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Effects of maternal dietary olive oil on pathways involved in diabetic embryopathy

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ABSTRACT

Maternal diabetes induces a pro-oxidant/pro-inflammatory intrauterine environment related to the induction of congenital anomalies. Peroxisome proliferator activated receptors (PPARs) are transcription factors that regulate antioxidant and anti-inflammatory pathways. We investigated whether maternal diets supplemented with olive oil, enriched in oleic acid, a PPAR agonist, can regulate the expression of PPAR system genes, levels of lipoperoxidation and activity of matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs) in embryos and decidua from diabetic rats. The embryos and decidua from diabetic rats showed reduced expression of PPARs and increased concentration of lipoperoxidation, MMPs and TIMPs, whereas the maternal treatments enriched in olive oil increased PPARδ in embryos and PPARγ and PPARγ-coactivator-1α expression in decidua, and increased TIMPs concentrations and decreased lipoperoxidation and MMPs activity in both tissues. Thus, maternal diets enriched in olive oil can regulate embryonic and decidual PPAR system genes expression and reduce the pro-oxidant/pro-inflammatory environment during rat early organogenesis.

1. Introduction

Maternal diabetes during early organogenesis induces embryonic alterations and increases the risk of congenital anomalies [1,2]. Animal models of diabetes and pregnancy are useful to study pathways involved in diabetic embryopathy and to find approaches to reduce its incidence [3]. Although the mechanisms of induction of embryo malformations in maternal diabetes are still not fully understood, they can be related to the intrauterine pro-inflammatory and pro-oxidant environment induced by the excess of metabolic substrates in maternal circulation [2,4,5].

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors involved in anti-inflammatory, antioxidant and developmental processes [6–8]. PPARδ is the only PPAR isotype expressed during early embryo organogenesis [9] and its protein levels have been found reduced in embryos from diabetic rats [10]. The maternal decidua surrounds the embryo during organogenesis, providing supporting, endocrinal and immunological functions [11–13]. PPARδ has also an important role during decidualization [14], whereas little is known about the roles of PPARγ and PPARα in the decidua. It is also unknown whether PPARγ coactivator-1α (PGC-1α), a relevant coactivator in PPARs signaling and a regulator of mitochondrial function and biogenesis [15], is altered in embryos and decidua in diabetic pregnancies.

The activity of PPARs depends on their endogenous ligands, many of which are unsaturated fatty acids such as essential fatty acids and their derivatives through the ω-6 and ω-3 pathways. PPAR target genes include desaturase enzymes [16,17]. Δ9 desaturase is required for the conversion of saturated fatty acids into monounsaturated fatty acids (MUFAs), such as oleic acid [18]. Δ5 and Δ6 desaturases catalyze the rate-limiting steps in the conversion of the essential fatty acids linoleic acid and α-linolenic acid into their derived ω-6 and ω-3 polyunsaturated fatty acids (PUFAs), respectively [19]. Desaturases are insulin dependent enzymes which are decreased in different tissues in the diabetic disease [19,20]. Whether maternal diabetes leads to changes in the expression of desaturases during development is still unknown.

Prostaglandins (PGs) are derived from arachidonic acid, a ω-6 PUFA. Deficiency in arachidonic acid and PGE_2 has been closely

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related to diabetic embryopathy [5,21,22]. Interestingly, PPARδ activation in the embryo has been shown to increase the production of PGI_2 and PGE_2, which are needed in optimal concentration for proper embryo development [10]. Moreover, maternal diets supplemented with olive oil, enriched in oleic acid, a MUFA capable of activating PPARs, have been shown to increase PGI_2 and PGE_2 production [23].

On the other hand, we and others have found that PPAR agonists are effective negative regulators of the activity of matrix metalloproteinases (MMPs) [24,25]. MMPs are proteolytic enzymes involved in the extracellular matrix remodeling that takes place during embryo and placenta development [26]. Indeed, both MMP-2 and MMP-9 are largely involved in physiological reproductive processes, although they are also markers of a pro-inflammatory state when produced in excess [27]. We have previously found that MMP-2 and MMP-9 activities are enhanced in embryos and decidua from diabetic rats [28].

Consumption of olive oil has demonstrated beneficial effects on pro-inflammatory diseases [29,30]. Besides, diets enriched in olive oil have been found to regulate metabolic, antioxidant and anti-inflammatory pathways in reproductive tissues, in a way similar to that evidenced when PPARs are activated by certain PGs, leukotrienes and pharmacological agonists [23,31,32]. Aiming to gain insights into the pathways involved in diabetic embryopathy and the effects of olive-oil supplementation on diabetic pregnancies during early organogenesis, we evaluated the expression of PPARs, PGC-1α and desaturases, as well as the concentration of oxidative stress markers and the activities of MMPs and their endogenous inhibitors (TIMPs), in embryos and decidua from control and diabetic rats either treated or not with a diet supplemented with 6% olive oil.

2. Materials and methods

2.1. 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 12 h light: 12 h dark. Diabetes was induced by 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) to female rats weighing 200–230 g, as previously described [10]. Control rats were injected with citrate buffer only. Glycemia was measured with Accu-Chek reagent strips and a glucometer (Bayer Diagnostics, Buenos Aires, Argentina) previous to mating and at 10.5 days of gestation. Females showing glycemia values higher than 250 mg/dl were considered diabetic. 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 smears and this day was designated day 0.5 of pregnancy. The reproductive characteristics of the diabetic model have been previously described [3]. 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 "Guide for the Care and Use of Laboratory Animals" US National Institutes of Health (NIH Publication, 8th Edition, 2011) http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader.

2.2. Dietary treatment

On day 0.5 of gestation, both control and diabetic rats were randomized into two groups: (A) rats fed ad libitum with the commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) (standard diet) and (B) rats fed ad libitum with the standard diet supplemented with 6% wt/wt olive oil. The feed was coated after manufacture with oil to produce the test diet. Both diets met the nutritional requirements of calories, fat, carbohydrates and proteins for maintenance and reproduction [33], and have been previously used during rat gestation [23]. The olive oil-supplemented diet is enriched mostly in oleic acid (354%) as well as in palmitic acid (167%) and linoleic acid (22%). The standard diet (caloric density: 324 kcal/100 g) was composed of (g/100 g): carbohydrates (50); proteins (25); fat (5), major fatty acids: 16:0 (0.58), 18:0 (0.16), 18:1 (1.27), 18:2 (1.99), 18:3 (0.73). The olive-oil supplemented diet (caloric density: 340 kcal/100 g) was composed of: carbohydrates (g/100 g): carbohydrates (48); proteins (24); fat (11), major fatty acids: 16:0 (1.55), 18:0 (0.26), 18:1 (5.77), 18:2 (2.41), 18:3 (0.57). The standard and olive oil-supplemented diets were given from day 0.5 until day 10.5 of gestation, a day corresponding to early embryo organogenesis in rats [21].

2.3. Tissue collection and evaluation of embryo morphology

Animals were euthanized in a CO_2 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 Na^+, 5.9 mM K^+, 2.2 mM Ca^{++}, 1.2 mM Mg^{++}, 127 mM Cl^-, 25 mM HCO_3^-, 1.2 mM SO_4^{2-} and 1.2 mM PO_4^{3-}. The balls of decidual tissue were explanted from each uterus and gently opened to free the conceptuses using a stereomicroscope and microsurgical dissecting instruments. The embryos were dissected out of the yolk sacs and evaluated morphologically under a stereomicroscope. Decidua was separated from the embryo, the extraembryonic tissues and the ectoplacental cone. Viability was established by the presence of a beating heart. Viable embryos were categorized as morphologically normal or as showing either neural tube defects or other malformations. Embryos in resorption stages received no further analyses. Viable embryos and their corresponding decidua were separately pooled (four or three embryos or decidua per rat) and preserved immediately according to the determinations described below.

2.4. Analysis of gene expression of PPARs, PGC-1α and desaturases

Expression of PPARs, PGC-1α and desaturases was evaluated by RT-PCR, as previously determined [34]. Four pooled embryos or decidua per rat ($n = 9$ rats) were kept in RNAlater® (Ambion, TX, USA) at −20 °C and RNA was extracted with Tri reagent (Genbiotech, Buenos Aires, Argentina) in accordance with the manufacturer's instructions. cDNA was synthesized incubating 2 μg of extracted RNA in a first-strand buffer containing MMLV enzyme from Promega (Buenos Aires, Argentina), random primer hexamers (Promega) and each of all four dNTPs (Invitrogen, CA, USA) in accordance with the MMLV manufacturer's instructions. The reaction mixture was incubated at 37 °C for 60 min and then at 70 °C for 15 min. cDNA (2 μl, selected to work within the linear range) was amplified by PCR in a buffer containing dNTPs, magnesium chloride solution, Taq polymerase (GoTaq Polymerase, Promega) and each specific primer in accordance with the Taq polymerase manufacturer's instructions. The primers used, detailed in Table 1, were purchased from Invitrogen. The primers for the ribosomal protein L30 were used as an internal control.

The initial step in the reaction was 95 °C for 5 min, followed, selected to work within the linear range, by 30 cycles for embryonic and decidual PPARδ, 36 cycles for decidual PPARγ, 32 cycles for decidual PPARα, 34 cycles for embryonic and decidual PGC-1α, 26 cycles for embryonic and 25 cycles for decidual Δ5 desaturase, 26 cycles for decidual Δ6 desaturase, 26 cycles for decidual Δ9 desaturase and 27 cycles for embryonic and 26 cycles for decidual L30. Each cycle consisted of denaturation at 95 °C for 15 s, primer annealing at 58 °C for 30 s and extension at 72 °C for 15 s.

Table 1
Primer sequences for each primer pair used for gene expression analysis by RT-PCR in embryos and decidua.

Genes	Primer sequence	Amplification product size	Source
PPARδ	Forward: 5′-GAGGGGTGCAAGGGCTTCTT-3′ Reverse: 5′-CACTTGTTGCGGTTCTTCTTCTG-3′	101-bp	[34]
PPARγ	Forward: 5′-CATGCTTGTGAAGGATGCAAG-3′ Reverse: 5′-TTCTGAAACCGACAGTACTGACAT-3′	131-bp	[54]
PPARα	Forward: 5′-TCACACAATGCAATCCGTTT-3′ Reverse: 5′-GGCCTTGACCTTGTTCATGT-3′	177-bp	[41]
PGC-1α	Forward: 5′-GTGCAGCCAAGACTCTGTATGG-3′ Reverse: 5′-GTCCAGGTCATTCACATCAAGTTC-3′	121-bp	[57]
Δ5 Desaturase (fatty acid desaturase 1)	Forward: 5′-AAGCACATGCCATACAACCA-3′ Reverse: 5′-CAGCGGCATGTAAGTGAAGA-3′	177-bp	[55]
Δ6 Desaturase (fatty acid desaturase 2)	Forward: 5′-ATCTGCCCTACAACCACCAG-3′ Reverse: 5′-GTGTGACCCACACAAACCAG-3′	249-bp	Primer 3 software
Δ9 Desaturase (stearoyl-CoA-desaturase 1)	Forward: 5′-CCTCATCATTGCCAACACCAT-3′ Reverse: 5′-AGCCAACCCACGTGAGAGAA-3′	144-bp	[56]
L30	Forward: 5′-CCATCTTGGCGTCTGATCTT-3′ Reverse: 5′-GGCGAGGATAACCAATTTC-3′	201-bp	[34]

The resulting products were separated on a 2% agarose gel and stained with SYBR® Safe (Invitrogen). The images were taken with the ImageQuant spectrophotometer and software (GE Healthcare, Buckinghamshire UK) and quantified with the Image J software.

2.5. Evaluation of lipid peroxidation

Lipid peroxidation in embryos was evaluated by measuring concentrations of the isoprostane 8-iso-$PGF_{2\alpha}$ (8-isoprostane) using a commercial enzyme immunoassay kit (Cayman Chemical Co., Ann Harbor, MI, USA) according to the manufacturer's instructions, as previously performed [28]. Briefly, three pooled embryos per rat ($n = 9$ rats) were homogenized in 2 M NaOH and an aliquot was separated for protein determination by the Bradford method using a protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were incubated at 45 °C for 2 h, neutralized by the addition of 2 M HCl (pH 7.5) and centrifuged at 3000 rpm for 10 min.

This assay is based on the competition between 8-isoprostane in the sample and an 8-isoprostane conjugate with a tracer for a limited number of 8-isoprostane-specific antiserum binding sites.

Lipid peroxidation in decidua was analyzed by measuring the concentrations of thiobarbituric acid reactive substances (TBARS), as previously [34], a method widely used to assess peroxidation of fatty acids [35]. Briefly, three pooled decidua per rat ($n = 9$ rats) were homogenized in 100 mM Tris–HCl buffer, pH 7.6. Trichloroacetic acid (40%) was added to the equivalent of 100 mg tissue in the homogenate and centrifuged at 3000 rpm for 15 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 at room temperature and quantified spectrophotometrically at 530 nm. A calibration curve was performed with malondialdehyde (Sigma–Aldrich) subjected to the same conditions as the tissue homogenates.

2.6. Analysis of gelatinase activity of matrix metalloproteinases

Zymography was performed to evaluate the presence of MMP-2 and MMP-9 gelatinase activity, as previously described [24]. Zymography allows the analysis of both MMPs and proMMPs, since the exposure to sodium dodecyl sulphate (SDS) induces changes in the conformation of proMMPs that promote their activation. Briefly, four pooled embryos or decidua per rat ($n = 9$ rats) were homogenized in 50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton X-100. Then, either 20 μg of protein of embryonic homogenates or 50 μg of protein of decidual homogenates (selected to work within the linear range) were 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. Gels were rinsed in 2.5% Triton X-100 for 60 min to remove SDS and incubated (for 96 h in the case of the embryonic tissue and for 24 h in the case of the decidual tissue, selected to work within the linear range) in 50 mM Tris buffer pH 7.4, containing 150 mM NaCl and 10 mM $CaCl_2$, at 37 °C. Then, gels were stained with Coomassie blue and the areas of proteolytic activity appeared as negatively stained bands in a dark background.

Identification of MMPs was based on their molecular weights and a positive internal control (conditioned medium of human fibrosarcoma HT-1080 cells), which was run in each gel to allow the standardization of the results obtained in the different zymograms. The enzymatic activity was quantified using ImageJ and expressed as arbitrary densitometric units. Data are shown as relative to a value of 1 assigned to the mean values for active MMP-9 in control embryos and decidua.

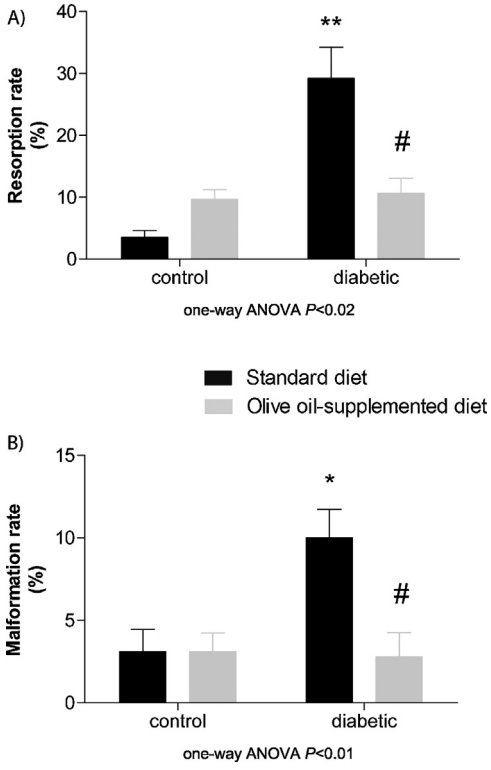

Fig. 1. Effect of maternal diet supplemented with 6% olive oil on (A) resorption rate and (B) malformation rate in control and diabetic rats. Values are the average of resorptions or malformations per rat (means ± SEM) obtained from 13 to 16 rats in each experimental group. One-way ANOVA with Tukey's post-test was performed on the data. Post-test significant results: $^{*}P < 0.05$, $^{**}P < 0.01$ vs control with standard diet; $^{\#}P < 0.05$ vs diabetic with standard diet.

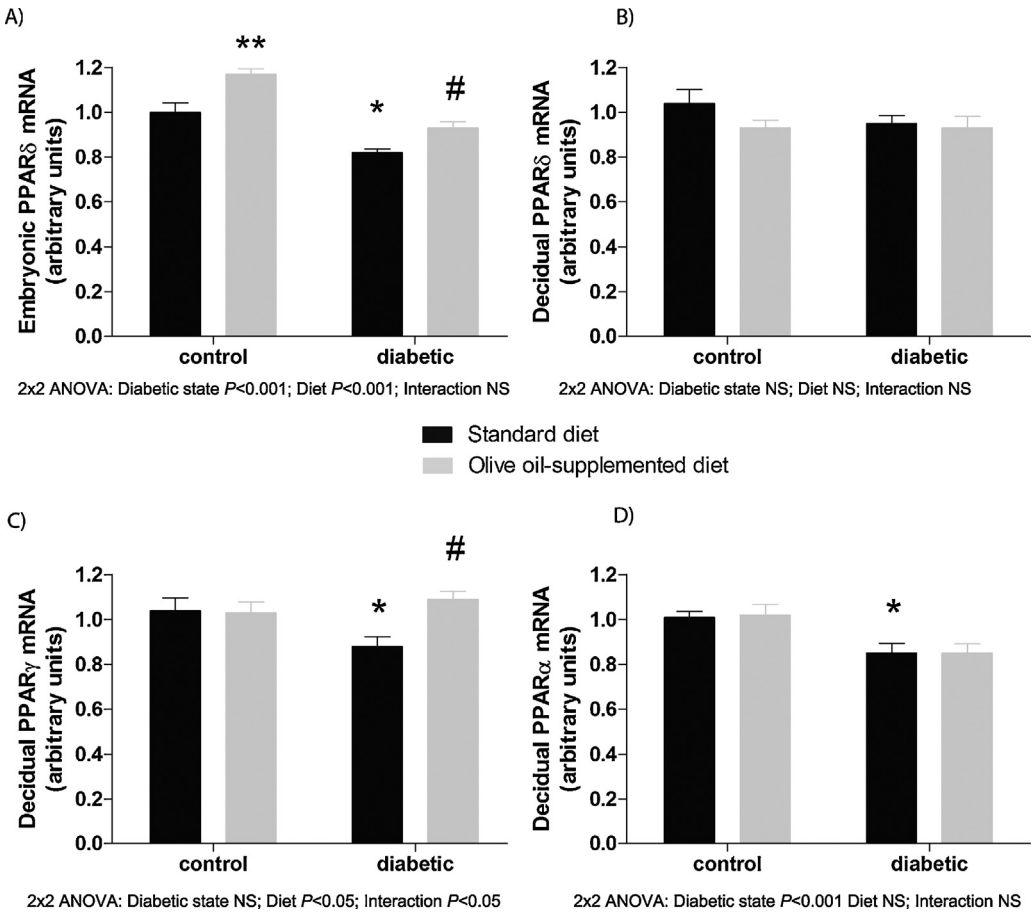

Fig. 2. Effect of maternal diet supplemented with 6% olive oil on PPARs gene expression in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) Values of PPARδ gene expression relative to those of L30 in embryos from control and diabetic rats. (B) Values of PPARδ gene expression relative to those of L30 in decidua from control and diabetic rats. (C) Values of PPARγ gene expression relative to those of L30 in decidua from control and diabetic rats. (D) Values of PPARα gene expression relative to those of L30 in decidua from control and diabetic rats. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{*}P<0.05$, $^{**}P<0.01$ vs control with standard diet; $^{\#}P<0.05$ vs diabetic with standard diet.

2.7. Evaluation of inhibitory capacity of TIMPs

Reverse zymography was performed to analyze the inhibitory capacity of TIMPs in embryos and decidua from control and diabetic rats, as previously described [24]. Briefly, this technique evaluates the ability of TIMPs to inhibit the degradation of gelatin by MMPs that were incorporated by the HT-1080 conditioned medium. Four pooled embryos or decidua per rat ($n = 9$ rats) were homogenized in 50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton X-100, followed by heat extraction (60 °C) with 50 mM Tris, 0.1 M $CaCl_2$ and 0.15 M NaCl. Either 20 μg of protein from embryonic tissues or 50 μg of protein from decidual tissues (selected to work within the linear range) were 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 source rich in various MMPs. After electrophoresis, gels were washed twice with 2.5% Triton X-100, and incubated at 37 °C for 24 h (selected to work within the linear range) in 50 mM Tris–HCl, 0.2 M NaCl and 5 mM $CaCl_2$, pH 8.0. Subsequently, gels were stained with Coomassie blue. The inhibition of MMPs gelatinolytic capacity by TIMPs appeared as dark bands on a clear background. TIMPs were identified based on their molecular weights, which were determined through prestained SDS-PAGE protein standards (prestained Full Range, GE Healthcare), run in the same gel. TIMP activity was quantified using ImageJ and expressed as arbitrary densitometric units. Data are shown as relative to a value of 1 assigned to the mean value for TIMP-1 in control embryos and decidua.

2.8. Statistical analysis

Data are presented as the mean ± standard error. The data obtained were checked for normality prior to the statistical analysis with the Shapiro–Wilk test (Statistix 10 software). Groups were compared by one-way ANOVA in conjunction with Tukey's test or two-way ANOVA in conjunction with Bonferroni's test (Prism 5 software). In all cases, differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Expression of PPARs and PGC-1α in embryos and decidua from control and diabetic rats fed an olive oil-supplemented diet during pregnancy

Glycemia was increased in diabetic rats fed either with the standard diet or the olive oil-supplemented diet when compared to the control group fed the same diet (control: 101 ± 8 mg/dl, diabetic: 415 ± 25 mg/dl, $P < 0.001$; control rats fed olive oil-supplemented diet: 93 ± 2 mg/dl, diabetic rats fed olive oil-supplemented diet: 361 ± 28 mg/dl, $P < 0.001$). Energy intake was similar in the control groups fed the standard (64 ± 2 kcal/day) and the olive oil-containing diet (61 ± 3 kcal/day), and was similarly increased in the diabetic rats fed the standard and the olive oil-supplemented diet when compared to the control groups (90 ± 7 and 93 ± 6 kcal/day, respectively, $P < 0.001$ vs control group fed the standard diet).

The resorption rate was increased in diabetic rats fed the standard diet when compared with control rats fed the same diet ($P < 0.01$). When the diabetic rats were fed with the olive oil-supplemented diet, the resorption rate was decreased to values similar to those found in the control groups ($P < 0.05$, Fig. 1A).

The malformation rate was increased in diabetic rats fed the standard diet when compared with control rats fed the same diet ($P < 0.05$). The malformations observed were mostly neural tube defects. Severe malrotation was also observed, although mainly concomitantly with embryonic neural tube defects. The malformation rate was decreased when the diabetic rats were fed the olive oil-supplemented diet and compared with the diabetic rats fed the standard diet ($P < 0.05$, Fig. 1B).

As PPARs expression may be regulated by their own ligands [34], and PGC-1α is a coactivator relevant for PPARs activity [15], we evaluated the expression of PPARs and PGC-1α in embryos and decidua from control and diabetic rats fed the olive oil-supplemented diet during pregnancy.

The expression of PPARδ, the only isotype expressed during early organogenesis [9], was decreased in embryos from diabetic rats fed the standard diet when compared with embryos from control rats fed the same diet ($P < 0.05$, Fig. 2A). Interestingly, PPARδ expression increased in embryos from both control ($P < 0.001$) and diabetic rats ($P < 0.05$, Fig. 2A) fed the olive oil-supplemented diet.

In contrast, PPARδ expression showed no differences in decidua from control and diabetic rats fed either the standard diet or the olive oil-supplemented diet (Fig. 2B). Differently, PPARγ expression was decreased in decidua from diabetic rats fed the standard diet when compared to the control group fed the same diet ($P < 0.05$, Fig. 2C). In addition, PPARγ expression was increased in decidua from diabetic rats fed the olive oil-supplemented diet when compared to the decidua from diabetic rats fed the standard diet ($P < 0.05$, Fig. 2C). Regarding PPARα, its expression was decreased in decidua from diabetic rats fed the standard diet when compared with the control group fed the same diet ($P < 0.05$, Fig. 2D), but showed no changes in the groups supplemented with olive oil (Fig. 2C).

The expression of the PPAR coactivator PGC-1α was similar in the embryos from control and diabetic groups fed the standard diet (Fig. 3A). PGC-1α expression increased only in embryos from control rats in the group supplemented with olive oil compared to the control group fed the standard diet ($P < 0.05$, Fig. 3A).

Interestingly, PGC-1α expression was decreased in the decidua from diabetic rats fed the standard diet when compared with decidua from control rats fed the same diet ($P < 0.05$, Fig. 3B). PGC-1α expression was increased in the decidua from both the control and diabetic groups fed the olive oil-supplemented diet when compared respectively with the control and diabetic groups fed the standard diet ($P < 0.05$, Fig. 3B).

3.2. Expression of desaturases in embryos and decidua from control and diabetic rats fed an olive oil-supplemented diet during pregnancy

As desaturases are targets of PPARs activation in different tissues [16,17] and insulin-dependent enzymes needed for the synthesis of arachidonic acid, substrate for the synthesis of eicosanoids [19,20], we evaluated their expression in embryos and decidua from control and diabetic rats fed the standard and olive oil-supplemented diets.

Δ5 desaturase expression showed no changes in embryos from diabetic rats fed the standard diet when compared to controls. In addition, it showed no changes in embryos from both control and diabetic rats fed the olive oil-supplemented diet when compared with the respective groups fed the standard diet (Fig. 4A).

Δ5 and Δ6 desaturase expressions were decreased in the decidua from diabetic rats fed the standard diet compared with

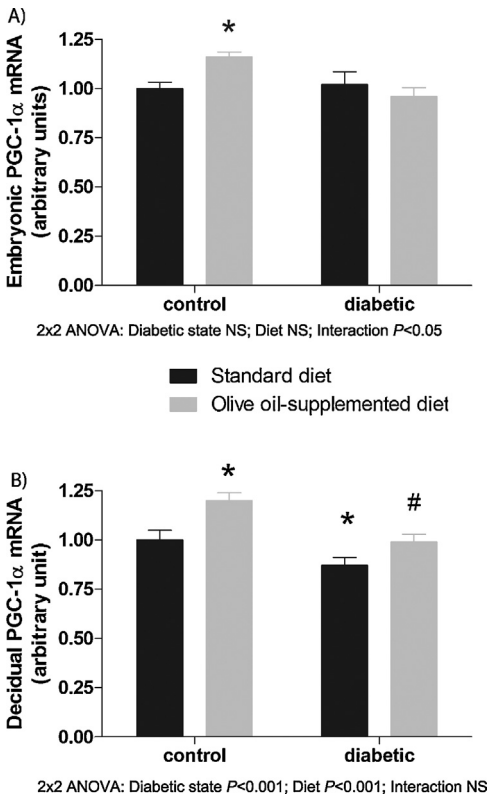

Fig. 3. Effect of maternal diet supplemented with 6% olive oil on PGC-1α gene expression in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) Values of PGC-1α gene expression relative to those of L30 in embryos from control and diabetic rats. (B) Values of PGC-1α gene expression relative to those of L30 in decidua from control and diabetic rats. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{*}P < 0.05$ vs control with standard diet; $^{\#}P < 0.05$ vs diabetic with standard diet.

control rats fed the same diet ($P < 0.05$, Fig. 4B and C). Δ5 and Δ6 desaturase expressions showed no changes in the decidua from control and diabetic rats fed the olive oil-supplemented diet when compared with the respective group fed the standard diet (Fig. 4B and C). Δ9 desaturase expression showed no changes in the decidua from the diabetic groups fed the standard diet when compared with the control group fed the same diet. In addition, it showed no changes in the decidua from either the control or the diabetic groups fed the olive oil-supplemented diet when compared with the respective groups fed the standard diet (Fig. 4D).

3.3. Lipid peroxidation in embryos and decidua from control and diabetic rats fed an olive oil-supplemented diet during pregnancy

As PPARs are negative regulators of oxidative stress in reproductive tissues [6], we evaluated markers of lipid peroxidation in embryos and decidua from control and diabetic animals fed the standard diet and the olive oil-supplemented diet.

The concentrations of 8-isoprostane were increased in the embryos from diabetic rats fed the standard diet when compared with the control rats fed the same diet ($P < 0.01$). The concentration of 8-isoprostane was decreased in embryos from diabetic rats fed the olive oil-supplemented diet ($P < 0.05$, Fig. 5A) when compared to embryos from diabetic rats fed the standard diet, but increased when the control rats were fed the olive oil-supplemented diet compared with the control rats fed the standard diet ($P < 0.05$, Fig. 5A).

TBARS concentrations were increased in the decidua from the diabetic group fed the standard diet when compared to the control

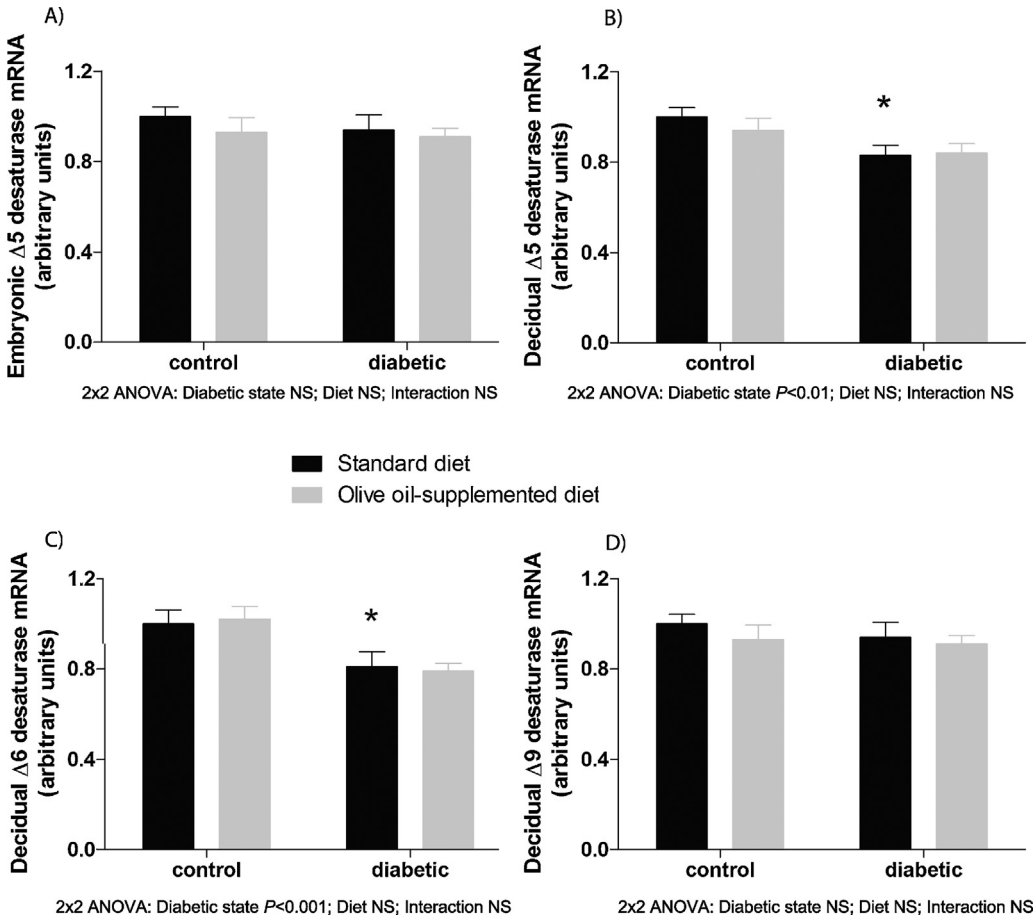

Fig. 4. Effect of maternal diet supplemented with 6% olive oil on the expression of desaturases in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) Values of Δ5 desaturase gene expression relative to those of L30 in embryos from control and diabetic rats. (B) Values of Δ5 desaturase gene expression relative to those of L30 in decidua from control and diabetic rats. (C) Values of Δ6 desaturase gene expression relative to those of L30 in decidua from control and diabetic rats. (D) Values of Δ9 desaturase gene expression relative to those of L30 in decidua from control and diabetic rats. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{*}P < 0.05$ vs control with standard diet.

group fed the same diet ($P < 0.05$, Fig. 5B). TBARS concentrations were decreased in the decidua from both diabetic ($P < 0.001$) and control ($P < 0.05$) rats fed the olive oil-supplemented when compared respectively with the control and diabetic groups fed the standard diet (Fig. 5B).

3.4. MMPs activities in embryos and decidua from control and diabetic rats fed an olive oil-supplemented diet during pregnancy

MMP-2 and MMP-9 play important roles in embryo and decidual development, and are overactivated in different gestational tissues in maternal diabetes [26,28,36].

Embryonic MMP-9 activity, which was detected only in its active form and was overactivated in the embryos from diabetic rats ($P < 0.001$), was decreased in the embryos from the diabetic group fed the olive oil-supplemented diet compared with the diabetic group fed the standard diet ($P < 0.01$, Fig. 6A and B).

Embryonic MMP-2 activity, which was detected both in its proenzyme (proMMP-2) and active forms (MMP-2), was increased in embryos from diabetic rats fed the standard diet (proMMP-2: $P < 0.001$, MMP-2: $P < 0.01$) when compared with the control group fed the same diet. Besides, both proMMP-2 and MMP-2 activities were decreased in embryos from diabetic rats ($P < 0.01$) fed the olive oil-supplemented diet when compared with the diabetic group fed the standard diet (Fig. 6A, C and D). ProMMP-2 ($P < 0.01$) and MMP-2 ($P < 0.001$) activities were also decreased in embryos from control animals fed the olive oil-supplemented diet when compared to the control group fed the standard diet (Fig. 6A, C and D).

Decidual MMP-9 activity was increased in diabetic animals fed the standard diet ($P < 0.001$) when compared with the control group fed the same diet, and decreased in decidua from both the diabetic ($P < 0.01$) and control ($P < 0.01$) groups fed the olive oil-supplemented diet when compared to the respective groups fed the standard diet (Fig. 6E and F).

ProMMP-2 activity was increased in the decidua from diabetic animals fed the standard diet when compared to decidua from control rats fed the same diet ($P < 0.001$, Fig. 6E and G), but showed no changes in the decidua from either the diabetic and the control rats fed the olive oil-supplemented diet when compared to the respective group fed the standard diet (Fig. 6E and G). Besides, the activity of the active MMP-2 form showed no changes in the decidua from diabetic rats fed the standard diet when compared with the decidua from control rats fed the same diet, and no changes in the decidua from either the control or diabetic groups fed with the olive-oil supplemented diets when compared to the respective groups fed the standard diet (Fig. 6G and H).

3.5. TIMPs inhibitory capacity in embryos and decidua from control and diabetic rats fed an olive oil-supplemented diet during pregnancy

TIMP-1 and TIMP-2 are endogenous MMPs inhibitors relevant in reproductive processes [37,38]. We found that TIMP-1 inhibitory

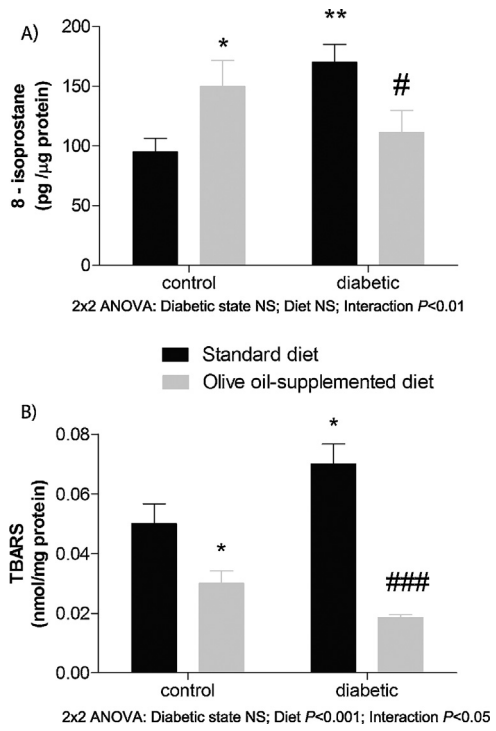

Fig. 5. Effect of maternal diet supplemented with 6% olive oil on lipid peroxidation in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) 8-Isoprostane concentrations in embryos from control and diabetic rats. (B) TBARS concentrations in decidua from control and diabetic rats. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{*}P < 0.05$, $^{**}P < 0.01$ vs control with standard diet; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ vs diabetic with standard diet.

capacity was increased in embryos from diabetic rats fed the standard diet when compared to embryos from control rats fed the same diet and also increased in the embryos from diabetic rats fed the olive oil-supplemented diet when compared to the embryos from diabetic rats fed the standard diet ($P < 0.05$, Fig. 7A and B). Similarly, TIMP-2 inhibitory capacity was increased in the embryos from diabetic rats fed the standard diet when compared to embryos from control rats fed the same diet and also increased in the embryos from diabetic rats fed the olive oil-supplemented diet when compared to the embryos from diabetic rats fed the standard diet ($P < 0.001$) (Fig. 7A and C).

On the other hand, TIMP-1 inhibitory capacity was increased in the decidua from diabetic rats fed the standard diet when compared to the control group fed the same diet ($P < 0.001$) and also increased in the decidua from both the control ($P < 0.001$) and the diabetic rats ($P < 0.05$) fed the olive oil-supplemented diet when compared with the respective group fed the standard diet (Fig. 7D and E). Similarly, TIMP-2 inhibitory capacity was increased in the decidua from diabetic rats fed the standard diet when compared to the control group fed the same diet ($P < 0.05$) and also increased in decidua from both the control ($P < 0.001$) and the diabetic group ($P < 0.05$) fed the olive oil-supplemented diet when compared with the respective groups fed the standard diet (Fig. 7D and F).

4. Discussion

The main finding of this work is that maternal diabetes-induced embryopathy can be partly prevented by maternal dietary treatments supplemented with olive oil, through pathways that involve the regulation of the expression of components of the PPAR system and that lead to antioxidant and anti-inflammatory effects. There are multiple diabetes-induced teratogenic pathways, most interconnected with each other [2,4,5,39]. Impaired PPAR signaling may be related with many of the altered mechanisms related to embryo dysmorphogenesis. Indeed, PPARs are involved in anti-inflammatory, antioxidant and developmental processes, energy homeostasis and regulation of key cellular functions [6,8].

We previously found that aberrant PPARδ signaling is involved in embryo dysmorphogenesis [10]. In this work, we found that the decreased expression of PPARδ in the embryo from diabetic rats can be increased when diabetic mothers are treated with a diet supplemented with 6% olive oil. Accordingly, it has been described that PPARα and PPARδ expression is positive-feedback regulated in the placenta of diabetic rats and that a network of different PPAR isoforms is involved in the regulation of PPARδ expression [34,40,41].

Previous studies have revealed a relevant function of PPARδ in decidualization [14], but, to our knowledge, this is the first time that PPARγ and PPARα expression is evaluated in the decidua. In the decidua from diabetic rats, we found that both PPARγ and PPARα expressions were decreased when compared to controls. The ability of PPARγ to modulate inflammatory and oxidative pathways is well documented [42,43], and the reduced expression of this receptor may be related to the increase in oxidative and pro-inflammatory pathways in the decidua from diabetic rats [28]. Interestingly, maternal dietary supplementation with olive oil was able to increase PPARγ expression in the decidua from diabetic rats, a treatment that also induces antioxidant and anti-inflammatory effects on this tissue, as discussed below.

Besides, in the decidua from diabetic rats, we also found a decreased expression of PGC-1α, which possibly further contributes to a dysfunctional PPAR system in this tissue. PGC-1α is highly expressed in tissues with high oxidative capacity, in which it promotes mitochondrial biogenesis and fatty acid oxidation [15], but little is known about its role in the embryo or its surrounding decidua. The ability of maternal treatments supplemented with olive oil to increase PGC-1α expression suggests that this treatment may regulate the PPAR system, and that other possible effects on mitochondrial and metabolic functions deserve to be further studied.

In this work, we found reduced expression of Δ5 and Δ6 desaturases in the decidua from diabetic rats. Desaturases are positively regulated by insulin and reduced in different diabetic tissues [16,17]. As the decidua are involved in the histotrophic nourishing of the embryo during rat early organogenesis, the reduced desaturase expression in the decidua from diabetic rats may be related to the deficiency in arachidonic acid involved in diabetic embryopathy [2,5,21]. On the other hand, although desaturases are PPAR target genes in different tissues, the maternal olive oil-supplemented diet had no effect on desaturase expression in embryos or decidua from control and diabetic rats. Whether desaturases can be regulated or not by other PPAR ligands during development will require further studies.

Oxidative and nitrative stress is one of the most studied pathways in diabetic embryopathy [2,5,21]. In this work, we found that oxidative stress in embryos and decidua from diabetic rats can be reduced by maternal diets enriched in olive oil. This effect may be mediated by the antioxidant capacity of oleic acid, a PPAR ligand present in increased concentrations in olive oil. Indeed, PPAR activation can lead to the transcription of antioxidant enzymes like catalase and superoxide dismutase [43–45]. On the other hand, olive oil is highly enriched in polyphenols, which can scavenge reactive oxygen species (ROS) [46]. Although 8-isoprostane is considered a reliable marker of oxidative stress [47], further studies are needed to clarify the

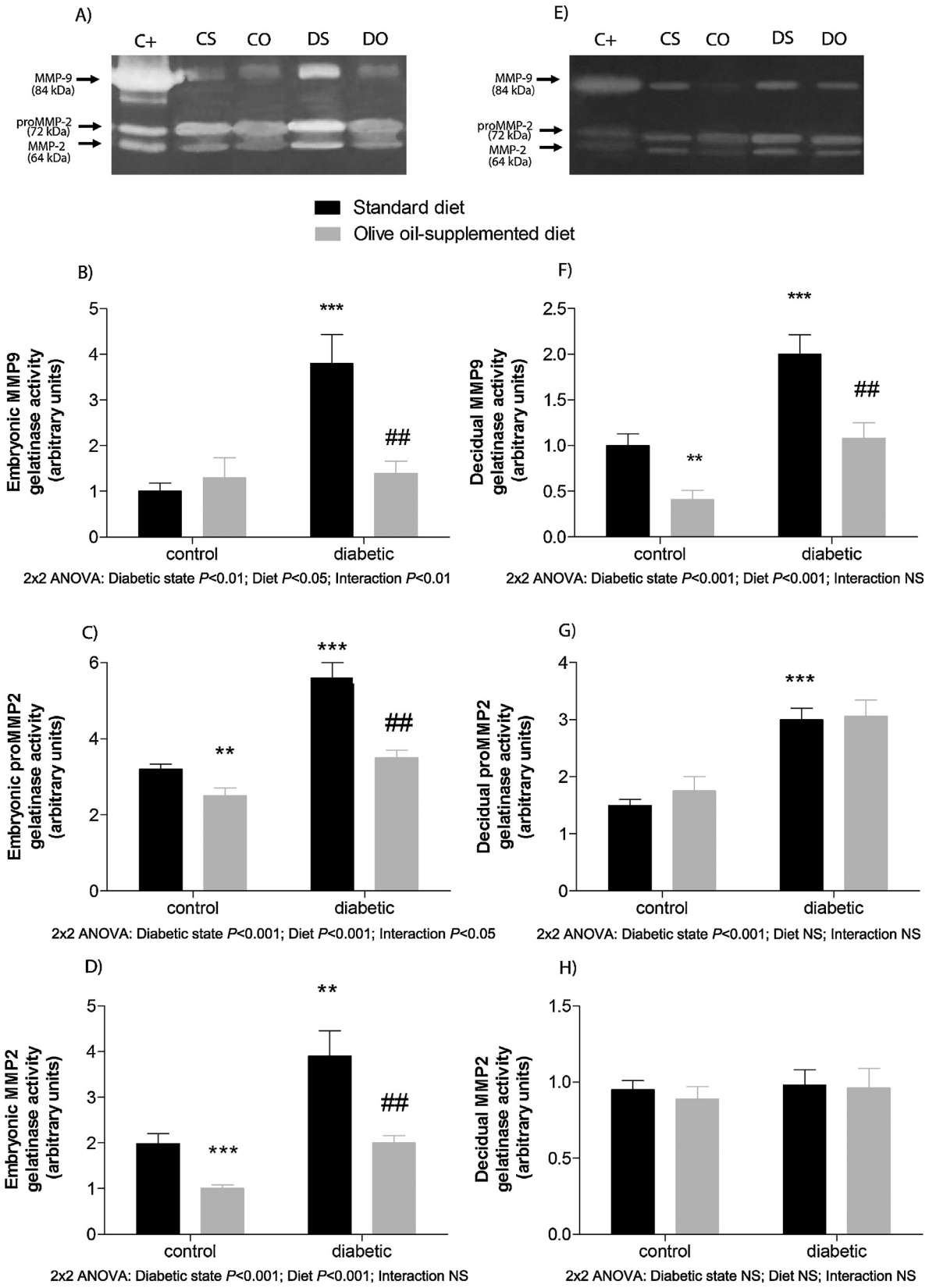

Fig. 6. Effect of maternal diet supplemented with 6% olive oil on MMPs activities evaluated in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) Representative zymogram exhibiting embryonic MMP-9, proMMP-2 and MMP-2 activities (CS: control – standard diet, CO: control – olive oil-supplemented diet, DS: diabetic – standard diet, DO: diabetic – olive oil-supplemented diet). (B) Densitometric analysis of embryonic MMP-9 activity. (C) Densitometric analysis of embryonic proMMP-2 activity. (D) Densitometric analysis of embryonic MMP-2 activity. (E) Representative zymogram exhibiting decidual MMP-9, proMMP-2 and MMP-2 activities (CS: control-standard diet, CO: control – olive oil-supplemented diet, DS: diabetic – standard diet, DO: diabetic – olive oil-supplemented diet). (F) Densitometric analysis of embryonic MMP-9 activity. (G) Densitometric analysis of decidual proMMP-2 activity. (H) Densitometric analysis of decidual MMP-2 activity. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{**}P < 0.01$, $^{***}P < 0.001$ vs control with standard diet; $^{\#\#}P < 0.01$ vs diabetic with standard diet.

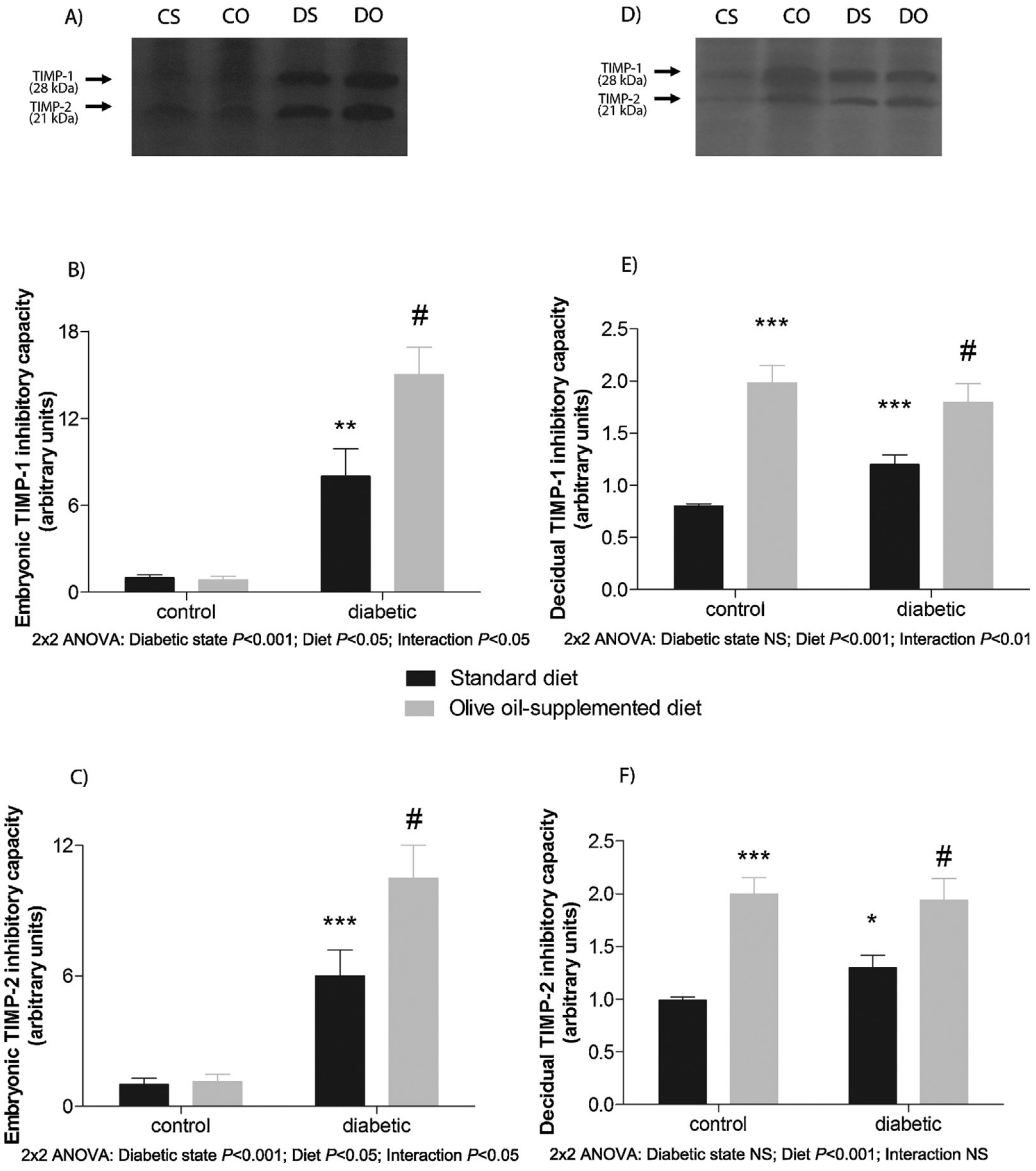

Fig. 7. Effect of maternal diet supplemented with 6% olive oil on TIMP-1 and TIMP-2 inhibitory capacity in embryos and decidua from control and diabetic rats on day 10.5 of gestation. (A) Representative reverse zymogram exhibiting embryonic TIMP-1 and TIMP-2 inhibitory capacity (CS: control – standard diet, CO: control – olive oil-supplemented diet, DS: diabetic – standard diet, DO: diabetic – olive oil-supplemented diet). (B) Densitometric analysis of embryonic TIMP-1 inhibitory capacity. (C) Densitometric analysis of embryonic TIMP-2 inhibitory capacity. (D) Representative reverse zymogram exhibiting decidual TIMP-1 and TIMP-2 inhibitory capacity (CS: control-standard diet, CO: control-olive oil-supplemented diet, DS: diabetic-standard diet, DO: diabetic-olive oil-supplemented diet). (E) Densitometric analysis of decidual TIMP-1 inhibitory capacity. (F) Densitometric analysis of decidual TIMP-2 inhibitory capacity. Values are means ± SEM obtained from 9 rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's post-test was performed. Post-test significant results: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control with standard diet; $^{\#}P < 0.05$ vs diabetic with standard diet.

increase in 8-isoprostane observed in control embryos fed the olive oil-supplemented diet, which may be due to the complex interactions existing between polyphenols, PPARs and the transporters of lipoperoxidation by-products. Indeed, although PPAR activation has been found to up-regulate members of the multidrug resistant protein family [48], highly expressed in trophoblasts and yolk sacs [49,50], polyphenols can induce isoprostane retention and can reduce expression and activity of transporters of the multidrug resistant protein families in different tissues [51,52].

MMP-2 and MMP-9 are closely related to a pro-oxidant and pro-inflammatory environment, can be activated by ROS and nitric oxide (NO), and their overactivation is involved in embryonic, decidual and placental damage in maternal diabetes [27,28,36]. Indeed, impaired endometrial extracellular matrix remodeling during decidualization, critical for the establishment of the maternal–fetal interface and successful pregnancy, has been found in maternal diabetes [53]. In this work, we found that maternal diets enriched in olive oil were able to decrease MMP-9 and MMP-2 overactivation in embryos and MMP-9 overactivation in the decidua from diabetic rats.

Besides, the activity of MMPs activity can be modulated by their endogenous inhibitors, TIMPs, and interestingly, we found that maternal diets enriched in olive oil increased TIMP-1 and TIMP-2 inhibitory capacity in embryos and decidua from diabetic rats. TIMPs concentrations are up-regulated by PPARγ activation in various tissues [24,25], and thus activation of the PPARγ isotype may be involved in the effects evidenced in the decidua from rats treated with olive oil during pregnancy.

5. Conclusions

Olive oil has a profound influence on health outcomes, showing modulatory effects on inflammatory states, cardiovascular diseases, dyslipemias and diabetes [29,30]. In this work, using animal models of diabetes, we provide evidence of the ability of an olive oil-supplemented diet to induce beneficial effects in the embryo and decidua. Indeed, this treatment ameliorates multiple pathways in diabetic embryopathy that range from modulating gene expression of several components of the PPAR system to regulate the enzymatic activity of MMPs and TIMPs. Thus, diabetic pregnancies may be a new possible target for the beneficial effects of diets enriched in olive oil, an issue that deserves to be clinically studied.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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