

# Combined Supplementation of Folic Acid and Vitamin E Diminishes Diabetes-Induced Embryotoxicity in Rats

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**BACKGROUND:** Oxidative stress and enhanced apoptosis may be involved in the induction of embryonic dysmorphogenesis in diabetic pregnancy. Administration of folic acid or vitamin E diminishes embryonic dysmorphogenesis. We aimed to evaluate the effect of combined treatment with folic acid and vitamin E on the disturbed development in embryos of diabetic rats. **METHODS:** Pregnant nondiabetic and diabetic rats were treated with daily injections of 15 mg/kg folic acid or with 5% vitamin E in the diet. A third group received combined treatment. Day 10 and day 11 embryos were evaluated for development and apoptotic profile. **RESULTS:** We found increased malformations, resorptions, and profound growth retardation in embryos of diabetic rats compared to control embryos. Vitamin E or folic acid alone, or the 2 compounds combined, normalized embryonic demise. Maternal diabetes caused decreased nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity and B-cell lymphoma 2 (Bcl-2) protein level, and increased Bcl-2-associated  $\times$  proteins (Bax) in embryos. Supplementation of vitamin E alone normalized the Bax protein level in a diabetic environment. Administration of folic acid to diabetic rats increased NF- $\kappa$ B activity and Bcl-2 protein level. Combined treatment normalized Bcl-2 and Bax protein level in a diabetic environment. **CONCLUSIONS:** Combined supplementation of folic acid and vitamin E to pregnant diabetic rats diminished diabetes-induced malformations and resorptions, concomitant with normalization of apoptotic protein levels. No treatment completely abolished the embryonic demise; therefore, other mechanisms than oxidative stress and apoptosis are likely to be involved in diabetic embryopathy. *Birth Defects Research (Part A) 76:483–490, 2006.* © 2006 Wiley-Liss, Inc.

**Key words:** embryopathy; rat; diabetes in pregnancy; folic acid; vitamin E; apoptosis

## INTRODUCTION

Maternal diabetes is associated with increased risk of growth disturbances and congenital malformations in the offspring (Mills, 1982; Becerra et al., 1990; Hawthorne et al., 1994; Penney et al., 2003). The risk of having a malformed child in diabetic pregnancy is 6–9% (Becerra et al., 1990; Hawthorne et al., 1994; Penney et al., 2003) compared to 2–3% (Mills, 1982) in normal pregnancy. The exact biological mechanisms behind diabetic embryopathy have not been fully elucidated. Increased oxidative stress, decreased antioxidative defense, or both conditions simultaneously, have been suggested as teratogenic agents in diabetic pregnancy (Eriksson and Borg, 1991; Eriksson et al., 1993; Eriksson and Simán, 1996; Forsberg et al., 1996; Cederberg and Eriksson, 1997; Wentzel et al., 1997). Several studies have tried to diminish oxidative stress by increasing the antioxidative defense, with good results (Cederberg et al., 2001; Bojunga et al., 2004).

In previous studies, we have successfully reduced embryonic dysmorphogenesis in offspring of diabetic rats by treating the mother with vitamin E (Simán and Eriksson,

1997) or folic acid (Wentzel et al., 2005). However, we were unable to completely normalize embryonic development with either of these therapies. Therefore, in this study we aimed to evaluate the effect of combined treatment with folic acid and vitamin E on the morphology of embryos from manifestly diabetic (MD) rats. Furthermore, we investigated the influence of these treatments on the levels of different proteins involved in apoptosis.

In a recent study we found that the intrinsic apoptosis pathway seems to be the predominant pathway of the increased apoptosis associated with embryonic maldevelop-

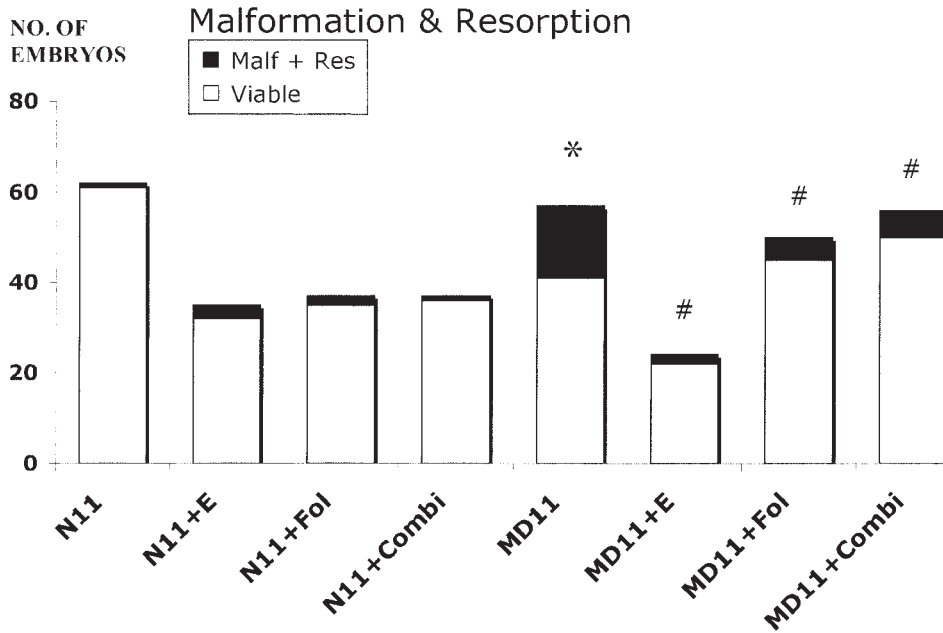
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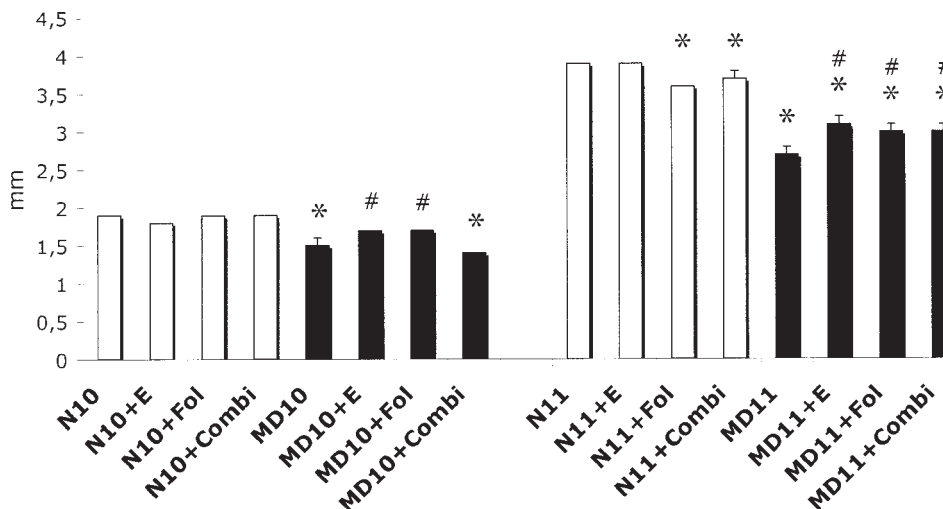
**Figure 1.** Malformations and resorptions. Embryos from nondiabetic and diabetic rats at GD 11 (N11, MD11), treated with vitamin E (N+E, MD+E) and folic acid alone (N+Fol, MD+Fol) or with a combination of vitamin E and folic acid (N+Combi, MD + Combi), were evaluated for malformations and resorptions calculated in real numbers of total implantations in each experimental group. We studied 4–6 pregnancies and evaluated 23–60 embryos of each group. Significances \* =  $P < .05$  versus N embryos, # =  $P < .05$  versus MD embryos ( $\chi^2$  test).

ment in the diabetic environment (Gäreskog M., Cederberg J., Eriksson U. J., Wentzel P. unpublished results). In this study we therefore measured several proteins involved in the activation of apoptosis through the intrinsic pathway, namely the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) and the proapoptotic marker Bcl-2-associated x protein (Bax). Furthermore, we measured activation of nuclear factor- $\kappa$  B (NF- $\kappa$ B). This transcription factor can be activated by several different agents, after which it translocates to the nucleus and enhances the transcription of several genes, including antioxidative enzymes and antiapoptotic proteins (Tamatani et al., 1999; Catz and Johnson, 2001; Tanaka et al., 2002).

Previous studies have suggested a role for changed apoptotic rate in the maldevelopment of embryos subjected to a diabetic milieu. Recently we found an association

between increased apoptosis and embryos exposed to a diabetic environment (Gäreskog M., Cederberg J., Eriksson U. J., Wentzel P. unpublished results). This finding suggested that increased apoptosis may contribute to the demise of the embryo and that enhanced cell death rate may also be involved in the malformations found in diabetic pregnancy. This notion has been supported by studies suggesting that hyperglycemia induces apoptosis in preimplantation embryos via cell-death effector pathways (Moley et al., 1998). Maternal diabetes has also been found to decrease the number of cells in the inner cell mass of preimplantation embryos (Pampfer et al., 1990). Furthermore, neural tube defects were associated with apoptosis in a postimplantation model of maternal diabetes (Phelan et al., 1997). These observations support the notion that increased apoptosis is associated with diabetic embryopathy.

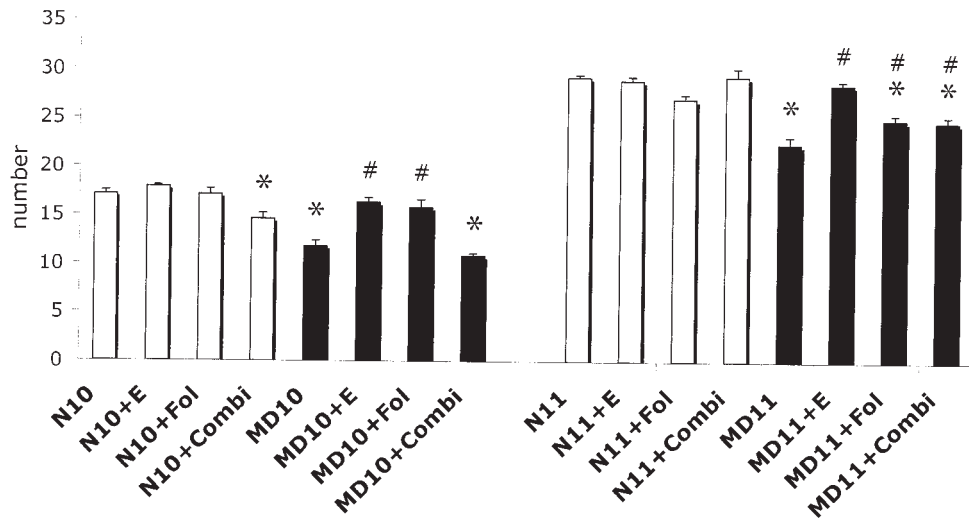
**Crown rump length**



**Figure 2.** Crown rump length of embryos. Embryos from nondiabetic and diabetic rats at GD 10 (N10, MD10) and GD 11 (N11, MD11), treated with vitamin E (N+E, MD+E) and folic acid alone (N+Fol, MD+Fol) or combined with vitamin E (N+Combi, MD + Combi), were measured in mm regarding crown rump length. We studied 3–6 pregnancies and evaluated 23–60 embryos from each group. Means + SEM. Significances \* =  $P < .05$  versus N embryos, # =  $P < .05$  versus MD embryos (ANOVA).

Somite numbers

**Figure 3.** Somite numbers of embryos. Embryos from nondiabetic and diabetic rats at GD 10 (N10, MD10) and GD 11 (N11, MD11), treated with vitamin E (N + E, MD + E) and folic acid alone (N + Fol, MD + Fol) or combined with vitamin E (N+Combi, MD + Combi), were evaluated regarding number of somites. We studied 4–6 pregnancies and evaluated 23–60 embryos of each group. Means + SEM. Significances \* =  $P < .05$  versus N embryos, # =  $P < .05$  versus MD embryos (ANOVA).



In this study we treated pregnant nondiabetic (N) and MD rats with daily folic acid injections, with vitamin E-supplemented food. In addition, we treated rats with both substances individually. Both substances have been used individually before, with beneficial effects on diabetes-induced dysmorphogenesis. We aimed to evaluate possible positive effects from a combined treatment on embryonic growth and malformation rate. We collected and examined embryos of N and MD rats with and without treatment. Furthermore, we wanted to investigate whether markers of apoptosis were influenced by the treatment in the same manner as the malformations. We measured the activity of NF- $\kappa$ B and the Bcl-2, Bax in the collected embryos.

MATERIALS AND METHODS

Animals

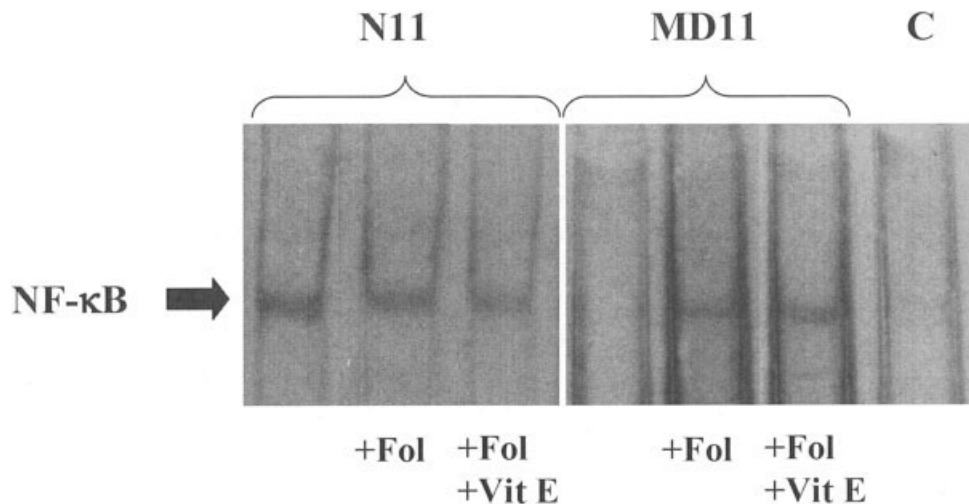
Embryos were obtained from nondiabetic and diabetic females of a local outbred Sprague-Dawley rat strain

with an increased incidence of congenital malformations in diabetic pregnancy (Eriksson et al., 1982). All rats were fed a commercial pellet diet (AB Analycen, Lidköping, Sweden). The rats had free access to food and tap water and were maintained at an ambient temperature of 22°C with a 12-hr light/dark cycle.

Diabetes was induced with a single injection of 40 mg/kg streptozotocin (SZ; Sigma-Aldrich Chemie GmbH, Stenheim, Germany) into a tail vein. Blood glucose was measured after 1 week with a MediSense glucose sensor (Abbott Scandinavia AB, Solna, Sweden). Rats with a glucose concentration of >20 mM were considered to be MD, and non-SZ-injected female rats of same age served as N controls. After establishing the diabetic state, the MD and N rats were mated with male N rats overnight. A positive vaginal smear (containing sperm) the next morning was designated gestational day (GD) 0.

N and MD pregnant rats were given 15 mg/kg folic acid (Sigma-Aldrich Chemie GmbH) by daily subcutaneous injections in the neck pouch of 0.5 ml of 10 mg/ml

**Figure 4.** Estimated activity of NF- $\kappa$ B in embryos. Representative picture from electromobility shift assays of NF- $\kappa$ B activity. Embryos from nondiabetic and diabetic rats at GD 11 (N11, MD11), treated with folic acid alone (N + Fol, MD + Fol) or combined with vitamin E (N + Combi, MD+Combi), were prepared as described in Materials and Methods, and electromobility shift assay of NF- $\kappa$ B activity was carried out. We studied 3–5 pregnancies and used 10–15 embryos of each group for the experimental procedure. The last lane, "C," represents negative control; a 100-fold excess of unlabeled oligonucleotide was added.



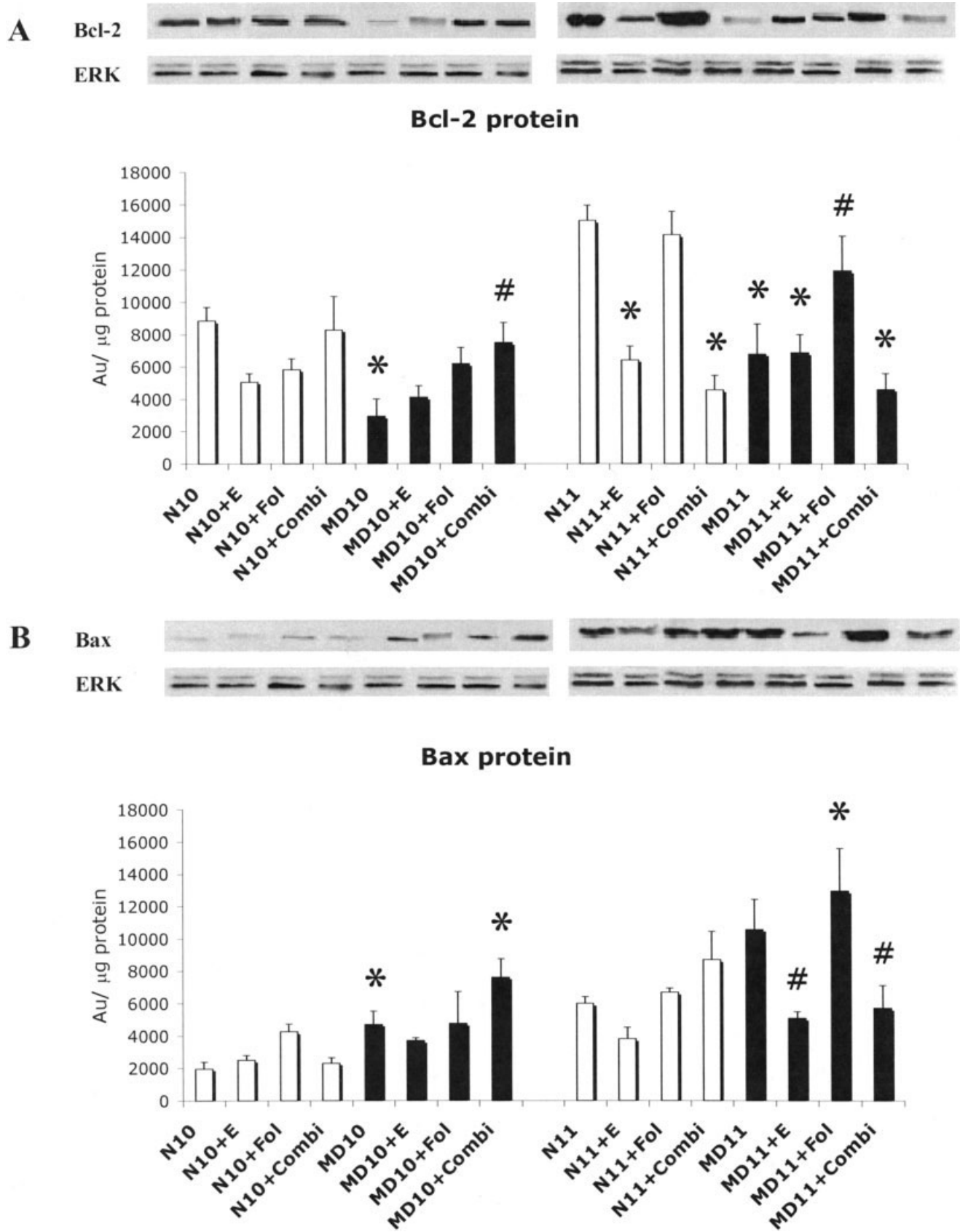


Figure 5.

folic acid (dissolved in redistilled water with pH adjustment to 7.8–7.9). The injections started on GD 0 and continued until termination of pregnancy on GD 10 or GD 11. A second group of N and MD rats were supplemented with 5% of vitamin E mixed in the food during the same period of time. A third group received a combined treatment with folic acid and vitamin E. Untreated N and MD pregnant rats served as controls.

On GD 10 and GD 11 the N and MD rats were killed by cervical dislocation after light ether anesthesia. Each embryo was carefully dissected out and examined in a stereomicroscope for malformations, crown rump length (CRL), and somite numbers. In particular, the occurrence of disturbed embryonic development was noted, such as open neural tube, tail twist, and somatic malrotation. Some of the embryos were processed for NF- $\kappa$ B activity measurement (see Electromobility Shift Assay below), and some were used for Western blot (see Western blot Analyses below).

#### Extraction of Nuclear Proteins

Embryos from GD 10 and GD 11 control and diabetic rats were pooled and processed to extract the nuclear proteins. Briefly, the embryos were homogenized with a handheld blender, pelleted, and lysed in 50  $\mu$ l of buffer A (10 mM Tris pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 2 mM dithiothreitol, and 0.4 mM Pefabloc; Roche, Mannheim, Germany). After centrifugation the pellet was resuspended of buffer A. Nuclei were pelleted and nuclear proteins were extracted by addition of 50  $\mu$ l buffer C (20 mM Tris pH 7.5, 1.5 mM MgCl<sub>2</sub>, 0.42 M KCl, 20% glycerol, 2 mM dithiothreitol, 0.4 mM Pefabloc) and sonication. After sonication the samples were kept on ice for 20 min. Nuclear protein extracts were stored at 70°C.

#### Electromobility Shift Assay

For electromobility shift assays (EMSA) of NF- $\kappa$ B, we used the following double-stranded oligonucleotide: 5'-

AGCTTCAGAGGGGACTTTCCGAGAGG-3'. The oligonucleotide was labeled with [<sup>32</sup>P] ATP using T4 polynucleotide kinase (USB Corporation, Cleveland, OH) and purified with Chroma spin columns (BD Biosciences, Palo Alto, CA). Nuclear protein extracts (4.5  $\mu$ l) were denatured with formamide and incubated in a 20  $\mu$ l reaction mixture (0.1 ng DNA; 14,000 cpm), 10 mM Tris pH 7.5, 40 mM NaCl, 1 mM EDTA, 0.2% deoxycholic acid, 4% glycerol, 1 mM  $\beta$ -mercaptoethanol, 2  $\mu$ g polydeoxyinosinic-deoxycytidylic acid) for 30 min at room temperature. A 100-fold excess of nonlabeled oligonucleotide was used as negative control. Samples were separated on 5% non-denaturing polyacrylamide gel in 0.5  $\times$  TBE (Tris-Borate-EDTA), dried and exposed to X-ray film (Kodak Industries, Cedex, France).

#### Western Blot Analyses

Tissue homogenates were lysed and fractionated by SDS-PAGE (12%) at 14 mA for 1 hr. Proteins were transferred to nitrocellulose membrane (Hybond-P; Amersham Biotech, Uppsala, Sweden) overnight at 30 V.

The membranes were blocked overnight with 5% non-fat dried milk and subsequently incubated with the primary antibody (Bcl-2 sc-492, 1:1000, Bax sc-526, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Unbound antibody was removed by washing with PBS-Tween. The membranes were then incubated with the secondary antibody (anti-rabbit Ig, Amersham Biosciences, Buckinghamshire, England) diluted 1:1000 in 25 ml of PBS-Tween + 2.5% BSA (Miles Scientific, Naperville, IL) for 30 min.

After extensive washing with PBS-Tween, the membranes were covered with Enhanced chemiluminescent (ECL)+plus Western blotting detection system fluid (RPN 2132; Amersham Biosciences, Buckinghamshire, UK). After 5 min excess fluid was removed with Whatman paper. The membranes were confined in plastic film, with the proteins upward, and placed in an exposure box. Film (Hyperfilm MP, 18  $\times$  24 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK) was applied in a darkroom and developed with Agfa Curix 60 (Agfa-Gevaert AB, Kista, Sweden).

The developed film was scanned into a computer and densitometrically evaluated with Kodak Digital Science 1D; the protein density was expressed in arbitrary units (AU). Total extracellular regulated kinase (ERK) was used as normalization control.

#### Measurement of Protein

Total protein content was estimated in each sample from an aliquot of 10  $\mu$ l of lysate by the method of Lowry et al. (1951) using BSA as a standard.

#### Ethical and Statistical Considerations

The Animal Ethical Committee of the Medical Faculty of Uppsala University approved the research protocol including all experimental procedures involving animals. In most comparisons of group means, statistical significance ( $P < .05$ ) was determined by ANOVA followed by Fisher's protected least significant difference post-hoc test. Differences between fractions of malformations and resorptions were analyzed with  $\chi^2$ -test. Analyses were performed using the program StatView for Macintosh (SAS Institute, Cary, NC).

**Figure 5. A:** Estimated Bcl-2 protein in embryos. Bcl-2 protein levels in GD 10 and GD 11 untreated embryos from normal (N) or diabetic (MD) rats, treated with vitamin E (N+E, MD+E) and folic acid alone (N+Fol, MD+Fol) or combined with vitamin E (N+Combi, MD+Combi), estimated by Western blot. Each fraction is normalized by dividing the densitometric reading (Arbitrary Unit, AU) with the protein content of the whole sample ( $\mu$ g), thereby expressing the protein amount in AU/ $\mu$ g. We studied 3–4 pregnancies and used 7–20 embryos of each group for the experimental procedure. Original Western blot bands are shown at the top of the picture, and below normalization control ERK, in the same order of categories as the bars. Means + SEM. Significances of protein activity: \* =  $P < .05$  versus N embryos, # =  $P < .05$  versus MD embryos (ANOVA). **B.** Estimated Bax protein in embryos. Bax protein in GD 10 and GD 11 untreated embryos from normal (N) or diabetic (MD) rats, treated with vitamin E (N+E, MD+E) and folic acid alone (N+Fol, MD+Fol) or combined with vitamin E (N+Combi, MD+Combi), estimated by Western blot. Each fraction is normalized by dividing the densitometric reading (AU) with the protein content of the whole sample ( $\mu$ g), thereby expressing the protein amount in AU/ $\mu$ g. We studied 3–4 pregnancies and used 7–20 embryos of each group for the experimental procedure. Original Western blot bands are shown at the top of the picture, and below normalization control ERK, in the same order of categories as the bars. Means + SEM. Significances of protein activity: \* =  $P < .05$  versus N embryos, # =  $P < .05$  versus MD embryos (ANOVA).

## RESULTS

### Morphological Evaluation

**Malformations and resorptions.** We found an increased number of malformations and resorptions in embryos from MD rats compared to embryos from N rats. Administration of vitamin E and folic acid individually to MD rats normalized malformations and resorptions. Supplementation of folic acid with vitamin E to MD rats also normalized the malformations and resorptions compared to untreated diabetic rats. The addition of vitamin E and injections of folic acid to N rats did not alter malformations and resorptions compared to untreated N rats, nor did combined administration of folic acid and vitamin E (Fig. 1).

**Crown rump length.** We found decreased CRL in MD embryos compared to N embryos, on both GD 10 and GD 11. Administration of vitamin E and folic acid to MD rats normalized CRL on GD 10 and almost normalized CRL on GD 11. Combined supplementation of folic acid and vitamin E to MD rats did not change CRL on GD 10, but partly normalized CRL on GD 11. Addition of vitamin E to N rats did not affect CRL. Administration of folic acid alone or in combination with vitamin E to N rats did not alter CRL on GD 10, but slightly decreased the CRL on GD 11 (Fig. 2).

**Somite numbers.** We found a decreased number of somites in embryos exposed to a diabetic milieu compared to embryos from normal pregnancy on GD 10 and GD 11. Addition of vitamin E and injections of folic acid to MD pregnant rats normalized somite numbers on GD 10. On GD 11 the somite numbers were normalized with vitamin E treatment and partly normalized with folic acid treatment compared to untreated MD rats. Combined administration of folic acid and vitamin E did not change somite numbers on GD 10, but increased the somite numbers on GD 11. Administration of vitamin E and folic acid individually to N rats did not alter somite numbers. Combined treatment of N rats decreased somite numbers at GD 10, but did not affect the somite numbers on GD 11 (Fig. 3).

### Estimation of NF- $\kappa$ B Activity

The result of the EMSAs showed undetectable levels of NF- $\kappa$ B in nuclear protein extracts of GD 10 embryos with or without diabetes regardless of treatment. At GD 11 the EMSA showed a decreased activation of NF- $\kappa$ B in embryos from a diabetic milieu compared to embryos from a normal environment. Administration of folic acid to the pregnant MD rats increased the NF- $\kappa$ B activity in offspring. Combined administration of folic acid and vitamin E increased the activation of NF- $\kappa$ B in the embryos from MD rats. Supplementation of folic acid alone and combined with vitamin E to N rats did not alter the NF- $\kappa$ B activity in the offspring compared to embryos from untreated N rats (Fig. 4).

### Estimation of Apoptosis-Related Proteins

**Bcl-2 protein.** The Bcl-2 protein level was decreased on GD 10 and GD 11 in embryos from MD rats compared to embryos from N rats. Treatment of the MD rats with vitamin E and folic acid individually did not affect the embryos on GD 10 but folic acid normalized the Bcl-2 protein levels on GD 11. Combined treatment of the MD

rats normalized Bcl-2 protein in GD 10 embryos, whereas Bcl-2 protein levels remained decreased in GD 11 embryos. Addition of vitamin E to N rats decreased Bcl-2 protein on GD 11 compared to untreated N rats. Supplementation of folic acid to N rats did not alter Bcl-2 protein on GD 10 or GD 11 embryos. Combined supplementation decreased Bcl-2 protein on GD 11 compared to untreated N rats (Fig. 5A).

**Bax protein.** The proapoptotic protein Bax was increased in the embryos from MD rats compared to levels in embryos from N rats on GD 10 and tended to increase on GD 11. Administration of vitamin E did not affect Bax protein level in embryos of MD rats at GD 10, but normalized Bax levels at GD 11. Treatment with folic acid did not affect embryos of MD rats, neither on GD 10 nor on GD 11. The combined treatment of the MD rats increased the Bax protein level at GD 10, whereas Bax protein level in GD 11 MD embryos was normalized. However, the Bax protein levels of the GD 11 MD embryos treated with folic acid differed from that of the MD11 embryos treated with vitamin E and folic acid ( $P < .05$ ). Supplementation of vitamin E alone and folic acid alone or combined with vitamin E to the N rats did not alter the Bax protein in the embryo, either on GD 10 or GD 11 (Fig. 5B).

## DISCUSSION

The most important findings in this study were the positive effects of combined administration of folic acid and vitamin E on diabetes-induced embryonic malformations and resorptions as well as the findings supporting a role for NF- $\kappa$ B in rat organogenesis. Along with the malformation- and resorption-lowering effect of the combined treatment, we found normalizing effects on Bcl-2 protein levels on GD 10 but not on GD 11. This complex pattern of effects on apoptosis-related proteins not only indicates a possible role for apoptosis in the demise occurring in the embryo due to a hyperglycemic environment, but also suggests that other mechanisms are involved in the diabetic embryopathy.

This study focused on rat embryonic development at GD 10 and GD 11. These 2 days were chosen because they are critical in neural tube closure, early heart development, and other somatic malformations found in diabetic pregnancy. GD 10 and GD 11 can be seen as representative of 2 separate embryonic periods where antiteratogenic treatments may exert completely different effects on the embryo, mainly because the shift from a yolk sac placenta to the permanent chorioallantoic placenta occurs during these 2 days.

It has been proposed that NF- $\kappa$ B is responsible for proapoptotic events in cells with NF- $\kappa$ B-regulated genes such as Fas, FasL, and p53 (Wu and Lozano, 1994; Matsui et al., 1998; Kasibhatla et al., 1999). However, several recent studies have demonstrated antiapoptotic products from NF- $\kappa$ B-regulated genes such as Bcl-2, Bcl-X<sub>L</sub>, and MnSOD (Chen et al., 2000; Catz and Johnson, 2001; Tanaka et al., 2002). Bcl-2, Bcl-X<sub>L</sub>, and Bax are important during embryonic development since they inhibit (Bcl-2 and Bcl-X<sub>L</sub>) and promote (Bax) apoptosis in cell populations (Metcalf et al., 2004). When the ratio of Bcl-2 to Bax changes to the advantage of Bax, the downstream effector in the apoptotic pathway, caspase-3, is activated, a process that is also important in fetal development (Woo et al., 1998). The present study showed strong acti-

vation of NF- $\kappa$ B in embryos of normal rats and decreased activity in embryos of diabetic rats, whereas the normalizing effect with both folic acid administration and combined supplementation of folic acid and vitamin E may indicate a connection between reactive oxygen species (ROS) and NF- $\kappa$ B. Indeed, a recent study shows that NF- $\kappa$ B inhibits ROS in part by upregulating the ferritin heavy chain (FHC), 1 of 2 subunits of ferritin and the primary iron-storage protein complex of the cell (Pham et al., 2004). Moreover NF- $\kappa$ B inhibits MKK7 in the JNK-signaling pathway by Gadd45 $\beta$ , preventing cell death (Papa et al., 2004).

In the present study, we found, in general, an attenuating effect on markers of apoptosis by treatment with vitamin E and folic acid. However, we were unable to demonstrate a clear additive or synergistic effect of the treatments. Furthermore, we noted inappropriate effects of the treatments, such as the combined administration of the 2 treatments not being able to normalize embryonic development on GD 10, although the Bcl-2 levels were normalized. Likewise, on GD 11, the combined treatment differed from folic acid administration with regard to Bcl-2 and Bax protein levels in the embryos. These findings may be the result of stochastic coincidence, although we cannot rule out the possibility of negative interaction between folic acid and vitamin E, at least in specific dosage intervals. The possible biochemical background of such negative interaction is not clear, however, and the main result of this study remains the positive effects on diabetes-induced developmental disturbances and apoptotic markers exerted by vitamin E and folic acid. The absence of an additive or synergistic effect suggests that these agents affect the same teratological pathway(s).

The fact that diabetic women still have an increased risk of giving birth to malformed children, despite intensive insulin therapy, obliges us to do further research in this field. Several suggestions have emerged to explain the reasons for the increased malformation rate, such as oxidative stress, abnormal activation of protein kinase C, apoptosis, disturbed arachidonic acid pathway and prostaglandin synthesis, and hypoxia (Goldman et al., 1985; Eriksson and Borg, 1991; Phelan et al., 1997; Gareskog and Wentzel, 2004; Li et al., 2005; Wentzel and Eriksson, 2005). It is likely that all of these suggestions could be involved in the etiology of diabetic embryopathy (Sadler et al., 1989).

The question is whether we can affect these disturbed pathways with 1 universal treatment. Based on the results from the present study it is probable that we need combined treatments to normalize the increased risk of fetal dysmorphogenesis associated with maternal diabetes.

In a previous study, we found a clear beneficial effect on malformations and growth retardation from folic acid administration to pregnant diabetic rats, both in vivo and in vitro (Wentzel and Eriksson, 2005). Other studies have shown beneficial effects on neural tube defects in clinical trials using folic acid (MRC, 1991; Berry et al., 1999). Similar results have been found in follow-up studies on the folic acid fortification of the U.S. food supply (Honein et al., 2001; Simmons et al., 2004). Several experimental studies have used supplementation of vitamin E alone and found protective effects against diabetic embryopathy for malformations and resorptions (Sivan et al., 1996; Viana et al., 1996; Simán and Eriksson, 1997). A recent study reported decreased malformation rate in embryos exposed to a diabetes-like milieu treated with

vitamin E in vivo and folic acid in vitro concomitant with a reverse of diabetes-induced diminished prostaglandin E<sub>2</sub> levels (Wentzel and Eriksson, 2005).

These results, in combination with the present results, may indicate that the doses of each individual antioxidant, rather than a combined synergistic effect of folic acid and vitamin E, determine the protective effect against diabetic embryopathy. Cederberg et al. (2001) treated diabetic rats with a combination of vitamin E and vitamin C, which successfully decreased fetal malformations and diminished oxygen radical-related tissue damage. However, the authors could find no synergistic effect between the 2 antioxidants.

Vitamin E is absorbed from the intestine along with dietary fats and is released into the circulation with chylomicrons (Traber, 1997). The  $\alpha$ -tocopherol transfer protein exists in the liver and facilitates the incorporation of  $\alpha$ -tocopherol (Sato et al., 1979). Fetal liver concentration of  $\alpha$ -tocopherol increases in a dose-dependent manner by maternal vitamin supplementation (Cederberg et al., 2001). Oral supplementation of the mother should therefore be a good means of increasing vitamin E concentration.

The cellular uptake of folate involves 2 distinct pathways: the reduced folate carrier pathway (Henderson et al., 1986) and the membrane-associated folate receptors pathway (Kamen et al., 1988). The folate receptors are abundant in human placental tissue, where they play a major role in maternal-fetal folate transport (Henderson et al., 1995).

There may be an issue of dose regarding combined antioxidants. A recently published article reported a dose-dependent decrease of malformed embryos when the diabetic dams were supplemented with vitamin E concentrations from 2 to 15% (Cederberg and Eriksson, 2005). However, they also found 1 malformation in the nondiabetic group of rats receiving 15% vitamin E, indicating the need for precaution with the antioxidative compounds in future human research.

In conclusion, folic acid and vitamin E, 2 compounds with antioxidative features (Ingold, 1961; Racek et al., 2005) diminish the increased oxidative stress in the diabetic milieu. This may be exerted by each compound alone as well as in combination. Since we could not demonstrate the additive or synergistic beneficial effects of the combined treatment, we conclude that both compounds block the same teratological processes. There are probably mechanisms involved in diabetic embryopathy separated from oxidative stress that these antioxidative properties cannot normalize. As a consequence, attempts to completely normalize fetal outcome in diabetic pregnancy may have to include some other mixtures and antiteratogenic substances.

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