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Original Contribution

OXIDATIVE DAMAGE IN PREGNANT DIABETIC RATS AND THEIR EMBRYOS

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Abstract—Free radical mechanisms may be involved in the teratogenesis of diabetes. The contribution of oxidative stress in diabetic complications was investigated from the standpoint of oxidative damage to DNA, lipids, and proteins in the livers and embryos of pregnant diabetic rats. Diabetes was induced prior to pregnancy by the administration of streptozotocin (45 mg/kg). Two groups of diabetic rats were studied, one without any supplementation (D) and another treated during pregnancy with vitamin E (150 mg/d by gavage) (D + E). A control group was also included (C). The percentage of malformations in D rats were 44%, higher than the values observed in C (7%) and D + E (12%) animals. D Group rats showed a higher concentration of thiobarbituric acid reactive substances in the mother's liver, however, treatment with vitamin E decreased this by 58%. The levels of protein carbonyls in the liver of C, D, and D + E groups were similar. The "total levels" of the DNA adducts measured, both in liver and embryos C groups were similar to the D groups. Treatment of D groups with vitamin E reduced the levels by 17% in the liver and by 25% in the embryos. In terms of the "total levels" of DNA adducts, the embryos in diabetic pregnancy appear to be under less oxidative stress when compared with the livers of their mothers. Graziewicz et al. (*Free Radical Biology & Medicine*, 28:75–83, 1999) suggested "that Fapyadenine is a toxic lesion that moderately arrests DNA synthesis depending on the neighboring nucleotide sequence and interactions with the active site of DNA polymerase." Thus the increased levels of Fapyadenine in the diabetic livers and embryos may similarly arrest DNA polymerase, and in the case of this occurring in the embryos, contribute to the congenital malformations. It is now critical to probe the molecular mechanisms of the oxidative stress-associated development of diabetic congenital malformations. © 2000 Elsevier Science Inc.

Keywords—Diabetes, Antioxidants, Vitamin E, Teratogenesis, Free radicals, DNA damage, Lipid peroxidation, Protein oxidation, Embryonic malformations

INTRODUCTION

In the early stages of diabetic pregnancy, poor metabolic control has been suggested to be associated with the increased rate of abortions and fetal malformations [1–4]. The administration of antioxidants (e.g., vitamin E or butylated hydroxytoluene) decreases the rate of embryo malformations and reabsorptions seen in experimental animal models of diabetes mellitus [5–8]. This observation intrinsically links free radicals and oxidative stress as important factors in the teratogenic effects of diabetes. The complications of diabetes and the role of oxidative stress/antioxidant balance in diabetes is a fertile research area [9–11].

Several mechanisms could be involved in the teratogenesis mediated by free radicals. The products of free radical-dependent fatty acids oxidation can induce chromosome breakage [12,13]. Thus, the reaction of these products with the embryo chromosomes could lead to severe malformations [14]. The free radicals can also react with DNA bases leading to alterations in their structure [15,16], such that they are no longer recognized as the homologous bases (by repair enzymes), leading to mutations [15–17]. In cell cultures of bacteria, it has been shown that the addition of products that can generate free radicals in the media increases the rate of mutation [18], if the lesions are misrepaired and processed by the cell. An increased rate of mutations has

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been shown in embryos from diabetic rats [19]. Such mutations could lead to malformations. Given that free radicals can induce alterations in gene expression [20, 21], it is conceivable that free radical species, through different mechanisms, could lead to embryo malformations and be implicated in faulty reproduction and development [22].

Previous studies performed in our laboratory [5] and in others [6] have shown that in diabetic pregnancies the administration of vitamin E decreases the rate of embryo malformation. This study investigated whether the administration of the dose of vitamin E that decreased the rate of embryo malformations could affect the levels of markers of oxidative DNA, and lipid and protein damage in diabetic rats.

EXPERIMENTAL PROCEDURE

Animals and experimental design

Female virgin Wistar rats from an in-house colony, weighing 190–220 g, were housed in a temperature-controlled room (22 ± 1°C) with alternating 12 h light and dark cycles, and fed a Purina Chow diet (containing 60IU of α tocopherol/kg diet) (Rat and Mouse Standard diet, Beekay Feeds, B.K.: Universal S.L., Barcelona, Spain). The feeding routine for the animals at best was the same. The rats were cared for and handled in accordance with the animal care law of the European Community (Strasburg, March 18, 1986). Diabetes was induced by the administration of a single intravenous dose of Streptozotocin (45 mg/kg) (Sigma Chemical Company, St. Louis, MO, USA) in citrate buffer (0.05 M) (pH 4.5). Five days later the state of insulin deficiency was confirmed by the presence of a positive reaction of urine chemi-strip for both glucose and ketone bodies, and insulin replacement was started. The insulin dose was chosen on the basis of previous laboratory experience [5,23]. One and one half UI/100 g Body weight of Insulin Lente MC (90% bovine, 10% porcine) (Novo Nordisk A/S, Bagsvaerd, Denmark) was administered subcutaneously, between 9 and 10 AM. The animals were mated with untreated control animals. The same day that sperm appeared in vaginal smears (day 0 of gestation) they were divided into the following experimental groups: two groups of diabetic animals were studied, one without any supplementation (D) and another one supplemented with 150 mg/d, [5] of vitamin E, administered orally by gavage (D + E). This represents a true supplementation independent of the dietary source mentioned above. In parallel a group of normal rats were also studied (C). Rats were decapitated on day 11.5 of gestation, which corresponds to the end of the embryo period. Blood was collected on EDTA (1 mg/ml) and plasma separated and kept at −20°C until processed. A piece of liver was obtained and placed in liquid N_2 and stored at −70°C, until the day of the assays. The two uterine horns were immediately dissected and immersed at room temperature in saline contained within a petri dish (100 mm). Embryos and investing membranes were teased apart with fine jeweler's forceps during visualization with a dissecting microscope (Carlzeiss Jena 212T OPM). 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 embryos were inspected under the microscope to determine whether the morphology of brain spheres, neural tube, optic and otic vesicles, limb buds, and axial curvature conformed to that expected on day 11.5 of gestation. Embryos not conforming to normal morphology in any of the above structures were considered dysmorphic. In the reabsorptions, the decidua was present but the yolk sacs or embryos were not found.

Determination of plasma glucose and vitamin E

Plasma glucose was determined using a GOD-Pad Enzymatic Colorimetric Test (Boehringer Mannheim, Mannheim, Germany) in the different experimental groups, confirming that the animals remained diabetic at the time of sacrifice. Vitamin E in plasma and liver were determined by HPLC following methods previously described [24,25].

Measurement of lipid oxidation

Lipid peroxidation was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS) [24]. The method was a modification of a previously published method [26]. In brief, approximately 100 mg of liver was homogenized in 1 ml of phosphate-buffered saline (PBS) and butylated hydroxytoluene (BHT, 5 μl of 200 mM solution in ethanol) was added to the homogenate, to give a final concentration of 1 mM. Two hundred μl of the homogenate was incubated with 1 ml mixture of thiobarbituric acid (26 mM), tricholoroacetic acid (15% w/v) and HCl (2.5% v/v) and the solution heated at 95°C for 20 min. The samples were cooled and equal volumes of butan-1-ol added to extract the colors. The mixtures were centrifuged at 3000 rpm for 10 min. The supernatant was measured fluorometrically at excitation wavelength 532 nm and emission wavelength 553 nm, using a Hitachi fluorimeter (F-2000). Malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxy-propane, submitted to the same conditions as the tissue homogenates, was used as standard. To determine the TBARS concentration in embryos, 15 of

them were pooled together, homogenized with 0.5 ml of PBS and the same procedure as for the liver was followed. The TBARS concentration in 100 μl of plasma was determined.

DNA isolation

The DNA from liver and embryos were isolated using the method described by Gupta [27], with some modification [28]. In brief, either 50 mg of liver or a pool of 10 embryos were homogenized in a solution of Tris buffer 1 M, pH 7.5, containing NaCl 4 M and EDTA 0.5 M. Proteinase K and SDS were added to the homogenate to have a final concentration of 0.2 mg/ml and 0.5%, respectively. The mixture was incubated at 60°C for 2 h. After this period an equal volume of chloroform and isopropyl alcohol (24:1, v/v) was added to the homogenate and mixed. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant collected. This solvent washing procedure was repeated twice. NaCl 4 M was then added to the supernatant (final concentration of 0.2 M) and mixed. The DNA was precipitated by adding twice the initial volume of 100% ethanol, kept at −20°C precipitated the DNA. The ethanol was discarded and the procedure repeated with 70% (v/v) ethanol. The DNA was stored in 70% (v/v) ethanol until analysis.

Assessment of oxidative DNA damage by GC/MS

The assessment of oxidative DNA damage including the preparation, hydrolysis, derivatization and analysis of samples gas chromatography-mass spectrometry were performed as described previously [29–31].

Measurement of protein carbonyls

Liver samples (200 mg) were homogenized in 2 ml of homogenizing buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.4, plus 0.1% digitonin). Streptomycin sulfate (final concentration 1%) was added to the homogenate to eliminate nucleic acids. The homogenized tissue was incubated at room temperature for 15 min and then centrifuged at 4,000 rpm. The supernatant was removed, and 0.4 ml were placed into eppendorf tubes. Protein carbonyls were analyzed essentially as previously described [32].

Statistical analysis

The means ± SEM are given. The significance of the difference between the means of two groups was obtained with the analysis of variance and Tuckey HSD test for multiple comparisons and by the quadratic trend analysis, using the SPSS 10.0 program. The χ^2 test was performed to detect differences in the rate of malformations and reabsorption between the different experimental groups.

Table 1. Levels of Glucose, Vitamin E and Thiobarbituric acid Reactive Materials in the Plasma of Pregnant Rats

Groups	Glucose (mg/dl)	Vitamin E (mg/dl)	Tbars (nmol/l)
C (n = 9)	126.2 ± 8.8	1217.5 ± 76.5	0.58 ± 0.05
D (n = 10)	612.5 ± 28.9 a	993.6 ± 64.2	0.55 ± 0.03
D + E (n = 9)	608.9 ± 52.1 a	2549.9 ± 288.5 a,m	0.57 ± 0.08

C vs. D and D + E: a $p < .001$; D vs. D + E: m: $p < .001$.

RESULTS

Diabetic rats had higher glucose concentration than control nondiabetic animals, and the administration of vitamin E to diabetic rats did not produce any changes in the plasma glucose levels compared to the nontreated diabetic rats (Table 1). The plasma concentration of vitamin E in the D group was 18% lower than the C group animals. As expected, the D + E group had plasma levels of vitamin E much higher than in the C and D animals. Interestingly, the plasma levels of TBARS in this study did not differ between C and D rats, nor did vitamin E treatment modify this variable (Table 1).

Embryos of D rats had a significantly lower crown-rump length, and somites number than the C embryos (Table 2). When vitamin E was administered to diabetic rats a slight improvement was observed in both parameters, although not reaching the values observed in the control embryos (Table 2). It was not clear how much of

Table 2A. Morphology of the Embryos

Groups	Crown-rump Mm	Somites
C (n = 227)	3.8 ± 0.02	27.5 ± 0.1
D (n = 261)	3.3 ± 0.03 a	25.2 ± 0.2 a
D + E (n = 167)	3.4 ± 0.03 a, p	25.7 ± 0.2 a, p

Table 2B. Rate of Malformations and Reabsorptions

Groups	Yolk sacs	Reabsorptions (%)	Embryos	Malformed (%)
C	243	6.6	227	6.6
D	378	31 a	261	44 a
D + E	205	19 a,m	167	12 m

The numbers of reabsorbed yolks are (C) 16, (D) 117, and for (D + E) 38. The number of malformed embryos are (C) 15, (D) 116, and for (D + E) 20.

C vs. D and D + E: a: $p < .001$; D vs. D + E: m: $p < .001$: D vs. D + E: p: $p < 0.05$.

Table 3. Levels of Thiobarbituric Acid Reactive Materials, Protein Carbonyls and Vitamin E in the Liver of Pregnant Rats

	Tbars		Carbonyl	Vitamin E
Groups	nmol/g tissue	nmol/mg lipid	nmol/mg protein	nmol/g tissue
C (n = 9)	5.8 ± 0.8	0.09 ± 0.01	21.3 ± 0.7	75.2 ± 7.0
D (n = 10)	13.8 ± 1.5 a	0.26 ± 0.0 a	22.1 ± 1.1	97.1 ± 11.6
D + E (n = 9)	6.2 ± 0.6 m	0.11 ± 0.02 m	21.4 ± 0.9	488.6 ± 56.8 a, m

C vs. D and D + E: a: $p < .001$; D vs. D + E: m: $p < .001$.

the administered vitamin E reached the embryo. The incidence of malformations and reabsorption was, respectively, 6.6 and 6.6% in C animals of our own colony (Table 2). The rate of both malformations and reabsorption raised dramatically in the diabetic animals, reaching values of 44.4 and 30.9%, respectively (Table 2). The administration of vitamin E decreased both parameters to 12 and 18.5%, respectively.

The liver homogenates of diabetic rats had a TBARS concentration significantly higher than the homogenates of C animals (Table 3). The administration of vitamin E to diabetic rats decreased the concentrations of TBARS in liver to values similar to that found in C animals and significantly lower than in the diabetic group (Table 3). These differences were independent of how the values of the TBARS are expressed, i.e., "per g of tissue" or "mg of total lipid content". Under the experimental conditions described, the TBARS concentration in homogenates of 15 embryos was not detectable. No difference was observed in the liver protein carbonyl, whereas liver vitamin E concentration was much higher in the D + E group than in the C + D animals (Table 3).

Using GC/MS, a wide range of different DNA adducts were determined, in DNA obtained from both embryos and livers of the experimental animals. Figures 1 and 2 show the levels of the different products measured in the liver and embryo of the diabetic rats. The products measured are 5-OH-Me-hydantoin, 5-OH-hydantoin, 5-OH-uracil, 5-OH,Me-uracil, 5-OH-cytosine, FapyAdenine, 8-OH-Adenine, 2-OH-Adenine, FapyGuanine, and 8-OH-Guanine. Majority of the individual base products measured showed a trend toward higher levels

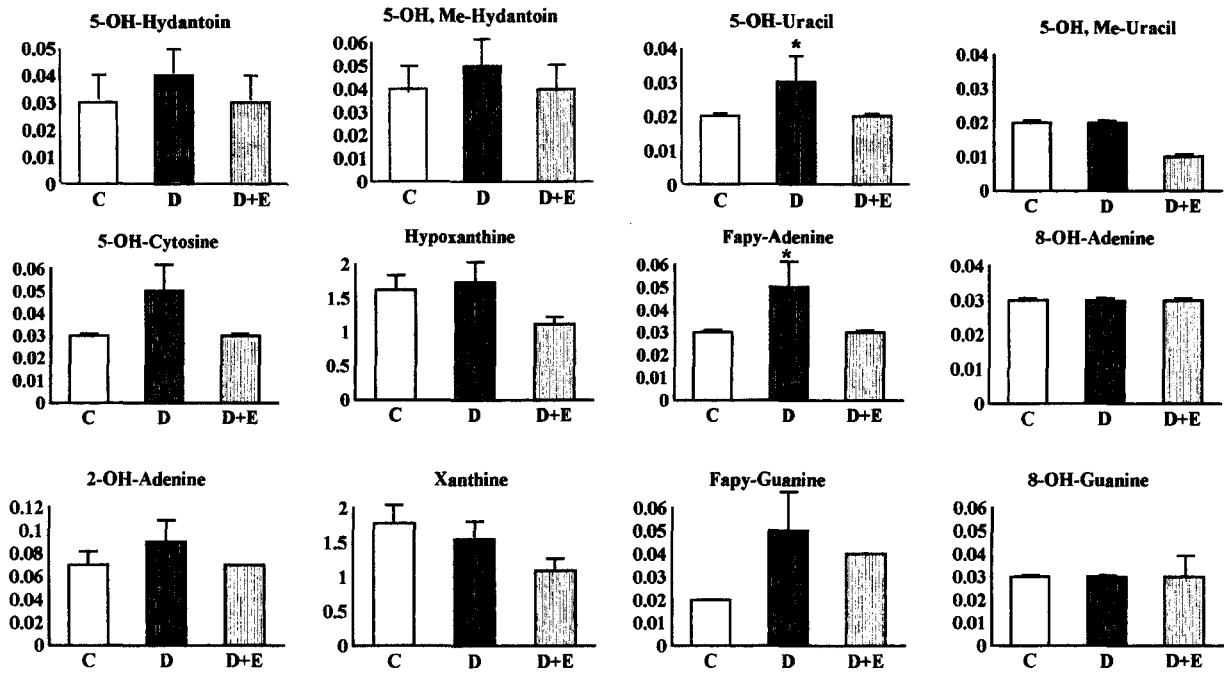

Fig. 1. Embryo DNA damaged bases, including 5-OH-Me-hydantoin, 5-OH-hydantoin, 5-OH-uracil, 5-OH-Me-uracil, 5-OH-cytosine, FapyAdenine, 8-OH-Adenine, 2-OH-Adenine, FapyGuanine, and 8-OH-Guanine are shown in this figure. The bases were determined in DNA embryos isolated from 12 different pools of DNA, as described in the Methods section and analyzed by gas chromatography-mass spectrometry. The differences between the bases isolated from embryos of the D group and the C or D + E groups were analysis both by using the analysis of the variance and the quadratic trend analysis. *$p < .05$.

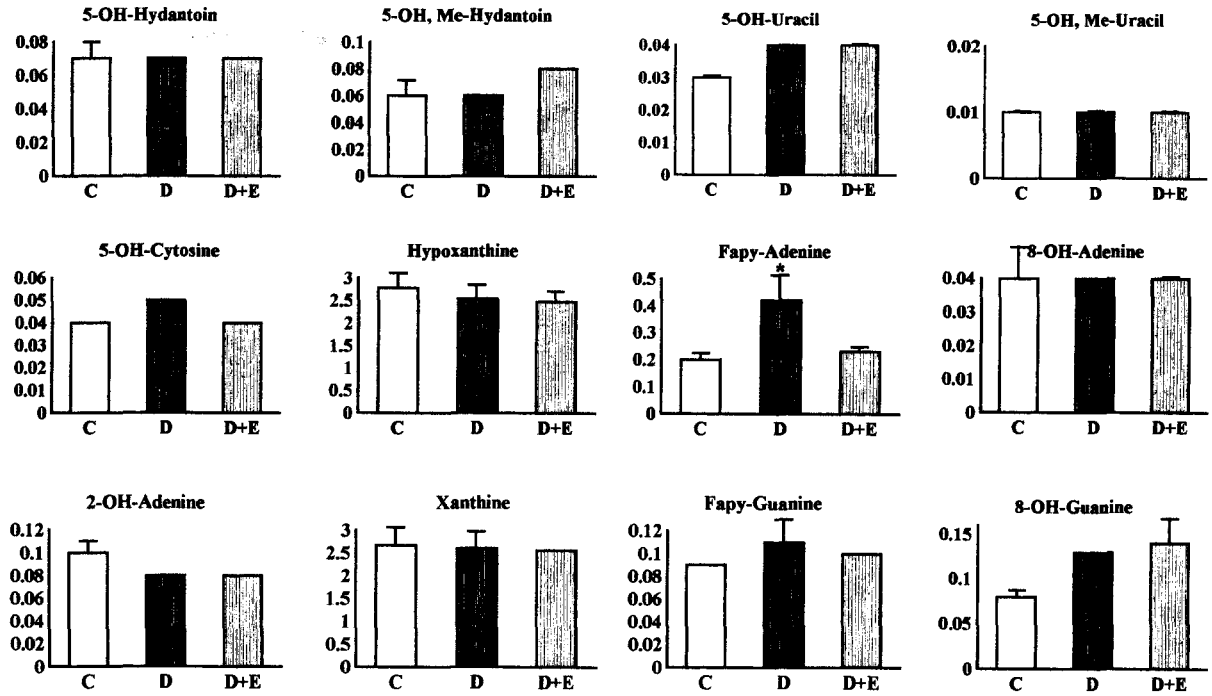

Fig. 2. Liver DNA damaged bases. Both the analysis of the bases and the differences between the three experimental groups were performed as described in Fig. 1.

in the livers (Fig. 2) and embryos (Fig. 1) of diabetic rats. Under our experimental conditions, only the lesions Fapyadenine and 5-OH-uracil reached statistical significance when the diabetic rat embryos were compared with the control or the D + E group. For the liver, only the Fapyadenine reached a statistical significance when the same groups were compared.

DISCUSSION

In diabetic rats there was a tendency towards an increased concentration of altered DNA bases in liver and embryos, when compared to nondiabetic animals. (Figs. 1 and 2). Although embryos are under more hypoxic states compared with their pregnant mothers, this is the first report of oxidative DNA damage in the diabetic rat embryos and we are currently extending the studies to other antioxidant supplements. Presently, with the administration of vitamin E, the DNA adduct levels in the embryos were reduced but the oxidative DNA damage in the liver of their mothers were unaffected. The high rate of embryo malformations and reabsorptions observed in diabetic rats decreases to values close to nondiabetic animals with the administration of vitamin E. Thus, the protection by vitamin E of the teratogenic effects of diabetes may be secondary to the reduction in the extent of liver lipid oxidation and of oxidative DNA damage. The apparent increases in DNA base lesions in the diabetic embryos may have mutagenic implications. In a transgenic mouse model, diabetes is associated with a higher rate of embryo mutations [19]. These mutations could lead to malformations if they affected genes involved in the development of the embryo. This finding would be in agreement with the fact that vitamin E administration to pregnant diabetic rats decreases both the concentration of altered DNA bases and the rate of embryo malformations. Deletions or rearrangements of human chromosome 22q11.2 lead to a variety of related clinical syndromes such as DiGeorge syndrome and velocardiofacial syndrome [33,34]. The malformations found in the fetus from diabetic rats have some similarity with the malformations found in DiGeorge syndrome. The malformations found in diabetic embryos are probably constant and affect the mesencephalo and the brachial arcs and may be secondary to chromosomal damage.

The levels of 8-OH Guanine, one of the most common markers used to study oxidative DNA damage, were almost identical in the three experimental groups, both in liver and embryos. Present results emphasize the importance of measuring a range of DNA adducts. Indeed, the technique of GC/MS provides the measure of a broad range of DNA adducts [35]. Interestingly, Collins et al. [36] studied 10 patients with insulin-dependent diabetes and matched controls using a modified comet assay and found that FAPy glycosylase-sensitive sites are elevated

in lymphocytes of diabetes compared with the normal, implicating an increase of 8-OH Guanine or FAPyGuanine.

One feature of this study centers on the observation that in diabetic pregnancy, the embryos appear to be under less oxidative stress compared with their mothers. It is conceivable that the small level of lesions might be related to the malformations observed. Graziewicz et al. [37] have suggested "that FapyAdenine is a toxic lesion which moderately arrests DNA synthesis depending on the neighbouring nucleotide sequence and interactions with the active site of DNA polymerase." It was further suggested that FapyGuanine might possess similar properties. Thus the increased levels of Fapyadenine in the diabetic livers and embyos may similarly arrest DNA polymerase and in the case of this occurring in the embryo, contribute to the congenital malformations.

The increased lipid peroxidation found in the diabetic liver (Table 3) may also play a role in the teratogenic effects of diabetes. In other clinical and experimental situations, increased production of lipid peroxides in different tissues induce the generation of clastogenic factors [9,10,38], which could lead to chromosome alterations and malformations if this were to occur in the embryos. It is possible that there may be increased concentrations of clastogenic factors in the plasma of diabetic rats as a consequence of the oxidative stress secondary to the diabetes. It is interesting to note that the teratogenic effects of the plasma from diabetic subjects is associated to different factors, other than glucose and ketone bodies [39,40]. Even more relevant is the fact that the teratogenicity of these factors is prevented with the administration of antioxidants [40].

Gopaul et al. [42] have shown that the levels of isoprostanes are increased in the plasma of individuals with non-insulin-dependent diabetes mellitus. In this study, levels of TBARS were used as markers of the lipid oxidative stress in plasma. This assay is probably either not sensitive enough to detect differences in plasma lipid peroxidation between diabetic and control animals, or the products of the lipid peroxidation are rapidly cleared by glomerular filtration. Nevertheless, the liver of pregnant diabetic rats (Table 3), showing an increased concentration of TBARS, is in agreement with the view that oxidative lipid damage is involved in diabetes [42–44]. Again, the administration of vitamin E decreased both the concentration of TBARS in liver and the rate of embryo malformations. This could be associated with the fact that, in states of increased formation of clastogenic factors, the administration of antioxidants decreases the plasma concentration of these factors as well as the incidence of chromosome alterations [38]. In a study that examined the human antioxidant status and lipid peroxidation in diabetic pregnancy, there was no evidence of greater lipid peroxidation in the serum of diabetic patients and total antioxidant capacity, as these were similar in diabetic mothers and matched control subjects before 12 weeks of gestation [45]. In view of the data in Table 1, it is suggested that plasma measures of lipid peroxidation is not a good index of lipid peroxidation in diabetes. Nevertheless, it is interesting to speculate that an increased production of lipid aldehydes in the liver could increase the release of clastogenic factors into the circulation, and if they reach the embryo, could cause chromosomal damage and malformations. Ongoing work in our laboratory is addressing whether the administration of thermally stressed oils to pregnant rats can increase the rate of malformations.

In summary, this is the first time that an oxidative DNA damage in the embryos of diabetic rats has been documented. The results presented link oxidative lipid and DNA damage as the two mechanisms associated with the free radical-induced teratogenic effects of diabetes. Thus the increased levels of Fapyadenine in the diabetic livers and embryos may similarly arrest DNA polymerase and, in the case of this occurring in the embryos, contribute to the congenital malformations. It is now critical to probe the molecular mechanisms of the oxidative stress-associated development of diabetic congenital malformations, and by so doing, establish the basis for antioxidant therapy in diabetic pregnancy that might help reduce free radical-induced incidence of embryo malformations.

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