Folic acid and safflower oil supplementation interacts and protects embryos from maternal diabetes-induced damage

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ABSTRACT: Maternal diabetes increases the risk of embryo malformations. Folic acid and safflower oil supplementations have been shown to reduce embryo malformations in experimental models of diabetes. In this study we here tested whether folic acid and safflower oil supplementations interact to prevent embryo malformations in diabetic rats, and analyzed whether they act through the regulation of matrix metalloproteinases (MMPs), their endogenous inhibitors (TIMPs), and nitric oxide (NO) and reactive oxygen species production. Diabetes was induced by streptozotocin administration prior to mating. From Day 0.5 of pregnancy, rats did or did not receive folic acid (15 mg/kg) and/or a 6% safflower oil-supplemented diet. Embryos and decidua were explanted on Day 10.5 of gestation for further analysis of embryo resorptions and malformations, MMP-2 and MMP-9 activities, TIMP-1 and TIMP-2 levels, NO production and lipid peroxidation. Maternal diabetes induced resorptions and malformations that were prevented by folic acid and safflower oil supplementation. MMP-2 and MMP-9 activities were increased in embryos and decidua from diabetic rats and decreased with safflower oil and folic acid supplementations. In diabetic animals, the embryonic and decidual TIMPs were increased mainly with safflower oil supplementation in decidua and with folic acid in embryos. NO overproduction was decreased in decidua from diabetic rats treated with folic acid alone and in combination with safflower oil. These treatments also prevented increases in embryonic and decidual lipid peroxidation. In conclusion, folic acid and safflower oil supplementations interact and protect the embryos from diabetes-induced damage through several pathways related to a decrease in pro-inflammatory mediators.

Key words: diabetes / pregnancy / folic acid / safflower oil / matrix metalloproteinases

Introduction

Diabetes during pregnancy is associated with increased risks of congenital malformations, mostly cardiac and neural tube defects (Martinez-Frias, 1994). Their induction occurs during early organogenesis and has been clearly related to the degree of maternal metabolic imbalance (Kitzmiller et al., 2010). Although still not completely understood, mechanisms of induction of embryo malformations in maternal diabetes are related to the intrauterine pro-inflammatory environment, reactive oxygen and nitrogen species formation, apoptotic events and deficiency in arachidonic acid derivatives (Eriksson, 2009; Jawerbaum and White, 2010; Zabih and Loeken, 2010). Nitric oxide (NO) overproduction and the resulting nitrative stress have been found in both embryos and decidua from diabetic rats during early organogenesis (Jawerbaum and Gonzalez, 2005; Higa et al., 2010). The decidua possesses nutritional functions during embryo organogenesis, as well as immunological functions in the regulation of trophoblast invasion and the formation of the placenta (Parr et al., 1990; Cohen et al., 2010). Experimental studies have shown that supplementation with safflower oil, which provides linoleic acid, a substrate for the synthesis of arachidonic acid and an endogenous activator of peroxisome proliferator activated receptors (PPARs), is capable of reducing maternal diabetes-induced developmental defects and of regulating pro-inflammatory pathways in the embryo, the decidua, the fetus and the placenta (Reece et al., 1996; Higa et al., 2010; Jawerbaum and Capobianco, 2011).

On the other hand, it is well known that folic acid supplementation highly reduces the induction of congenital malformations in both
human pregnancies and experimental diabetic models (Wentzel, 2009). Indeed, in human studies, folic acid supplementation reduces the induction of congenital defects with doses ranging 0.4–4 mg/day by 50–70% (Goh and Koren, 2008). During development, antioxidant and anti-apoptotic effects of folic acid have been described and possibly related to their protective effects during embryo organogenesis (van der Put et al., 2001; Wentzel, 2009). As mechanisms involved in the reduction of congenital malformations of both folic acid and safflower oil supplemenations in maternal diabetes are possibly related to anti-inflammatory effects, we hypothesized that they may cause beneficial effects by similar mechanisms of action, and thus, that joint effects of these treatments could be observed. Folic acid supplementation reduces apoptosis and the concentration of isoprostanes, sensitive markers of oxidative stress, in embryos and yolk sacks from diabetic rats (Wentzel and Eriksson, 2005; Gareshog et al., 2006; Zabihi et al., 2007; Jia et al., 2008). On the other hand, safflower oil supplementation reduces NO production and oxidative stress in embryos and decidua from diabetic rats (Reece and Wu, 1997; Higa et al., 2010). Moreover, folic acid has been shown to prevent neural tube defects caused by excessive NO (Weil et al., 2004). Both NO and reactive oxygen species (ROS) are positive regulators of matrix metalloproteinases (MMPs) activity (Ra and Parks, 2007).

MMPs are zinc-dependent endopeptidases capable of cleaving most components of the extracellular matrix (Mott and Werb, 2004). MMPs activities have been found to be clearly related to relevant developmental processes such as implantation, decidualization, embryo organogenesis and placental formation (Vu and Werb, 2000; Cohen et al., 2010). MMPs are regulated at multiple levels, including transcription, activation of the zymogen and inhibition of their active forms by the endogenous inhibitors of matrix metalloproteinases (TIMPs) (Nagase et al., 2006). Both MMP-2 and MMP-9 are MMPs largely involved in reproductive processes, although they are also markers of a pro-inflammatory state when produced in excess (Vu and Werb, 2000; Mandal et al., 2003; Huang et al., 2009). We have recently found an increased activity of MMP-2 and MMP-9 as well as an impaired MMPs/TIMPs balance that occurred despite increased TIMP-1 and TIMP-2 concentrations in both embryos and decidua from diabetic rats during early organogenesis (Higa et al., 2011). MMPs overactivities are possibly related to the induction of diabetic embryopathy, indeed, increased MMP-2 and MMP-9 have been involved in the pathogenesis of several diseases, and are considered markers of a pro-inflammatory environment (Mandal et al., 2003; Huang et al., 2009). Whether folic acid and safflower oil treatments alone or in combination can regulate embryonic MMPs overactivity in maternal diabetes is unknown.

Therefore, this work aimed to address whether maternal treatments with folic acid and/or safflower oil regulate the activity of MMP-2 and MMP-9 and modulate TIMP-1 and TIMP-2 in embryos and decidua from diabetic rats during early organogenesis, as well as to analyze whether these joint treatments provide greater protection from MMPs, NO and ROS overproduction and from the induction of congenital malformations.

Materials and Methods

Animals

Albino Wistar rats were bred in the laboratory with free access to commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) and water, in a lighting cycle of 14 h light: 10 h dark. Female rats weighing 200–230 g were made diabetic with a single i.p. injection of streptozotocin (55 mg/kg) (Sigma-Aldrich, St Louis, MO, USA) in citrate buffer (0.05 M, pH 4.5), as previously described (Higa et al., 2007). Control rats were injected with citrate buffer alone. One week after diabetes induction, control and diabetic females were mated with control male rats. Mating was confirmed by the presence of sperm in vaginal smear and the noon of the day on which the mating was identified was designated Day 0.5 of pregnancy. The reproductive characteristics of the diabetic model have been previously described (Jawerbaum and White, 2010). Glycemia was measured with Accu-Chek reagent strips and a glucometer (Accu-Chek, Bayer Diagnostics, Buenos Aires, Argentina) 1 week after streptozotocin administration and on Day 10.5 of gestation. Females showing glycemia values higher than 250 mg/dl were considered diabetic. The study was carried out in accordance with the guidelines for the care and use of animals approved by the local institution, according to the ‘Principles of laboratory animal care’ (NIH publication No. 85–23, revised 1985, http://grants1.nih.gov/grants/olaw/reference/pspol.htm).

Treatments

On Day 0.5 of gestation, both control and diabetic rats were randomized into four groups (Table I): (A) rats fed ad libitum with the commercial rat chow that did not receive any treatment except a daily saline solution s.c. injection (‘non-treated rats’); (B) rats fed ad libitum with the standard diet supplemented with safflower oil (6% wt/wt) that received a daily saline solution s.c. injection (‘safflower oil-treated rats’); (C) rats fed ad libitum with the commercial rat chow that received a daily dose of folic acid (15 mg/kg s.c., vehicle: saline solution) (‘folic acid-treated rats’); and (D) rats fed ad libitum with the standard diet supplemented with safflower oil (6% wt/wt) that received a daily dose of folic acid (15 mg/kg s.c., vehicle: saline solution) (‘safflower oil plus folic acid-treated rats’). All diets met the nutritional requirements of calories, fat, carbohydrates and proteins for maintenance and reproduction (Council SoLANNR, 1995), and were previously used during rat gestation (Higa et al., 2010). The safflower oil-supplemented diet has similar amounts of carbohydrates, proteins and calories, and a 6% increase in lipid content, mostly linoleic acid (226%) as well as palmitic acid (67%) and oleic acid (42%), when compared with the standard diet (Table I). All treatments were given until Day 10.5 of gestation, a period corresponding to early embryo organogenesis in rats (Jawerbaum and Gonzalez, 2005).

Tissue collection and evaluation of embryo morphology

Animals were euthanized in a CO2 chamber on Day 10.5 of pregnancy, and the uteri were transferred to Petri dishes with Krebs Ringer Bicarbonate (KRB) solution: 5 mM glucose, 145 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl2·2H2O, 1.2 mM MgCl2·6H2O, 127 mM Cl−, 25 mM HCO3−, 1.2 mM SO42− and 1.2 mM PO43−. Blood was obtained from the aorta artery and serum obtained after blood centrifugation and preserved at −20°C for further determination of glucose, non-esterified fatty acids (NEFA) and triglyceride concentrations.

By the use of a stereomicroscope and microsurgical disecting instruments, the balls of decidual tissue were explanted from each uterus, and gently opened to free the conceptuses. The embryos were dissected out of the yolk sacs and evaluated morphologically under a stereomicroscope. Decidua were separated from the embryo, the extraembryonic tissues and the ectoplacental cone. Embryo viability was established by the presence of a beating heart. The embryos were categorized as morphologically normal or as showing malformations. Malformations were mostly neural tube defects. Severe malrotation was also observed although...
mainly present concomitantly with embryonic neural tube defects. By using another group of treated and untreated control and diabetic animals, the malformation rate was corroborated on Day 12.5 of gestation at which all non-malformed embryos showed a closed neural tube. Embryos in resorption stages received no further analyses. Viable 10.5 day embryos and their corresponding decidua were immediately prepared according to the determinations described below.

**Biochemical measurements**

Serum triglycerides and NEFA were, respectively, measured by commercial colorimetric kits from Wiener Lab. Rosario, Argentina and Randox, Antrim, UK.

**Analysis of MMPs gelatinase activity**

Zymography was performed to evaluate the presence of MMP-2 and MMP-9 gelatinase activity, as previously described (Pustovrh *et al.*, 2009). Both MMPs and pro-MMPs were analyzed by zymography, since the exposure to sodium dodecyl sulfate (SDS) induces changes in pro-MMPs conformation, which are associated with their activation. Briefly, this technique evaluates the capacity of MMPs (separated from other molecular weight proteins through an SDS gel) to degrade the gelatin which was incorporated in the gel. Three pooled embryos or one decidua obtained from each rat (*n* = 7–9 rats in each experimental group) were homogenized in 50 mM Tris, 5 mM CaCl₂, 1 mM ZnCl₂, 1% Triton X-100. Either 20 μg of protein of embryonic homogenates or 50 μg of protein of decidual homogenates were mixed with loading buffer (2% SDS), 10% glycerol, 0.1% bromophenol blue, 50 mM Tris–HCl, pH 6.8 and subjected to a 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), in which 1 mg/ml gelatin (type A from porcine skin) had been incorporated. Following electrophoresis, gels were washed in 2.5% Triton X-100 for 60 min to remove SDS. The gels were then incubated for 96 h in the case of the embryonic tissue and for 24 h in the case of the decidual tissue, in 50 mM Tris Buffer pH 7.4, containing 150 mM NaCl and 10 mM CaCl₂ at 37°C. Then, gels were stained with Coomassie blue and destained with 10% acetic acid and 30% methanol in water. The areas of proteolytic activity appeared as negatively stained bands in the dark background.

The identities of MMPs were based on their molecular weights and a positive internal control (conditioned medium of human fibrosarcoma HT-1080 cells). The enzymatic activity was quantified using an image analysis program (ImageJ), and expressed as arbitrary densitometric units, which were normalized to an internal control. Data are shown as relative to a value of 1 assigned to the mean values for active MMPs in control embryos and decidua.

**Evaluation of tissue inhibitors of MMPs**

To analyze the inhibitory capacity of TIMP proteins in embryos and decidua from control and diabetic rats, reverse zymography analysis was performed, as previously described (Pustovrh *et al.*, 2009). Briefly, this technique evaluates the capacity of TIMPs (separated

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**Table I Experimental design and diet composition of the experimental groups.**

<table>
<thead>
<tr>
<th>Diet composition</th>
<th>Experimental design Pre-pregnancy Day 0.5 of gestation Pregnancy Day 10.5 of gestation</th>
</tr>
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<tbody>
<tr>
<td>STZ administration</td>
<td>Non-treated rats; Folic acid treated rats</td>
</tr>
<tr>
<td>Carbohydrates (g/100 g diet)</td>
<td>50</td>
</tr>
<tr>
<td>Proteins (g/100 g diet)</td>
<td>25</td>
</tr>
<tr>
<td>Fat (g/100 g diet)</td>
<td>5</td>
</tr>
<tr>
<td>Energy content (Kcal/100 g diet)</td>
<td>324</td>
</tr>
<tr>
<td>Major fatty acid content (g/100 g diet)</td>
<td></td>
</tr>
<tr>
<td>16:0 (palmitic)</td>
<td>0.58</td>
</tr>
<tr>
<td>18:0 (eicosaen)</td>
<td>0.16</td>
</tr>
<tr>
<td>18:1 (oleic)</td>
<td>1.27</td>
</tr>
<tr>
<td>18:2 (linoleic)</td>
<td>1.99</td>
</tr>
<tr>
<td>18:3 (linolenic)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Streptozotocin (STZ), 55 mg/kg or vehicle was given to non-pregnant rats 1 week before mating. Both control and STZ-induced diabetic animals were randomized into four groups on Day 0.5 of gestation and evaluated on Day 10.5 of gestation.
from different molecular weight proteins through an SDS-gel) to inhibit the degradation of gelatin by MMPs that were incorporated in the culture media. Three pooled embryos or one decidua obtained from each rat (n = 7–9 rats in each experimental group) were homogenized in 50 mM Tris, 5 mM CaCl2, 1 μM ZnCl2, 1% Triton X-100, followed by a heat extraction (60°C) with 50 mM Tris, 0.1 M CaCl2 and 0.15 M NaCl. Either 20 μg of protein from embryonic tissues or 50 μg of protein from deciduous tissues were mixed with loading buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM Tris–HCl, pH 6.8) and applied to 15% polyacrylamide gels containing 0.1% SDS and 1 mg/ml gelatin plus 25% conditioned medium of human fibrosarcoma HT-1080 cells, which is a rich source of various MMPs. After electrophoresis, gels were rinsed twice with 2.5% Triton X-100, and then incubated at 37°C for 24 h in Tris buffer (50 mM Tris–HCl, 0.2 M NaCl, 5 mM CaCl2, pH 8.0). Subsequently, gels were stained with Coomassie blue and then destained with 10% acetic acid and 30% methanol in water. MMPs provided by the HT-1080 conditioned medium digested the gelatin within the gel, whereas the TIMPs in the samples inhibited the MMP action, allowing the identification of the inhibitory capacity of TIMPs as dark bands on a clear background. The identities of TIMPs were based on their molecular weights, determined through prestained SDS–PAGE protein standards (prestained Full Range, GE Healthcare, Buckinghamshire UK), which were run in the same gel. TIMP activity was quantified using an image analysis program (ImageJ), and expressed as arbitrary densitometric units. Data are shown as relative to a value of 1 assigned to the mean value for TIMPs in control embryos and decidua.

**Analysis of NO production**

NO production was evaluated in embryos and decidua by the determination of the concentration of its stable metabolites nitrates/nitrates, as previously performed (Higa et al., 2010), by using a commercial assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly, four embryos or one decidua obtained from each rat (n = 7–9 rats in each experimental group) were homogenized in Tris–HCl solution (0.1 mM, pH 7.4) and an aliquot was separated to determine protein content by the Bradford method. Nitrates in the supernatant were reduced to nitrates using nitrite reductase, and total nitrates were then quantified by the Griess reaction. Different amounts of sodium nitrates and nitrates were used as standards. Optical densities were measured at 540 nm. Results are expressed as nanomoles/milligram protein.

**Evaluation of lipid peroxidation**

Lipid peroxidation in embryos was evaluated by measuring 8-isoprostanes (8-iso-PGF_{2α}) concentrations, as previously performed (Higa et al., 2011) by using a commercial enzyme immunoassay kit (Cayman Chemical Co.). Briefly, three pooled embryos were homogenized in 2 M NaOH (n = 7–9 rats in each experimental group) and an aliquot separated for protein determination by the Bradford method using a protein assay reagent (Bio-Rad Laboratories Inc.). Samples were incubated at 45°C for 2 h, neutralized by the addition of 2 M HCl (pH 7.5) and centrifuged at 1000 g for 10 min. The assay was developed according to the manufacturer’s instructions. Briefly, the kit uses a polyclonal antibody against 8-iso-PGF_{2α}, which binds, in a competitive manner, either the 8-isoprostane in the sample or an 8-iso-PGF_{2α} molecule which has an acetylcholinesterase covalently attached to it. After a simultaneous incubation, a reagent consisting of acetylthiocholine and 5,5dithio-bis-(2-nitrobenzoic acid) was added. The hydrolysis of acetylthiocholine by acetylcholinesterase yields thiocholine, which reacts with 5,5dithio-bis-(2-nitrobenzoic acid), generating a yellow color which was evaluated on a microplate reader at 412 nm. Results are expressed as picogram/microgram protein.

Lipid peroxidation in decidua was analyzed by measuring the concentrations of thiobarbituric acid reactive substances (TBARS), as previously performed (Kurtz et al., 2010), a method widely used to assess peroxidation of fatty acids (Ohkawa et al., 1979). Briefly, ~100 mg of tissue was homogenized in 100 mM Tris–HCl buffer, pH 7.6 (n = 7–9 rats in each experimental group). The homogenate was added with trichloroacetic acid (40%) and centrifuged at 1.8g for 10 min. The supernatant was added with an equal volume of thiobarbituric acid (46 mM), and the solution was heated at 95°C for 15 min. Then, the samples were cooled and quantified spectrophotometrically at 330 nm. Malondialdehyde (Sigma-Aldrich) subjected to the same conditions as the tissue homogenates was used as a standard. TBARS are expressed as picomoles/milligram protein.

**Statistical analysis**

Data are presented as the mean ± standard error. Groups were compared by two-way analysis of variance (ANOVA) in conjunction with Bonferroni’s test. For malformation and resorption rates, data were compared using χ² contingency tests. In all cases, differences were considered statistically significant at P < 0.05.

**Results**

**Resorption and malformation rate in diabetic rats treated with safflower oil and/or folic acid during pregnancy**

All diabetic animals, non-treated or treated with safflower oil (6%) and/or folic acid (15 mg/kg) during pregnancy showed increased serum glucose, NEFA and triglycerides on Day 10.5 of gestation when compared with their respective controls (Table II). No changes in serum glucose and NEFA were found when treated and non-treated diabetic rats were compared. A decrease in serum triglycerides concentration was found in both the control and diabetics (P < 0.01) and diabetic (P < 0.05) groups treated with folic acid alone but not in combination with safflower oil when compared with the non-treated groups (Table II).

When we analyzed the resorption rate, which was increased 5.7 times in the non-treated diabetic animals when compared with controls (P < 0.001), we found that the three diabetic animal treated groups showed a decreased resorption rate when compared with the non-treated diabetic animals (safflower oil 64.4%, P < 0.001, folic acid 84.2%, P < 0.001, safflower oil plus folic acid 80%, P < 0.001, Table II).

When we evaluated the malformation rate, which was increased 13 times in the non-treated diabetic animals when compared with controls (P < 0.001), we found that both safflower and folic acid treatments were able to significantly decrease the malformation rate in diabetic animals (57.5%, P < 0.05 and 62.4%, P < 0.05, respectively). Of note, only the combination of both safflower and folic acid treatments was able to decrease the malformation rate in diabetic rats to values similar to those found in the control animals (84%, P < 0.01, Table II).
Embryonic and decidual MMPs activities in diabetic rats treated with safflower oil and/or folic acid during pregnancy

Considering the relevance of the embryonic and decidual MMP-2 and MMP-9 in embryo and decidual remodeling throughout development, and the overactivity of these MMPs previously found in diabetic animals (Vu and Werb, 2000; Cohen et al., 2010; Higa et al., 2011), we analyzed MMP-2 and MMP-9 activities in embryos and decidua from diabetic rats treated with safflower oil (6%) and/or folic acid (15 mg/kg) during pregnancy.

We found that MMP-2, which was overactivated in the embryos from diabetic rats in its active (P < 0.01) and proenzyme forms (P < 0.001), was decreased in the embryos from safflower oil (MMP-2: P < 0.01, proMMP-2: P < 0.001) and safflower oil plus folic acid (MMP-2: P < 0.05, proMMP-2: P < 0.001) treated diabetic rats when compared with the non-treated diabetic group (Fig. 1). No changes in embryonic MMP-2 activity were found when diabetic animals treated with folic acid alone were compared with the non-treated ones. Embryos from control animals treated with safflower oil also showed a decrease in the active form of MMP-2 when compared with the non-treated control group (P < 0.001), and no other changes in embryonic MMP-2 were observed in the control groups (Fig. 1).

On the other hand, as regards MMP-9, in the embryos we detected only its active form, which was increased in the embryo from non-treated diabetic rats (P < 0.001), and decreased in embryos from diabetic rats treated with safflower oil alone (P < 0.05) and in combination with folic acid (P < 0.01), but not with folic acid alone, compared with the non-treated diabetic group (Fig. 2).

When we analyzed the gelatinolytic activity of MMP-2 in the decidua, we did not detect changes in its active form in any of the control and diabetic experimental groups evaluated. Differently, proMMP-2, which was increased in the decidua from non-treated diabetic animals (P < 0.001), was decreased in the decidua from safflower oil plus folic acid diabetic-treated animals (P < 0.001), but not when either safflower oil or folic acid were given alone (Fig. 3).

On the other hand, as regards MMP-9 gelatinolytic activity, we found that its active form was enhanced in the decidua from non-treated diabetic animals (P < 0.001), and also in this case, only the combined treatment with safflower oil and folic acid was able to decrease MMP-9 activity in diabetic rats when compared with the non-treated diabetic group (P < 0.01; Fig. 4).

Embryonic and decidual TIMPs in diabetic rats treated with safflower oil and/or folic acid during pregnancy

TIMP-1 and TIMP-2 are endogenous MMPs inhibitors involved in embryo development and decidual remodeling, previously found increased in embryos and decidua from diabetic rats (Alexander et al., 1996; Higa et al., 2011). Therefore, we analyzed TIMP-1 and TIMP-2 MMPs inhibitory capacity in embryos and decidua from diabetic rats treated with safflower oil (6%) and/or folic acid (15 mg/kg) during pregnancy.

We found that maternal treatments with safflower oil (P < 0.05), folic acid (P < 0.001) and their combination (P < 0.05) led to further increases in TIMP-1 inhibitory capacity in embryos from diabetic animals compared with the non-treated diabetic group (Fig. 5B). In embryos from control rats, only the folic acid treatment alone led to increases in TIMP-1 inhibitory capacity compared with the non-treated group (P < 0.001).

On the other hand, TIMP-2 inhibitory capacity was found further increased in the embryos from diabetic rats treated with safflower oil and folic acid alone (P < 0.05 and P < 0.01, respectively), although not in combination, when compared with the non-treated diabetic group (Fig. 5C).

When TIMPs were analyzed in the decidua, we found that both in the control and in the diabetic group, the only treatment that induced significant changes was the safflower oil treatment, which led to increases in decidual TIMP-1 and TIMP-2 inhibitory capacity in the control (TIMP-1: P < 0.001, TIMP-2: P < 0.05) and the diabetic (P < 0.001) groups, respectively compared with the non-treated control and diabetic groups (Fig. 6).

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### Table II: Maternal serum glucose, NEFA and triglycerides concentrations, as well as resorption and malformation rates in control and diabetic rats treated or not with safflower oil and/or folic acid.

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>Safflower oil</th>
<th>Folic acid</th>
<th>Safflower oil + folic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Maternal glycemia (mg/dl)</td>
<td>105 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>439 ± 27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 ± 40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>356 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maternal Serum NEFA (mM)</td>
<td>0.64 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80 ± 0.05&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.64 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maternal triglyceridemia (g/l)</td>
<td>1.09 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.69 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.95 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resorption rate: Embryo resorptions/total implantation sites (%)</td>
<td>10/173&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>51/154&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13/142&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>19/161&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malformation rate: Malformed embryos/ total viable embryos (%)</td>
<td>2/163&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17/103&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/129&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10/142&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
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</table>

Serological data are shown as means ± SEM and evaluated by two-way ANOVA in conjunction with Bonferroni’s test. Resorption and Malformation rates are shown as percentage and evaluated by χ<sup>2</sup> contingency tests. Values were obtained from 10 to 13 rats in each experimental group. Different letters denote significant differences (P < 0.05) between groups.
Embryonic and decidual NO production and lipid peroxidation in diabetic rats treated with 6% safflower oil and/or folic acid during pregnancy

As NO and ROS are closely related to diabetes-induced embryopathy and are capable of activating MMPs (Jawerbaum and Gonzalez, 2005; Ra and Parks, 2007), nitrates/nitrites (an index of NO production) and lipid peroxidation (an index of ROS-induced damage), were evaluated in embryos and decidua from control and diabetic rats treated with safflower (6%) and folic acid (15 mg/kg) alone or in combination during pregnancy. We found that embryonic NO production, which was enhanced in the non-treated diabetic rats when compared with controls ($P < 0.001$), was significantly decreased by the safflower oil treatment ($P < 0.05$), although not in combination with folic acid (Fig. 7A).

The concentration of 8-isoprostane, which was increased in the embryos from non-treated diabetic rats when compared with non-treated controls ($P < 0.05$), was significantly decreased in embryos from diabetic rats treated with folic acid either alone ($P < 0.001$) or in combination with safflower oil ($P < 0.001$), although not in the diabetic group treated only with safflower oil, when compared with the non-treated diabetic group (Fig. 7B). In control rats, safflower oil treatment led to an increase in embryonic 8-isoprostane concentrations and folic acid treatment led to a decrease in embryonic 8-isoprostane concentrations when compared with the non-treated group, although no other changes were detected in embryonic NO production and lipoperoxidation.

On the other hand, in the decidua, NO production, which was enhanced in the non-treated diabetic group ($P < 0.001$), was highly decreased by the folic acid treatment alone ($P < 0.001$) and in
combination with the safflower oil treatment when compared with the non-treated diabetic group ($P < 0.001$; Fig. 8A). Decidual lipid peroxidation, evaluated through the measurement of TBARS, which was increased in the decidua from non-treated diabetic rats ($P < 0.05$), was decreased in the diabetic animals treated with safflower oil ($P < 0.001$), folic acid ($P < 0.001$) and their combination ($P < 0.001$) when compared with the non-treated diabetic group (Fig. 8B). Decreases in lipid peroxidation were also evident in the decidua from control rats treated with safflower oil in combination with folic acid when compared with the non-treated control group ($P < 0.05$, Fig. 8B).

**Discussion**

In this study, we demonstrated that joint treatments with folic acid and safflower oil of diabetic pregnant rats are more effective in preventing neural tube defects than each treatment alone. As the most important findings, we observed that these maternal treatments induced beneficial changes on embryonic and decidual MMPs and TIMPs activities, which together with the regulation of NO and ROS production, are likely to be involved in the prevention of diabetic embryopathy.

Previous studies addressing folic acid or safflower oil supplementation in diabetic embryopathy have shown the capacity of these treatments to reduce embryo resorptions and malformations (Wentzel et al., 2005; Reece et al., 2006; Oyama et al., 2009; Higa et al., 2010). Indeed, it is well known that folate supplementation for the general population can reduce the incidence of congenital malformations by 50–70% (Goh and Koren, 2008). In this work, we found that diabetic rats treated with folic acid or safflower oil during pregnancy showed, respectively, a 57–62% reduction in the induction of neural tube defects, while the combined treatment led to an 84% reduction of embryo malformations. This suggests a possible useful combination for further improving the prevention of congenital malformations in maternal diabetes. A limitation of this study is that we did not test lower doses of folic acid. Indeed, the folic acid

![Figure 3](https://academic.oup.com/molehr/article-abstract/18/5/253/1073446/3.png)

**Figure 3** MMP-2 activities evaluated in decidua from treated and non-treated control and diabetic rats on Day 10.5 of gestation. (A) Representative zymogram exhibiting decidual MMP-2 activities. (B) Densitometric analysis of proMMP-2 activities. (C) Densitometric analysis of MMP-2 activity. Values are means ± SEM. $n = 7–9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni’s test was performed. Different letters denote significant differences ($P < 0.05$) between groups.

![Figure 4](https://academic.oup.com/molehr/article-abstract/18/5/253/1073446/4.png)

**Figure 4** MMP-9 activities evaluated in decidua from treated and non-treated control and diabetic rats on Day 10.5 of gestation. (A) Representative zymogram exhibiting decidual MMP-9 activities. (B) Densitometric analysis of MMP-9 activities. Values are means ± SEM. $n = 7–9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni’s test was performed. Different letters denote significant differences ($P < 0.05$) between groups.
**Figure 5** TIMP-1 and TIMP-2 inhibitory capacity in embryos from treated and non-treated control and diabetic rats on Day 10.5 of gestation. (A) Representative reverse zymogram exhibiting embryonic TIMP-1 and TIMP-2 inhibitory capacity. (B) Densitometric analysis of TIMP-1 inhibitory capacity. (C) Densitometric analysis of TIMP-2 inhibitory capacity. Values are means ± SEM. n = 7–9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni’s test was performed. Different letters denote significant differences (P < 0.05) between groups.

**Figure 6** TIMP-1 and TIMP-2 inhibitory capacity in decidua from treated and non-treated control and diabetic rats on Day 10.5 of gestation. (A) Representative reverse zymogram exhibiting decidual TIMP-1 and TIMP-2 inhibitory capacity. (B) Densitometric analysis of TIMP-1 inhibitory capacity. (C) Densitometric analysis of TIMP-2 inhibitory capacity. Values are means ± SEM. n = 7–9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni’s test was performed. Different letters denote significant differences (P < 0.05) between groups.
doses that should be used in diabetic gestations are still unclear (Mathiesen et al., 2011) and recent results have shown possible adverse effects of high doses of folates in embryo development (Pickell et al., 2011).

The diabetic model used has been previously characterized during gestation in our laboratory (Higa et al., 2010; Jawerbaum and White, 2010), and has an increased energy intake (Control: 65 ± 2 kcal/day, Diabetic: 89 ± 5 kcal/day, P < 0.001) and a decreased weight gain during Days 0.5–10.5 of pregnancy (Control: 30 ± 2 g, Diabetic: 26 ± 1 g, P < 0.001) when compared with controls. As metabolic changes, we found that serum glucose, triglycerides and NEFA were increased in diabetic animals when compared with controls, and that folic acid alone (although not in combination with safflower oil) led to a decrease in triglyceridemia. Studies performed in pregnant and non-pregnant animals have reported that supplementation with folic acid reduces triglyceridemia and that deficiency in methyl donors increases tissue triglyceride concentrations (McNeil et al., 2008; Ojeda et al., 2008).

As the main novel findings of this work, we found that both safflower and folic acid treatments have the capacity to regulate MMPs and TIMPs activities. These activities were evaluated separately, and down-regulation of MMPs and/or up-regulation of TIMPs were observed in embryos and decidua from diabetic rats treated with safflower and/or folic acid. We found differences between the capacity of the two treatments alone and in combination to regulate MMPs and TIMPs in embryonic and decidual tissues from diabetic rats, showing a very different pattern of expression and regulation of MMPs and TIMPs in the embryo and its surrounding decidua.
Indeed, the MMPs evaluated were highly decreased by the maternal treatment with safflower oil alone and in combination with folic acid in the embryos, although only with the combined treatment in the decidua. This may suggest that maternal treatments capable of ameliorating the pro-inflammatory environment in both the embryo and the decidua during early organogenesis can lead to greater beneficial effects in the embryo from diabetic rats.

Diets supplemented with safflower oil are enriched in linoleic acid, an essential n-6 polyunsaturated fatty acid (PUFA) that is transferred through the yolk sac and the placenta for proper embryonic and fetal development, capable of activating the three PPAR isotypes (Xu et al., 2007). Although n-3 PUFAs, present in high concentrations in fish oil, can also activate PPARs and possess anti-inflammatory effects, they could lead to a decrease in embryonic arachidonic acid concentrations, highly required during embryo organogenesis (Amusquivar et al., 2000; Jawerbaum and Gonzalez, 2005).

There are relevant differences in PPARs expression in embryonic and decidual tissues, as during early organogenesis the embryo expresses only the PPARα isotype while maternal tissues expresses the three PPAR isofoms (Higa et al., 2007; Abbott, 2009), a difference that may be involved in the different responses observed in decidual and embryonic tissues and that would require further evaluation. Although several works have found that activation of PPARγ is involved in the restoration of MMPs/TIMPs balance in various tissues (Hua et al., 2009; Pustovrh et al., 2009), there are still few data reporting MMPs/TIMPs regulatory pathways by the activation of PPARα, a relevant PPAR isotype during embryo organogenesis (Higa et al., 2007; Kim et al., 2009).

Folic acid treatments have also shown to be capable of regulating MMPs/TIMPs balance in different tissues (Guo et al., 2006; Qipshidze et al., 2010). Indeed, besides the complex and pleiotropic functions and mechanisms of action of folic acid during development (van der Put et al., 2001), a close interaction between PPARs and folic acid regulatory pathways has been described and related to their anti-inflammatory effects (Hayden and Tyagi, 2004). Therefore, the interaction between folic acid and safflower oil treatments may be the result of both common and independent effects of each of the treatments. In agreement, in this study we found that the folate treatment alone, although not in combination with safflower oil, induced changes in triglycerides concentrations that could benefit embryo development in maternal diabetes. NEFA concentrations were unchanged with the performed treatments, although increased in the sera from diabetic rats. On the other hand, in this work we found that relevant post-transductional mechanisms capable of regulating MMPs activities were differentially regulated in embryos and decidua by the safflower and folic acid treatments alone and in combination. Indeed, both the safflower oil and folic acid treatments were able to increase embryonic TIMPs while only the safflower oil treatment led to increased TIMPs in the decidua. The observed increased TIMPs concentrations suggest an increased capacity to inhibit MMPs overactivity in both the embryo and the decidua. TIMPs concentrations are up-regulated by PPARγ activation in various tissues (Hua et al., 2009; Pustovrh et al., 2009), and there are some recent reports of the capacity of folic acid to up-regulate tissue TIMPs concentrations (Qipshidze et al., 2010). TIMPs are relevant due to their capacity to inhibit MMPs activity, but also possess several functions related to cell proliferation and differentiation (Stetler-Stevenson, 2008). Therefore, the impact of the changes observed in embryonic and decidual TIMPs inhibitory capacity on the treated diabetic animals will require further research to be fully elucidated.

Various previous works have addressed the involvement of NO in neural tube formation and the relevance of NO overproduction in diabetic embryopathy (Plachta et al., 2003; Jawerbaum and Gonzalez, 2005). Besides, the protective effects of folic acid have been found related to the prevention of NO-derived oxidative molecules in various tissues (Weil et al., 2004; McCarty et al., 2009). Nevertheless, in this study we found that in maternal diabetes, folic acid supplementation was not able to reduce NO production in the embryos, although it clearly regulated NO production in the decidua. In contrast, safflower oil decreased NO production in embryos and not in decidua, suggesting specific embryonic and decidual effects of safflower oil and folic acid treatments on NO production both possibly helping to prevent diabetes-induced developmental defects. On the other hand, supplementation with folic acid alone and in combination with safflower oil was able to reduce ROS formation in the embryos, while all treatments were able to reduce lipoperoxidation in decidua from diabetic animals. Previous works demonstrated a clear involvement of oxidative stress in diabetic embryopathy and showed the capacity of folic acid treatments to reduce embryonic oxidative stress (Eriksson, 2009; Wentzel, 2009; Zabihi and Loeken, 2010). Our results suggest that the combined treatment of folic acid and safflower oil could constitute an effective antioxidant treatment in maternal diabetes, with regulatory effects on both the embryo and the decidua. Nevertheless, it should be taken into account that fat requirements are lower in rats than in humans (Council SoLANNR, 1995), and thus difficulties may arise in the translation from animals to human studies.

In conclusion, we found that safflower oil and folic acid treatments alone and in combination lead to the regulation of MMPs and TIMPs activities, together with changes in the formation of NO and ROS, suggesting anti-inflammatory and antioxidant effects helpful in the prevention of diabetic embryopathy. Since in most measurements the combined treatment of safflower oil and folic acid was the most effective and led to a greater reduction in embryo malformations, this combination may be considered for further evaluation in clinical trials.

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**Authors’ roles**

R.H. was involved in experimental design and procedures, data analysis and interpretation; M.K., M.B.M., D.M. played a role in experimental procedures and manuscript revision; V.W. took part in Data analysis and interpretation; A.J. was involved in Concept and design, interpretation and article draft.

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Conflict of interest

None declared.

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