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Diet enriched in olive oil attenuates placental dysfunction in rats with gestational diabetes induced by intrauterine programming.

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Abbreviations: CTGF, connective tissue growth factor; GDM, gestational diabetes mellitus; MMP, matrix metalloproteinase; mTOR, mechanistic target of rapamycin; PPAR, Peroxisome proliferator activated receptor; SUPPL, supplemented.

Abstract

Scope: Offspring from rats with mild diabetes develop gestational diabetes (GDM). We tested the hypothesis that an olive oil-supplemented diet attenuates placental oxidative stress/inflammation, activation of mTOR signaling and inhibition of PPARγ and fetal overgrowth in GDM offspring from mild diabetic rats. **Methods and results:** Female offspring from rats with mild diabetes (group that developed GDM) and controls were fed with either a standard diet or a 6% olive oil-supplemented diet during pregnancy. On day 21 of pregnancy, plasma glucose levels in mothers and fetuses were increased in the GDM group independently of the diet. Fetal overgrowth and activation of placental mTOR signaling were partially prevented in the olive oil-treated GDM group. Placental PPARγ protein expression was decreased in GDM rats independently of the diet. However, increases in placental lipoperoxidation, connective tissue growth factor and matrix metalloproteinase 2 levels were prevented by the olive oil-enriched diet. **Conclusion:** Diets enriched in olive oil attenuate placental dysfunction and fetal overgrowth in rats with GDM induced by intrauterine programming.

Graphical abstract text

The effect of a maternal olive oil-supplemented diet in the placenta in a GDM rat model induced by intrauterine programming is investigated. The olive oil- supplemented diet partially prevented fetal overweight and activation of placental mTOR signaling. Increases in

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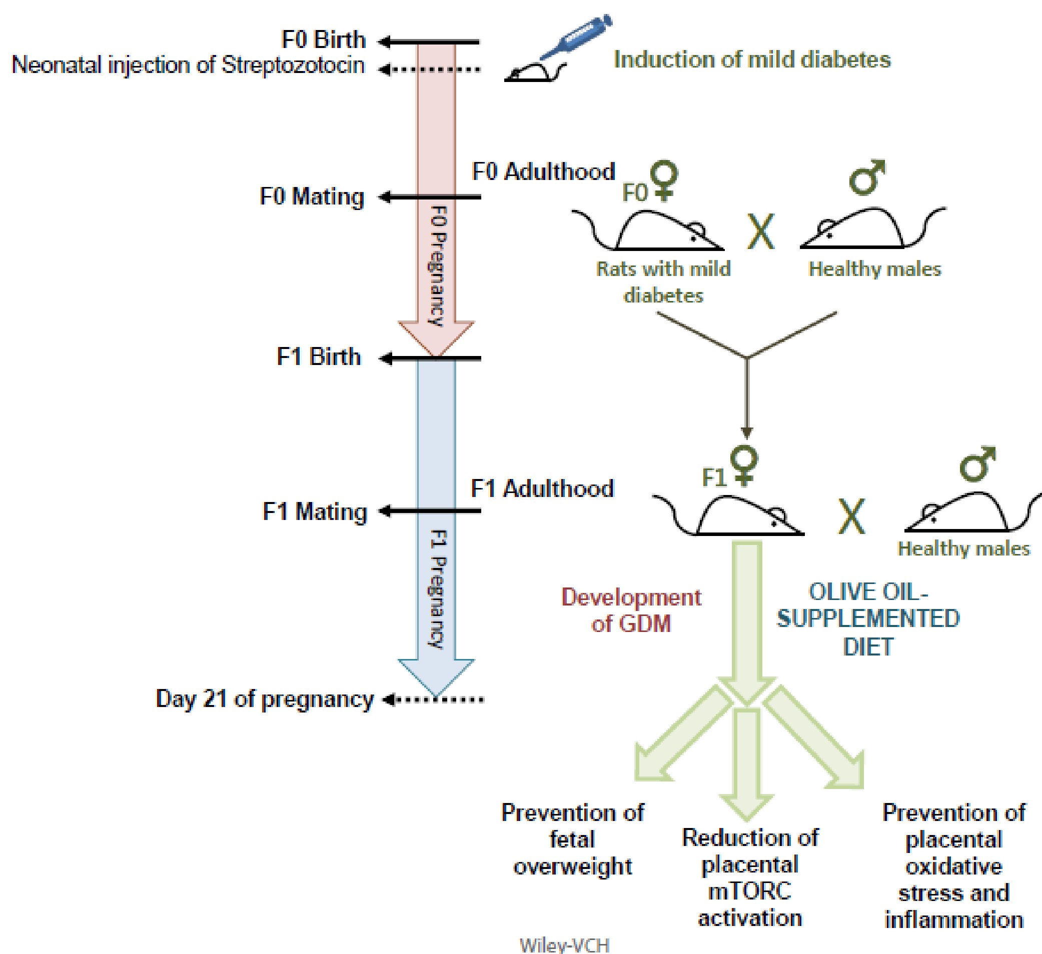
markers of the prooxidant/proinflammatory state were prevented in the placenta of GDM rats fed the olive oil-supplemented diet, highlighting the ability of this diet to attenuate GDM-induced placental dysfunction and fetal overgrowth.



1. Introduction

Gestational diabetes mellitus (GDM) is a prevalent disease associated with health risks for both the mother and the child [1]. In addition to the short-term fetal and neonatal adverse outcomes, programming of metabolic diseases in the offspring has been shown in women with pregestational and gestational diabetes mellitus as well as in experimental models of diabetes and pregnancy [1-5].

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Previously, we have reported that female offspring from rats with mild diabetes develop GDM, as these animals have normal glucose and insulin circulating levels before mating, and increased fasting glucose and insulin circulating levels at late pregnancy [6]. In this GDM model, the placental mechanistic target of rapamycin (mTOR) signaling is activated [6]. mTOR is a serine/threonine kinase that regulates cell survival, metabolism and proliferation [7]. mTOR forms two protein complexes named mTORC1 and mTORC2. Both mTORC1 and mTORC2 have been found to positively regulate transporters of amino acids in the placenta, stimulating fetal growth [8, 9]. Ribosomal protein S6 (rpS6) and 4E-binding protein-1 (4EBP-1) are downstream effectors of mTORC1 that control protein synthesis and cell growth [7]. Protein kinase Cα (PKCα) as well as serum and glucocorticoid-inducible kinase 1 (SGK1) are downstream effectors of mTORC2 involved in cell proliferation and metabolic processes [7, 10]. In GDM rats, both mTORC1 and mTORC2 signaling is increased in the placenta, which is associated with the fetal overgrowth observed in this experimental model [6].

The levels of placental pro-oxidant/pro-inflammatory markers in GDM rats are increased, which is associated with decreased protein expression of peroxisome proliferator-activated receptor γ (PPARγ) [6]. PPARγ is a ligand activated transcription factor with an important role in the control of inflammation and oxidative stress in several diseases and in different tissues [11-13]. Moreover, PPARγ regulates numerous trophoblast functions and is essential for placental development [14, 15]. PPARγ agonists reduce lipoperoxidation, a marker of oxidative stress, in placental explants from diabetic patients [16]. In placental explants from diabetic rats, PPARγ activation induces a decrease in the levels and activity of matrix metalloproteinases (MMPs) [17], proteolytic enzymes important for placental remodeling and trophoblast function, and markers of a pro-inflammatory environment when produced in excess [18, 19]. On the other hand, connective tissue growth factor (CTGF) is a non-structural extracellular matrix protein associated with tissue development and remodeling, and believed to participate in the pathophysiology of different diseases when produced in excess [20]. In

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the placenta, CTGF levels are increased in diverse clinical conditions, including preeclampsia and intrauterine growth restriction, although not previously studied in maternal diabetes [21].

Our previous studies have shown that maternal diets enriched in mono- and poly-unsaturated fatty acids, which are known to activate the three PPAR isoforms, reduce the levels of pro-oxidant and pro-inflammatory markers in placentae of rats with mild diabetes [13, 22]. More recently, we reported that treatments with polyunsaturated fatty acids during the pregnancy of the initial parent generation (F0) of rats with mild diabetes, which will lead to GDM induction in their offspring, normalize PPARɣ and mTOR signaling and reduce oxidative stress in the placenta, and prevent fetal overgrowth in the F1 pregnant offspring that develop GDM [23]. In the current work, we focused in the F1 generation susceptible to develop GDM and hypothesized that an olive oil-supplemented diet in this F1 generation attenuates placental dysfunction. The rationale to study supplementation with olive oil is that it is enriched in oleic acid, a PPAR activator, and has potent antioxidant properties both as a PPAR ligand and due to its polyphenol content [24, 25]. Thus, the aim of the present study was to assess fetal weight, placental oxidative stress/inflammation and placental PPARγ and mTOR signaling in the offspring from rats with mild diabetes that develop GDM.

2. Experimental Section

2.1. Animals

Albino Wistar rats were purchased from the certified animal facilities of the School of Exact and Natural Sciences, University of Buenos Aires (UBA, Argentina) and housed in the animal facilities of our Institute (CEFYBO-CONICET-UBA, Argentina), with a 12h light/12h dark cycle. Animals received food (commercial rat chow, Asociación Cooperativa Argentina, Buenos Aires, Argentina) and water *ad libitum*. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD Nº 3170/2015; School of Medicine, UBA, Argentina), and conducted following the Guide for the Care and Use of Laboratory Animals, US National

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Institutes of Health (NIH Publication, 8th Edition, 2011) http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader.

The experimental design is presented in **Figure 1**. We used a model of mild diabetes (F0) thoroughly characterized during pregnancy [26], induced by the injection of 2-day-old neonates with streptozotocin (90 mg/kg, s.c, Sigma-Aldrich) diluted in vehicle (citrate buffer, 0.05 M, pH 4.5, Sigma-Aldrich). Control animals were injected with vehicle alone. Blood glucose levels were evaluated in 2-month-old female rats prior to mating. Rats were considered diabetic when the 6 h fasting blood glucose levels were > 130 mg/dl. Control and diabetic female rats (F0) were mated with healthy controls purchased for this purpose. Day 1 of pregnancy was determined by the presence of sperm cells in vaginal smears. All rats were fed with a standard diet (commercial rat chow) during F0 pregnancy and allowed to deliver and nurse their pups. The offspring were fed with the standard diet until 3 months of age when blood glucose levels were determined. Then, the F1 females were mated with healthy males. From day 1 to day 21 of the F1 pregnancy, the offspring from rats with mild diabetes and controls were fed with a) a standard diet (commercial rat chow) or b) a standard diet (commercial rat chow) supplemented with 6% olive oil. The diet composition, analyzed by gas chromatography-mass spectrometry as previously [27] was: a) Standard diet (g/100 g): carbohydrates (50); proteins (25); fat (5), major fatty acids 16:0 (0.58), 18:0 (0.16), 18:1 n-9 (1.27), 18:2 n-6 (1.99) and 18:3 n-3 (0.73), and b) Olive oil-supplemented diet (g/100 g): carbohydrates (48); proteins (24); fat (11), major fatty acids 16:0 (1.55), 18:0 (0.26), 18:1 n-9 (5.77), 18:2 n-6 (2.41) and 18:3 n-3 (0.57). The F1 pregnant rats were euthanized at day 21 of gestation. Maternal blood and pooled fetal blood were collected and processed for determination of blood glucose. Placentae of F1 rats (*n=6-8* in each experimental group, each dam from a different F0 mother) were homogenized in ice-cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, pH 7.4 with protease and phosphatase inhibitors), as previously [23], and stored at −80°C for determination of protein expression of PPARγ and of total and phosphorylated mTOR downstream targets using Western blot. Placental tissues were also frozen

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and stored at -80ºC for subsequent determination of lipoperoxidation or prepared for immunohistochemical analysis of CTGF and MMP2. Plasma from F1 mothers was also stored at -80ºC for zymography assay to evaluate MMP2 activity.

2.2. Plasma analyses

Blood glucose levels were determined by Accu-Chek reagent strips and a glucometer Accu-Chek (Bayer Diagnostics) in tail vein samples. Plasma maternal and fetal glucose concentrations were determined by an enzymatic colorimetric commercial kit (Wiener lab). Plasma MMP2 activity was determined by zymography. Briefly, as previously described [28], 40 µg of protein was 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. Gels were rinsed in 2.5% Triton X-100 for 60 min to remove SDS and incubated for 24 h in 50 mM Tris buffer pH 7.4, containing 150 mM NaCl and 10 mM $CaCl_2$, at 37ºC. Subsequently, 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 on a dark background. Identification of MMP2 was based on its molecular weight and a positive internal control (conditioned medium of human fibrosarcoma HT-1080 cells), which was run in each gel. Enzymatic activity was quantified by means of ImageJ software and expressed as arbitrary densitometric units.

2.3. Western Blot analysis

Placental homogenates were prepared and proteins separated by SDS-PAGE and transferred to PVDF membranes (35V constant, overnight at 4°C), as previously described [23]. The membranes were

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stained with Amido Black staining solution for total proteins (Sigma-Aldrich) to confirm equal loading and transfer. Blocking was carried out for 1 h at room temperature in 5% non-fat milk in TBS-Tween and membranes were incubated with the primary antibody overnight at 4°C. The antibodies used were against total and phosphorylated rpS6 (S256/236), total and phosphorylated 4EBP-1 (T70), total and phosphorylated PKCα (S657), total and phosphorylated SGK1 (S422) (Cell Signaling Technology), and PPARγ (Cayman Chemical). Membranes were then washed and incubated with the appropriate peroxidase conjugated secondary antibody and visualized using ECL detection solution (Thermo Scientific). The images were captured in a G: BOX gel imaging system (Syngene) and densitometrically analyzed with the ImageJ software. The expression of the target protein in each individual lane was normalized for total protein staining to adjust for unequal loading and transfer.

2.4. Lipoperoxidation assay

Lipoperoxidation was evaluated by measuring the concentrations of thiobarbituric acid reactive substances (TBARS) as previously described [6]. Briefly, placentae were homogenized in 100 mM Tris-HCl buffer (0.1 mM, pH 7.4) and the homogenates then added with trichloroacetic acid (40%, Merck Darmstadt, Germany). The supernatant was centrifuged and then mixed with an equal volume of thiobarbituric acid (46 mM) (Sigma-Aldrich). The solution was heated at 95ºC, cooled, and quantified using spectrophotometry at 540 nm. Different concentrations of malondialdehyde (Sigma-Aldrich) subjected to the same conditions as the tissue homogenates were used as standards.

2.5. Immunohistochemistry

To perform immunostaining for CTGF and MMP2, placental tissues were fixed in buffered formaldehyde solution 4% v/v, then paraffinized and serially cut in 5-µm-thick sections. Then, as previously [29], sections were deparaffinized, rehydrated through a graded series of ethanol and the

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endogenous peroxidase activity was blocked. The sections were processed using the corresponding primary antibodies: anti-CTGF (goat polyclonal antibody, 1:300 dilution, Santa Cruz Biotechnology), and anti-MMP2 (mouse polyclonal antibody, 1:200 dilution, Santa Cruz Biotechnology). Negative controls were performed in the absence of primary antibody and by replacing the primary antibody by a pooled serum of the same species containing a spectrum of the IgG subclasses (Vector Laboratories Burlingame, CA, USA). Biotinylated antibodies (anti-goat IgG or anti-mouse IgG-rat absorbed), 1:200 PBS-T / 1.5% normal horse serum (Vector Laboratories Burlingame, CA, USA) were then applied, followed by a 60-min incubation with the avidin–biotin complex (Vector Laboratories). Staining was visualized by adding 40% 3,3´-diaminobenzidine tetrahydrochloride chromogen-buffer plus 0.02% (v/v) hydrogen peroxide in 0.05 M Tris (pH 7.6) for 10 min. Three sections per placenta were evaluated by two skilled blinded observers, a semiquantitative score was performed and the immunoreactivity intensity was quantified by means of the ImageProPlus software. Data are shown as relative to a value of 1, arbitrarily assigned to the control.

2.6. Statistical analysis

Data are presented as means ± SEM. Groups were compared by two-way ANOVA followed by Bonferroni's test. A *p* value lower than 0.05 was considered statistically significant.

3. Results

3.1. Effects of 6% olive oil supplementation in F1 pregnant control and GDM rats on glucose levels and feto-placental weights

Before mating, blood glucose levels in the offspring from control rats (101± 4 mg/dl) and the offspring from rats with mild diabetes (111± 4 mg/dl) were similar. On day 21 of the F1 pregnancy, maternal plasma glucose was increased by 33% in the offspring from rats with mild diabetes, consistent with the development of GDM and independently of the diet ($p<0.001$ v. control, **FIGURE**

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2). Similarly, fetuses of the GDM group had increased plasma glucose levels, independently of the diet ($p<0.001$ v. control, **FIGURE 2).** Fetal weights were significantly increased in the GDM group compared to the control group ($p<0.01$ v. control), but not in the olive oil-supplemented GDM group (**FIGURE 2**). Placental weight was similar in the control and diabetic groups supplemented or not with olive oil (**FIGURE 2**).

3.2. Effects of 6% olive oil dietary supplementation in F1 pregnant control and GDM rats on placental mTOR signaling

As previous works have identified a link between activation of placental mTOR signaling and fetal overgrowth in maternal obesity and GDM [8], protein expression of total and phosphorylated mTOR downstream targets were determined in the placenta. No differences were found in placental levels of total or phosphorylated rpS6 between the GDM and the control group, independently of the maternal diet (**FIGURE 3**). No differences were found in placental phosphorylated 4EBP-1 levels between the groups. Whereas total 4EBP-1 placental levels were decreased in the GDM group ($p<0.05$ v. control), the olive oil-supplemented diet markedly increased total 4EBP-1 levels in the GDM group ($p<0.001$ v. GDM and $p<0.01$ v. control) leading to a decreased phosphorylated/total 4EBP-1 ratio in the placenta of the olive oil-supplemented GDM group ($p<0.001$ v. GDM and $p<0.01$ v. control, **FIGURE 3**).

Downstream proteins in the mTORC2 pathway were also evaluated. Whereas placental levels of total PKCα were decreased in the GDM group ($p<0.05$ v. control), the olive oil-supplemented diet in the GDM group was associated with an increase in placental levels of total PKCα ($p<0.001$ v. GDM-standard diet, $p<0.05$ v. control groups, **FIGURE 4**). Thus, although phosphorylation of PKCα was increased in both the placentae of the control ($p<0.05$) and the GDM groups ($p<0.01$) treated with the olive oil-supplemented diet compared to the respective standard diet groups, an unchanged phosphorylated/total PKCα ratio was observed in the placentae of the olive oil-supplemented GDM

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group compared to the standard diet control group (**FIGURE 4**). Although there were no differences in total SGK1 levels in the four experimental groups, increases in SGK1 phosphorylation and the phosphorylated/total SGK1 ratio were observed in the placentae of the GDM group, an effect that was not modified by the olive oil dietary treatment ($p<0.01$ vs control-standard diet, **FIGURE 4**).

3.3. Effects of 6% olive oil dietary supplementation in F1 pregnant control and GDM rats on the levels of placental PPARγ and pro-oxidant/pro-inflammatory markers

As previous works have shown that PPARγ is involved in the induction of a pro-oxidant/pro-inflammatory intrauterine environment [13, 19], we determined placental PPARγ levels. Reduced PPARγ levels were observed in the placentae of GDM rats fed both with the standard and the olive oil-supplemented diet compared with their respective controls ($p<0.01$, **FIGURE 5**). Nevertheless, lipoperoxidation, as assessed by TBARS levels, was increased in the placentae of GDM rats fed the standard diet ($p<0.05$) and decreased both in the placentae of GDM rats fed the olive oil-supplemented diet ($p<0.05$) and in the placentae of control rats fed the olive oil-supplemented diet ($p<0.05$) compared to the control group fed the standard diet (**FIGURE 5**). Furthermore, the levels of CTGF were also increased in the placentae of GDM rats fed the standard diet compared to controls ($p<0.01$) and decreased when GDM rats were fed with the diet enriched in olive oil ($p<0.05$ v. GDM-standard diet, **FIGURE 6**). In addition, MMP2 levels were increased in GDM placentae compared to controls ($p<0.05$), an increase that was prevented by the olive oil-supplemented diet ($p<0.05$, **FIGURE 7**). In contrast, the increased maternal plasma MMP2 levels in GDM rats were not affected by the diet enriched in olive oil ($p<0.01$ v. control, **FIGURE 7**).

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4. Discussion

Using a GDM model induced by intrauterine programming in the female offspring from rats with mild diabetes, we demonstrated that a maternal diet supplemented with 6% olive oil, albeit not preventing the development of GDM and the decreased placental PPARγ levels, resulted in normalization of pup weight, attenuation of placental mTOR activation and prevention of placental oxidative stress and inflammation. It is possible that these changes contribute to improving the health of the F2 generation.

Placental function is critical for fetal development [30]. The adverse metabolic environment in GDM is associated with placental dysfunction, fetal overgrowth and later diseases in the offspring [2, 31]. We have previously found that female offspring from rats with mild diabetes develop GDM, associated with fetal overgrowth, activation of mTOR signaling, impaired PPAR signaling and increased levels of pro-oxidative and pro-inflammatory markers in the placenta [6]. In the current study, supplementation of the diet with 6% olive oil in F1 pregnant offspring from rats with mild diabetes was not able to prevent the induction of GDM, as shown by the increased maternal and fetal plasma glucose levels at term. Similarly, we previously found that an olive oil-supplemented diet in F0 pregnant rats with mild diabetes did not normalize maternal blood glucose levels [32] and did not prevent development of type 2 diabetes associated with aging in the non-pregnant F1 offspring [27]. In a recent study in pregnant women, a Mediterranean diet added with extra virgin olive oil and pistachios reduced the incidence of GDM and fetal overgrowth [33]. In contrast, a recent meta-analysis found no association between PUFAs supplementation during pregnancy and the risk of developing GDM [34], although PUFAs supplementation in obese pregnant women has been reported to reduce the levels of pro-inflammatory markers in the placenta [35, 36].

Maternal obesity in mice as well as obesity and GDM in women are associated with activation of mTOR signaling in the placenta [37-39]. In this work, although the activation of placental mTORC1

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signaling in GDM rats was prevented, as represented by the increase in total expression of 4EBP-1, placental mTORC2 signaling remained activated in GDM rats treated with the olive oil-supplemented diet. Because mTORC1 is a positive regulator of amino acid transport in the placenta [8, 9], it is possible that the attenuated fetal overgrowth in response to supplementation of the maternal diet with 6% olive oil is due to reduced placental mTORC1 signaling and subsequent decreased amino acid availability. In a recent study, prevention of fetal overgrowth and normalization of placental mTOR signaling were observed in GDM rats whose mothers (F0 generation) were fed with a PUFA-enriched diet in the F0 generation [23], suggesting that supplementation with unsaturated fatty acids in the F0 pregnancy may be required to completely prevent placental dysfunction in the F1 pregnancy in which GDM develops. Further work is needed to address possible sex-dependent changes in the parameters evaluated, although our previous studies showed no sex-dependent changes in fetal weight [40] or placental weight (unpublished data) in healthy and GDM rats.

It is now established that an intrauterine pro-oxidative and pro-inflammatory environment is linked to impaired feto-placental development and fetal programming [19]. In this work, the expression of PPARγ, a PPAR isotype with potent antioxidant/anti-inflammatory actions [41], was found to be reduced in the placentae of GDM rats, but this reduction was not prevented by olive oil supplementation. Nevertheless, olive oil contains high amounts of oleic acid, a PPARγ agonist [24], and is rich in polyphenols, which have potent antioxidant properties [25]. The antioxidant effects of these factors in olive oil likely prevented the GDM-induced placental oxidative stress, as indicated by the decrease in lipid peroxidation. In addition, this dietary treatment prevented both the increase in CTGF and MMP2 in GDM placentae, suggesting a reduction of the pro-inflammatory state and the restoration of a proper extracellular matrix remodeling, which are required for normal placental function [42]. In agreement with this, *in vitro* studies have shown that oleic acid protects trophoblast cells from impaired autophagy and from increases in MMPs induced by saturated fatty acids [43]. Moreover, previous studies performed in rats with pre-gestational diabetes at mid-gestation have

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shown that placental MMP2 levels and activity are positively correlated to serum MMP2 activity, and that MMPs increases are prevented by a maternal olive oil-enriched diet [22]. In the current study of GDM rats at term, MMP2 levels were increased in both the placenta and maternal plasma of the GDM dams. However, although the increase in placental MMP2 was prevented by a diet supplemented with olive oil, maternal plasma MMP2 activity was unaffected. It is possible that the rapid uptake and accretion of unsaturated fatty acids in term placentae [44] favors the local antioxidant/anti-inflammatory effects in the placenta, although further studies are needed to specifically address this question.

Although this work is an *in vivo* animal study and thus the implications of the results in humans remain to be established, our findings may have implications for women with GDM given that dietary supplementation with olive oil is feasible in humans.

In conclusion, although GDM originates from intrauterine programming in a previous generation, a diet enriched in olive oil during the F1 pregnancy attenuated placental dysfunction in rats with GDM.

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Conflict of interest

The authors have declared no conflicts of interest.

Acknowledgements

A.J. and E.C designed the research; E.C., D.G.R., D.F. and C.L. conducted the research; E.C., D.G.R., T.L.P, T.J and A.J analyzed and interpreted the data; A.J. wrote the manuscript; T.P and T.J critically revised the manuscript.

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Figure legends

Figure 1. Experimental design. A rat model of mild diabetes was induced in Wistar rats by neonatal administration of streptozotocin (F0). F0 female rats with mild diabetes and controls were mated and their female offspring (F1) were studied at three months of age. F1 females were mated, and fed with a standard diet or a 6% olive oil-supplemented diet from days 1 to 21 of pregnancy. On day 21 of pregnancy, the placenta of F1 control rats and F1 rats that develop GDM rats was evaluated.

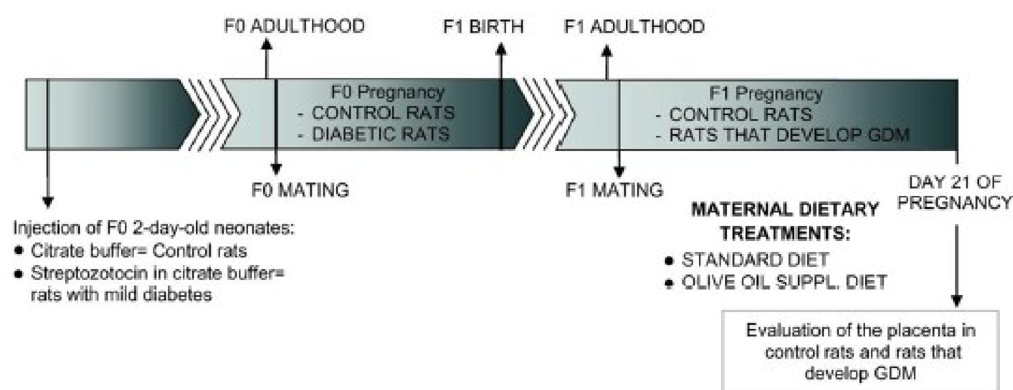

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Figure 2. Maternal and fetal plasma glucose concentrations and feto-placental weights A) Maternal plasma glucose concentrations, B) Fetal plasma glucose concentrations, C) Fetal weight and D) Placental weight in control and GDM rats on day 21 of pregnancy. Animals were fed a standard or a 6% olive oil-supplemented diet during the F1 pregnancy. Values are mean ± SEM, representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.

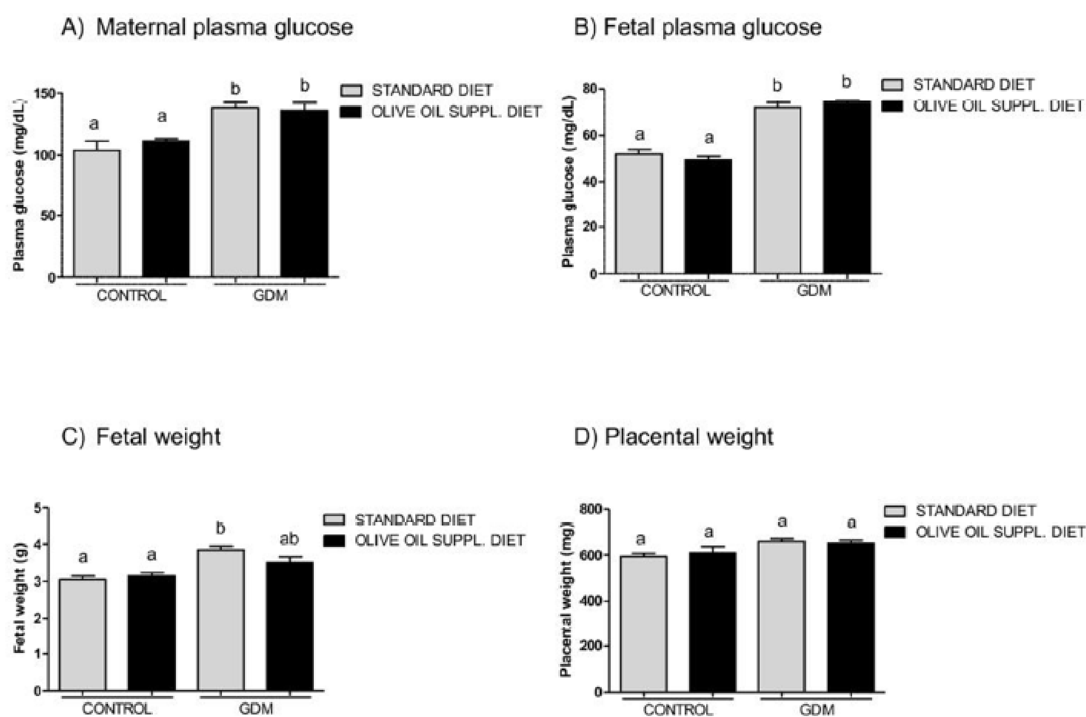

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Figure 3. Placental mTORC1 signaling. Expression and phosphorylation of proteins involved in the mTORC1 signaling pathway in the placenta of control and GDM rats on day 21 of pregnancy. Animals were fed a standard (STD) or a 6% olive oil-supplemented diet (OO) during the F1 pregnancy **A.** Representative Western blots, data summary of total and phosphorylated rpS6 and of phosphorylated/total rpS6 ratio. **B.** Representative Western blots and data summary of total and phosphorylated 4EBP-1 and of phosphorylated/total 4EBP-1 ratio. Values are mean ± SEM representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.

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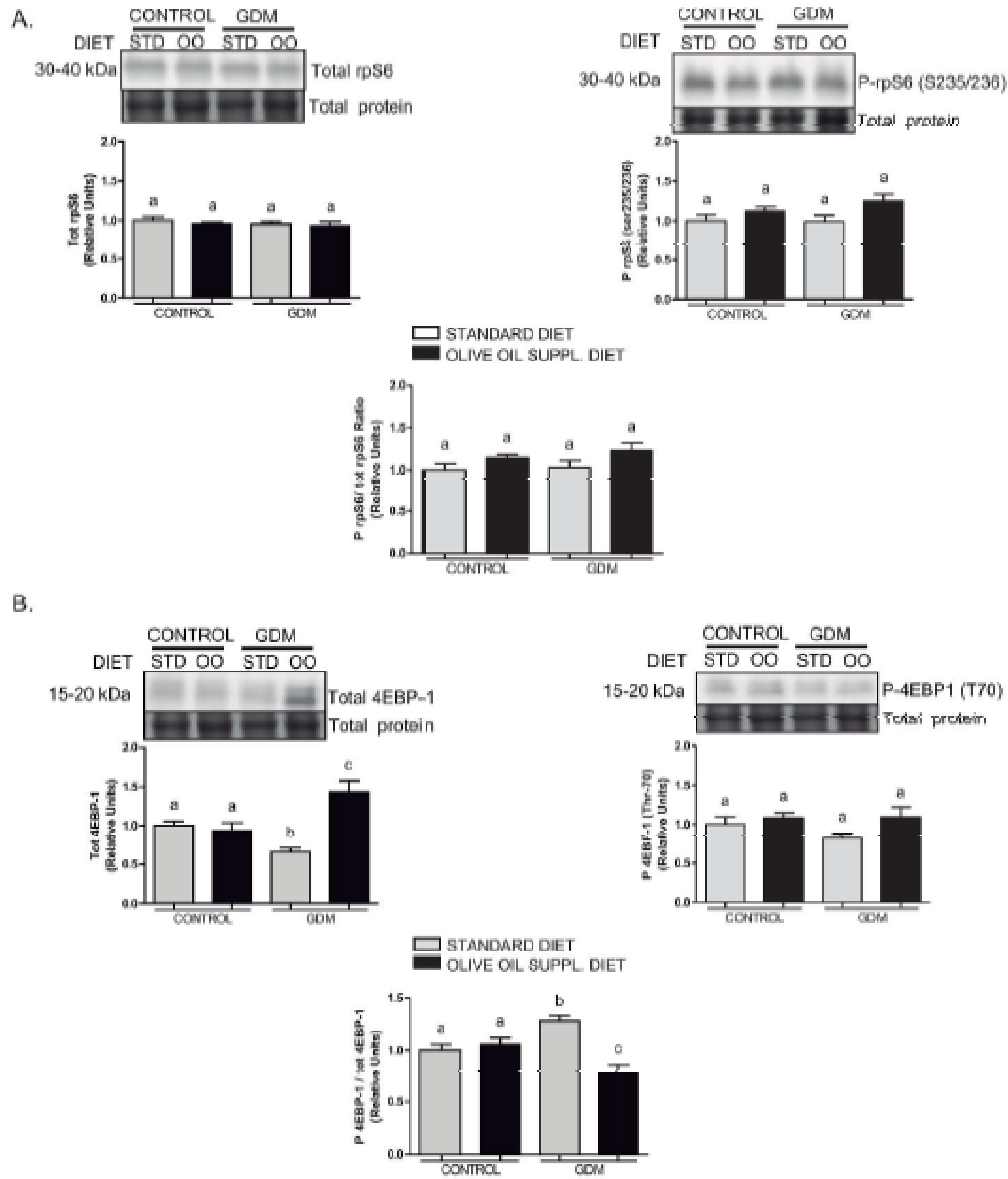

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Figure 4. Placental mTORC2 signaling. Expression and phosphorylation of proteins involved in the mTORC2 pathway in the placenta of control and GDM rats on day 21 of pregnancy. Animals were fed a standard (STD) or a 6% olive oil-supplemented diet (OO) during the F1 pregnancy. **A.** Representative Western blots and data summary of total and phosphorylated PKCα and of phosphorylated/total PKCα ratio. **B.** Representative Western blots and data summary of total and phosphorylated SGK1 and of phosphorylated/total SGK1 ratio. Values are mean ± SEM representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.

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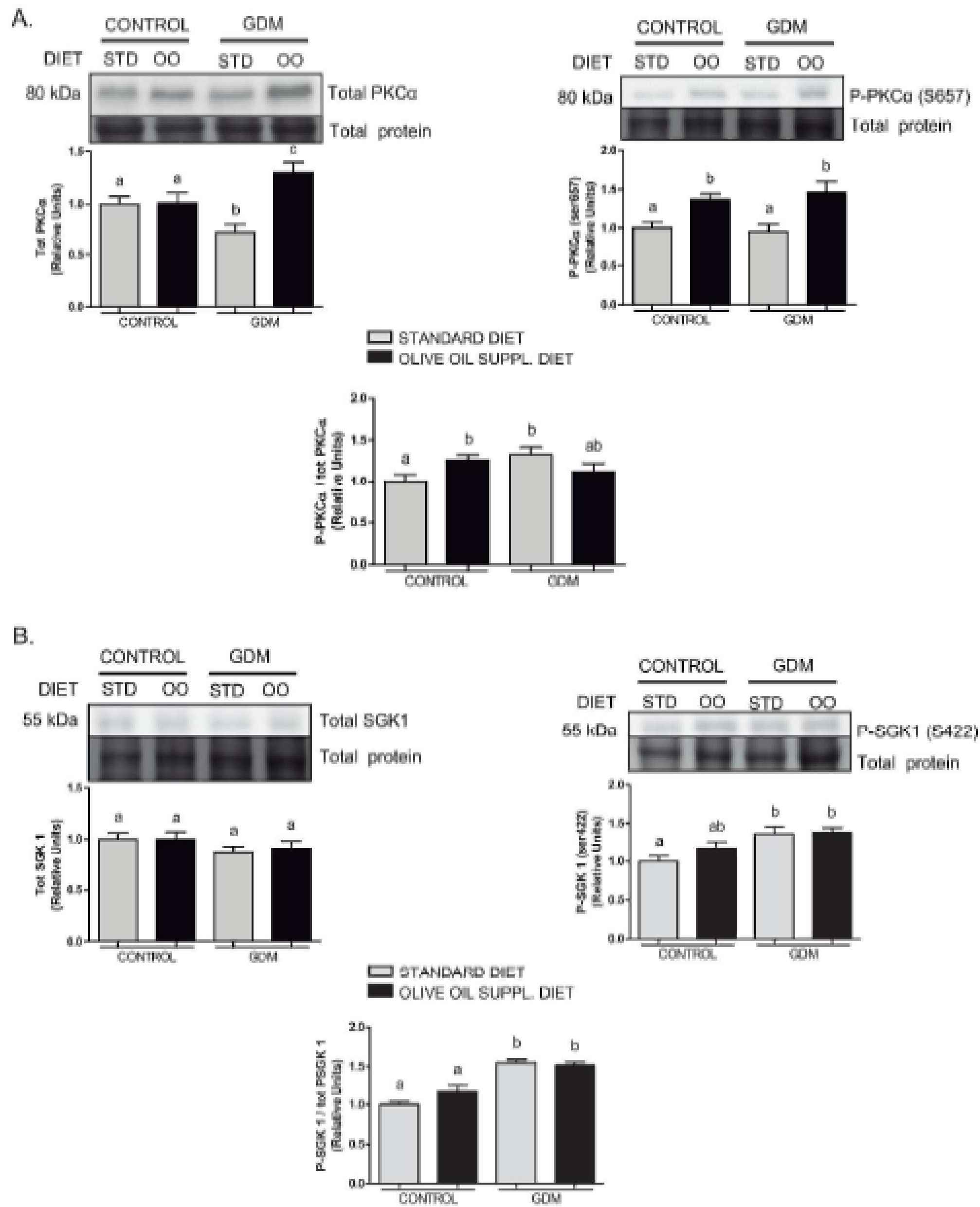

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Figure 5. Placental PPARγ protein expression and lipoperoxidation. A) PPARγ protein expression and B) Lipoperoxidation in placentae of control and GDM rats on day 21 of pregnancy. Animals were fed a standard (STD) or a 6% olive oil-supplemented diet (OO) during the F1 pregnancy. Values are mean ± SEM, representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.

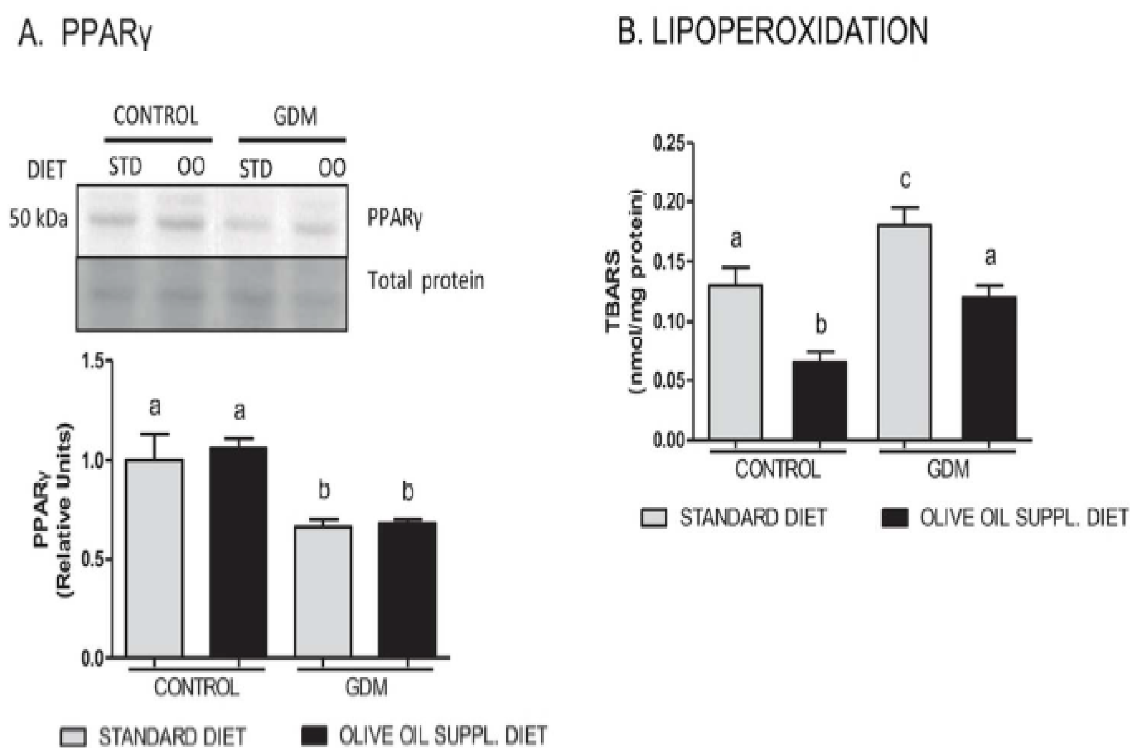

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Figure 6. Placental CTGF levels. CTGF levels in placentae of control and GDM rats on day 21 of pregnancy. Animals were fed a standard (STD) or a 6% olive oil-supplemented diet (OO) during the F1 pregnancy. Values are mean ± SEM, representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.

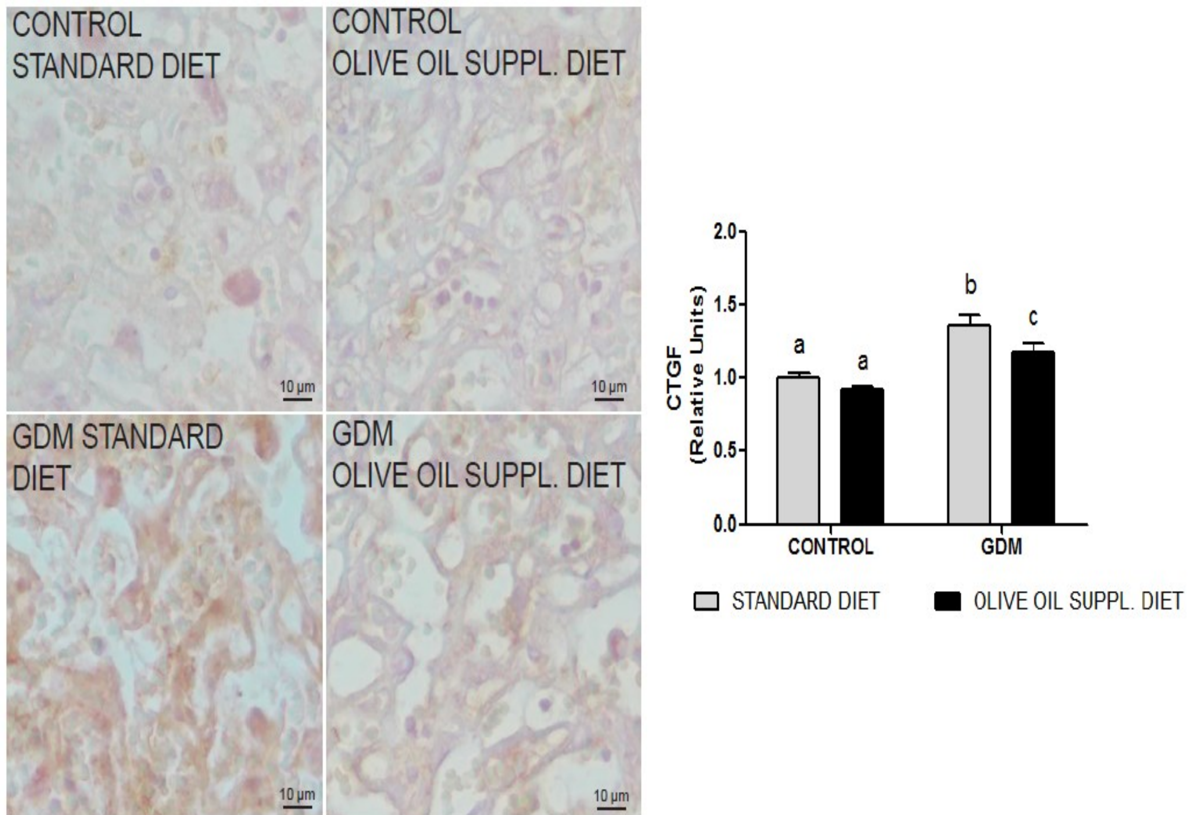

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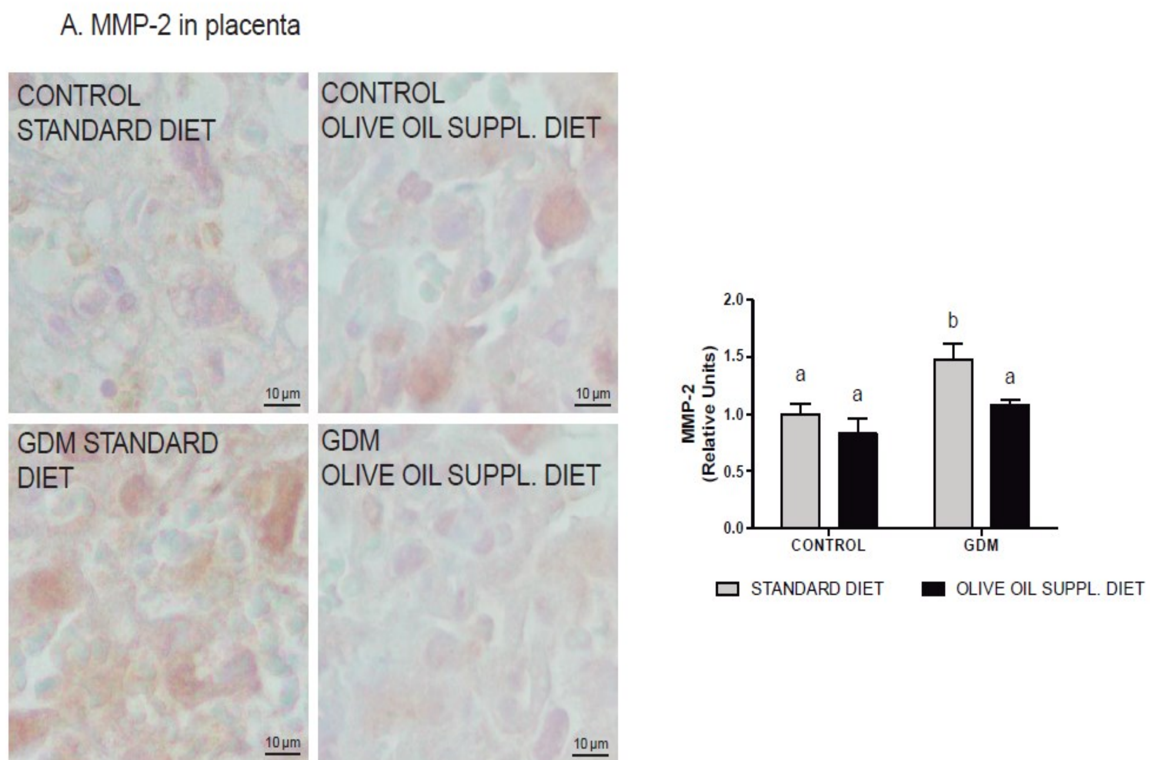
Figure 7. Placental MMP2 levels and plasma MMP2 activity A) MMP2 levels in the placenta and B) MMP2 activity in plasma from control and GDM rats on day 21 of pregnancy. Animals were fed a standard (STD) or a 6% olive oil-supplemented diet (OO) during the F1 pregnancy. Values are mean ± SEM, representing *n=6-8* F1 rats from different F0 mothers in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed. Different letters indicate significant differences between groups, $p < 0.05$.



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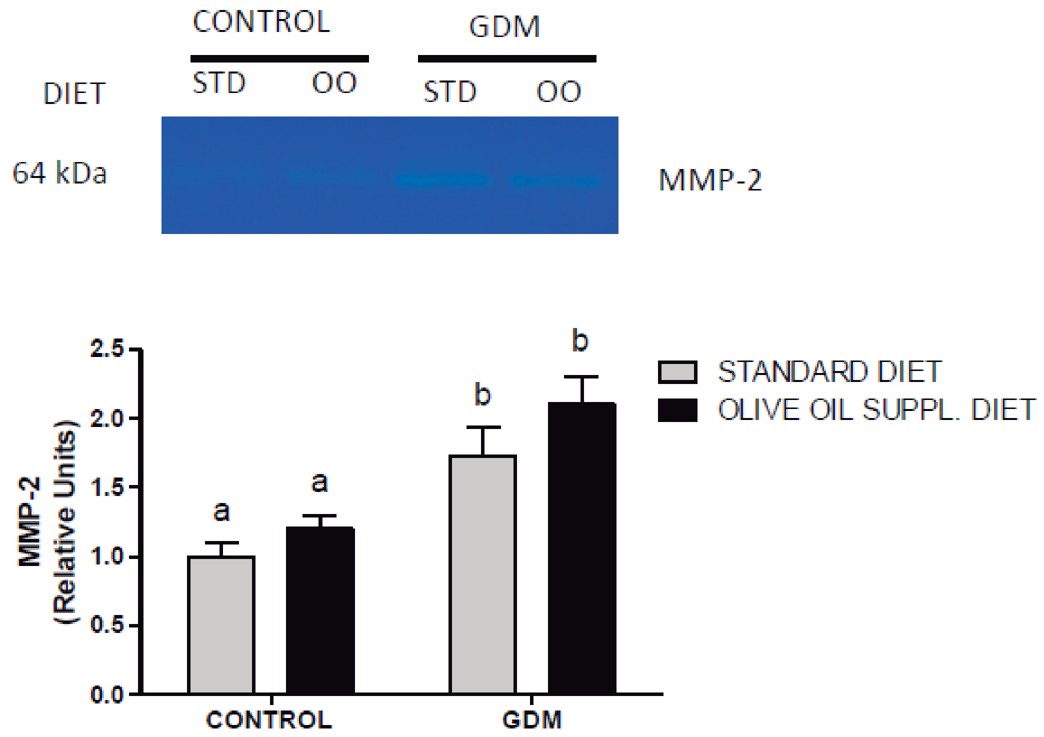

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