural tissues in the brain regions of the E10.5 embryos were isolated and individually collected using fine scissors under a stereo-microscope in cold PBS. The tissues were homogenized in a lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, IL). The tissue homogenates were centrifuged at 14,000 rpm for 15 min at 4°C to obtain supernatants.

Protein samples were resolved in 10% polyacrylamide gel using electrophoresis in presence of SDS and blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). After blocking with 10% non-fat milk or bovine serum albumin, the membranes were incubated with primary antibodies for 16 hours at 4°C. The primary antibodies were against Nos2, Sod1, immunoglobulin-binding protein (Bip), phosphorylated (p) eukaryotic initiation factor 2 α (p-eIF2 α), phosphorylated (p) inositol-requiring enzyme 1 α (p-Ire1 α), p65, Ikk α , Ikk β , and p-Ikk β (Cell Signaling Technology, Beverly, MA). After incubation with horseshoe radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature, signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), captured using a CCD Chemi-camera, and analyzed using the program in UVP Bioimage system (UVP, Upland, CA).

The same membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and probed again with an antibody against β -actin (Abcam, Cambridge, MA) to control equal loading of protein samples. The values of β -actin were used to normalize the data of the corresponding bands of interest.

Statistical analyses

NTD rate was calculated as a percentage of the embryos with NTDs out of the total number of embryos. Log binomial models for clustered data were applied to compare the NTD rates between groups. Data of protein levels relative to those of β -actin on Western blots, presented as mean $\pm$ standard deviation (SD), were analyzed using the exact Wilcoxon rank sum test. $p < 0.05$ was considered statistically significant.

Results

Effect of Q3G on embryonic malformations in diabetic pregnancies

Diabetic pregnant mice were treated with Q3G from E6.5 to E9.5. The NTD rate at E10.5 in the DM+Q3G group was significantly lower than that in the DM+VEH group (fed with water alone), and similar to that in the ND group (ND; Table 1; $p < 0.001$; Figure 1).

Decreases in cell apoptosis in the neural tube by Q3G treatment

NTD formation is associated with increased apoptosis in the neuroepithelium.²⁸ The phenomenon was seen in the DM+VEH group, manifested as high levels of TUNEL-positive signals (apoptosis) in the dorsal region of the neural tube in the sites of NTDs (Figure 2B). In the DM+Q3G group, the apoptotic signals were lower than those in the DM+VEH group in the similar regions of the neural tube, but similar as in the ND group (Figure 2C).

Restoration of Nos2 and Sod1 expression by Q3G treatment

Maternal hyperglycemia has been shown to increase the expression of Nos2 and suppress the expression and activity of Sod1 in the embryo.^{4-6, 21, 31} To address the question of whether Q3G affects these enzymes, the levels of these proteins in the malformed embryos from the DM+VEH group, and normal embryos from the ND group and DM+Q3G group were assessed and compared. Nos2 expression was significantly increased in the DM+VEH group, compared with that in the ND group (Figure 3A). Q3G treatment significantly reduced the levels of Nos2 expression to the levels in the ND group, compared with those in the DM+VEH group ($p < 0.05$; Figure 3A).

Maternal hyperglycemia (DM+VEH group) decreased the levels of Sod1 in the embryos, compared with the ND group (Figure 3B). Q3G treatment significantly increased Sod1 expression to the similar levels in the ND group, compared with those in the DM+VEH group ($p < 0.05$; Figure 3B).

Alleviation of intracellular stress conditions by Q3G treatment

In association with the changes in Nos2 and Sod1 expression after Q3G treatment, the levels of 3-nitrotyrosine (3-NT), a marker for nitrosative and oxidative stresses, were also decreased in the DM+Q3G group, compared with those in the DM+VEH group ($p < 0.05$), to the similar levels in the ND group (Figure 3C).

Nitrosative and oxidative stress can perturb the activity of the ER, leading to ER stress.^{16, 21} Significant increases in the ER stress markers Bip, p-eIF2 α , and p-Ire1 α were observed in the embryos of the DM+VEH group, compared with the ND group (Figure 4). Q3G

treatment alleviated ER stress, indicated by significant decreases in these same markers in the DM+Q3G group, compared with the DM+VEH group ($p<0.05$; Figure 4).

Modulation of NF- κ B signaling by Q3G treatment

The expression of the *NOS2* gene is regulated by the transcription factors in the NF- κ B signaling system, specifically p65.^{17, 18} In the embryos of diabetic mice (DM+VEH group), the expression p65 was significantly increased, compared with the ND group (Figure 5A). Treatment with Q3G (DM+Q3G group) significantly decreased the expression of p65 to the levels in the ND group ($p<0.05$; Figure 5A).

Activation of NF- κ B transcription factors requires IKK to phosphorylate I κ B for degradation.¹⁹ The expression of Ikka was significantly increased in the embryos of the DM +VEH group, compared with that in the ND group ($p<0.05$; Figure 5B). Q3G treatment (DM +Q3G group) significantly decreased the levels of Ikka, compared with the DM+VEH group ($p<0.05$; Figure 5B). In association with the upregulation of Ikka, the levels of phosphorylated I κ B (p-I κ B α) were increased and those of total I κ B α were significantly decreased in the embryos of the DM+VEH group ($p<0.05$), compared with those in the ND group (Figure 5C). Treatment with Q3G (DM+Q3G group) significantly reduced the levels of pI κ B α and increased the levels of total I κ B α , compared with the DM+VEH group ($p<0.05$), to those similar to the ND group (Figure 5C).

Comment

Principal findings of the study

Maternal hyperglycemia-induced cell apoptosis and NTDs in the embryo are associated with nitrosative stress which is mediated by high levels of NO and RNS produced by Nos2.^{16, 21} Blocking Nos2 activity with inhibitors can alleviate nitrosative stress and reduce embryonic malformations in diabetic embryopathy.^{5, 16} Here, we showed that the naturally occurring phytochemical Q3C inhibited Nos2 expression, ameliorated nitrosative stress, and reduced NTDs in diabetic embryopathy. In addition, Q3G upregulated Sod1, thereby enhancing the antioxidative capacity of the cells. These effects resulted in the alleviation of oxidative stress and ER stress and decreased apoptosis in the developing neural tube.

Regulation of NOS2 expression by Q3G in diabetic embryopathy

The expression of NOS2 is regulated by transcription factors in the NF- κ B family, notably the p50/p65 dimer complex.^{17, 18} In the neural tube of the embryos of diabetic mice, p65 expression is upregulated, suggesting that the NF- κ B signaling responds to hyperglycemia. The p50/p65 dimer is detained in the cytoplasm by I κ B. IKK phosphorylates I κ B for degradation, and thus, releases the p50/p65 complex to allow them to translocate into the nucleus to regulate gene expression (Figure 6).¹⁹ Maternal hyperglycemia may stimulate the inflammation-like response in the embryo by increasing Ikk expression. The upregulation of Ikka leads to removal of I κ B α , indicated by the increases in I κ B α phosphorylation and decreases in total I κ B α levels. The increases in p65 expression and nuclear translocation potentially upregulate Nos2 transcription, and ultimately elevate of RNS levels and nitrosative stress (Figure 6). Treatment with Q3G ameliorated the effects of hyperglycemia

on the factors in the NF- κ B system and restore their levels to those in the non-diabetic (“normal”) conditions (Figure 6). The effects of Q3G treatment may result in downregulation of Nos2 expression and alleviation of intracellular stresses. Future investigations are aimed at delineating the action of Q3G in the modulation of the NF- κ B system.

Q3G as a candidate agent for preventing embryonic malformations in diabetic pregnancy

Q3G is a glucoside derivative of QC, which has been shown to reduce NTDs in diabetic embryopathy.²¹ Compared with QC and other derivatives, Q3G has higher water solubility, higher absorption rate in the gastrointestinal tract, and higher bioavailability in organ systems.^{22, 24, 25} Unlike other QC derivatives, Q3G can also be transported via glucose carriers, increasing its bioavailability in cells.³² Q3G has been shown to possess antioxidative and anti-inflammatory properties,²⁶ protect neurons from ischemia-reperfusion injury, and ameliorate neurodegenerative diseases.^{27, 33} The present study demonstrated that Q3G reduced NTDs in the embryos of diabetic mice; however, the beneficial effects of Q3G may extend beyond only preventing malformations in the central nervous system. Animal studies have shown that hyperglycemia induces similar molecular dysregulation in the cardiovascular system, especially in terms of NOS expression and nitrosative stress.^{5, 13, 16} Thus, it is conceivable that a Q3G supplement may have broad action against diabetic embryopathy.

One of the criteria for selecting a therapeutic agent for use in pregnancy is its safety for the embryo and fetus. Studies in humans and animals have shown that flavonoids, including Q3G, have no adverse effects when used in low dosages (chronic use) or high dosages (acute treatment).^{34, 35} Although flavonoids are present in foods,^{36, 37} therapeutic effects of certain form of flavonoid require increasing the amount of intake through dietary supplementation. In the present study, the dose of Q3G is comparable to the high levels of human intake of flavonoids.^{36, 37} The data from the animal study provide a scientific basis for clinical trials to further optimize the dosage for human pregnancies.

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Figure 1. Effect of Q3G on NTD formation. Embryos at E10.5 were examined for NTDs after Q3G treatment. (A) ND. (B) DM+VEH. (C) DM+Q3G. Arrows indicate open neural tube. Scale bar = 2 mm.

Figure 2.

Effect of Q3G on apoptosis in dorsal regions of the neural tube. TUNEL assay (green signals) of neural tube at E10.5 with counterstaining with DAPI. (A) ND. (B) DM+VEH. (C) DM+Q3G. Arrowheads indicate apoptotic bodies at the defect site. Inset: open neural tube. Scale bar = 10 μ m.

Figure 3.

Effects of Q3G on expression of Nos2, Sod1, and markers of nitrosative and oxidative stresses. Western blot assay of protein levels of (A) Nos2, (B) Sod1, and (C) 3-NT in the neural tube at E10.5. (Upper panels) Western blots. (Lower panels) Quantifications of the bands. Data are presented as mean $\pm$ SD. * $p < 0.05$ (DM+VEH vs. ND; DM+VEH vs. DM+Q3G); $n = 3$ embryos.

Figure 4.

Effects of Q3G on expression of ER stress markers. Western blot assay of ER stress markers in the neural tube at E10.5. (A) Bip. (B) p-eIF2α. (C) p-Ire1α. (Upper panels) Western blots. (Lower panels) Quantifications of the bands. Data are presented as mean ± SD. * $p < 0.05$ (DM+VEH vs. ND; DM+VEH vs. DM+Q3G); $n = 3$ embryos.

Figure 5. Effects of Q3G on expression of NF-κB factors. Western blot assay of proteins in the neural tube at E10.5. (A) p65. (B) Iκkα. (C) p-IκBα and total IκBα. (Upper panels) Western blots. (Lower panels) Quantifications of the bands. Data are presented as mean ± SD. * $p < 0.05$ (DM+VEH vs. ND; DM+VEH vs. DM+Q3G); $n = 3$ embryos.

Figure 6.

Schematic illustration of potential actions of Q3G in suppression of NOS2 expression. Maternal hyperglycemia increases the expression of p65, which forms a complex with p50, to activate *NOS2* transcription. Hyperglycemia also upregulates IKK, which phosphorylates IκB for degradation. The p50/p65 complex is released and translocates into the nucleus to regulate gene transcription. Q3G suppresses the expression of p65 and IKK, and thus, decreases the expression NOS2, and consequent nitrosative stress.

Table 1
Effect of Q3G treatment on NTD rate

	ND	DM+VEH	DM+Q3G
NTD/total embryos (rate)(litter)	0/61(0%)(8)	19/77(24.7%)(10)	2/64(3.1%)(9) *
Maternal glucose (E7.5-E10.5; mg/dl; Mean + SD)	103.20 ± 24.11	318.85 ± 94.74	307.78 ± 79.23

* $p < 0.001$,
DM+VEH vs. DM+Q3G (95% CI 0.0373, 0.4296). DM, diabetes; Q3G, quercetin-3-glucoside; NTD, neural tube defect; VEH, vehicle