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Olive oil supplementation prevents extracellular matrix deposition and reduces prooxidant markers and apoptosis in the offspring´s heart of diabetic rats.

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Highlights

- Maternal diabetes programs cardiovascular diseases
- Diabetic rats were fed an olive oil-enriched diet during pregnancy
- This diet prevented increased collagen deposition in the offspring´s heart
- This diet reduced prooxidant markers in the offspring´s heart
- This diet prevented increased apoptosis in the offspring´s heart

Abstract

Maternal diabetes induces fetal programming of cardiovascular diseases. Diabetes induced-cardiac fibrosis is a process that may start *in utero* and may be related to the prooxidant/proinflammatory environment. The aim of this study was to investigate the effect of a maternal diet enriched in olive oil on the levels of components and regulators of the extracellular matrix, on prooxidant markers and on apoptosis rate in the heart of 21-day-old offspring of diabetic rats. Maternal diabetes was induced by neonatal administration of streptozotocin. During pregnancy, diabetic and control rats were fed with diets supplemented or not with 6% olive oil. The heart of the offspring was studied at 21 days of age. We found increased deposition of collagen IV and fibronectin in the offspring´s heart of diabetic rats, which was prevented by the maternal diets enriched in olive oil. Increases in connective tissue growth factor were also prevented by the maternal diets enriched in olive oil. Prooxidant markers as well as apoptosis, which were increased in the heart of the offspring of diabetic rats, were prevented by the maternal olive oil dietary treatment. Our findings identified powerful effects of a maternal diet enriched in olive oil on the prevention of increased extracellular matrix deposition and increased prooxidant markers in the heart of 21-day-old offspring of diabetic rats.

Abbreviations: Matrix metalloproteinase, MMP; connective tissue growth factor, CTGF; peroxisome proliferator receptor, PPAR.

Keywords: Maternal diabetes, fetal programming, offspring's heart, extracellular matrix, fibrosis.

1. Introduction

Diabetes in pregnancy increases the risks of adverse outcomes in the mother, the placenta, the fetus and the offspring, both in the perinatal period and in the long term [1, 2]. In different gestational diseases, including maternal diabetes, cardiovascular diseases are programmed *in utero* [3-5]. Experimental models of diabetes and pregnancy are useful to address the underlying mechanisms of fetal programming [6]. Our previous studies performed in rats have shown that maternal diabetes induces a prooxidant/proinflammatory intrauterine environment, which affects the development of different fetal organs, including the fetal heart, leading to adverse consequences in the offspring´s later life [7]. In human diabetic pregnancies, the intrauterine compartment is characterized by a prooxidant/proinflammatory state [8]. As a prooxidant/proinflammatory state changes the activity of enzymes involved in

extracellular matrix remodeling [9, 10], a different deposition of extracellular matrix components may be programmed by maternal diabetes.

The extracellular matrix is crucial in cardiac development and function, and its altered remodeling has been found profoundly involved in cardiovascular diseases [10]. Fibrosis is increased in the heart of diabetic patients, and the mechanisms involved in the deposition of extracellular matrix components are still unclear [11]. A main regulator of deposition of extracellular matrix components is the matricellular protein connective tissue growth factor (CTGF), while main regulators of their degradation are matrix metalloproteinases (MMPs) [12, 13].

In recent studies, we addressed the effects of the administration of the mitochondrial antioxidant idebenone to diabetic pregnant rats, showing its ability to prevent maternal diabetes-induced changes in CTGF levels and MMPs in the heart of 21-day-old offspring [14]. Whether maternal diets enriched in olive oil can exert these beneficial effects is unknown. In humans, diets enriched in extra-virgin olive oil have been shown to exert potent antioxidant/anti-inflammatory effects in cardiovascular diseases [15, 16]. The PREDIMED study showed that enhanced extra-virgin olive oil consumption is associated with reduced risks of cardiovascular diseases and mortality in patients with high cardiovascular risks [17]. Indeed, due to the cardiovascular benefits of the Mediterranean diet, with olive oil as a main component, olive oil has been proposed as a valuable health-promoting nutrient [16, 17]. In pregnancy, diets enriched in olive oil have been shown to reduce the risk of gestational diabetes in the general population [18]. In experimental models of diabetes and pregnancy, treatments with olive oil have been found to activate peroxisome proliferator activated receptors (PPAR) pathways and lead to antioxidant and metabolic effects in adult offspring [19], beneficial effects that may be evident at earlier postnatal stages. In this work, we hypothesized that

maternal diets enriched in olive oil are able to prevent maternal diabetes-induced changes in components and regulators of the extracellular matrix, to reduce prooxidant markers and to prevent increased apoptosis in the heart of the 21-day-old offspring of diabetic rats.

2. Materials and methods

2.1. Animals

Twenty-eight Albino Wistar rats were obtained from the certified animal facilities of the School of Exact and Natural Sciences, University of Buenos Aires (UBA), Argentina. The rats were housed in the animal facilities of our Institution (CEFYBO-CONICET-UBA), with a 12 h light and 12 h dark cycle, and fed *ad libitum* with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina). A mild diabetic rat model was induced by injecting 2-day-old neonates with streptozotocin (90 mg/kg, s.c, Sigma-Aldrich, St. Louis, MO, USA, diluted in citrate buffer 0.05M pH 4.5, Sigma-Aldrich), as previously [6, 20]. Control rats were injected with citrate buffer alone. The diabetic state was confirmed in 2-month-old rats prior to mating. Rats were considered diabetic when they presented fasting glycemia values higher than 130 mg/dl. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD Nº 1497/2013), School of Medicine, UBA, complied with the ARRIVE guidelines and was conducted 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. Experimental design and sample collection

Fourteen control and fourteen diabetic female adult rats were mated with control adult males. The presence of sperm cells in vaginal smears confirmed the first day of pregnancy. From the first day of pregnancy until parturition, the control and diabetic rats received or not the following diets: a) a standard diet (commercial rat chow) or b) a standard diet enriched in 6% olive oil. The diet composition was: a) Standard diet (g/100 g): carbohydrates (50); proteins (25); fats (5), major fatty acids 16:0 (0.58), 18:0 (0.16), 18:1 (1.27), 18:2 (1.99), 18:3 (0.73) and b) Olive oil-supplemented diet (g/100 g): carbohydrates (48); proteins (24); fats (11), major fatty acids 16:0 (1.55), 18:0 (0.26), 18:1 (5.77), 18:2 (2.41), 18:3 (0.57). After parturition, all animals received the described standard diet. Each litter was adjusted to 10 offspring, which were euthanized at 21 days of age. Pooled blood per sex per litter was collected in citrate buffer tubes for further determination of glucose levels in plasma. The hearts of male and female offspring from each control and diabetic mother were explanted (n=7 litters, 36 male and 34 female offspring in the control group; 35 male and 35 female offspring in the olive oil-supplemented control group; 36 male and 34 female offspring in the diabetic group; and 36 male and 34 female offspring in the olive oil-supplemented diabetic group). From each litter and sex, hearts explanted from two offspring, randomly selected, were prepared for immunohistochemical and TUNEL analyses. The remaining hearts of each sex and litter were conserved at -80ºC for the determination of nitrates/nitrites (an index of nitric oxide (NO) production) and thiobarbituric acid reactive substances (TBARS, an index of lipoperoxidation and marker of oxidative stress).

2.3. Immunohistochemical analysis

Immunostaining of collagen IV, fibronectin, CTGF, MMP-2 and nitrotyrosine (an index of peroxynitrite-induced damage [8]) was performed in the heart of 21-day-old offspring of control and diabetic rats from the olive oil-supplemented and non-supplemented experimental groups (randomly selected hearts from n=7 different F0 mothers in each group). The hearts were paraffinized and serially sliced in 5-μm-thick sections. Then, sections were deparaffinized with xylol, rehydrated through a graded series of ethanol and the endogenous peroxidase activity was blocked with H_2O_2 0.3%. The sections were marked using the corresponding primary antibodies: anti-collagen type IV (rabbit polyclonal antibody, 1:400, Santa Cruz Biotechnology, CA, USA), anti-fibronectin (rabbit polyclonal antibody, 1:250, DAKO Diagnostics, CA, USA), anti-CTGF (goat polyclonal antibody, 1:200, Santa Cruz Biotechnology), anti-MMP-2 (goat polyclonal antibody, 1:200 dilution, Santa Cruz Biotechnology) and anti-nitrotyrosine (mouse monoclonal antibody, 1:4000 dilution, Millipore, Darmstadt, Germany). The Avidin-Biotin Complex system (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was then followed using the corresponding biotinylated antibodies (anti-goat IgG, anti-mouse IgG- rat absorbed or anti-rabbit IgG, 1:200 dilution, Vector Laboratories) and developed with 3,3'-diaminobenzidine, as reported previously [20]. Control sections were performed by omitting the primary antibody and by replacing the primary antibody by a pooled serum of the same species that contains a spectrum of the IgG subclasses (Vector Laboratories). The slides immunostained with anti-CTGF antibody were also stained with hematoxylin. Using light microscopy, three entire sections per heart were examined by two skilled blinded observers.

Immunoreactivity intensity was quantified with the ImageProPlus software. Data are shown as relative to a value of 1, assigned to the control.

2.4. Nitric oxide production

The production of NO was determined by measuring the concentration of its stable metabolites nitrates/nitrites in the heart of 21-day-old offspring of control and diabetic rats from the olive oil-supplemented and non-supplemented experimental groups (2-4 pooled hearts from n=7 different F0 mothers in each group). Briefly, as previously done [21], each heart was homogenized in 1 ml Tris-HCl buffer (0.1 M, pH: 7.4), and an aliquot was separated for protein measurement. Nitrates in the supernatant were reduced to nitrites by using nitrate reductase, and then, total nitrites were determined using a commercial assay kit (Cayman Chemical Co. Ann Arbor, MI, USA), according to the manufacturer´s instructions.

2.5. Lipoperoxidation analysis

Lipoperoxidation was assessed by evaluating the concentrations of TBARS, a method widely used to assess peroxidation of fatty acids [22], in the heart of 21-day-old offspring of control and diabetic rats from the olive oil-supplemented and non-supplemented experimental groups (2-4 pooled hearts from n=7 different F0 mothers in each group). Briefly, as previously done [21], each heart was homogenized in Tris-HCl buffer (0.1 M, pH: 7.4). The homogenate was added with 40% trichloroacetic acid (Merck Darmstadt, Germany). After centrifugation, an equal volume of thiobarbituric acid (46 mM) (Sigma-Aldrich) was added to the supernatant and the solution was

heated at 95ºC. After cooling, TBARS were measured spectrophotometrically at 540 nm. Different concentrations of malondialdehyde (Sigma-Aldrich) subjected to the same conditions as the tissue homogenates were used as standards.

2.6. Apoptosis analysis

To study apoptosis in the heart of 21-day-old offspring of control and diabetic rats from the olive oil-supplemented and non-supplemented experimental groups, we used the same paraffinized samples prepared for immunohistochemical studies (randomly selected hearts from n=7 different F0 mothers in each group), a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using an *in situ* cell apoptosis detection kit (DeadEnd Fluorometric TUNEL System, Promega, Durham, NC, USA) according to the manufacturer's instructions, as previously [23]. Three entire sections per heart were examined by two skilled blinded observers under fluorescent microscopy (Nikon Eclipse E200) and photographed (Nikon DS-Fi1). The number of positive cells was quantified using the Image J software (NIH, Bethesda, MA, USA).

2.7. Statistical analysis

Data are presented as the mean ± SD. Groups were compared by two-way ANOVA in conjunction with Bonferroni's post hoc tests. A p value lower than 0.05 was considered statistically significant.

3. Results

3.1. Effect of maternal olive oil supplementation on extracellular matrix components and deposition regulators in the offspring´s heart of diabetic rats

A mild diabetic rat model induced by neonatal streptozotocin administration, previously characterized during pregnancy [6], was used to evaluate the offspring´s heart. Glycemia values in the pregnant diabetic rats at term were increased in both the non-supplemented and olive oil-supplemented diabetic groups compared to controls ($p<0.001$, **Table 1**). The offspring of diabetic animals showed sex-dependent changes in glycemia at 21 days of age. Female offspring showed no changes in glycemia in both the olive oil-supplemented and non-supplemented groups, compared to controls (**Table 1**). Differently, the male offspring of diabetic rats showed increased glycemia ($p<0.05$ vs control), an alteration that was prevented by the maternal dietary olive oil supplementation during pregnancy ($p<0.01$ vs non-supplemented diabetic groups) (**Table 1**).

In the heart of the 21-day-old offspring, main components of the extracellular matrix were evaluated. The deposition of collagen IV was increased in the heart of female and male offspring of diabetic rats ($p<0.05$ vs control), an alteration prevented by the maternal dietary supplementation with olive oil ($p<0.01$ vs non-supplemented diabetic group) (**Fig. 1**). Fibronectin levels showed no changes in the heart of female offspring of diabetic rats in both the olive oil-supplemented and non-supplemented groups compared to controls (**Fig. 2**). Differently, fibronectin levels were increased in the heart of male offspring of diabetic rats compared to controls ($p<0.01$), an alteration prevented by the maternal dietary supplementation with olive oil ($p<0.001$ vs non-supplemented diabetic group) (**Fig. 2**).

CTGF, a profibrotic matricellular protein [12], was found increased in the heart of female offspring of diabetic rats ($p<0.05$ vs control), although no changes were observed when the olive oil-supplemented diabetic group was compared to controls (**Fig. 3**). A 2-fold increase in CTGF was observed in the heart of male offspring of diabetic rats ($p<0.001$ vs control), an alteration prevented by the maternal dietary supplementation with olive oil ($p<0.001$ vs non-supplemented diabetic group) (**Fig. 3**). On the other hand, the protein levels of the proteolytic enzyme MMP-2 showed no changes in the heart of male and female offspring of the non-supplemented diabetic groups (**Fig. 4**), but were reduced in the heart of male and female offspring of the olive oil-supplemented diabetic groups ($p<0.01$ vs non-supplemented diabetic group) (**Fig. 4**).

3.2. Effect of maternal olive oil supplementation on prooxidant markers and on apoptosis in the offspring´s heart from diabetic rats

Aberrant extracellular matrix remodeling is highly related to the prooxidant/proinflammatory environment [9, 13]. In this work, we measured the levels of nitrated proteins, an index of the damage induced by the potent oxidant peroxynitrites [24], finding no changes in the heart of female offspring of diabetic rats compared to controls, but a reduction in the heart of female offspring of the olive oil-supplemented diabetic group ($p<0.001$ vs non-supplemented diabetic group) (**Fig. 5**). Differently, a 3-fold increase in nitrated protein levels was found in the heart of male offspring of diabetic rats ($p<0.001$ vs control), an alteration prevented by maternal dietary supplementation with olive oil ($p<0.001$ vs non-supplemented diabetic group) (**Fig. 5**). As peroxynitrites are formed by the reaction of NO and reactive oxygen

species [24], we next measure nitrates/nitrites, an index of nitric oxide production, and TBARS, a measurement of lipoperoxidation, a marker of oxidative stress. In the heart of the female offspring, no changes in nitrates/nitrites were observed either in the non-supplemented or the olive oil-supplemented diabetic groups compared to controls (**Fig. 6**). Differently, in the heart of the male offspring, nitrates/nitrites showed increases in the non-supplemented diabetic group ($p<0.05$ vs control), although no changes were observed in the olive oil-supplemented diabetic group compared to controls (**Fig. 6A**). TBARS showed increased levels in the heart of both the female and male offspring of diabetic rats compared to controls ($p<0.01$), alterations prevented by the maternal dietary supplementation with olive oil ($p<0.01$ vs non-supplemented diabetic group) (**Fig. 6B**).

Finally, regarding apoptosis, the number of apoptotic cells was increased in the heart of both the female and male offspring of diabetic rats compared to controls ($p<0.05$), an alteration prevented by the maternal dietary supplementation with olive oil ($p<0.001$ vs non-supplemented diabetic group) (**Fig. 7**).

4. Discussion

The current study demonstrates sex-dependent alterations in the heart of the offspring of diabetic rats at a young age, which include aberrant deposition of components of the extracellular matrix, increased levels of the matricellular protein CTGF, enhanced prooxidant mediators and increased apoptosis, alterations prevented by the maternal diets enriched in olive oil.

The burden of metabolic diseases and their recognized involvement in the programming of metabolic and cardiovascular diseases in the offspring require efforts to

find feasible treatments to prevent fetal programming in metabolic gestational diseases [1, 25]. In experimental models of diabetes and pregnancy, programming of metabolic and cardiovascular diseases is induced, allowing to address putative maternal treatments to prevent anomalies in the offspring´s heart [7]. In this study, we evaluated the offspring of diabetic rats at postnatal day 21 and found that males but not females showed hyperglycemia, a metabolic alteration that was prevented by the dietary treatment with olive oil. In agreement, in the male adult offspring of diabetic animals, we have previously shown that hyperglycemia is prevented by the maternal olive oil-supplemented diet, although hyperglycemia remains in the female adult offspring [19]. Many studies have shown the increased susceptibility in males compared to females to metabolic diseases, the role of sex hormones associated with these differences and the different effects of pharmacological treatments according to sex [26-29]. Moreover, several studies have shown that sex-dependent changes arise before puberty, and even *in utero* in a wide range of diseases, including metabolic diseases [30]. It has also been shown that the levels of growth factors and adipokines in cord blood change according to the fetal sex, suggesting different responses to maternal glycemia and different mechanisms involved in the programming of metabolic diseases [31]. Indeed, mechanisms of programming have been found related to sex in humans and different animal models of metabolic diseases [32]. Likely involved in programming of metabolic diseases, the pancreas is highly susceptible to maternal hyperglycemia and the development *in utero* of this organ is influenced by sex hormones [28, 33]. Whether the pancreas is differentially affected by sex during development in the experimental model evaluated deserves further research. In this study, we focused in the offspring´s heart, and found more marked changes in males, compared to females, in the parameters

evaluated, likely generated *in utero* and possibly influenced by the increased glycemia evidenced in the 21-day-old male offspring of diabetic rats.

In the heart, the extracellular matrix is crucial in cardiac homeostasis, being fibrosis associated with the diabetic disease in both patients and experimental models [10, 11]. In this work, we focused in the putative changes in extracellular matrix components and deposition regulators induced in the offspring´s heart by maternal diabetes. Increased collagen deposition in the heart of males and females and fibronectin deposition in the heart of males were prevented by the maternal diets enriched in olive oil in the 21-day-old offspring of diabetic rats. Out of pregnancy, many cardiovascular benefits of an olive oil-enriched diet have been identified [15, 17], although its effects as a regulator of components of the extracellular matrix had not been previously addressed. In this work, we identified the ability of the olive oil-enriched diet to prevent aberrant deposition of extracellular matrix in the offspring´s heart of diabetic rats, a result possibly relevant in the prevention of programming of cardiovascular diseases.

To address putative regulators of the observed changes in the extracellular matrix components, we addressed the levels of CTGF, which were increased in the heart of the male and female offspring from diabetic rats compared to controls, an increase that was more marked in males and was prevented by the maternal diet enriched in olive oil. These results are significant, as CTGF is considered a marker of cardiac dysfunction, and its levels are increased in different models of heart failure, fibrosis and cardiac hypertrophy [34, 35]. As a limitation of this work, we were not able to perform functional analysis of the offspring´s hearts. Previous studies have shown the ability of a maternal treatment with idebenone to prevent increased CTGF levels in the heart of the offspring of diabetic rats [14], suggesting that the antioxidant effect of olive oil is

involved in the beneficial effects observed. Indeed, out of pregnancy, many studies have addressed the antioxidant effects of olive oil in cardiovascular diseases [15, 16]. Moreover, in experimental models of diabetes and pregnancy, diets enriched in olive oil have shown antioxidant effects in different fetal organs, including the fetal heart [21, 36], beneficial effects that may be involved in the antioxidant effects observed in the heart of the 21-day-old offspring of diabetic rats. In this work, we found no changes in MMP-2 levels in the heart of 21-day-old offspring of diabetic rats, although their reduction in the groups that received the olive oil supplementation suggests their putative contribution to the extracellular matrix remodeling observed under this maternal treatment. MMP-2 is involved in development as well as in tissue remodeling and many different pathological processes [37]. Our previous studies have shown increased MMP-2 activity in placentas and fetuses from diabetic rats at mid pregnancy, an alteration regulated by maternal diets enriched in olive oil [20]. Increased MMP-2 activity is involved in pathogenic mechanisms in the heart and related to increased oxidative stress [10, 38, 39]. Although unchanged at the offspring age evaluated, MMP-2 has been previously found increased in the heart of adult offspring of diabetic rats [38]. Thus, more marked impairments as those that arise in youth are likely needed to alter the levels of MMP-2 in the offspring of the mild diabetic rat model evaluated.

Prooxidant/proinflammatory markers, including nitrated proteins and NO overproduction, which are enhanced only in the heart of male offspring of diabetic rats, and TBARS, which are increased in the heart of male and female offspring of diabetic rats, showed reduced levels with the maternal treatment with olive oil, indicating the ability of this treatment to exert potent antioxidant and anti-inflammatory effects in the offspring´s heart at the postnatal stage evaluated. Antioxidant/anti-inflammatory effects of olive oil have been previously reported in multiple studies and tissues [15, 40].

Moreover, possibly related to the ability of the olive oil-enriched diet to prevent a prooxidant/proinflammatory environment, we observed that the increased number of apoptotic cells in the heart of the 21-day-old offspring of diabetic rats was prevented by the maternal diet enriched in olive oil. In this regard, there is a close link between oxidative stress, inflammation and apoptosis [41, 42]. Previous studies have shown that diabetes induces cardiomyocyte apoptosis, whereas *in vitro* studies have addressed the ability of oleic acid to protect vascular smooth muscle cells from apoptosis induced by prooxidant/proinflammatory mediators [41, 43, 44]. As cardiac apoptosis is believed to play a causal role in heart diseases [42], the observed effects of the olive oil-enriched maternal diet to reduce apoptosis in the offspring´s heart are likely relevant.

The ability of the olive oil-enriched diet to exert beneficial effects on the offspring´s heart is likely the result of the *in utero* effects of this diet. Indeed, prooxidant and proinflammatory markers as well as PPARs and mTOR signaling pathways are regulated by this dietary treatment in the placenta, which may indirectly benefit the fetal heart [20, 45]. Moreover, we have previously found that, at the fetal stage, both lipoperoxidation, which is increased in hearts from male fetuses of diabetic rats, and nitric oxide overproduction, which is increased in hearts from male and female fetuses of diabetic rats, are prevented by maternal diets enriched in olive oil [21]. This beneficial effect has been related to the ability of this diet to activate PPAR pathways and induce antioxidant/anti-inflammatory pathways [21, 36]. Thus, possibly, the observed ability of the olive oil-supplemented diet to reduce oxidative stress markers in the heart of the 21-day-old offspring is the result of antioxidant effects exerted *in utero*, which in turn lead to the prevention of the adverse programming observed in the offspring´s heart.

5. Conclusions

The current study demonstrates the ability of a maternal diet enriched in olive oil to prevent anomalies in deposition of components of the extracellular matrix, to regulate the levels of the profibrotic mediator CTGF, to reduce prooxidant markers and to prevent increased apoptosis in the heart of the offspring of diabetic rats at an early postnatal stage, suggesting putative long-term cardiovascular benefits. As an olive oil-enriched diet has been found highly beneficial to cardiovascular health in non-pregnant subjects, the possibility that an olive oil-enriched diet prevents long-term cardiovascular diseases in the offspring of diabetic subjects is an issue that warrants further research.

Conflict of interest

The authors report no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

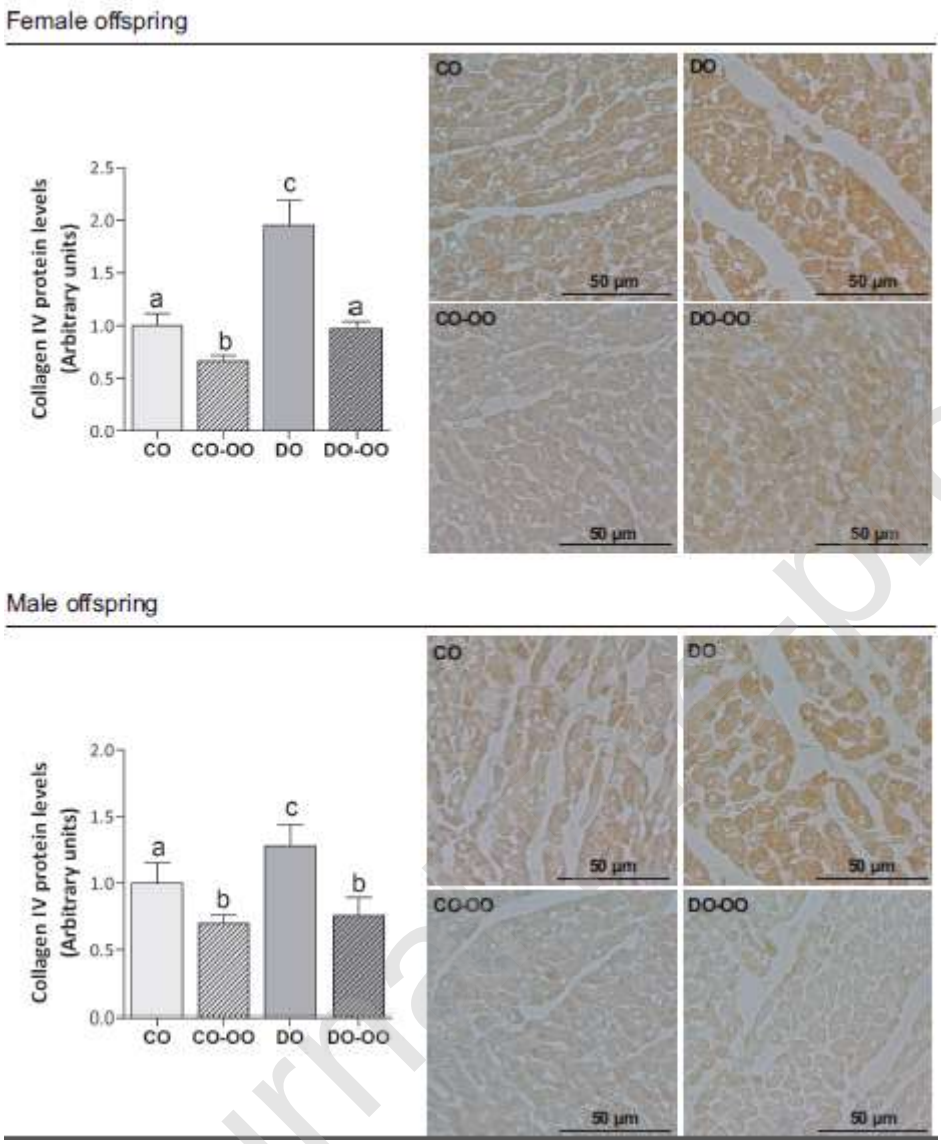

Fig. 1. Immunostaining of collagen IV in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-

supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from different F0 mothers in each experimental group. Different letters denote significant differences between groups, $p < 0.05$.

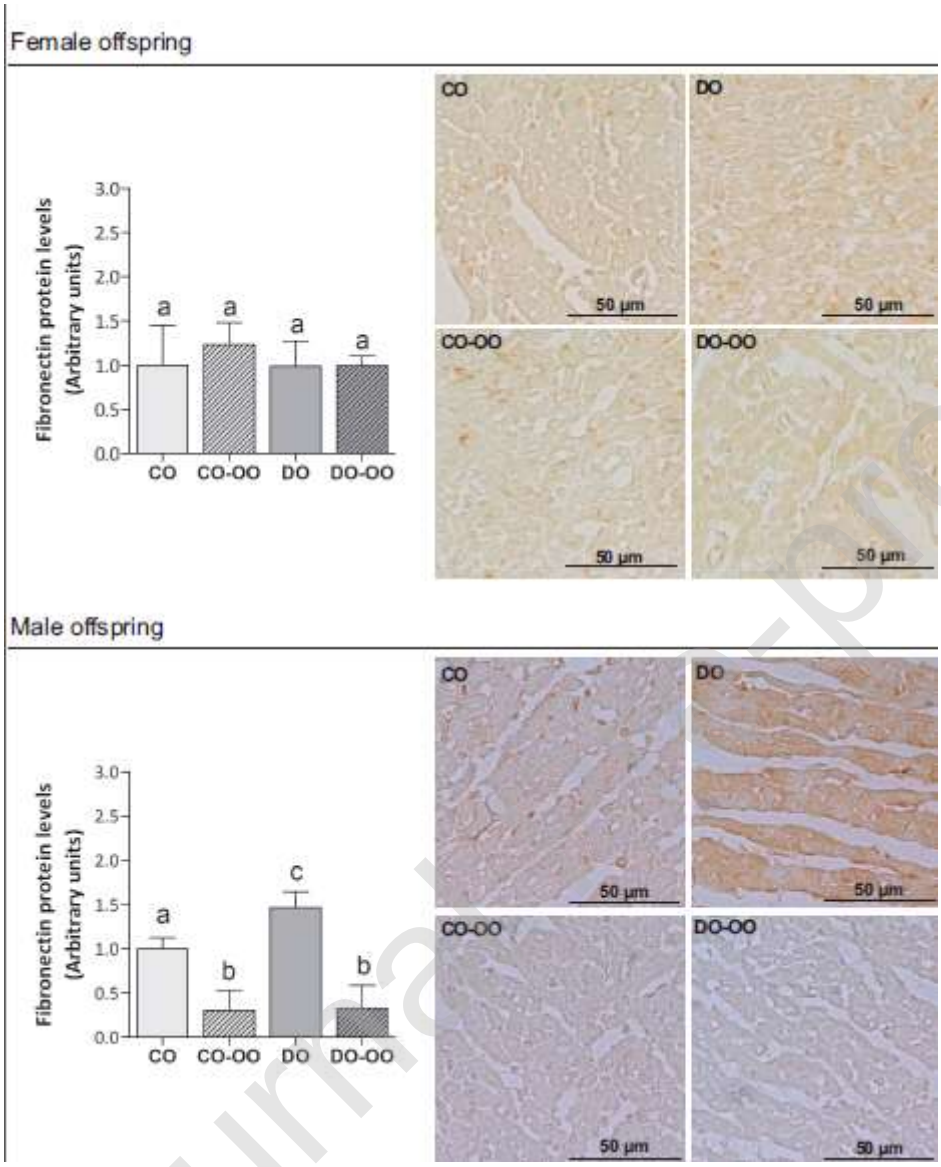

Fig. 2. Immunostaining of fibronectin in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from

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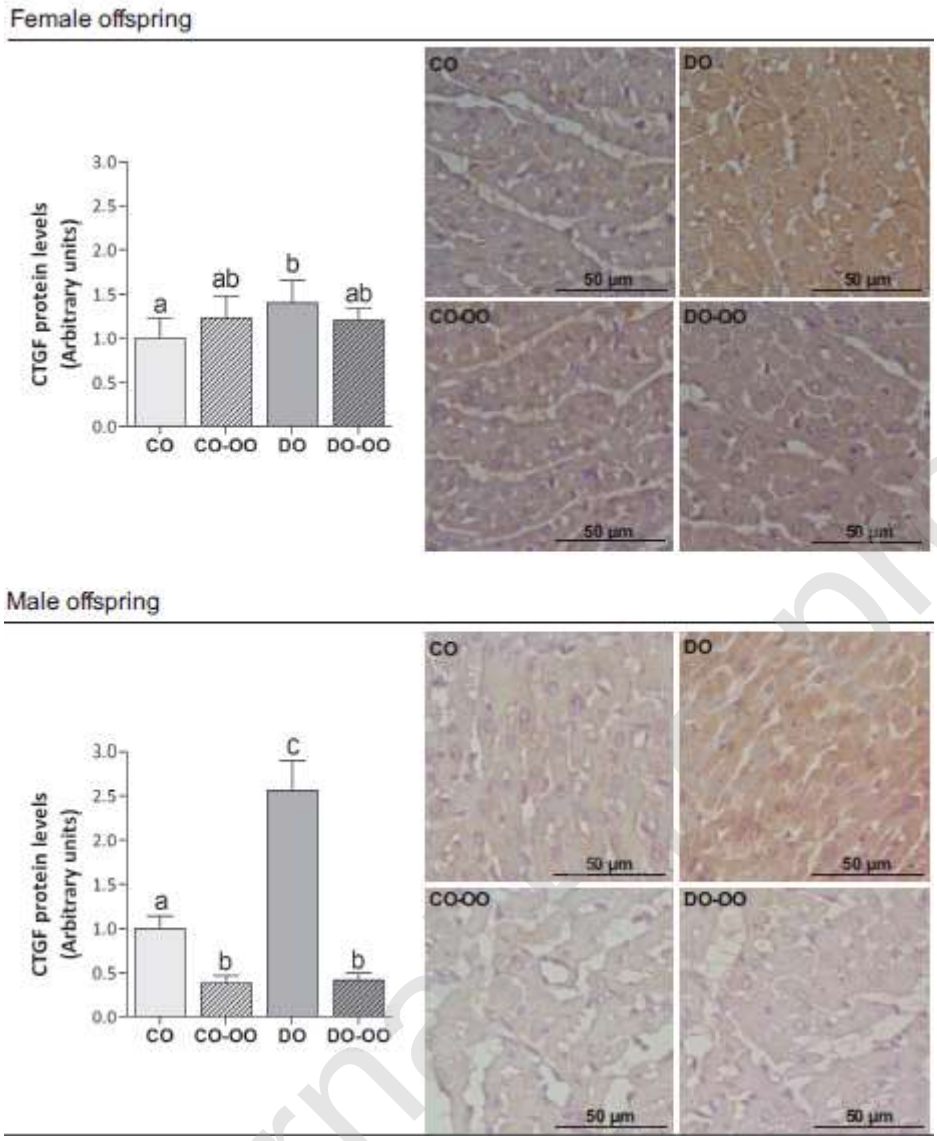

Fig. 3. Immunostaining of CTGF in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from different F0 mothers in each

experimental group. Different letters denote significant differences between groups, $p < 0.05$.

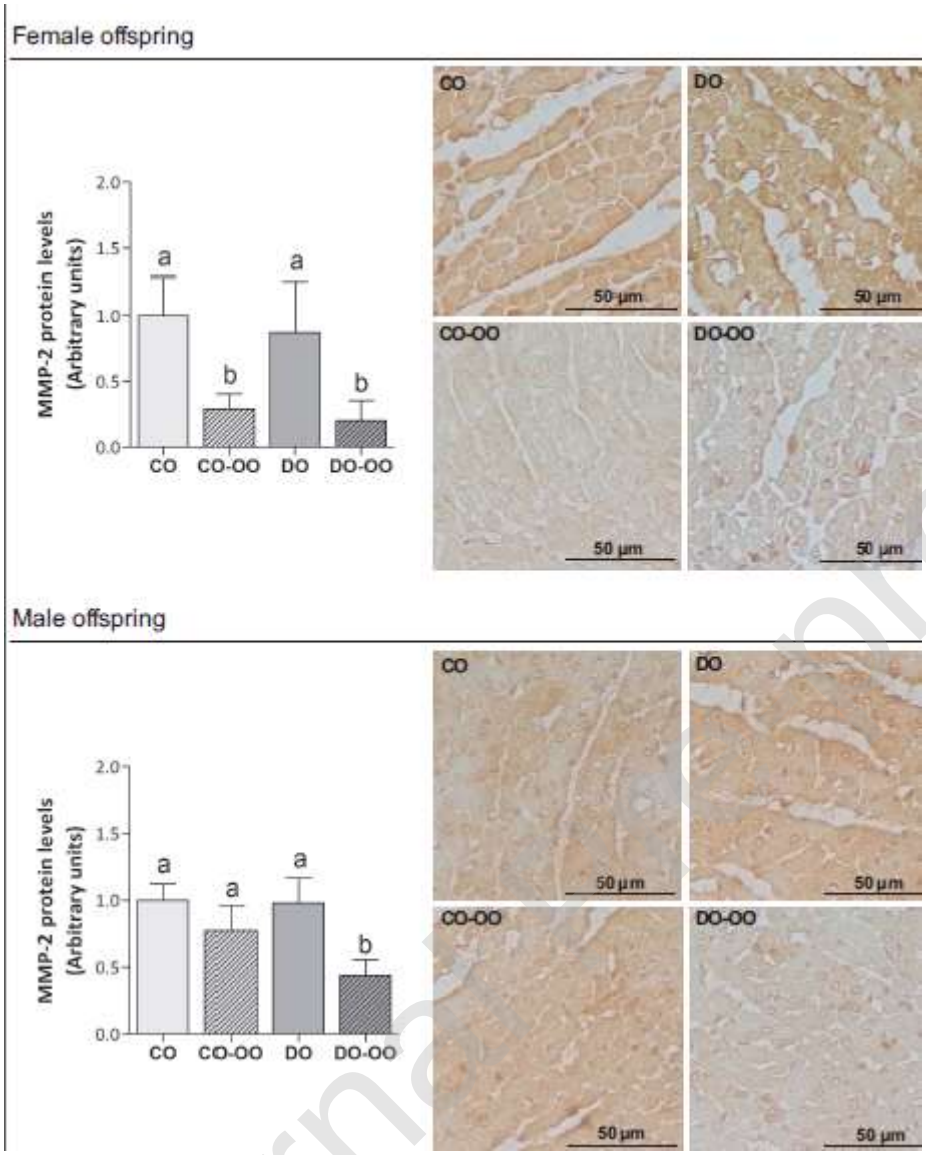

Fig. 4. Immunostaining of MMP-2 in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from different F0 mothers in each

experimental group. Different letters denote significant differences between groups, $p < 0.05$.

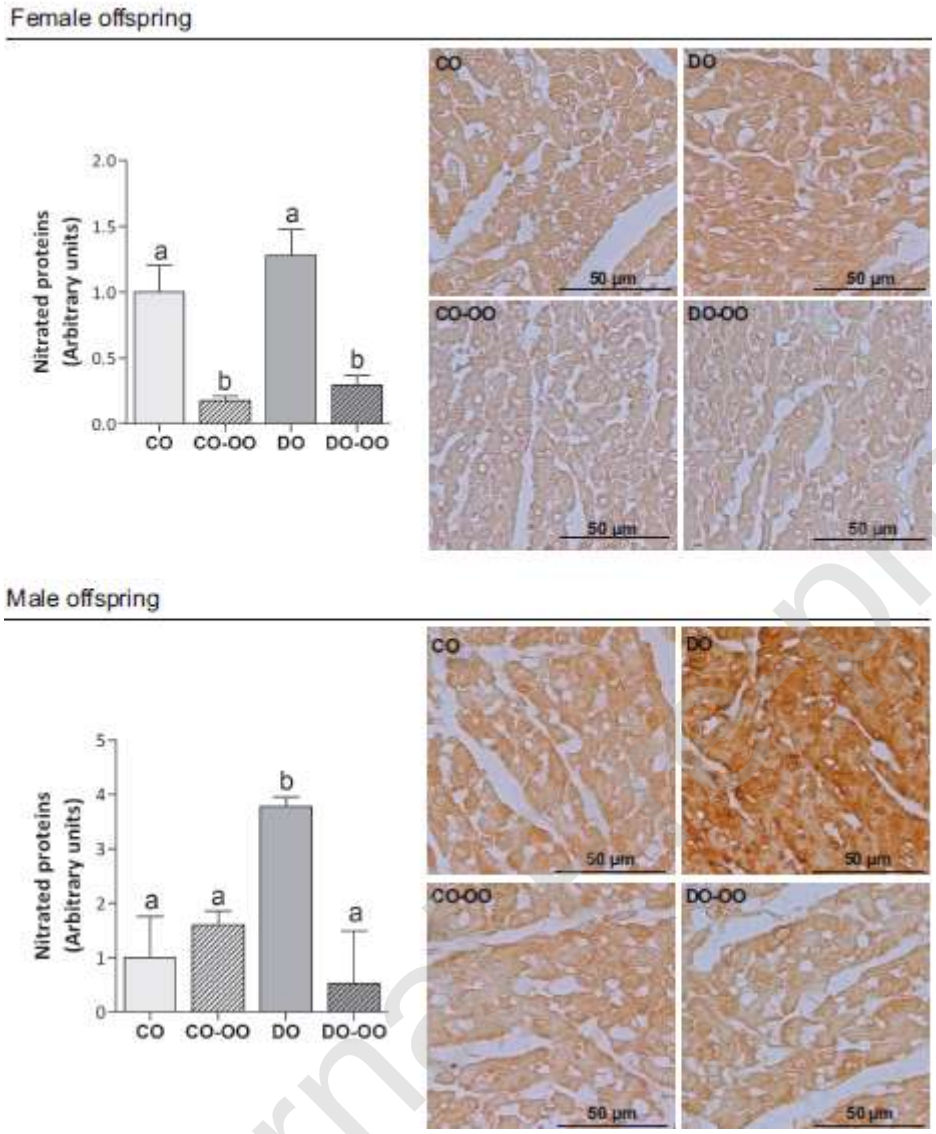

Fig. 5. Immunostaining of nitrated proteins, an index of peroxynitrite-induced damage in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD

obtained from 7 rats from different F0 mothers in each experimental group. Different letters denote significant differences between groups, $p < 0.05$.

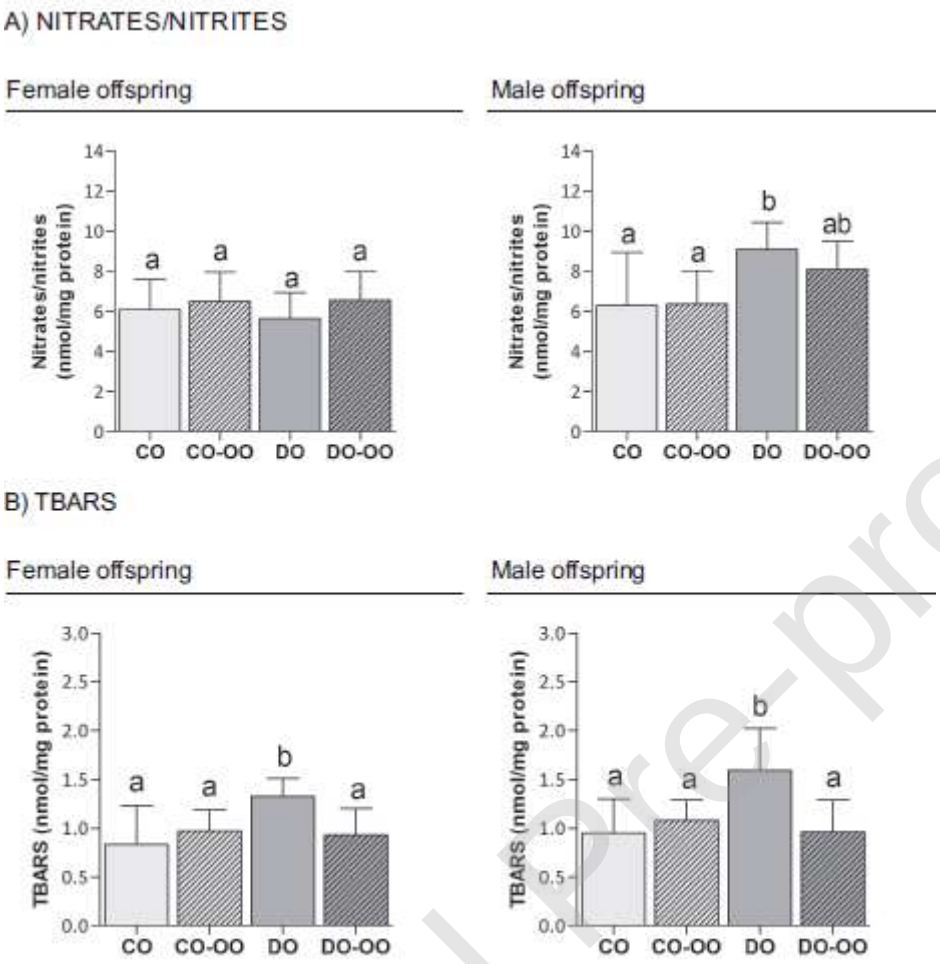

Fig. 6. The production of NO (measured as the concentrations of nitrates/nitrites, stable NO metabolites) and lipoperoxidation (measured as TBARS concentrations) in the heart of 21-day-old female and male offspring from control and diabetic rats. **A.** nitrates/nitrites levels. **B.** TBARS concentrations. CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from different F0

mothers in each experimental group. Different letters denote significant differences between groups, $p < 0.05$.

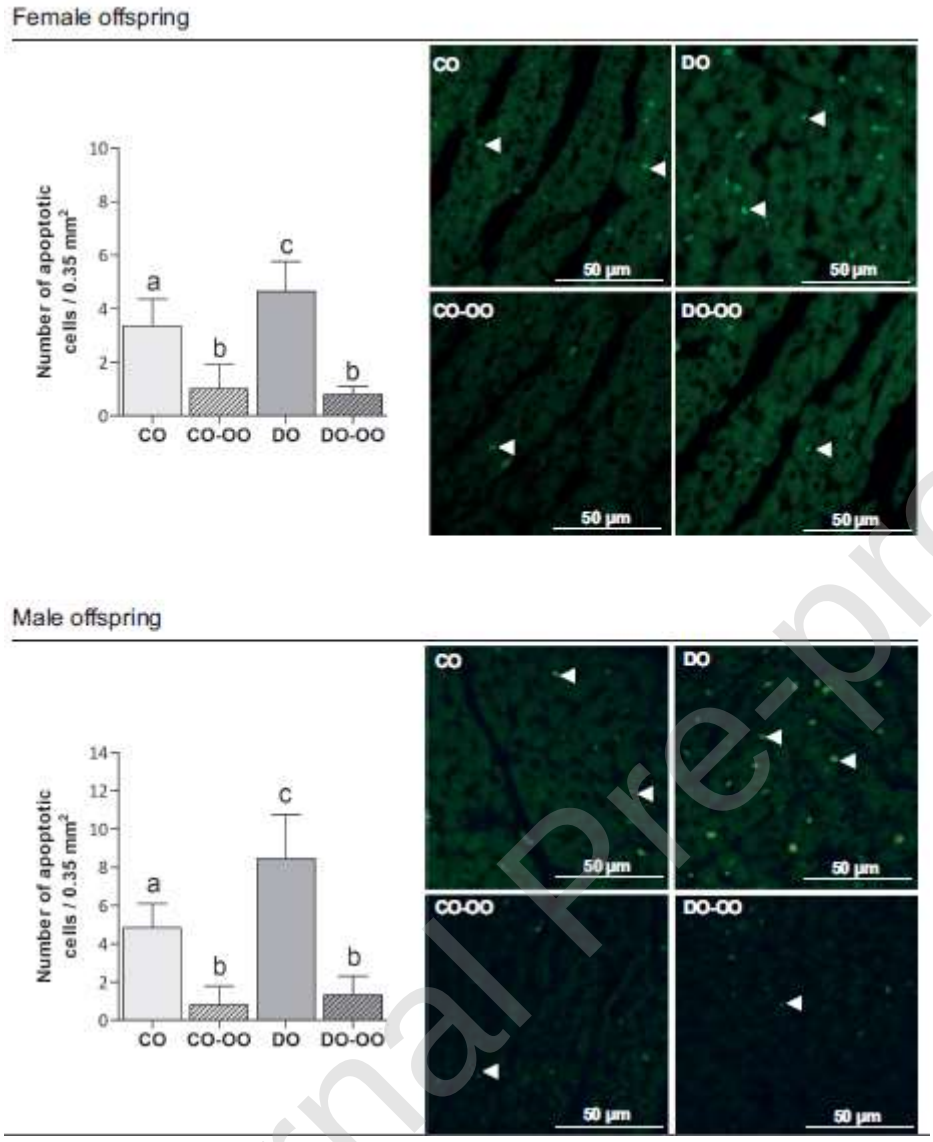

Fig. 7. Apoptosis in the heart of 21-day-old female and male offspring from control and diabetic rats. Representative microphotographs of the ventricles and densitometry analysis are shown (400X). Apoptotic cells were labeled in green. CO: Offspring of non-supplemented control rats, CO-OO: Offspring of Olive Oil-supplemented control rats, DO: Offspring of non-supplemented diabetic rats, DO-OO: Offspring of Olive Oil-supplemented diabetic rats. Values represent mean ± SD obtained from 7 rats from

different F0 mothers in each experimental group. Different letters denote significant differences between groups, $p < 0.05$.

	CONTROL GROUP		DIABETIC GROUP	
	Standard diet	6% Olive oil-enriched diet	Standard diet	6% Olive oil-enriched diet
Maternal data	94 ± 18 [a]	103 ± 18 [a]	228 ± 32 [b]	224 ± 37 [b]
Female offspring data	103 ± 16 [a]	101 ± 8 [a]	110 ± 16 [a]	100 ± 11 [a]
Male offspring data	99 ± 18 [a]	107 ± 11 [a]	132 ± 16 [b]	82 ± 11 [c]

Table 1. Glycemia values in control and diabetic rats (day 21 of pregnancy) and in their 21-day-old female and male offspring. Values represent mean ± SD obtained from 7 rats from different F0 mothers in each experimental group. Different letters denote significant differences between groups, $p<0.05$.