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Superoxide dismutase 2 overexpression alleviates maternal diabetes-induced neural tube defects, restores mitochondrial function and suppresses cellular stress in diabetic embryopathy

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Abstract

Pregestational diabetes disrupts neurulation leading to neural tube defects (NTDs). Oxidative stress resulting from reactive oxygen species (ROS) plays a central role in the induction of NTD formation in diabetic pregnancies. We aimed to determine whether mitochondrial dysfunction increases ROS production leading to oxidative stress and diabetic embryopathy. Overexpression of the mitochondrion-specific antioxidant enzyme superoxide dismutase 2 (SOD2) in a transgenic (Tg) mouse model significantly reduced maternal diabetes-induced NTDs. SOD2 overexpression abrogated maternal diabetes-induced mitochondrial dysfunction by inhibiting mitochondrial translocation of the pro-apoptotic Bcl-2 family members, reducing the number of defective mitochondria in neuroepithelial cells, and decreasing mitochondrial membrane potential. Furthermore, SOD2 overexpression blocked maternal diabetes-increased ROS production by diminishing dihydroethidium staining signals in the developing neuroepithelium, and reducing the levels of nitrotyrosine-modified proteins and lipid hydroperoxide level in neurulation stage embryos. SOD2 overexpression also abolished maternal diabetes-induced endoplasmic reticulum stress. Finally, caspase-dependent neuroepithelial cell apoptosis enhanced by oxidative stress was significantly reduced by SOD2 overexpression. Thus, our findings support the hypothesis that mitochondrial dysfunction in the developing neuroepithelium enhances ROS production, which leads to oxidative stress and endoplasmic reticulum (ER) stress. SOD2 overexpression blocks maternal diabetes-induced oxidative stress and ER stress, and reduces the incidence of NTDs in embryos exposed to maternal diabetes.

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Keywords

SOD2; neural tube defects (NTDs); mitochondria dysfunction; oxidative stress; caspase activation

Introduction

Pregestational diabetes increases the risk for congenital anomalies, especially neural tube defects (NTDs), in a process termed diabetic embryopathy [1–3]. The exact mechanism of this process remains unclear. However, translational studies using rodent models of pregestational diabetes have highlighted oxidative stress as one of the central causes [4–13]. Oxidative stress results from an altered balance involving the overproduction of reactive oxygen species (ROS) and a low antioxidant enzyme capacity. Both processes have been implicated in pregestational diabetes-associated birth defects [14–19].

ROS are chemically reactive molecules containing oxygen that are formed as natural byproducts of aerobic respiration and substrate oxidation and that have important roles in cell signaling and homeostasis. ROS usually exist in cells at low levels; however, higher levels due to either increased production or decreased removal may be malicious to the cell [20]. Diabetes is associated with increased ROS levels [21, 22]; however, the origin of which is not entirely understood. Potential enzymatic sources of ROS include the mitochondrial respiratory chain and cellular enzymes such as the cytochrome p450 monooxygenases, lipooxygenase, nitric oxide synthase (NOS) and the NADPH oxidase [23]. Regarding the source of ROS, mitochondrial ROS (mtROS) have drawn considerable interest because mtROS have been shown to directly contribute to a number of diseases [24–26].

Superoxide dismutase (SOD) enzymes are responsible for the majority of a cell's ability to scavenge and reduce cellular ROS levels. This family consists of three isoforms that catalyze the dismutation of $O_2^{\bullet -}$ into oxygen and hydrogen peroxide (H_2O_2). SOD1 is the major intracellular SOD enzyme (cytosolic Cu/ZnSOD) and is mainly localized to the cytosol, but a small fraction is present in the intermembrane space of the mitochondria [27, 28]. SOD1 has also been reported to be present in the nucleus, lysosomes and peroxisomes and is widely distributed in a variety of cells [29]. Our group revealed that the overexpression of SOD1 in diabetic mouse models blocks endoplasmic reticulum stress and reduces the rates of NTDs [30, 31]. However, SOD1 is located primarily in the cytosol, and it may not be the mitochondrion's principal antioxidant enzyme in diabetic embryopathy.

Several studies have implicated the mitochondria in the teratogenicity of diabetes *in vivo* and high glucose *in vitro*. Changes in mitochondrial morphology are known to occur in rat embryos subjected to diabetic environments, which implies that the origin of ROS may be in the embryonic mitochondria [32, 33]. Unlike SOD1, SOD2 is localized solely in the mitochondrial matrix where it dismutates the $O_2^{\bullet -}$ – generated by the respiratory chain and is essential for maintaining the role of the mitochondria in the cell [16, 34]. Furthermore, a substantial amount of evidence indicates that SOD2 deficiency causes numerous mitochondrial dysfunctions in gene knockout mice [35]. Based on the mtROS scavenging function of SOD2, we hypothesized that ROS enhancement due to maternal diabetes-induced mitochondrial dysfunction is responsible for diabetic embryopathy and that the

overexpression of SOD2, which is allocated to the mitochondria only, would scavenge those ROS and ameliorate diabetes-induced NTD formation.

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 (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar harbor, ME). The SOD2-Tg mice overexpressing the human mitochondrial SOD2 under the β -actin promoter were obtained from Dr. Robia Pautler at Baylor College of Medicine [36]. Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in 0.1 M citrate buffer (pH 4.5).

Mouse model of diabetic embryopathy and morphologic assessment of the NTDs

Our mouse model of diabetic embryopathy has been described previously [6, 7]. Briefly, WT female mice were intravenously (IV) injected with 75 mg/kg streptozotocin (STZ) daily for 2 days to induce diabetes. Vehicle-injected nondiabetic WT mice served as controls. Diabetes was defined by a 12-h fasting blood glucose level of ≥ 250 mg/dL, which normally occurred at 3–5 days after the STZ injections. There were no residual toxic effects caused by STZ in this animal model. The mice were then mated with SOD2-Tg male mice at 3:00 PM to generate WT (SOD2-negative) and SOD2-overexpressing embryos under nondiabetic and diabetic conditions. We designated noon of the day which a vaginal plug was present as embryonic day (E) 0.5. On E8.75, the mice were euthanized, and the conceptuses were dissected out of the uteri for analysis. The embryos were harvested on embryonic day (E) 8.75 (2 PM at E8.5) for analysis and on E12.5 for NTD examination.

At E12.5, the embryos were examined under a Leica MZ16F stereomicroscope to identify the NTDs. Images of the embryos were captured with a DFC420 5-megapixel digital camera with software (Leica). Normal embryos were classified as possessing completely closed neural tubes, and no evidence of other malformations. Embryos exhibiting evidence of failed closure of the anterior neural tubes resulting in exencephaly, which is a major type of NTD, were classified as malformed. Spina bifida was not observed in this study.

Genotyping of the embryos

The embryos from the WT diabetic dams mated with the SOD2-Tg male mice were genotyped according to the protocol previously described [36] using the yolk sac deoxyribonucleic acid (DNA).

Western blotting

Western blotting was performed as previously described [37]. To extract protein, the embryos were sonicated in ice-cold lysis buffer (Cell Signaling Technology, Beverly, MA) with a protease inhibitor cocktail (Sigma-Aldrich). The mitochondria were isolated from the embryos using the Pierce mitochondria isolation kit. Equal amounts of protein from each group or the Precision Plus Protein Standard (BioRad) were resolved by SDS-PAGE, transferred onto PVDF membranes and then immunoblotted with primary antibodies at

1:1000 dilutions in 5% nonfat milk. Antibodies for the pro-apoptotic Bcl-2 family members: Bak, Bax, p53 upregulated modulator of apoptosis (Puma), Bim, tBid, phosphorylated Bcl-2-associated death promoter (pBad), kinase RNA-like ER kinase (PERK), phosphorylated PERK, eIF2 α , phosphorylated eIF2 α , C/EBP-homologous protein (CHOP), IRE1 α and nitrotyrosine were acquired from Cell Signaling Technology. Anti-4-hydroxynonenal (4-HNE) and anti-caspase 3 were obtained from Millipore. The antibody for phosphorylated IRE1 α was obtained from Abcam (Cambridge, MA), and the antibody for caspase 8 (mouse-specific) was obtained from Alexis Biochemicals (San Diego, CA). HRP-conjugated goat anti-rabbit and goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-rat (Chemicon, Temecula, CA) secondary antibodies were also used. The intensities of the target protein bands were assessed by densitometry and normalized to the densities of β -actin (Abcam, Cambridge, MA). The signals were detected by the SuperSignal West Femto Maximum Sensitivity Substrate kits (Thermo Scientific, Rockford, IL), and chemiluminescence emitted from the bands was captured by the UVP Bioimage EC3 system (Upland, CA). All experiments were repeated three times with independently prepared tissue lysates.

Electron microscopy

Mitochondrial structures were examined by transmission electron microscopy (EM) in our university's EM core facility. Thick sections (1 μ m) were cut and visualized at 100 $\times$ magnification to identify the neuroepithelia of the E8.75 embryos. Thin sections (80 nm) of identified neuroepithelia were cut and viewed with an electron microscope (Joel JEM-1200EX; Tokyo, Japan) at high resolution (10, 12 and 25 K) to identify the cellular organelle structures.

JC-1 dye staining

Mitochondria from the embryos were extracted using the Pierce mitochondria isolation kit, and incubated with JC-1 (2 μ M final concentration, Life Technologies, Carlsbad, CA) at 37 °C for 30 min. Images were acquired using a fluorescence microscope (Nikon Eclipse Ni). Membrane potential monitored as the mean fluorescent intensity ratio of red fluorescence (590 nm) to green fluorescence (529 nm). Fluorescent intensity was determined by the NIH software Image J.

Measurement of Lipid hydroperoxide (LPO)

The abundance of lipid peroxidation in embryos were determined by the LPO assay kit (Millipore, Bedford, MA) as previously described [38]. Briefly, embryos were homogenized in HPLC-grade water. Lipid hydroperoxides in embryos were extracted using deoxygenated chloroform and then reacted with chromogen. The optical density was measured as the absorbance at 500 nm. The results are expressed as μ mol/L lipid hydroperoxides per μ g protein. The protein concentrations were determined with the BioRad DC protein assay kit (BioRad, Hercules, CA).

Real-Time PCR (RT-PCR)

mRNA was isolated from E8.75 embryos using the Trizol reagent (Invitrogen, Calsbad, CA), and then reversed transcribed using the high-capacity cDNA archive kit (Applied Biosystem, Grand Island, NY). RT-PCR for *Sod2* and β -*actin* were performed using the Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, IL) in the StepOnePlus system (Applied Biosystem, Grand Island, NY). Primer sequences are listed below, *Sod2*, forward: 5'-GTTGGGGTTGGCTTGGTTT-3'; reverse: 5'-TAAGGCCTGTTGTTCCCTTG-3'; β -*actin*, forward: 5'-GTGACGTTGACATCCGTAAAGA-3' reverse: 5'-GCCGGACTCATCGTACTCC-3'.

TUNEL assay

TUNEL assays were performed as previously described [7, 39]. Briefly, 5 μ m serial coronal sections through the forebrain-midbrain (FB-MB) boundary of the anterior neuroepithelium were fixed with 4% PFA in PBS and incubated with TUNEL reaction agents. Closure at the FB-MB boundary is an essential step in the closure of the anterior neural tube, and the failed closure in this area results in exencephaly [40, 41]. The TUNEL-positive cells were counted in the neuroepithelium of each section. Based on the total cell nuclei counts, the sizes of the neuroepithelium of each section were relatively constant. Thus, the numbers of apoptotic cells are expressed as the total TUNEL-positive cells per neuroepithelium. The TUNEL assays were performed by observers who were blinded to the experimental groups of the examined embryos.

Statistical analysis

The data are presented as the means $\pm$ the standard errors (SE). Experiments were repeated at least three times, and the embryonic samples from each replicate were from different dams. One-way ANOVA was performed using the SigmaStat 3.5 software (Systar Software Inc., San Jose, CA) followed by a *Tukey* test to estimate significance of the results ($P < 0.05$) [42].

RESULTS

SOD2 overexpression blocks maternal diabetes-induced neural tube defect formation

To determine whether maternal diabetes affects SOD2 expression, we analyzed mRNA and protein levels of SOD2 (Fig. 1A). Maternal diabetes did not affect endogenous SOD2 expression (Fig. 1A). To assess the effect of SOD2 overexpression on maternal diabetes-induced NTDs, we took advantage of the SOD2 transgenic (SOD2-Tg) mice [36]. The NTD incidences in the wild-type (WT) and SOD2 overexpressing embryos from WT females mated with SOD2 transgenic (Tg) males were summarized in Table 1. A total of 105 embryos from 17 female mice, including eight diabetic dams and nine nondiabetic dams, were evaluated (Table 1). Under nondiabetic conditions, SOD2 overexpression did not affect embryonic neurulation (Fig. 1B, Table 1). However the blood glucose level in diabetic dams was several-fold higher than that in nondiabetic dams (Table 1) and maternal diabetes significantly induced NTDs in mouse embryos (Fig. 1B, Table 1). The greatest NTD rate was observed in WT embryos from the diabetic dams mated with the SOD2-Tg males (7/21,

25% NTDs, Table 1). In contrast, the NTD rate in the SOD2-overexpressing embryos from diabetic dams mated with the SOD2-Tg males was significantly lower (1/24, 4% NTDs, Table 1). These data suggest that SOD2 overexpression alleviates maternal diabetes-induced NTD formation.

SOD2 overexpression inhibits maternal diabetes-induced mitochondrial translocation of the pro-apoptotic Bcl-2 family members

Maternal diabetes activates the pro-apoptotic Bcl-2 family members leading to mitochondrial dysfunction [43–45]. To determine whether SOD2 overexpression blocks the translocation of several key pro-apoptotic Bcl-2 members, including Bax, Puma, Bak and Bim, mitochondria were isolated from embryos, and the amounts of mitochondrial Bax, Puma, Bak and Bim were assessed. Obviously Bax, Puma, Bak and Bim were translocated to and accumulated in the mitochondria of embryos exposed to maternal diabetes but not in WT embryos of nondiabetic dams (Fig. 1C, D, E, F). Mitochondrial translocation of Bax, Puma, Bak and Bim was diminished in SOD2-overexpressing embryos from both nondiabetic and diabetic dams (Fig. 1C, D, E, F).

Furthermore, we examined the activation of two other pro-apoptotic Bcl-2 members, Bid and Bad, in whole embryonic lysates. The level of tBid, cleaved (activated) Bid, was significantly increased, and Bad phosphorylation (inactivation) was decreased, in embryos of diabetic dams compared with WT embryos of nondiabetic dams and SOD2-overexpressing embryos of nondiabetic and diabetic dams (Fig. 1G, H). These results collectively suggest that SOD2 overexpression inhibits maternal diabetes-induced mitochondrial translocation and activation of the pro-apoptotic Bcl-2 family members.

SOD2 overexpression preserves mitochondrial function by reducing the number of defective mitochondria and mitochondrial membrane potential

Mitochondrial dysfunction is associated with diabetic embryopathy. In a previous study, we found that maternal diabetes significantly increases the number of defective mitochondria but does not affect the total number of mitochondria [43]. Under maternal diabetic conditions, SOD2 overexpression significantly reduced the number of defective mitochondria to a level comparable to that in the nondiabetic control group (Fig. 2A, B). Additionally the mitochondrial membrane potential provides a valuable indicator of the health and functional status of the cells. Therefore, we also determined the mitochondrial membrane potentials using the JC-1 dye staining. Increased membrane potentials (green fluorescence) were observed in the mitochondria extracted from embryos exposed to maternal diabetes but not in WT embryos of nondiabetic dams (red fluorescence) (Fig. 2C, D). Moreover SOD2 overexpression abrogated maternal diabetes-increased JC-1 green fluorescence in isolated mitochondria (Fig. 2C, D). Above data revealed that SOD2 overexpression restores mitochondrial function in embryos exposed to maternal diabetes.

SOD2 overexpression blocks maternal diabetes-induced oxidative stress

Mitochondrial dysfunction enhances ROS production. ROS induced by maternal diabetes react with inducible nitric oxide synthase-induced nitric oxide to generate reactive nitrogen species, which result in a severe form of oxidative stress [46]. Previous studies have

demonstrated that oxidative stress plays a key role in maternal diabetes-induced NTD formation [30, 44, 47]. Embryonic sections were staining with DHE to detect $O_2^{\bullet-}$ in the developing neuroepithelium. Compared with that of embryos from nondiabetic dams, the neuroepithelia of embryos from diabetic dams exhibited significantly higher DHE staining signal (Fig. 3A). Next, we assessed the protein levels of the lipid peroxidation marker 4-HNE and the nitrotyrosine-modified proteins that are indicative of nitrosative stress. Under diabetic conditions, the levels of 4-HNE- and nitrotyrosine-modified proteins were higher than those in the embryos from nondiabetic dams (Fig. 3B, C). Additionally, maternal diabetes also increased the level of lipid hydroperoxide (LPO), which is also an index of lipid peroxidation (Figure 3D). However SOD2 overexpression suppressed maternal diabetes-increased DHE staining, lipid peroxidation and nitrosative stress (Fig. 3A, B, C, D). These results demonstrate that SOD2 overexpression alleviates maternal diabetes-induced oxidative and nitrosative stress.

SOD2 overexpression abrogates maternal diabetes-induced ER stress

Our previous study has demonstrated that oxidative stress causes ER stress [7, 30]. To determine whether SOD2 overexpression abolishes maternal diabetes-induced ER stress, we measured the protein levels of several ER stress markers. The levels of phosphorylated eIF2 α , phosphorylated IRE1 α , phosphorylated PERK and C/EBP-homologous protein (CHOP) were significantly increased in embryos from diabetic dams compared with those in embryos from nondiabetic dams (Fig. 4A, B, C, D). In contrast, SOD2 overexpression abrogated the increases in these ER stress markers due to maternal diabetes but did not affect the expression of ER stress markers in embryos of nondiabetic dams (Fig. 4A, B, C, D). Consistent with the decrease of maternal diabetes-induced oxidative stress, SOD2 overexpression also reduces ER stress enhanced by maternal diabetes.

SOD2 overexpression reduces maternal diabetes-induced apoptosis

Mitochondrial dysfunction, oxidative stress and ER stress converge on the induction of neuroepithelial cell apoptosis, and maternal diabetes induces apoptosis in the neuroepithelium via a caspase-dependent signaling pathway [48, 49]. We first used the TUNEL assay to detect apoptotic cells. We found robust levels of apoptotic cells in WT embryos from diabetic dams (Figure 5A, B). Under diabetic conditions, the neuroepithelia of SOD2-overexpressing embryos exhibited significantly lower numbers of apoptotic cells compared to those in the neuroepithelia of WT embryos (Figure 5A, B). To determine whether maternal diabetes induces caspase activation in the neuroepithelium, cleaved caspase 3 and caspase 8 were assessed. Maternal diabetes triggered caspase 3 and caspase 8 cleavage in WT embryos, but the cleaved caspase products were significantly reduced in SOD2-overexpressing embryos (Figure 5C, D). These results collectively suggest that SOD2 overexpression inhibits maternal diabetes-induced apoptosis.

DISCUSSION

There are multiple sources of ROS in the cell, including the NOX family of NADPH oxidases, xanthine oxidase, cytochrome P450 enzymes and the mitochondrial electron transport chain. However, extensive studies have focused on mitochondrial ROS, which

directly contribute to the etiology of many diseases, including inflammatory diseases, cancers and cardiovascular disease [23]. Because mitochondrial dysfunction is manifested in diabetic embryopathy [43], it is reasoned that mitochondrial dysfunction-induced ROS is responsible for cellular stress and may contribute to NTD formation. An in-depth understanding of how to block mitochondrial ROS production may enable promising therapies for combating pregestational diabetes-induced NTDs.

ROS are toxic by-products of cellular metabolism; thus, mammalian cells have evolved many antioxidant enzymes to scavenge mtROS. SOD2 is a mitochondrial manganese-containing enzyme that is only located in the mitochondrial matrix [50]. Many studies have indicated that SOD2 deficiency causes early neonatal death associated with mitochondrial dysfunctions including reduced aconitase activity and increased ultrastructural abnormalities of the mitochondrion in cells of *Sod2* gene knockout mice [51, 52]. In contrast, SOD2 overexpression can enhance mitochondrial tolerance [53]. Total SOD activity is reduced in embryos exposed to maternal diabetes [14], suggesting that SOD2 function may be impaired along with mitochondrial dysfunction in diabetic embryopathy. Because SOD2 promotes mitochondrial function, we determined whether SOD2 overexpression would block maternal diabetes-induced mitochondrial dysfunction in the developing embryo. Indeed, SOD2 overexpression inhibits maternal diabetes-induced oxidative stress and ER stress, and restores mitochondrial function leading to blockage of neuroepithelial cell apoptosis and reduction of NTD formation. These results support the involvement of mitochondrial ROS in the etiology of diabetic embryopathy.

One of the prominent features of mitochondrial dysfunction is the mitochondrial structural abnormalities. Mitochondria in neuroepithelial cells of embryos exposed to maternal diabetes exhibit structural abnormalities including disarrayed or disruptive cristae, decreased electronic density of the matrix and swollen mitochondria with entire loss of cristae [43, 54]. Consistent with these previous findings in defective mitochondrial morphology, in the present study, maternal diabetes increases the number of defective mitochondria in neuroepithelial cells, and SOD2 overexpression prevents defective mitochondrial morphology, suggesting that increased mitochondrial ROS causes the damage of mitochondrial morphology. Defective mitochondrial morphology leads to the loss of membrane potential [55, 56] and causes the release of mitochondrial ROS into the cytoplasm. The loss of mitochondrial potential activates the intrinsic mitochondrial apoptosis and the pro-apoptotic Bcl-2 family members [57]. SOD2 overexpression abrogates the mitochondrial apoptosis pathway triggered by maternal diabetes. Thus, antioxidants targeting the mitochondrion may be effective in preventing diabetic embryopathy.

Oxidative stress also induces ER stress [30, 45]. We have previously reported that both maternal diabetes *in vivo* and high glucose *in vitro* induce the unfolded protein responses (UPR) by activating the UPR-sensor IRE1 α and PERK signaling pathways, which contribute to neuroepithelial cell apoptosis and NTD formation [6, 47, 58–62]. Treatment with the ER stress inhibitor, 4-phenylbutyric acid, abrogates high glucose-induced ER stress in cultured neurulation stage embryos and significantly reduces NTD formation [54], supporting the hypothesis that ER stress plays a causal role in maternal diabetes-induced NTD formation. It has been demonstrated that antioxidants can block ER stress [45]. Our finding that SOD2

overexpression suppresses ER stress further supports the causal role of oxidative stress in UPR activation and ER stress. Our finding also suggests the interlinked relationship between mitochondrial dysfunction and ER stress in diabetic embryopathy.

Mitochondrial ROS oxidize the mitochondrial pores, causes mitochondrial *cytochrome c* release and ultimately initiates apoptosis [63]. Our studies have also indicated that both oxidative stress and ER stress converge on the induction of apoptosis [6, 40, 58]. Oxidative stress can induce caspase activation and subsequent apoptosis by suppressing pro-survival signaling [58], activating pro-apoptotic signaling [37] or both in embryos exposed to maternal diabetes. SOD2 protects against apoptosis in mouse embryos by decreasing oxidative stress. Both the initiator caspase, caspases 8, and the executive caspase, caspase 3, are activated by maternal diabetes, and these activations are blocked by SOD2 overexpression.

High glucose is the first identified pathogenic stressor that induces mitochondrial ROS in epithelial cells including neuroepithelial cells, and a number of studies have indicated that high glucose-induced mitochondrial ROS are risk factors for epithelial dysfunction [64]. One of the primary function of mitochondria in epithelial cells may be the regulation of the ROS level for cell signaling purposes [65]. In the present study, high glucose-induced mitochondrial ROS regulate neuroepithelial cell apoptosis. A previous study has shown that high glucose of maternal diabetes also impairs neuroepithelial cell differentiation into neurons [43]. Future studies may determine whether SOD2 overexpression-restored mitochondrial function could alleviate the inhibitory effect of high glucose on neuroepithelial cell differentiation.

While it is impossible to ascertain which early stage embryo exposed to maternal diabetes eventually forms a NTD, our study focused on E8.75, a critical time of murine neurulation which only encompasses from E8.0-E9.5. Because no structural hallmarks were identified for precisely predicting NTD formation in our study, distinguishing the difference between NTD- or non-NTD-like embryos is not possible before NTDs manifest. Under maternal diabetic conditions, every embryo exhibit molecular alterations but a threshold of such alterations is required for NTD formation. Concerns on the cause-and-effect relationship between observed changes and NTDs were raised on our previous studies using embryos after NTDs manifested [44]. In our published data [43] and current finding, maternal diabetes increased ROS, apoptosis and defective mitochondria only in the neuroepithelium but not in the adjacent non-neural tissues. Based on published findings [66] that antioxidant supplements ameliorate NTDs in diabetic pregnancy, our current findings support the hypothesis that SOD2 overexpression-blocked mitochondrial dysfunction and ROS production may contribute to the inhibitory effect of SOD2 overexpression on maternal diabetes-induced NTDs.

Due to the extreme small size of neurulation stage embryos, our study was able to detect embryonic gene and protein expression in the whole embryo. It is impossible to isolate sufficient amount of the developing neuroepithelia for some of the indices analyzed in our studies. However, when it is possible, the anterior neuroepithelium was used for analysis. Observations from the whole embryo are consistent with the findings in the neuroepithelium

[37, 43]. At neurulation stages, susceptible organs such as the heart are at their primordial stages. Thus, the changes observed at the whole embryo level largely reflect those in the anterior neuroepithelium.

In conclusion, SOD2 overexpression reverses maternal diabetes-induced mitochondrial dysfunction by suppressing mitochondrial ROS production and oxidative stress. SOD2 overexpression blocks the activation of pro-apoptotic Bcl-2 members, ER stress and caspase cleavage leading to reduction of neuroepithelial cell apoptosis, and ameliorates NTD incidence in the developing embryo.

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Highlights

- SOD2 overexpression ameliorates maternal diabetes-induced neural tube defects
- SOD2 overexpression restores mitochondrial function by blocking oxidative stress
- SOD2 abrogates maternal diabetes-induced endoplasmic reticulum stress and neuroepithelial cell apoptosis

Experiments were repeated three times using three embryos from three different dams. WT: Wild-type embryos; SOD2: SOD2 overexpressing embryos. ND: nondiabetic dams; DM: diabetic mellitus dams. * indicates significant difference ($P < 0.05$) compared with other groups.

Figure 2. SOD2 overexpression restores mitochondrial function by reducing the number of defective mitochondria and mitochondrial membrane potential

A: Representative EM images of mitochondria in E8.75 neuroepithelial cells sections, and the quantification of DHE mean fluorescence intensity. Defective mitochondria with disarrayed or disruptive cristae and decreased electron densities in the matrix. Scale bar: 200 nm. B: Defective mitochondria rates in neuroepithelial cells from E8.75 embryos. Defective mitochondria rate = the number of defective mitochondria divided by the total number of mitochondria. C: Representative images of JC-1 dye staining for detection of mitochondrial membrane potential in mitochondria isolated from E8.75 embryos. Green fluorescence indicates increased membrane potential while red fluorescence represents normal potential. Scale bar: 3.5 μ m. D: Ratios of green versus red fluorescent intensity in mitochondrial JC-1 dye staining. Membrane potential monitored as the mean fluorescent intensity ratio of green fluorescence to red fluorescence. Experiments were repeated three times. WT: Wild-type embryos; SOD2: SOD2 overexpressing embryos. ND: nondiabetic dams; DM: diabetic mellitus dams. * indicate significant difference ($P < 0.05$) compared with other groups.

Figure 3. SOD2 overexpression diminishes maternal diabetes-induced oxidative stress

A: Representative DHE staining images of E8.75 neuroepithelia sections. DHE reacts with the $O_2^{\bullet-}$ – present in cellular components such as protein and DNA, and is manifested by bright red fluorescence. All cell nuclei were stained with DAPI (blue). B, C: 4-HNE- and nitrotyrosine-modified protein levels in E8.75 embryos. Human SOD2 (hSOD2), the transgene product, was detected by a specific human SOD2 antibody. Experiments were repeated three times using three embryos from three different dams. D: Levels of lipid hydroperoxide (LPO) in E8.75 embryos, expressed as μM per gram of proteins. Embryos

from five different dams ($n = 5$) per group were analyzed. WT: Wild-type embryos; SOD2: SOD2 overexpressing embryos. ND: nondiabetic dams; DM: diabetic mellitus dams. * indicate significant difference ($P < 0.05$) compared with other groups.

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Figure 4. SOD2 overexpression abrogates maternal diabetes-induced endoplasmic reticulum stress

Protein abundance of phosphorylated and non-phosphorylated eIF2α (A), IRE1α (B), PERK (C) and CHOP (D) in E8.75 embryos. Human SOD2 (hSOD2), the transgene product, was detected by a specific human SOD2 antibody. Three embryos from three dams (n = 3) per group were analyzed. WT: Wild-type embryos; SOD2: SOD2 overexpressing embryos. ND: nondiabetic dams; DM: diabetic mellitus dams. * indicate significant difference ($P < 0.05$) compared with other groups.

Figure 5. SOD2 overexpression inhibits maternal diabetes-induced cell apoptosis in the developing neuroepithelium

A: Representative TUNEL assay images showing apoptotic cells (red dots) in the V-shape E8.75 neuroepithelia. Cell nuclei were stained with DAPI (blue). B: Quantification of TUNEL positive cells per section (three serial sections per embryo, and three embryos from three dams were analyzed). Protein abundance of cleaved caspase 8 (B) and caspase 3 (C) in E8.75 embryos. Human SOD2 (hSOD2), the transgene product, was detected by a specific human SOD2 antibody. Experiments were repeated three times using three embryos from three different dams (n = 3) per group. WT: Wild-type embryos; SOD2: SOD2

overexpressing embryos. ND: nondiabetic dams; DM: diabetic mellitus dams. * indicate significant difference ($P < 0.05$) compared with other groups.

SOD2 overexpression significantly reduces maternal diabetes-induced neural tube defects (NTDs)

Table 1

Experimental groups	Embryo Genotype	Blood glucose Lev (mg/dL)	Total number of embryos	Number of NTD embryos	NTD rate, %
ND	SOD2-Tg ♂ x WT ♀ (n = 9)	WT	30	0	0.0
		SOD2	30	1	3.3
DM	SOD2-Tg ♂ x WT ♀ (n = 8)	WT	21	7	25.0 [*]
		SOD2	24	1	4.0

ND: nondiabetic; DM: diabetes mellitus; WT: wild-type; Tg: transgenic; ♂: male and ♀: female (dams).

^{*} indicates significant difference compared with SOD2-overexpressing embryos from ND or DM dams, and WT embryos from ND dams.