Reduction in Embryonic Malformations and Alleviation of Endoplasmic Reticulum Stress by Nitric Oxide Synthase Inhibition in Diabetic Embryopathy

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Abstract

Maternal diabetes-induced neural tube defects (NTDs) are associated with increased programmed cell death (apoptosis) in the neuroepithelium, which is related to intracellular nitrosative stress. To alleviate nitrosative stress, diabetic pregnant mice were fed via gavage an inhibitor of nitric oxide (NO) synthase (NOS) 2, L-N⁶-(1-iminoethyl)-lysine (L-NIL; 80 mg/kg), once a day from embryonic (E) day 7.5 to 9.5 during early stages of neurulation. The treatment significantly reduced NTD rate in the embryos, compared with that in vehicle (normal saline)-treated diabetic group. In addition to alleviation of nitrosative stress, endoplasmic reticulum (ER) stress was also ameliorated, assessed by quantification of associated factors. Apoptosis was reduced, indicated by caspase 8 activation. These results show that nitrosative stress is important in diabetes-induced NTDs via exacerbating ER stress, leading to increased apoptosis. Oral treatment with NOS-2 inhibitor alleviates nitrosative and ER stress, decreases apoptosis, and reduces NTDs in the embryos, providing information for further interventional studies to reduce diabetes-associated birth defects.

Keywords

diabetic embryopathy, neural tube defects, nitrosative stress, endoplasmic reticulum stress, nitric oxide synthase, apoptosis

Introduction

Diabetes mellitus (DM) in pregnancy is one of the maternal diseases that cause congenital birth defects in infants.¹⁻³ Diabetes-induced fetal anomalies are commonly present in the central nervous system known as neural tube defects (NTDs).^{4,5} Manifestation of these developmental malformations is a result of failure in formation of the neural tube during the neurulation, via fusion of the neural grooves along the dorsal midline.^{6,7}

In diabetic embryopathy, NTDs are associated with excessive programmed cell death (apoptosis) in the neuroepithelium during neurulation.^{1,3,8} Although the factors involved in execution of apoptosis have been characterized, such as members of the B-cell lymphoma 2 (Bcl-2) and caspase families, the mechanisms by which these intermediaries induce apoptosis are not fully understood.^{9,10} Evidence suggests that oxidative stress—an imbalance between high level of reactive oxygen species (ROS) and low level of antioxidative buffering—and nitrosative stress, the result of a high level of reactive nitrogen species (RNS) derived from nitric oxide (NO), play an essential role in hyperglycemia-induced NTDs.¹¹⁻¹³

Nitric oxide is a signaling molecule that regulates a wide range of cellular activities, including proliferation, differentiation, and apoptosis.^{14,15} However, in pathological conditions, high levels of NO can stimulate apoptosis by itself or form highly reactive radicals with ROS, known as reactive nitrogen

species (RNS). The resultant nitrosative stress perturbs intracellular signaling, leading to the activation of apoptotic regulators.¹⁴

Nitric oxide is generated by NO synthases (NOSes), including neuronal (nNOS; NOS-1), inducible (iNOS; NOS-2), and endothelial (eNOS; NOS-3).¹⁶ NOS-1 and NOS-3 are constitutively expressed and do not vigorously respond to extracellular stimulation.¹⁷ In contrast, NOS-2 actively responds to extracellular changes, with a marked upregulation in expression and activity.¹⁸

In the embryos of diabetic pregnancy, high levels of NO and RNS are generated.^{12,19} While the expression of NOS-1 and NOS-3 is decreased,^{11,20} NOS-2 is dramatically upregulated and plays a major role in NO generation.¹¹ Using a *nos2* gene knockout animal model and NOS-2 inhibitor, the role for

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nitrosative stress in embryonic malformations in diabetic pregnancies has been demonstrated.¹¹

Both NO and RNS disturb the function of the endoplasmic reticulum (ER). Dysfunction of this organelle, including deficient protein folding and processing, causes prolonged retention of polypeptides in its lumen, resulting in ER stress.^{21,22} In response to ER stress, a number of chaperone proteins, including calnexin, are induced to facilitate protein folding.²¹ When ER stress reaches a certain threshold level, cells undergo apoptosis by switching on apoptotic machinery, including the unfolded protein response (UPR), ROS, stress-response kinase (apoptosis signal-regulating kinase 1 [ASK1] and c-jun N-terminal kinases [JNKs] cascades.^{23,24} The ER stress is one of the major intracellular factors involved in apoptotic induction in various pathological conditions, although it has not been characterized in diabetic embryopathy.²³

Both NO and RNS induce ER stress by disturbing ER calcium (Ca^{2+}) homeostasis, which is essential for chaperone proteins to function properly in protein folding.²⁵ They also influence the activity of factors in the UPR cascade. Both NO and RNS activate eukaryotic initiation factor 2 α (eIF2 α) and double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) via phosphorylation.²⁶ These factors interact with activating transcription factors (ATFs) to regulate apoptosis-associated genes, including members of the Bcl-2 and caspase families.^{23,24}

The ultimate goal of research in diabetic embryopathy is to develop interventions to prevent birth defects associated with diabetes-complicated pregnancies. Animal research has shown that alleviation of oxidative stress via oral treatment with antioxidants, such as vitamins C and E, reduces malformations in the embryos of diabetic rodents.²⁷⁻³⁰ However, clinical trials using these vitamins to treat similar diseases in humans have produced unsatisfactory results.³¹⁻³³ Inhibition of NOS-2 via injection of NOS-2 inhibitor (ONO-1714) to diabetic pregnant mice has been shown to reduce malformation rate in the embryos, suggesting that inhibition of NOS-2 is a strategy for disease prevention.¹¹ In the present study, we investigate the efficacy of oral treatment with a well-characterized NOS-2 inhibitor, L-N6-(1-iminoethyl)-lysine (L-NIL),³⁴ in reducing embryonic malformations in diabetic pregnant mice. We also study the impact of NOS-2 inhibition on ER stress and apoptosis.

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 (CAS 18883-66-4; Sigma-Aldrich, St Louis, Missouri) at a 65 mg/kg dosage to eliminate insulin-producing β -cells in pancreas.³⁵ Diabetes mellitus was defined as blood glucose levels reaching ≥ 14 mmol/L (250 mg/dL). Before mating with normal male mice, the glucose levels were

restored to the normal range (4.5-8.5 mmol/L) by subcutaneous implantation of insulin pellets (Linshin Canada).^{36,37} Presence of the vaginal plug in the next morning after pairing was designated as embryonic (E) day 0.5. At E5.5, insulin implants were removed to make the female mice hyperglycemic again (denoted as diabetes mellitus [DM] group), which usually occurs at E7.5, one day before neurulation begins.³⁸ A group of mice with insulin pellets retained were used as nondiabetic mellitus (Non-DM) control.

Pregnant mice were fed L-N6-(1-iminoethyl)-lysine dihydrochloride (L-NIL; CAS 159190-45-1; dissolved in saline; Enzo Life Sciences, Plymouth Meeting, Pennsylvania; 80 mg/kg body weight) or normal saline (NS; 0.1 mL) once a day from E7.5 to E9.5.^{39,40} Four groups were included in this study, non-DM + NS (control [CON]), non-DM + L-NIL, DM + NS, and DM + L-NIL. At E10.5, which is the late stage of neurulation,³⁸ the mice were euthanized and the embryos were collected for examination. Maternal blood glucose levels were monitored every day between E7.5 and E10.5.

Immunohistochemistry

The embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 16 hours at 4°C and dehydrated through a series of concentrations of ethanol. They were then embedded in paraffin wax through mediation steps of xylene. Tissue sections in 6 μm thickness were cut using a microtome. Tissue sections were dewaxed in xylene and rehydrated through a reverse ethanol concentration series to water, boiled in a citrate buffer (10 mmol/L citrate acid, pH 6.0) for 5 minutes to unmask antigens, and equilibrated in PBS (pH 7.4). The sections were incubated with antibodies against phosphorylated calnexin and eIF2 α (p-calnexin; Abcam, Cambridge, Massachusetts) and p-eIF2 α (Cell Signaling Technology, Beverly, Massachusetts) for 16 hours at 4°C, followed by an incubation of alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, California). Signals were detected using phosphatase substrates (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate; Sigma-Aldrich). The sections were counterstained with fast red (Vector Laboratories, Burlingame, California). A negative control (ie, without incubation of a primary antibody) was included to control nonspecific binding of antibodies to tissue sections. At least 3 normal and malformed embryos from Non-DM and DM groups, respectively, were used for the immunohistochemical assay.

Western Blot Assay

The neural tissues in the cranial regions (forebrain, midbrain, and hindbrain) of the embryos were isolated and individually collected using fine tungsten needles under a stereo-microscope in cold PBS (pH 7.4). The tissues were homogenized in a lysis buffer (25 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L EDTA, 1% NP-40) containing protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford,

Figure 1. Neural tube defects (NTDs) in the embryos (E10.5) of diabetic mice. (A) CON; (B) DM + NS; Arrow indicates open neural tube in the brain region. (C) DM + L-NIL. fb indicates forebrain; hb, hindbrain; mb, midbrain. CON indicates control; DM, diabetes mellitus; NS, normal saline; L-NIL, L-N6-(1-iminoethyl)-lysine. Scale bar = 2 mm.

Illinois). The tissue homogenates were centrifuged at 14 000 rpm for 15 minutes at 4°C to obtain supernatants.

Samples containing 40 µg of protein from normal embryos in the CON and DM + L-NIL groups and malformed embryos in the DM + NS group were resolved in 10% polyacrylamide gel using electrophoresis in the presence of sodium dodecyl sulfate (SDS) and blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts). After blocking with 10% nonfat milk, the membranes were incubated with primary antibodies for 16 hours at 4°C. The primary antibodies included antibodies against eIF2α, p-eIF2α, PERK, p-PERK, and calnexin (Cell Signaling Technology); p-calnexin (Abcam); caspase 8 (Enzo Life Sciences); caspase 12 (EMD Biosciences, Gibbstown, New Jersey), and 3-nitrotyrosine (3-NT; Millipore). This was followed by incubations with horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) 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, California). The same membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and probed again with an antibody against β-actin (Abcam) to control equal loading of protein samples. The fluorescence intensity of the Western blot bands was measured using the UVP Bioimage system (UVP). The values of β-actin were used to normalize the data of the corresponding bands of interest.

Statistical Analysis

The NTD rate was calculated as a percentage of the embryos with NTDs in total number of embryos in the same litter. The embryos of all litters in the same group were pooled for analysis because of no indication of heterogeneity among litters in the same group (Pearson statistic: $\chi^2 = 7.71$ with $df = 12$). The exact unconditional test was performed to compare the NTD rates between groups. The confidence interval of the NTD rate difference was calculated. The relative levels of proteins of interest to those of β-actin were analyzed using the exact

Wilcoxon rank sum test, with $P < .05$ as a standard of significant difference.

Results

Reduction of NTD in the Embryos of Diabetic Mice Treated With L-NIL

To investigate whether alleviation of nitrosative stress via inhibition of NOS-2 suppresses embryonic malformation in diabetic mice, pregnant mice were treated with the NOS-2 inhibitor L-NIL via gavage. The effects of L-NIL on embryonic development in non-DM (“normal”) condition were examined. No NTDs were observed in this non-DM + L-NIL group, which was similar as in the CON group (non-DM + NS; Figure 1A; Table 1). In the DM + NS group, 19.5% of the embryos displayed NTDs (Figure 1B; Table 1), and this was reduced to 3.85% in the DM + L-NIL mice (Figure 1C; Table 1). The NTD rate in the DM + L-NIL group was significantly lower than that in the DM + NS group ($P = .017$; Table 1).

Alleviation of Nitrosative Stress in the Neural Tubes of the Embryos of Diabetic Mice Treated With L-NIL

To address whether treatment with L-NIL alleviates nitrosative stress, protein-associated 3-NT level was evaluated using immunoblot assay. 3-NT levels, quantified in the whole lane on the blot, in the embryos with NTDs in the DM + NS group were higher than those in the CON group (Figure 2). Levels of 3-NT in the embryos of the DM + L-NIL group were significantly lower than that in the DM + NS group and similar to that in the CON group (Figure 2).

Amelioration of ER Stress in the Neural Tubes of the Embryos of Diabetic Mice Treated With L-NIL

The ER stress has been implicated in many pathological conditions. To investigate whether ER stress is increased in the embryos of diabetic pregnancies, ER stress was examined at 2 levels: (1) tissue localization of ER stress factors, p-eIF2α

Table 1. Neural Tube Defect (NTD) Rate in the Embryos of Diabetic Mice Treated With L-NIL

Group	Total Number of Embryos/(litters)	Embryos With NTDs	NTD rate (%)	95% CI	P Value	Res	Blood glucose ^b (mg/dL)
Non-DM + NS	27/(4)	0	0			0	115.7 ± 14.8
Non-DM + L-NIL	28/(4)	0	0			0	124.5 ± 11.6
DM + NS	41/(6)	8	19.5			3	352.9 ± 21.0
DM + L-NIL	52/(8)	2	3.85	(0.029, 0.31)	.017 ^a	1	351.0 ± 17.5

Abbreviation: CI, confidence interval; Res, resorption; L-NIL, L-N6-(1-iminoethyl)-lysine; DM, diabetes mellitus; NS, normal saline; E, embryonic; SEM, standard error of the mean.

^a Comparison between DM + NS and DM + L-NIL.

^b Average values (mean ± SEM) from E day 7.5 to E day 10.5.

Figure 2. Changes in nitrosative stress in the neural tubes of embryos of diabetic mice treated with L-NIL. (Left panel) Western blots of 3-NT. β -actin, sample loading control. (Right panel) Quantification of Western blot bands. Data are presented as mean ± SD. * represents a significant difference between the DM + NS and DM + L-NIL groups ($P < .05$; $n = 5$). DM indicates diabetes mellitus; NS, normal saline; L-NIL, L-N6-(1-iminoethyl)-lysine; SD, standard deviation; 3-NT, 3-nitrotyrosine.

and p-calnexin, in the neural tube of the embryos using immunohistochemistry; and (2) quantification of the level of these factors by immunoblot assay.

Presence of ER stress in the neural epithelium was, first, examined in the embryos without treatment with either L-NIL or NS. Both p-eIF2 α and p-calnexin were detected in the neuroepithelium of embryonic neural tube (Figure 3). More intense staining was observed in the embryos displaying NTDs in the DM group, compared to normal embryos in the non-DM group. Immunoblot assays showed increases in the levels of p-PERK, p-eIF2 α , and p-calnexin in the DM group, while no significant changes in the total proteins of PERK, eIF2 α , and calnexin (data not shown; similar to those in Figure 4).

To address the question whether nitrosative stress influences ER stress, the levels of the ER stress factors were assessed in the embryos treated with L-NIL. Significant decreases in p-PERK, p-eIF2 α , and p-calnexin were observed, compared

with the DM + NS group (Figure 4). The levels were similar to those in the CON group (Figure 4).

Decreases in Caspase 8 Activation in the Neural Tubes of the Embryos of Diabetic Mice Treated With L-NIL

To determine whether the reduction of NTDs by NOS-2 inhibition is associated with changes in cell death, apoptosis in the neural tube was examined by monitoring caspase 8 cleavage. A significant decrease in the level of cleaved caspase 8 was observed in the DM + L-NIL group, compared with that in the DM + NS group (Figure 5). In contrast, the level of cleaved caspase 12 was not altered in any of these groups (Figure 5).

Discussion

Nitrosative stress is a crucial intracellular condition in the embryos of diabetic pregnancy causing developmental malformations.¹¹ This study demonstrates that dietary inhibition of NOS-2 significantly alleviates nitrosative stress and reduces NTDs in the embryos of diabetic mice. This suggests that inhibition of NOS-2 function may be a therapeutic strategy. Further studies using animal models are warranted.

In diabetic embryopathy, intracellular nitrosative and oxidative stresses have been well characterized.^{11,13} However, ER stress, a critical factor in induction of programmed cell death (apoptosis and autophagy) in many pathological conditions,²³ has not been studied in diabetic embryopathy. Our present study provides evidence that ER stress is present in diabetic mouse embryos. Furthermore, suppression of NOS-2 activity is associated with reduced ER stress and reduced numbers of NTDs. These observations suggest the existence of complex molecular interactions in embryonic cells challenged with hyperglycemia.

The ER stress is associated with inappropriate protein folding and processing, which require calcium (Ca^{2+}) homeostasis.⁴¹ The level of Ca^{2+} in the ER is normally tightly regulated⁴¹ but can be regulated by NO and RNS.⁴² The increases in activated (phosphorylated) calnexin, a Ca^{2+} -dependent chaperone protein, implicate changes in Ca^{2+} concentration in the ER. The NOS-2 inhibition restores the levels of p-calnexin to the control range, suggesting a potential involvement of nitrosative stress in the regulation of ER Ca^{2+} homeostasis. Ca^{2+} is transported into

Figure 3. Tissue localization of ER stress factors in the embryos of diabetic mice. Immunohistochemistry of neural tissue sections in the mid-brain region at E day 10.5. Blue indicates positive signals. Red, counterstaining with fast red. (A, C) Normal embryo in non-DM. (B, D) Abnormal embryo in DM. (A, B) p-eIF2 α ; (C, D) p-calnexin. (E) Negative control (without primary antibody). ne indicates neural epithelium; E, embryonic; ER, endoplasmic reticulum; DM, diabetes mellitus; eIF2 α , eukaryotic initiation factor 2 α .

the ER via its transmembrane channels, such as sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) and released from the ER via inositol triphosphate receptor (IP3R) and/or ryanodine receptor (RyR).²⁵ Nitric oxide has been shown to influence the activity of these Ca²⁺ channels.^{43,44} However, the underlying mechanisms in diabetic embryopathy remain to be delineated.

The ER stress also activates the ASK1/JNK stress response pathway.^{24,45} JNK2 has been clearly shown to be an essential mediator of the effects of maternal hyperglycemia on embryonic malformations.^{27,46} However, since JNKs also respond to oxidative stress, it remains to be determined which stress condition plays the predominant role in JNK activation.⁴⁷

Under severe ER stress, cells turn on apoptotic machinery, including the UPR. In this pathway, PERK phosphorylates eIF2 α , which, in turn, represses protein translation and activates ATF4 transcription factor-dependent expression of apoptosis-associated genes.^{48,49} We show in this study that the PERK-eIF2 α pathway is activated in diabetic embryopathy.

Further investigation into the role of this pathway in regulation of apoptosis in diabetic embryopathy should be pursued.

Alleviation of nitrosative stress by NOS-2 inhibition leads to decreases in apoptosis in the embryonic neural tube of diabetic mice. This is associated with the suppression of caspase 8 activation, which plays a role in diabetic embryopathy.⁹ This finding suggests an involvement of the caspase-8-regulated apoptotic cascade in NO- and/or RNS-induced apoptosis in diabetic embryopathy. Whether ER stress mediates the effects of nitrosative stress in caspase 8 activation remains to be addressed. The ER stress has been shown to activate caspase 12, which resides on the ER in many types of animal cells.⁵⁰ In the present study, we show that caspase 8, but not caspase 12, is activated in embryos that have NTDs.

Nitric oxide appears to be essential for normal embryonic and fetal development. When NO donor sodium nitroprusside or the pan-NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA; 200-600 μ mol/L) is injected into the amniotic fluids of rat E10.5 conceptuses in culture in vitro, they cause embryonic

Figure 4. Changes in ER stress factors of the neural tubes of embryos of diabetic mice treated with L-NIL. (Left panel) Western blots. β -actin, sample loading control. (Right panel) Quantification of the Western blot bands. Data are presented as Mean $\pm$ SD. *Represents a significant difference between the DM + NS and CON as well as DM + L-NIL groups ($P < .05$; $n = 5$ [p-PERK and p-calnexin]; $n = 4$ [p-eIF2 α]). Note: The images of p-PERK and PERK are from a different blot with similar levels of β -actin in the 3 lanes. ER, endoplasmic reticulum; DM, diabetes mellitus; eIF2 α , eukaryotic initiation factor 2 α ; L-NIL, L-N6-(1-iminoethyl)-lysine; SD, standard deviation; PERK, protein kinase (PKR)-like ER kinase;

Figure 5. Activation of caspases 8 and 12 in the neural tubes of embryos of diabetic mice treated with L-NIL. (Left panel) Western blot assay of cleaved caspases 8 and 12. β -actin, sample loading control. (Right panel) Quantification of caspase 8. No significant differences were seen in caspase 12 (data not shown). Data are presented as Mean $\pm$ SD. *Represents a significant difference between the DM + NS and DM + L-NIL groups ($P < .05$; $n = 4$). L-NIL indicates L-N6-(1-iminoethyl)-lysine; DM, diabetes mellitus; NS, normal saline; SD, standard deviation.

malformations.⁵¹ However, in vivo treatment with a similar pan-NOS inhibitor, N(G)-nitro-(L)-arginine methyl ester (L-NAME), produces no significant increases in fetal malformations.¹¹

Fetal limb abnormalities have been observed in pregnant rats injected with L-NAME (~500 mg/kg) at late fetal development.^{52,53} Interestingly, the limb reduction phenotype is present

in mouse fetuses lacking the *nos3* (*enos*) gene,^{54,55} but no abnormal phenotypes are seen in mice lacking the other NOS genes.⁵⁶⁻⁵⁸ It has been suggested that the effects of L-NAME are on fetal vascular homeostasis secondary to NO deficiency.^{53,59}

Treatments of pregnant rats with NOS-2 inhibitors (eg, L-NIL, phenyl-N-t-butyl nitron, and aminoguanidine)

generate no fetal abnormalities, rather reduce the incidence and severity of hemorrhage caused by L-NAME.^{60,61} The NOS-2 inhibitors ONO-1714¹¹ and L-NIL (this report) have been shown to exert no teratogenic effect, although the cause of higher resorption rate by ONO-1714 treatment remains to be further examined. In our present studies, the lack of teratogenic effect of L-NIL suggests that either L-NIL did not significantly act on NOS-1 and/or NOS-3 or the concentration of L-NIL used (80 mg/kg) did not reduce the NO level to an extent that influences development. Therefore, the use of NOS-2-selective inhibitors will be a preferred strategy, since the level of NOS-2 is high when embryos are challenged by maternal hyperglycemia.

The interaction among nitrosative, ER, and oxidative stress appears to be critical in the manifestation of NTDs in diabetic embryopathy. Alleviation of oxidative stresses using antioxidants, such as vitamins C and E, has been shown to reduce embryonic malformations in diabetic animals.^{11,27-29} However, clinical trials using these vitamins have shown unsatisfactory results in the amelioration of oxidative stress-related human diseases.³¹⁻³³ Similar outcome could likely be produced in diabetic pregnancies when the same approaches are applied.

Optimistically, amelioration of nitrosative stress appears to be associated with reduction in embryonic malformations in diabetic embryopathy when pregnant animals are injected with L-NAME or ONO-1714¹¹. Our study further demonstrates that dietary supplementation with an appropriate NOS inhibitor may be a viable approach to suppress fetal malformations. Furthermore, combination of agents to alleviate multiple stress conditions (oxidative, nitrosative, and ER) may be a more effective strategy to achieve the ultimate goal of preventing birth defects in diabetic pregnancies.

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Declaration of Conflicting Interests

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References

1. Zhao Z, Reece EA. Experimental mechanisms of diabetic embryopathy and strategies for developing therapeutic interventions. *J Soc Gynecol Investig*. 2005;12(8):549-557.
2. Loeken MR. Challenges in understanding diabetic embryopathy. *Diabetes*. 2008;57(12):3187-3188.
3. Eriksson UJ, Borg LA, Cederberg J, et al. Pathogenesis of diabetes-induced congenital malformations. *Ups J Med Sci*. 2000;105(2):53-84.
4. Correa A, Gilboa SM, Besser LM, et al. Diabetes mellitus and birth defects. *Am J Obstet Gynecol*. 2008;199(3):237. e1-e9.
5. Mills JL. Malformations in infants of diabetic mothers. *Teratology*. 1982;25(3):385-394.
6. Sadler TW. Embryology of neural tube development. *Am J Med Genet*. 2005;135C(1):2-8.
7. Wallingford JB. Neural tube closure and neural tube defects: studies in animal models reveal known knowns and known unknowns. *Am J Med Genet*. 2005;135C(1):59-68.
8. Chappell JH Jr, Wang XD, Loeken MR. Diabetes and apoptosis: neural crest cells and neural tube. *Apoptosis*. 2009;14(12):1472-1483.
9. Zhao Z, Yang P, Eckert RL, Reece EA. Caspase-8: a key role in the pathogenesis of diabetic embryopathy. *Birth Defects Res B Dev Reprod Toxicol*. 2009;86(1):72-77.
10. Gareskog M, Cederberg J, Eriksson UJ, Wentzel P. Maternal diabetes in vivo and high glucose concentration in vitro increases apoptosis in rat embryos. *Reprod Toxicol*. 2007;23(1):63-74.
11. Sugimura Y, Murase T, Oyama K, et al. Prevention of neural tube defects by loss of function of inducible nitric oxide synthase in fetuses of a mouse model of streptozotocin-induced diabetes. *Diabetologia*. 2009;52(5):962-971.
12. Jawerbaum A, Higa R, White V, et al. Peroxynitrites and impaired modulation of nitric oxide concentrations in embryos from diabetic rats during early organogenesis. *Reproduction*. 2005;130(5):695-703.
13. Loeken MR. Free radicals and birth defects. *J Matern Fetal Neonatal Med*. 2004;15(1):6-14.
14. Dimmeler S, Zeiher AM. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide*. 1997;1(4):275-281.
15. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43(2):109-142.
16. Groves JT, Wang CC. Nitric oxide synthase: models and mechanisms. *Curr Opin Chem Biol*. 2000;4(6):687-695.
17. Zhou L, Zhu DY. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide*. 2009;20(4):223-230.
18. Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem*. 2003;384(10-11):1343-1364.
19. Jawerbaum A, Sinner D, White V, Pustovrh C, Capobianco E, Gonzalez E. Modulation of nitric oxide concentration and lipid metabolism by 15-deoxy δ 12,14-prostaglandin J2 in embryos from control and diabetic rats during early organogenesis. *Reproduction*. 2002;124(5):625-631.
20. Kumar SD, Yong SK, Dheen ST, Bay BH, Tay SSW. Cardiac malformations are associated with altered expression of vascular endothelial growth factor and endothelial nitric oxide synthase genes in embryos of diabetic mice. *Exp Biol Med*. 2008;233(11):1421-1432.
21. Maattanen P, Gehring K, Bergeron JJ, Thomas DY. Protein quality control in the ER: the recognition of misfolded proteins. *Semin Cell Dev Biol*. 2010;21(5):500-511.
22. Hampton RY. ER stress response: getting the UPR hand on misfolded proteins. *Curr Biol*. 2000;10(14):R518-R521.

23. Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. *Nat Rev Drug Discov.* 2008;7(12):1013-1030.
24. Malhotra JD, Kaufman RJ. The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol.* 2007;18(6):716-731.
25. Wuytack F, Raeymaekers L, Missiaen L. Molecular physiology of the SERCA and SPCA pumps. *Cell Calcium.* 2002;32(5-6):279-305.
26. Hsieh YH, Su IJ, Lei HY, Lai MD, Chang WW, Huang W. Differential endoplasmic reticulum stress signaling pathways mediated by iNOS. *Biochem Biophys Res Commun.* 2007;359(3):643-648.
27. Reece EA, Wu YK, Zhao Z, Dhanasekaran D. Dietary vitamin and lipid therapy rescues aberrant signaling and apoptosis and prevents hyperglycemia-induced diabetic embryopathy in rats. *Am J Obstet Gynecol.* 2006;194(2):580-585.
28. Reece EA, Wu YK. Prevention of diabetic embryopathy in offspring of diabetic rats with use of a cocktail of deficient substrates and an antioxidant. *Am J Obstet Gynecol.* 1997;176(4):790-797; discussion 797-798.
29. Cederberg J, Siman CM, Eriksson UJ. Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. *Pediatr Res.* 2001;49(6):755-762.
30. Viana M, Aruoma OI, Herrera E, Bonet B. Oxidative damage in pregnant diabetic rats and their embryos. *Free Radic Biol Med.* 2000;29(11):1115-1121.
31. Villar J, Purwar M, Merialdi M, et al. World health organisation multicentre randomised trial of supplementation with vitamins C and E among pregnant women at high risk for pre-eclampsia in populations of low nutritional status from developing countries. *BJOG.* 2009;116(6):780-788.
32. Robinson I, de Serna DG, Gutierrez A, Schade DS. Vitamin E in humans: an explanation of clinical trial failure. *Endocr Pract.* 2006;12(5):576-582.
33. Polyzos NP, Mauri D, Tsappi M, et al. Combined vitamin C and E supplementation during pregnancy for preeclampsia prevention: a systematic review. *Obstet Gynecol Surv.* 2007;62(3):202-206.
34. Moore WM, Webber RK, Jerome GM, Tjoeng FS, Misko TP, Currie MG. L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem.* 1994;37(23):3886-3888.
35. Junod A, Lambert AE, Orci L, Pictet R, Gonet AE, Renold AE. Studies of the diabetogenic action of streptozotocin. *Proc Soc Exp Biol Med.* 1967;126(1):201-205.
36. Zhao Z. Cardiac malformations and alteration of TGF β signaling system in diabetic embryopathy. *Birth Defects Res B Dev Reprod Toxicol.* 2010;89(2):97-105.
37. 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(1):130. e1-e7.
38. Copp AJ, Greene ND, Murdoch JN. The genetic basis of mammalian neurulation. *Nat Rev Genet* 2003;4(10):784-793.
39. Fujimoto M, Shimizu N, Kunii K, Martyn JA, Ueki K, Kaneki M. A role for iNOS in fasting hyperglycemia and impaired insulin signaling in the liver of obese diabetic mice. *Diabetes.* 2005;54(5):1340-1348.
40. Carvalho-Filho MA, Ropelle ER, Pauli RJ, et al. Aspirin attenuates insulin resistance in muscle of diet-induced obese rats by inhibiting inducible nitric oxide synthase production and S-nitrosylation of IRbeta/IRS-1 and Akt. *Diabetologia.* 2009;52(11):2425-2434.
41. Burdakov D, Petersen OH, Verkhatsky A. Intraluminal calcium as a primary regulator of endoplasmic reticulum function. *Cell Calcium.* 2005;38(3-4):303-310.
42. Gotoh T, Mori M. Nitric oxide and endoplasmic reticulum stress. *Arterioscler Thromb Vasc Biol.* 2006;26(7):1439-1446.
43. Eu JP, Xu L, Stamler JS, Meissner G. Regulation of ryanodine receptors by reactive nitrogen species. *Biochem Pharmacol.* 1999;57(10):1079-1084.
44. Adachi T, Weisbrod RM, Pimentel DR, et al. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med.* 2004;10(11):1200-1207.
45. Sekine Y, Takeda K, Ichijo H. The ASK1-MAP kinase signaling in ER stress and neurodegenerative diseases. *Curr Mol Med.* 2006;6(1):87-97.
46. Yang P, Zhao Z, Reece EA. Involvement of c-Jun N-terminal kinases activation in diabetic embryopathy. *Biochem Biophys Res Commun.* 2007;357(3):749-754.
47. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J. Redox control of cell death. *Antioxid Redox Signal.* 2002;4(3):405-414.
48. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene.* 2003;22(53):8608-8618.
49. Raven JF, Koromilas AE. PERK and PKR: old kinases learn new tricks. *Cell Cycle.* 2008;7(9):1146-1150.
50. Yoneda T, Imaizumi K, Oono K, et al. Activation of caspase-12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J Biol Chem.* 2001;276(17):13935-13940.
51. Lee QP, Juchau MR. Dysmorphogenic effects of nitric oxide (NO) and NO-synthase inhibition: studies with intra-amniotic injections of sodium nitroprusside and NG-monomethyl-L-arginine. *Teratology.* 1994;49(6):452-464.
52. Fantel AG, Nekahi N, Shepard TH, Cornel LM, Unis AS, Lemire RJ. The teratogenicity of N(omega)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, in rats. *Reprod Toxicol.* 1997;11(5):709-717.
53. Tiboni GM, Clementini E. Teratological consequences of nitric oxide synthesis inhibition. *Curr Pharm Des.* 2004;10(22):2759-2767.
54. Gregg AR, Schauer A, Shi O, Liu Z, Lee CG, O'Brien WE. Limb reduction defects in endothelial nitric oxide synthase-deficient mice. *Am J Physiol.* 1998;275(6 pt 2):H2319-H2324.
55. Hefler LA, Reyes CA, O'Brien WE, Gregg AR. Perinatal development of endothelial nitric oxide synthase-deficient mice. *Biol Reprod.* 2001;64(2):666-673.

56. Laubach VE, Shesely EG, Smithies O, Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci U S A*. 1995;93(23):10688-10692.
57. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science*. 1994;265(5180):1883-1885.
58. Wei XQ, Charles IG, Smith A, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*. 1995;375(6530):408-411.
59. Fantel AG, Person RE. Further evidence for the role of free radicals in the limb teratogenicity of L-NAME. *Teratology*. 2002;66(1):24-32.
60. Fantel AG, Stamps LD, Tran TT, Mackler B, Person RE, Nekahi N. Role of free radicals in the limb teratogenicity of L-NAME (N(G)-nitro-(L)-arginine methyl ester): a new mechanistic model of vascular disruption. *Teratology*. 1999;60(3):151-160.
61. Bustamante SA, Pang Y, Romero S, et al. Inducible nitric oxide synthase and the regulation of central vessel caliber in the fetal rat. *Circulation*. 1996;94(8):1948-1953.