Folic Acid Supplementation Diminishes Diabetes Induced Neural Tube Defects by Recovering Impaired Embryo Gene Expression Through Its Antioxidant Activity

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors KL and XC contribute equally to this work. All authors read and approved the final manuscript.

ABSTRACT

Aims: This study aimed to determine whether folic acid supplementation could decrease the neural tube defects (NTDs) in embryos caused by hyperglycemia and the mechanism is associated with its antioxidative activity.

Study Design: Diabetic pregnancy model were made and folic acid, Vitamin E and control were given to mice. Then the neural tube defects rate, oxidative stress markers and embryos size were measured and analyzed.

Methodology: We injected folic acid to diabetic mice to score NTDs rate, measured embryos size, assayed ROS scavenging enzymes and Pax3 expression and oxidative stress marker including malondiadehyde and H2O2 as well as the vitamin E controls.

Results: Injection of folic acid during gestation could diminish neural tube defect rate of diabetic mice and recover the Pax3 expression inhibition which is similar to vitamin E, but no effect was observed on nondiabetic mice. Folic acid supplementation also normalized the embryos size, decreased oxidative stress level including malondiadehyde and H2O2 in diabetic mice and...
decreased the intracellular ROS of embryonic cells on day 8.5 induced by high glucose incubation in a dose-dependent manner. No effects were observed on ROS scavenging enzymes activity by folic acid supplementation.

**Conclusion:** Overall, the conclusion is that folic acid supplementation could diminish NTDs induced by hyperglycemia, the mechanism is associated with its antioxidant activity which can reduce the oxidative stress and recover the inhibition of Pax3 expression.

**Keywords:** Diabetic embryopathy; embryo; folic acid; neural tube defect; oxidative stress.

### ABBREVIATIONS

MDA : malondiadehyde  
NTDs: Neural tube defects

### 1. INTRODUCTION

Maternal diabetes has some adverse effects on embryogenesis and fetal development which could cause multiple congenital malformations. The incidence rate of major malformations in diabetic pregnancy is two to three times higher than the normal pregnancy [1]. Among the various defects induced by high glucose, neural tube defects could be the most common malformations [2-3]. Neural tube defects are very serious development malformations include spina bifida and anencephaly. The rate of diabetic malformation could not decrease to the non-diabetic level no matter how good clinic therapy strategy they take. So it should be necessary to explore the possible biochemical mechanism under diabetic embryopathy. Pax3 gene encodes a transcription factor that is essential for neural tube development. Homozygous Splotch embryos (Sp/Sp) which carry loss of functional Pax3 alleles develop neural tube defects, neural crest, and skeletal muscle with 100% penetrance[4-7]. Previous studies have indicated that maternal diabetes induced by streptozotocin (STZ), or transient induction of hyperglycemia through glucose injection on embryonic day 7.5 leads to decreased expression of Pax3 on day 8.5 and increased incidence of neural tube defect (NTD), which can be recognized on day 10.5[8-9]. Studies have suggested that diabetic pregnancy is associated with oxidative stress which is caused by maternal diabetes has been demonstrated to be the reason for the inhibition of Pax3 expression and increased NTDs [10]. Moreover, administration of antioxidants such as vitamin E or Glutathione (GSH) ethyl ester can prevent hyperglycemia-induced developmental defects through decreasing oxidative stress, recovering the inhibition of Pax3 [11]. Supplementary folic acid intake during human pregnancy can diminish the fetal rate of defects [12-18]. However, the antiteratogenic mechanism of folic acid is still unclear. The studies results show that folate could enhance the production of methionine from homocystine, thereby preventing the accumulation of homocysteine which is an oxidant[19-20]. In addition, folic acid has antioxidant properties of its own [21]. Thus the folic acid supplementation might diminish the neural tube defects caused by hyperglycemia by reducing the oxidative stress in the embryo, at least in part, diminishing the inhibition of Pax3.

In this study, we tested the hypothesis that folic acid supplementation could decrease the inhibition of Pax3 gene expression and the NTDs caused by hyperglycemia, and whether this is associated with its antioxidative activity to decrease oxidative stress.

### 2. MATERIALS AND METHODS

#### 2.1 Animal

ICR mice used in the experiments were purchased from animal center of Xi’an Jiaotong University of China. All ICR mice were fed with commercial food and sterile water. Male and female ICR mice were mated and checked the plug next morning. The day which copulation plug was found is considered the gestational 0.5 day. Mice were killed to recover embryos for assay of markers of oxidative stress MDA and H$_2$O$_2$ on day 7.5, for assay of genes expression on day 8.5, for score the NTD on day 10.5. We also measure the crown-rump length, biaparietal diameter, abdominal anteroposterior diameter and abdominal transverse diameter of embryos on day 10.5.

#### 2.2 Induction of Diabetes

Diabetes was induced in 5 to 7 weeks old female ICR mice with 100 mg/Kg streptozotocin (STZ Sigma) dissolved in 10 mM sodium citrate (pH 4.5). Blood glucose was monitored daily with ACCU-CHEK (Roche). STZ-diabetic mice
maintained euglycemia with insulin pellets before pregnancy but developed hyperglycemia beginning on day 4.5 of pregnancy, thereby exposing the embryo to hyperglycemia during the entire post implantation period. Nondiabetic mice were injected in parallel with PBS.

2.3 Injection of Folic Acid

Control and diabetic pregnant ICR mice were given 10 mg/Kg folic acid (Sigma) by daily subcutaneous injections. The injections commenced on gestational day 0.5 and continued up to termination of pregnancy on gestational day 7.5, 8.5 or 10.5. Controls were injected in parallel with PBS. Vitamin E-treated animals were supplemented with 0.5% (w/w) vitamin E succinate beginning on day 0.5 of pregnancy continued to the animals were killed.

2.4 Preparation of Total RNA and cDNA

The total RNA from 50 embryos (n=5) of each group were extracted with a total RNA isolation kit TRIzol reagent (Invitrogen) according to the manufacture’s instruction. The reaction mixture containing 2 μg of RNA, 2.5 μM random primer, 200 U of molony murine leukemia virus reverse transcriptase (MMLV, Promega), 2 mM of each dNTPs, 5 U of RNasin in a total volume of 25 μl was incubated for 1 h at 42°C to synthesize cDNA.

2.5 Analysis of SOD, CAT, GPX and Pax3 mRNA Levels

Specific primers encoding CuZnSOD, MnSOD, CAT, GPX and Pax3 were used (Table 1). Each 25 μl of Realtime-PCR reaction (Takara) volume included 25 ng of cDNA 2μl, 12.5 μl of 2× SYBR Green Premix Ex Taq buffer, 1μl forward and reverse primer and 8.5 μl H₂O. PCR cycle parameters were 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 30 s.

2.6 Preparation of Embryonic Cell Suspensions and Assessment of Intracellular ROS Production

Embryos on day 8.5 of gestation were dissected from uteri and extraembryonic structure in PBS and placed in DMEM medium. Whole embryos were minced then by passing through cell strainer with 40µM nylon mesh (BD Falcon TM), to generate single cell suspensions. The suspension was prepared at a density of 2 × 10⁷ cells/ml. The embryonic cells were cultured in DMEM medium under different conditions for 24 hours: 0 mmol/L glucose medium, 30 mmol/L glucose medium, 30mmol/L with 1 mmol/L and 3 mmol/L folic acid. Cells were then washed twice with PBS. Intracellular ROS production was measured with 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probes). Embryonic cells were incubated with DCF-DA (5 μmol/L) in serum-free medium at 37°C for 30 minutes and then washed with PBS. DCF fluorescence was excited at 488 nm, and emission at 530nm was measured on Gemini EM Reader (Molecular Devices). Fluorescence intensity values were presented as the percentage of the control value, after subtraction of background fluorescence (Fig 3).

2.7 Assay of Malondialdehyde and H₂O₂

Embryos were recovered on day 7.5 for the assay of malondialdehyde (MDA) and H₂O₂. MDA was assayed spectrophotometrically using the thiobarbituric acid (TBA) test, taking purified MDA (Sigma) to generate a standard curve. H₂O₂ was assayed using kits of Cayman Chemicals according to the instructions. MDA and H₂O₂ were normalized to protein concentrations in cell extracts by using Bio-Rad protein reagent (Bio-Rad).

2.8 Statistical Analysis

Data were analyzed by SPSS software. The results are expressed as mean ± SD. Student’s t-test was used to compare difference between two groups. A p value less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1 Folic acid diminished the inhibition of Pax3 expression and NTD induced by hyperglycemia in vivo

Diabetic mice display serious NTD malformation. Among the defects of diabetic mice, 53.6% of defects are single exencephaly located at forebrain, midbrain or hindbrain. 21.4% defects are combined exencephaly. 25% defects are spina bifida. Subcutaneous injection of folic acid during gestation could diminish the NTD rate of diabetic mice. Injection of folic acid lowered the NTD rate from 44% to 17% similar to vitamin E supplementation which decreased to 16% (Fig 1A). The malformation in folic acid injection
group have similar formation to diabetic group (55.6% for single exencephaly, 22.2% for combined exencephaly and 22.2% for spina bifida) (Table 2). Injection of folic acid could recover the Pax3 inhibition by high glucose and it has similar effect to vitamin E which had been proved to prevent the NTD from diabetic mice through the decreasing of oxidative stress induced by hyperglycemia (Fig 1B). For both NTD incidence and Pax3 expression level, folic acid injection has no effects on nondiabetic mice. There is no effect for folic acid to change the mice glucose level (Table 3 and Table 4).

3.2 Folic Acid Could Normalize the Fetal Size in Diabetic Mice

We measured the size of embryos day 10.5 including crown-rump length (CRL), biaparietal diameter (BPD), abdominal anteroposterior diameter (APD) and abdominal transverse diameter (ATD) under different treatment conditions. We found the decreased size including CRL, BPD, APD and ATD in diabetic mice embryos compared with nondiabetic mice (p < 0.01). Injection of folic acid could normalize the decreased size in diabetic mice significantly compared with the diabetic mice (p < 0.01). There are still difference about the CRL, BPD and APD between folic acid group and nondiabetic mice. But there is no difference on ATD of D/FA compared with ND ATD (Table 5). There is also no difference on somite number between each group.

3.3 Folic Acid Decreased the Intracellular ROS Caused by Hyperglycemia in Embryonic Cells in Situ

ROS production of embryonic cells on day 8.5 were measured by DCF fluorescence, there was significantly increased ROS after 24 hours incubation with 30 mmol/L glucose medium. Embryonic cells on day 8.5 cultured in 30 mmol/L glucose medium increased intracellular ROS production by 142 ± 11.2 % compared with control which is 0 mmol/L glucose medium (100%; p < 0.01; n = 6). Addition of 1mmol/L and 3 mmol/L folic acid to 30 mmol/L glucose medium could decrease the ROS production compared with 0 mmol/L folic acid in a dose-dependent manner (decreased from 142 ± 11.2% to 130 ± 7.84% and 118 ± 6.68% versus control 100% n=6 Fig. 2).

3.4 Folic Acid Reduced the Oxidative Stress Marker Level

To further test whether the effect of reducing neural tube defect and inhibition of Pax3 expression by the supplementation of folic acid is the result of blocking of oxidative stress induced by the hyperglycemia, we assayed the oxidative stress markers of MDA and H$_2$O$_2$ with and without the supplementation of folic acid. As shown in Fig. 4, MDA, a marker of lipid peroxidation, was significantly increased by hyperglycemia. H$_2$O$_2$ was also increased. On the other hand, there was no effect of folic acid on MDA in nondiabetic pregnancies, but the increase in MDA observed in diabetic mice was significantly reduced by folic acid, although it was still significantly increased compared with nondiabetic mice. Similarly, there was no effect of folic acid on H$_2$O$_2$ of nondiabetic pregnancies. The increase in H$_2$O$_2$ observed in diabetic mice was significantly suppressed by supplementation of folic acid, although it remained significantly increased compared with nondiabetic mice (Fig. 3).

Table 1. Nucleotide sequences of the primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZnSOD forward [23]</td>
<td>AAGGCGGTGTGGTGGCTGAA</td>
</tr>
<tr>
<td>CuZnSOD reverse [23]</td>
<td>CAGGTCTCAAACATGCCTCT</td>
</tr>
<tr>
<td>MnSOD forward [23]</td>
<td>GCACATTACCGCGCAGTCA</td>
</tr>
<tr>
<td>MnSOD reverse [23]</td>
<td>AGCCCTCAGCAACCTCCTT</td>
</tr>
<tr>
<td>CAT forward [23]</td>
<td>GCAGATACCTGTGAACTGT</td>
</tr>
<tr>
<td>CAT reverse [23]</td>
<td>GTGAAATGTCCGGACACTGAG</td>
</tr>
<tr>
<td>GPX forward [23]</td>
<td>CCTTCAATAGTACGTCCGACCTG</td>
</tr>
<tr>
<td>GPX reverse [23]</td>
<td>CAAATTGCATTGCACCAAC</td>
</tr>
<tr>
<td>Pax3 forward [24]</td>
<td>CCA ACC ATA TCC GCC ACA A</td>
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<tr>
<td>Pax3 reverse [24]</td>
<td>TCT TAG AGA CGC AAC CAT GGG</td>
</tr>
<tr>
<td>GAPDH forward [24]</td>
<td>TGTGTCCGTCGTGGAATCTGA</td>
</tr>
<tr>
<td>GAPDH reverse [24]</td>
<td>CCTGCTTACACCACCTCT TGA</td>
</tr>
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</table>
Fig. 1. Effects of folic acid or vitamin E on Pax3 expression or NTD induced by maternal hyperglycemia. ICR mice were induced diabetic mice (D) and nondiabetic mice (ND), they were given folic acid injection or vitamin E supplementation as described in MATERIALS AND METHODS. A: * p < 0.01 vs. ND; ** p < 0.05 vs. all other treatment groups. B: * p < 0.05 vs. all other treatment groups; ** p < 0.05 vs. all other treatment groups. n=6 ND: non diabetic pregnancy D: diabetic pregnancy ND/FA: non diabetic pregnancy giving folic acid D/FA: diabetic pregnancy giving folic acid ND/Vit E: diabetic pregnancy giving vitamin E.
Fig. 2. Effects of folic acid on the decreased ROS production on day 8.5 embryonic cells caused by hyperglycemia. Embryonic cells on day 8.5 were cultured in 30 mmol/L DMEM medium for 24 hours shows increased ROS production (142% vs 100%, * p < 0.01 n=6). Addition of 1 mmol/L and 3 mmol/L folic acid decreased the ROS significantly (G30F1 vs G30F0 ** p < 0.05; G30F3 vs G30F0 *** p < 0.01; G30F3 vs G30F1 *** p < 0.05; G30F3 vs G0F0 *** p > 0.05; n=5). G0F0: 0 mmol/L glucose medium with no folic acid G30F0: 30 mmol/L glucose medium with no folic acid G30F1: 30 mmol/L glucose medium with 1 mmol/L folic acid G30F3: 30 mmol/L glucose medium with 3 mmol/L folic acid
Fig. 3. MDA and H$_2$O$_2$ in diabetic mice and nondiabetic mice under different treatments. 

A: MDA * $p < 0.001$ vs. ND; ** $p < 0.05$ vs. all other treatment groups. 
B: H$_2$O$_2$ * $p < 0.01$ vs. all other treatment groups; ** $p < 0.05$ vs. ND. $n=6$
Fig. 4. Effects of folic acid on ROS scavenging enzymes activity expression in diabetic and nondiabetic mice. ICR mice were induced diabetic mice (D) and nondiabetic mice (ND)DS. There are not statistically difference (p > 0.05 n = 6) between every two groups. ND: non diabetic pregnancy D: diabetic pregnancy ND/FA: non diabetic pregnancy giving folic acid D/FA: diabetic pregnancy giving folic acid

### Table 2. Morphology of day 10.5 mice embryos

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Normal</th>
<th>Exencephaly (single)</th>
<th>Exencephaly (combined)</th>
<th>Spina bifida</th>
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<tbody>
<tr>
<td>ND</td>
<td>58</td>
<td>55</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N/FA</td>
<td>56</td>
<td>53</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>62</td>
<td>34</td>
<td>15</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>D/FA</td>
<td>54</td>
<td>45</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D/VitE</td>
<td>56</td>
<td>46</td>
<td>7</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Morphology of day 10.5 embryos from normal (ND) and diabetic (D) mice, some of which were supplemented with folic acid (N/FA and D/FA) and vitamin E (D/VitE). Exencephaly (single): only one malformation located on forebrain, midbrain or hindbrain. Exencephaly (combined): more than two malformation in one embryo*
Table 3. Mean blood glucose levels of pregnancies on day 7.5 in Fig 1A

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>ND/FA</th>
<th>ND/Vit E</th>
<th>D</th>
<th>D/FA</th>
<th>D/Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose, mM</td>
<td>7.1±0.07</td>
<td>6.8±0.03</td>
<td>7.3±0.08</td>
<td>21.5±0.22</td>
<td>23.4±0.19</td>
<td>20.2±0.18</td>
</tr>
</tbody>
</table>


Table 4. Mean blood glucose levels of pregnancies in Fig 2B

<table>
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<tr>
<th></th>
<th>ND</th>
<th>ND/FA</th>
<th>ND/Vit E</th>
<th>D</th>
<th>D/FA</th>
<th>D/Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose, mM</td>
<td>6.8±0.04</td>
<td>7.2±0.05</td>
<td>7.1±0.09</td>
<td>20.1±0.12</td>
<td>22.7±0.15</td>
<td>19.8±0.12</td>
</tr>
</tbody>
</table>


Table 5. Embryo size on gestational day 10.5

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Somite number</th>
<th>CRL(µm)</th>
<th>BPD(µm)</th>
<th>APD(µm)</th>
<th>ATD(µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>58</td>
<td>36.2 ± 0.6</td>
<td>5038±258</td>
<td>2022±188</td>
<td>2366±148</td>
<td>1397±178</td>
</tr>
<tr>
<td>D</td>
<td>62</td>
<td>35.6 ± 0.5</td>
<td>4074±249</td>
<td>1494±172</td>
<td>1852±130</td>
<td>1207±127</td>
</tr>
<tr>
<td>D/FA</td>
<td>54</td>
<td>34.9 ± 0.8</td>
<td>4691±242*</td>
<td>1865±152**</td>
<td>1987±87*</td>
<td>1368±129***</td>
</tr>
</tbody>
</table>

Value are mean ± SE. All D $p < 0.01$ vs ND. All D/FA $p < 0.01$ vs D. For D/FA, * $p < 0.01$ vs ND, ** $p < 0.05$ vs ND, *** $p > 0.05$ vs ND. CRL: crown-rump length. BPD: biaparital diameter. APD: abdominal anteroposterior diameter. ATD: abdominal transverse diameter. ND: non diabetic pregnancy. D: diabetic pregnancy. D/FA: diabetic pregnancy giving folic acid.
3.5 Folic Acid didn’t Affect the ROS Scavenging Enzymes Activity in Diabetic Embryos

We measured the ROS scavenging enzymes activity in diabetic embryos, diabetes didn’t affect embryos ROS scavenging enzymes on day 8.5, we didn’t find any significant effect of folic acid on CuZnSOD, MnSOD, CAT, GPX expression level on day 8.5. There are no significant difference between the diabetic embryos and normal embryos on enzymes activity (p > 0.05). There is no difference in diabetic embryos with or without folic acid treatment (p > 0.05) (Fig 4).

4. DISCUSSION

To investigate the effect of folic acid on preventing the NTD induced by hyperglycemia and its molecular mechanism, we treated diabetic mice with folic acid in vivo. The results reported here support the hypothesis that folic acid may decrease the risk of NTD induced by high glucose and the mechanism maybe related to its antioxidant activity that may reduce oxidative stress and reduce the inhibition of Pax3 induced by high glucose.

Folic acid supplementation could reduce the incidence of neural tube defects in many cases, it has been applied as an effective preventive teratogenic method for many years[17]. Many studies have shown that about 50-70% of NTDs can be prevented by folic acid supplementation before and during pregnancy[22-23]. The question is how about the preventive effect of folic acid against NTDs in diabetic pregnancies which has a higher NTDs incidence rate compared with non-diabetic pregnancy. Folic acid injection to diabetic mice indicates that folic acid supplementation could reduce the NTDs caused by hyperglycemia (Fig. 1A, Fig. 5). Folic acid supplementation not only can diminish the malformation rate in diabetic mice but also can normalize the smaller size of embryos caused by high glucose (Table 5). So folic acid addition could defeat the glucose’s effect on embryo development. But the molecular mechanism is still unclear. Some studies show that the addition of extra folic acid corrects subtle alterations in the folic acid metabolism, but folic acid could not reduce the NTD rate to zero no matter in a clinic or the animal model[24-25]. At the same time, maternal folate levels in most affected pregnancies are within the normal range[26]. The abnormal folate metabolism is another point involved in the mechanism of folic acid against malformation. But there is no correlation between folic acid levels in the mother and incidence of NTDs in infants and no detectable abnormal metabolism of folate in diabetic pregnant women compared with normal pregnant women[27-30]. The study using the rat diabetic pregnancy model and embryo culturing shows that the diabetic environment induces a state of functional folic acid deficiency in the embryo. This deficiency is only partly a transport and concentration deficiency, as the high glucose cultured embryos did not show decreased levels of folic acid despite the presence of developmental malformation [11]. So it seems there must be some other folic acid beneficial activity involved in its supplementation effect.

More and more pieces of evidence suggest that the gene-environment interactions are etiology of NTDs. There are more than 100 single-gene defects that could cause NTDs in the mice. Folate status is a key environmental factor. Genes whose expression is altered in diabetes-exposed embryos might represent excellent candidates for folate-responsive genes and may mediate the beneficial effect of folate in the prevention of neural tube and other developmental defects [31-33]. Study shows that folate deficiency does not cause NTDs in wild-type mice, but caused a significant in cranial NTDs among Sp2H embryos which carry a mutation in Pax3, which is crucial for the neural tube closure[26]. So it appears reasonable to conclude that Pax3 which was inhibited by hyperglycemia might be a target for the folic acid effect. In vivo experiments shows that subcutaneous injection of folic acid to the STZ treated diabetic mice recovered partly the Pax3 expression level compared with normal mice embryos. We also observed that the folic acid injection did not alter the malformation rate and Pax3 expression in the control mice (Fig. 1B). It seems that the folic acid will not affect the Pax3 expression in the normal condition, but it can correct the Pax3 expression in high glucose. From the data provided here, it appears reasonable to conclude that the folic acid did not take effect on Pax3 directly. The effect must be taken by other mechanisms.

A growing body of research shows that oxidative stress plays a very important role in the development of diabetes-induced development defects such as NTDs [34-40]. Oxidative stress disrupts the expression of the pax3 gene during diabetic pregnancy. Our previous research has shown that diabetics suffer from oxidative
stress caused by hypoxia, which negatively impacts Pax3 expression and development. Experimental animals treated with antioxidants, such as Vitamin E, had a reduced level of oxidative stress and were protected from diabetes defects [11].

Folic acid's antioxidant activity is cited as a mechanism by which it can reduce NTD rate and recover Pax3 expression. In spite of the study results that showed folate was able to increase methionine production from homocysteine, preventing the accumulation of homocysteine, which is an oxidant [41-44], Folic acid was found to have its own antioxidant properties independent of homocysteine metabolism in another study [45].

This led us to look forward to folic acids preventive effects. We investigated the effect of folic acid on oxidative stress in embryonic cells cultured with or without folic acid first. Folic acid was capable of decreasing the oxidative stress caused by high glucose, and it is a dose-dependent effect (Fig.2). We measured some other oxidative stress markers. Our results showed that folic acid injection decreased MDA and H$_2$O$_2$ in diabetic embryos (Fig. 3). Vitamin E had a similar effect, as a positive control. Combined with the findings that folic acid supplementation did not protect the fetuses from neural tube defects in splotch mice which with a defect Pax3 function and a high incidence of NTDs [46], it is now reconfirmed that at least some of the mechanism by which the folic acid reduces NTDs is by decreasing oxidative stress. Furthermore, in diabetic and non-diabetic mice, the activity of ROS scavenging enzymes was not altered by folic acid treatment.

5. CONCLUSION
As a consequence of decreasing oxidative stress and recovering NTDs, folic acid reduced the inhibition of Pax3 expression in diabetic pregnancy. Does this apply to all folate-responsive genes whose expression is altered in diabetes-exposed embryos, or does this apply specifically to the Pax3 gene? The mechanism of folic acid's prevention of malformations during normal pregnancy is the same as it is during diabetic pregnancy, or is it different? These questions require further investigation.

CONENT
It is not applicable.

ETHICAL APPROVAL
All authors hereby declare that all experiments have been examined and approved by the ethics committee of Northwest University

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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