

Teratogenic effects of diabetes mellitus in the rat. Prevention by vitamin E

M. Viana^{1,2}, E. Herrera², B. Bonet^{1,2}

¹ Hospital del Niño Jesús. Sección de Endocrinología, Madrid, Spain

² Facultad de Ciencias Experimentales y Técnicas, Universidad de San Pablo CEU, Madrid, Spain

Summary We wanted to determine whether administration of vitamin E could reduce the production of free radicals which could play a role in the teratogenic effects of diabetes mellitus. Diabetes was induced in Wistar rats by the intravenous administration of streptozotocin. The animals were divided into six groups: one with no supplement (D) and two, supplemented during pregnancy either with oral vitamin E (150 mg/day) (D + E) or with a placebo (safflower oil) (D + O). Three other groups were kept under the same conditions, but were treated with insulin: D + I, D + I + E and D + I + O. There were three groups of matched controls: C, C + E and C + O. All animals were killed on day 11.5 of pregnancy. In C animals the percentages of reabsorptions and malformations were 1.3 and 2%, respectively, compared with 23.6, 24.3, 6.2 and 13.2%, respectively in D and D + I groups. The crown-rump length, number

of somites, and protein and DNA content were higher in C animals than in the diabetic rats, independent of insulin treatment. When vitamin E was administered no changes in these parameters were observed in C and D + I animals; however, in the D mothers it reduced the rate of embryo malformations to 4.6% and increased the crown-rump length and the number of somites. However, vitamin E did not modify the protein and DNA content and the percentage of reabsorptions. In conclusion, administration of vitamin E to diabetic animals decreases the rate of embryo malformations and increases their size and maturation, supporting a role for free radicals in the teratogenic effects of diabetes. [Diabetologia (1996) 39: 1041–1046]

Keywords Diabetes mellitus, teratogenesis, free-radicals, pregnancy, vitamin E.

Poor metabolic control in pre-conception diabetes mellitus is associated with an increased incidence of abortions and congenital malformations [1–4]. Several metabolic parameters, including glucose, ketone bodies and triglycerides, could be involved in the teratogenic effects [5–8]. In the embryo culture model both hyperglycaemia and hyperketonaemia cause embryo malformations [9–11]. Very little is known about the pathophysiological mechanisms involved, which makes any attempt to reduce the rate of

embryo malformations, other than by achieving optimal metabolic control, very difficult.

Recently it has been proposed that enhanced production of free radicals could play a role in the teratogenic effects of diabetes [12, 13]. In the embryo culture model, the rate of malformations linked to hyperglycaemia and hyperketonaemia decreases when superoxide dismutase or butylated hydroxytoluene, two free radical scavengers, are added to the culture media [12, 13]. Furthermore, diabetes per se is a state of increased oxidative stress. The concentration of lipid peroxides is increased in cell membranes and lipoproteins of diabetic rats [14, 15]. LDL from pregnant diabetic women is more susceptible to oxidation [16]. Protein glycation, a phenomenon commonly found in diabetes, could generate free radicals [17, 18]. Finally, the concentration of antioxidant vitamins

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Corresponding author: Dr. B. Bonet, Centro de Ciencias Experimentales y Técnicas, Universidad de San Pablo, CEU, P.O. Box 67, E-28660 Boadilla del Monte (Madrid), Spain

is decreased both in the diabetic experimental animal and in humans [19–21].

Despite these considerations, there are no *in vivo* studies on the potential benefits of dietary antioxidant treatment for prevention of the teratogenic effects of diabetes. The present study addresses the question whether adding vitamin E, a liposoluble free radical scavenger, to the diet of pregnant diabetic rats could decrease the rate of fetal malformations.

Materials and methods

Animals and experimental design. Female virgin Wistar rats from our own colony, weighing 190–220 g, were housed in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with alternating 12-h light and dark cycles, and fed a Purina Chow diet (Rat and Mouse Standard diet; Beekay Feeds, B.K: Universal S.L., Barcelona, Spain). The care and handling of the animals throughout the study followed the current animal care law of the European Union.

Diabetes was induced by a single intravenous dose of streptozotocin (45 mg/kg) (Sigma Chemical Co., St. Louis, Mo., USA) in citrate buffer (0.05 mol/l) (pH 4.5). Five days later a state of insulin deficiency was confirmed by a positive reaction of a urine chemistrip for both glucose and ketone bodies, and insulin replacement was started. The insulin dosage was chosen on the basis of our previous laboratory experience [22, 23]; 1.5 IU/100 g body weight of Insulin Lente MC (90% bovine, 10% porcine) (Novo Nordisk A/S, Bagsvaerd, Denmark) was injected subcutaneously, between 09.00 and 10.00 hours. The animals were mated and the same day that sperm appeared in vaginal smears (day 0 of gestation) the animals were divided into their experimental groups.

Three groups of diabetic animals, with no insulin administration during pregnancy, one without any supplement (D), another supplemented with 150 mg vitamin E (Alfa-Tocopherol acetate, Sigma) per day, dissolved in safflower oil, total volume 250 μl , administered by gavage from day 1 of pregnancy (D + E) and a third group that received 250 μl safflower oil by gavage (D + O). Three additional groups of diabetic animals were treated with insulin during pregnancy: one with no other supplement (D + I), one receiving the daily vitamin E treatment (D + I + E) and another receiving placebo (safflower oil) (D + I + O), as described above. Finally three groups of normal rats were also studied: an untreated group (C) and two groups supplemented with either vitamin E (C + E) or the placebo.

Rats were decapitated on day 11.5 of gestation, which corresponds to the end of the embryo period. Blood was collected in EDTA (1 mg/ml) and plasma separated and kept at -20°C until processed. The two uterine horns were immediately dissected and

immersed in a petri dish (100 mm) at room temperature in saline. Embryos and investing membranes were teased apart with fine jewellers forceps during visualization with a dissecting microscope (Carlzeiss Jena 212T OPM, Germany). The yolk sac was isolated from the surrounding decidua and the embryo removed. In all embryos the crown-rump length and the number of somites were determined. All the embryos were inspected to determine whether the morphology of brain spheres, neural tube, heart, optic and otic vesicles, limb buds and axial curvature conformed to that expected on day 11.5 of gestation (Fig. 1). Embryos which did not conform to normal morphology in any of the above structures were considered dysmorphic (Fig. 1). Reabsorptions were considered when the decidua was present but the yolk sac or embryo were not found. After visual inspection, all the embryos were introduced into 0.1 N NaOH and analysed for total protein [24] and DNA [25].

Processing and analysis of the samples. Aliquots of plasma were used to determine glucose (Glucose God-Pad Enzymatic Colorimetric Test; Boehringer Mannheim, Mannheim, Germany), non-esterified fatty acids (NEFA C ACS. Acod Method; Wako Chemicals, Neuss, Germany), triglycerides (Triglycerides Enzymatic Trinder Method; Menarini Diagnostics, Florence, Italy), cholesterol (Cholesterol H.F., Enzymatic Trinder Method, Menarini Diagnostics) and fructosamine (Fructosamine, Hoffmann-La Roche AG, Basel, Switzerland: interassay coefficient of variation 3%). An aliquot was deproteinized with $\text{Ba}(\text{OH})_2$ and ZnSO_4 [26] and used for β -hydroxybutyrate determination [27, 28].

Statistical analysis

The mean \pm SEM are given. The significance of the difference between the means of two groups was obtained with the analysis of variance and Tuckey test for multiple comparisons, using the Systat program (Systat Inc. Evanston, Ill., USA).

Results

Metabolic parameters. Plasma glucose, β -hydroxybutyrate and fructosamine were measured as an index of the metabolic condition of the animals. As shown in Table 1, plasma concentration of these three parameters was higher in the non-insulin-treated pregnant diabetic rats (D, D + E and D + O) than in any of the control groups (C, C + E and C + O) or the insulin-treated pregnant diabetic rats (D + I, D + I + E and D + I + O) (Table 1). The administration of either vitamin E or the placebo had no effect

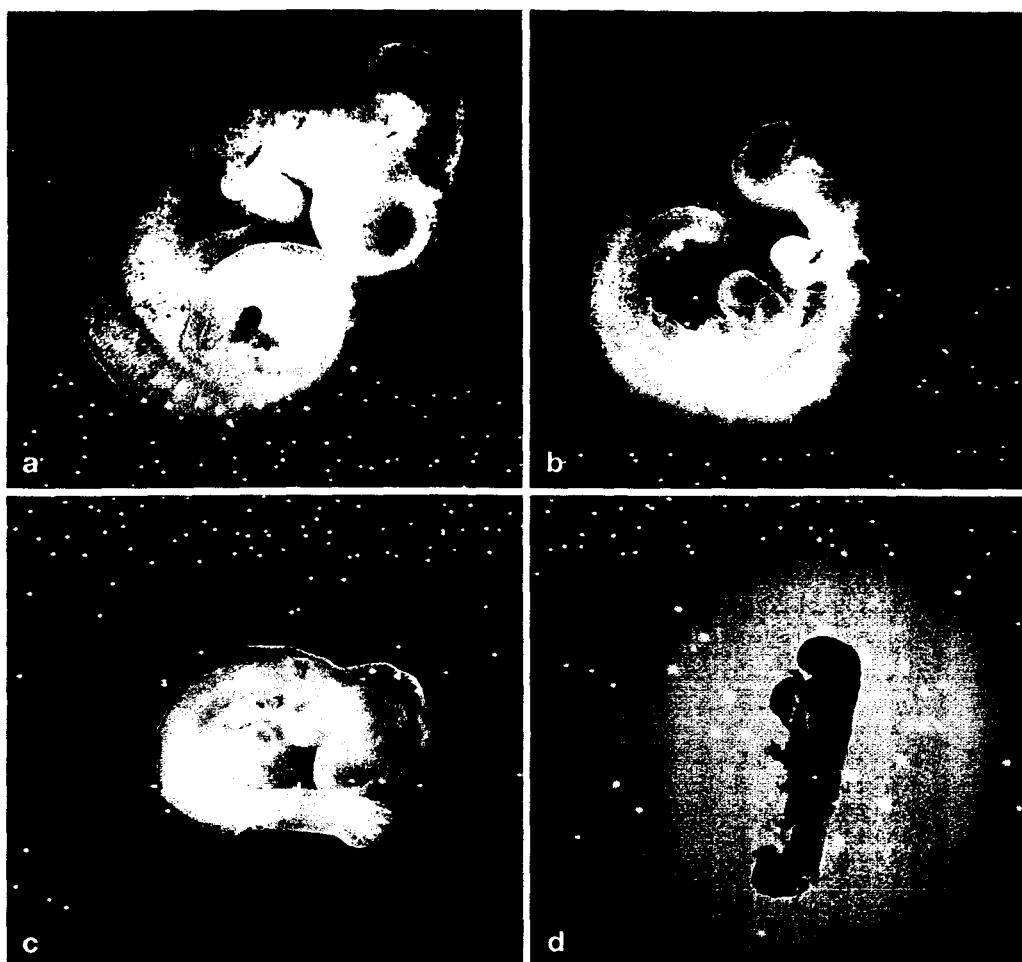


Fig. 1. a–d Rat embryos at day 11.5. a) Normal embryo from control animal ($\times 10$). b, c and d) Malformed embryos from diabetic rats: (b) embryo with microcephaly ($\times 10$); (c) Small embryo with severely malformed head and posterior neural pore open ($\times 10$); (d): Severely malformed embryo ($\times 16$)

on the plasma levels of glucose, β -hydroxybutyrate or fructosamine in either the control or diabetic groups not receiving insulin treatment (Table 1). Although insulin treatment reduced plasma glucose levels in the three diabetic groups, the effect was higher in rats receiving either vitamin E or placebo treatment, which attained levels that did not differ from their respective control groups. However, neither plasma β -hydroxybutyrate nor fructosamine levels differed between any of the diabetic groups receiving insulin treatment and their respective control groups (Table 1). However, although the NEFA concentration did not differ between D + I and control rats, it was lower in D + I + E and D + I + O rats (Table 1).

The concentration of plasma triglycerides and cholesterol was similar in all the experimental groups studied, both diabetic and controls (Table 1). Only the D + O group showed levels of triglycerides higher than those in the remaining groups (Table 1), suggesting that the administration of safflower oil could increase the plasma triglycerides, although this effect was only observed in this group. The administration of vitamin E had no effect on either parameter (Table 1).

Analysis of embryos. The embryos of D rats had a shorter crown-rump length, and lower number of somites and DNA and protein concentration than both C and D + I embryos ($p < 0.001$) (Table 2). In pregnant diabetic rats insulin treatment increased the crown-rump length, the number of somites and the DNA and protein content, although these did not reach the values observed in embryos of control animals (Table 2).

In diabetic rats vitamin E administration (D + E) increased the crown-rump length and the number of somites to values which were statistically higher than those of D embryos ($p < 0.01$), and reaching values similar to those of D + I embryos (Table 2), although still lower than those of the control animals (Table 2). No effects were observed in the protein and DNA content of D + E embryos compared to D embryos (Table 2).

The administration of placebo to D rats (D + O) had no effect on the crown-rump length or the number of somites but decreased both the embryo protein and DNA content when compared to D embryos ($p < 0.001$) (Table 2). A similar finding was observed in the embryos of insulin-treated diabetic rats when placebo was administered (D + I + O), as

Table 1. Metabolic parameters in diabetic rats. Effects of insulin, vitamin E and placebo

| Group | Number of rats (n) | Glucose (mmol/l) | β -OH-Butyrate (μ mol/l) | NEFA (μ mol/l) | Fructosamine (mmol/l) | Triglycerides (mmol/l) | Cholesterol (mmol/l) |
|-----------|--------------------|-------------------------------|-------------------------------------|-----------------------------|-------------------------------|--------------------------------|----------------------|
| C | 12 | 6.5 \pm 0.2 | 133.5 \pm 25 | 574.4 \pm 53 | 174.4 \pm 3 | 1.7 \pm 0.1 | 1.6 \pm 0.07 |
| C + E | 7 | 6.3 \pm 0.3 | 191.3 \pm 46 | 609.6 \pm 87 | 176.7 \pm 9 | 2.2 \pm 0.3 | 1.7 \pm 0.11 |
| C + O | 7 | 6.7 \pm 0.3 | 141.9 \pm 22 | 575.7 \pm 64 | 180.6 \pm 12 | 1.2 \pm 0.1 | 1.6 \pm 0.11 |
| D | 11 | 27.8 \pm 3.8 ^{a,z} | 284.1 \pm 54 | 568.4 \pm 49 | 277.0 \pm 31 ^c | 2.6 \pm 0.5 | 1.8 \pm 0.12 |
| D + E | 9 | 24.4 \pm 4.1 ^{a,x} | 355.3 \pm 98 ^r | 522.1 \pm 51 ^z | 274.8 \pm 27 ^{b,z} | 1.6 \pm 0.1 | 1.4 \pm 0.08 |
| D + O | 7 | 32.8 \pm 2.5 ^{a,x} | 327.0 \pm 61 | 698.4 \pm 73 ^x | 291.6 \pm 10 ^{b,z} | 3.7 \pm 1.2 ^{b,x,r} | 1.8 \pm 0.18 |
| D + I | 14 | 18.0 \pm 2.2 ^b | 136.1 \pm 35 | 441.3 \pm 27 | 230.2 \pm 28 | 2.2 \pm 0.2 | 1.7 \pm 0.09 |
| D + I + E | 9 | 8.3 \pm 0.8 | 99.3 \pm 27 | 269.2 \pm 35 ^a | 132.0 \pm 6 | 1.5 \pm 0.3 | 1.6 \pm 0.09 |
| D + I + O | 11 | 8.6 \pm 1.4 | 182.1 \pm 40 | 286.2 \pm 17 ^b | 198.4 \pm 14 | 1.2 \pm 0.1 | 1.6 \pm 0.07 |

Control, diabetic and insulin-treated diabetic animals, with no supplementation (C, D and D + I), supplemented with vitamin E (C + E, D + E and D + E + I) or with placebo (safflower oil) (C + O, D + O and D + I + O). Differences between diabetic rats, treated or not with insulin vs control animals, given the same supplement (vitamin E or placebo) ^a $p < 0.001$; ^b $p < 0.01$; ^c $p < 0.05$. Differences between diabetic rats treated

with insulin vs those not treated, given the same supplement (vitamin E or placebo) ^x $p < 0.001$; ^y $p < 0.01$; ^z $p < 0.05$. Differences between animals of the same experimental group (control, diabetic and insulin-treated diabetic animals) with no supplement vs rats given vitamin E or placebo; ^m $p < 0.001$; ⁿ $p < 0.01$; ^r $p < 0.05$

Table 2. Number of somites, crown-rump length, total protein and total DNA content in 11.5-day-old rat embryos

| Group | Embryos (n) | Somites (n) | Crown-rump (mm) | Protein (μ g) | DNA (μ g) |
|-----------|-------------|-------------------------------|-------------------------------|---------------------------------|------------------------------|
| C | 148 | 29.4 \pm 0.1 | 4.1 \pm 0.03 | 334.4 \pm 8.1 | 134.7 \pm 8.3 |
| C + E | 104 | 29.9 \pm 0.1 | 4.2 \pm 0.03 | 348.6 \pm 4.5 | 108.5 \pm 3.6 |
| C + O | 88 | 29.4 \pm 0.2 | 4.3 \pm 0.03 | 348.6 \pm 6.5 | 104.2 \pm 3.1 |
| D | 107 | 27.7 \pm 0.3 ^{a,x} | 3.7 \pm 0.06 ^{a,z} | 309.7 \pm 13.9 | 107.5 \pm 7.8 ^y |
| D + E | 82 | 28.8 \pm 0.5 ^{a,n} | 3.8 \pm 0.05 ^{a,n} | 298.6 \pm 8.8 ^b | 96.2 \pm 3.7 |
| D + O | 35 | 27.4 \pm 0.5 ^a | 3.6 \pm 0.08 ^a | 229.7 \pm 10.7 ^{a,m} | 66.2 \pm 4.9 |
| D + I | 167 | 28.6 \pm 0.1 ^c | 3.9 \pm 0.03 ^c | 340.7 \pm 11.4 | 141.0 \pm 13.1 |
| D + I + E | 118 | 28.8 \pm 0.2 ^b | 4.0 \pm 0.04 ^a | 289.9 \pm 9.1 ^a | 94.4 \pm 2.8 |
| D + I + O | 133 | 28.4 \pm 0.2 ^b | 3.9 \pm 0.07 ^b | 282.1 \pm 6.5 ^c | 88.4 \pm 3.0 |

Control, diabetic and insulin-treated diabetic animals, with no supplementation (C, D and D + I), supplemented with vitamin E (C + E, D + E and D + E + I) or with placebo (safflower oil) (C + O, D + O and D + I + O). Differences between diabetic rats, treated or not with insulin vs control animals, given the same supplement (vitamin E or placebo) ^a $p < 0.001$; ^b $p < 0.01$; ^c $p < 0.05$. Differences between diabetic rats treated

with insulin vs those not treated, given the same supplement (vitamin E or placebo) ^x $p < 0.001$; ^y $p < 0.01$; ^z $p < 0.05$. Differences between animals of the same experimental group (control, diabetic and insulin-treated diabetic animals) with no supplement vs rats given vitamin E or placebo; ^m $p < 0.001$; ⁿ $p < 0.01$; ^r $p < 0.05$

compared to those receiving no other treatment (D + I).

The incidence of reabsorptions and malformations was respectively 1.3 and 2% in the control group (C) (Table 3). In the D group the rate of reabsorptions and malformations increased to 23.6 and 24.3%, respectively. Administration of insulin to diabetic rats (D + I) decreased the incidence of reabsorptions to 6.2% and of malformations to 13.2%.

In D animals, vitamin E administration from day 1 of pregnancy had no effect on the incidence of reabsorptions (30.1% in D + E vs 23.6% in D rats), but drastically decreased the number of malformations in embryos of the D group from 24.3 to 4.6% (Table 3). However, vitamin E administration had no effect on the rate of reabsorptions and malformations in control or insulin-treated pregnant diabetic rats (Table 3).

The administration of placebo (safflower oil) had a negative effect in C animals, where an increase in malformations (2% in C vs 5.7% in C + O) and

reabsorptions (1.3% in C vs 6.4% in C + O) was observed. A similar effect was found in diabetic animals where the administration of safflower oil (D + O) increased the rate of malformations and reabsorptions to 48.6 and 56.8%, respectively from 24.3 and 23.6% in D animals (Table 3). No changes were observed in insulin treated pregnant diabetic rats when given with placebo (Table 3).

Discussion

In rats from our colony, as would be expected, poorly controlled diabetes is highly teratogenic. Fifty percent of the embryos are affected when considering malformations (24.3%) and reabsorptions (23.6%). The rate of embryo malformations in diabetic rats varies widely among different colonies [29]. Our colony therefore offers an excellent model with which to study the teratogenic effects of diabetes in vivo.

Table 3. Rate of malformations and reabsorptions

| Group | Yolk sacs | | Yolk sac with reabsorptions (%) | Embryos | | |
|-----------|-----------|----------|---------------------------------|----------------|--------------------|--------|
| | <i>n</i> | <i>n</i> | | Total <i>n</i> | Malformed <i>n</i> | (%) |
| C | 150 | 2 | (1.3) | 148 | 3 | (2.0) |
| C + E | 104 | 0 | (0) | 104 | 2 | (1.9) |
| C + O | 94 | 6 | (6.4) | 88 | 5 | (5.7) |
| D | 140 | 33 | (23.6) | 107 | 26 | (24.3) |
| D + E | 119 | 37 | (30.1) | 82 | 4 | (4.6) |
| D + O | 81 | 46 | (56.8) | 35 | 17 | (48.6) |
| D + I | 178 | 11 | (6.2) | 167 | 22 | (13.2) |
| D + I + E | 130 | 12 | (7.8) | 118 | 18 | (15.2) |
| D + I + O | 145 | 12 | (8.3) | 133 | 21 | (15.8) |

The embryos were studied as described in the methods section. Control, diabetic and insulin-treated diabetic animals, with no supplementation (C, D and D + I), supplemented with vitamin E (C + E, D + E and D + E + I) or with placebo (safflower oil) (C + O, D + O and D + I + O)

In diabetic rats, oral administration of vitamin E, from day 1 of pregnancy, decreased the rate of embryo malformations from 24.3 to 4.6%. These results demonstrate that in the experimental animal, vitamin E administration early in pregnancy reduces the incidence of embryo malformations secondary to diabetes. It remains to be determined whether vitamin E could have similar effects on human diabetic pregnancies, where the incidence of embryo malformations is still two- to threefold higher than in non-diabetic pregnancies, particularly when optimal metabolic control is not achieved [1–3].

Our results *in vivo* are in agreement with the findings in the embryo culture model, where the rate of malformations induced by high glucose and ketone body concentrations decreased when free radical scavengers, superoxide dismutase or butylated hydroxytoluene, were added to the media [12, 13]. All of these findings support the hypothesis of free radical-mediated teratogenesis.

The number of reabsorptions did not decrease in diabetic rats treated with vitamin E, which suggests that the damage produced by diabetes in the early phase of development was not reversed by this treatment. This agrees with the reported finding that diabetes can have negative effects on blastocyst development, decreasing the number of inner cells [30, 31], which are precursors of the embryo, and these effects are not reversible [30]. Furthermore, it cannot be ruled out that some components of diabetic embryopathy, leading to embryo reabsorptions, are not mediated by free radicals, and therefore are not suitable for targeting with antioxidants.

Administration of insulin to diabetic rats before and during pregnancy was found to decrease the rate of reabsorptions and embryo malformations to one-half the values observed in non-treated diabetic rats; however, incidence was still higher than in the control

group. This took place despite achieving good metabolic control, as indicated by a decreased plasma glucose, and NEFA, β -hydroxybutyrate and fructosamine levels which were similar to values observed in the control animals. Short periods of hypo- or hyperglycaemia could have occurred during the 24-h period between insulin administrations and they could have been responsible for the elevated number of reabsorptions and malformations observed in the D + I groups, since it has been shown that short periods of hypo- or hyperglycaemia may be teratogenic when occurring during critical periods of embryo development [32, 33]. Also, other teratogenic effects secondary to diabetes may not involve free radical generation or even be directly related to glucose or hydroxybutyrate. In fact, other authors have shown that in the embryo culture model plasma from diabetic rats remains teratogenic, despite normalization of plasma glucose and ketone bodies [10].

Administration of safflower oil as placebo increased the rate of malformations and reabsorptions in both normal and diabetic pregnancies. Fatty acids, especially polyunsaturated, are rapidly oxidized in pro-oxidative situations [34], such as the diabetic environment. Therefore, it is possible that administration of oil increased the generation of free radicals, leading to embryo malformations.

In summary, we have shown that oral administration of vitamin E, to diabetic rats from day 1 of pregnancy has no effect on the rate of embryo reabsorptions but decreases the high incidence of embryo malformations. To our knowledge these are the first results which demonstrate, *in vivo*, the protective effects of a dietary antioxidant on the teratogenic effects of diabetes, despite the fact that vitamin E treatment did not improve metabolic control in the rats studied. Clinical trials where vitamin E is administered either before or in early pregnancy are needed in order to demonstrate similar effects in human diabetic pregnancies.

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