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Adiponectin agonist treatment in diabetic pregnant rats

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Abstract

Gestational diabetes mellitus (GDM) reduces maternal adiponectin and docosahexaenoic acid (DHA) materno-fetal transfer, which may have negative consequences for the offspring. Our aim was to evaluate the effects of the administration of a novel adiponectin agonist (AdipoRon) to GDM rats on the long-term consequences in glycaemia and fatty acids (FA) profile in the offspring. Pregnant rats were randomized to three groups: GDM rats (GDM, n = 8), GDM rats treated with AdipoRon (GDM + ADI, n = 9), and control rats (n = 10). Diabetes was induced with streptozotocin (50 mg/kg) on day 12 of gestation. GDM+ADI received 50 mg/kg/day AdipoRon from day 14 until delivery. Glycaemia and FA profile were determined in mothers and adult offspring (12 weeks old). AdipoRon tended to reduce fasting glucose in diabetic mothers. Diabetic rats presented the foetus with intrauterine growth restriction and higher adiposity, which tried to be counteracted by AdipoRon. In the adult offspring, both GDM + ADI and control animals showed better glucose recovery after oral glucose overload with respect to GDM. DHA in offspring plasma was significantly reduced in both GDM and GDM + ADI compared to controls (P = 0.043). Nevertheless, n-6/n-3 polyunsaturated FA (PUFA) ratio improved in plasma of GDM + ADI adult offspring (GDM: 14.83 ± 0.85%, GDM + ADI: 11.49 ± 0.58%; control: 10.03 ± 1.22%, P = 0.034). Inflammatory markers and oxidative stress were reduced in the adult offspring of AdipoRon-treated mothers. In conclusion, AdipoRon administration to pregnant diabetic rats improved glycaemia in the mothers and long-term glucose tolerance in the offspring. In addition, it tended to reduce excessive foetal fat accumulation and improved n-6/n-3 PUFA ratio significantly in offspring at the adult state.

Introduction

Gestational diabetes mellitus (GDM) prevalence is increasing in women due to the obesity epidemic; however, the ideal management of GDM remains controversial (Mack & Tomich 2017). It is estimated that one out of six live births is affected by hyperglycaemia in pregnancy, most of those due to GDM (International Diabetes...
Federación 2017). GDM is associated with neonatal adverse outcomes such as large for gestational age infants and increased risk of obesity and cardiometabolic disease later in life (Boney et al. 2005, Lawlor et al. 2010), contributing to the perpetuation of the vicious cycle of obesity, diabetes, and metabolic syndrome. There is also growing evidence of the GDM long-term consequences for mothers, who have a higher risk of developing type 2 diabetes and cardiovascular disease (Bellamy et al. 2009, Kramer et al. 2019).

Several cohort studies reported decreased adiponectin concentrations in GDM women compared to their peers without diabetes (Fasshauer et al. 2014). Adiponectin is an insulin-sensitizing adipokine that promotes FA oxidation (liver and muscle) and lowers the expression of genes involved in hepatic gluconeogenesis via activation of AMP-activated protein kinase (AMPK) and via peroxisome proliferator-activated receptor α (PPARα) (Yamauchi et al. 2001, Kadokaki & Yamauchi 2005). Low adiponectin levels combined with high insulin resistance in GDM might contribute to enhance GDM-associated macrosomia (Fasshauer et al. 2014). Moreover, hypoadiponectinemia in pregnancy is a predictor of postpartum insulin resistance, beta-cell dysfunction, and elevated fasting glycaemia and may play a role in the subsequent development of type 2 diabetes (Retnakaran et al. 2010). These observations raised the question about the utilization of adiponectin as a new therapeutic target for insulin resistance in diabetic subjects.

There are two strategies to reverse hypoadiponectinemia, one is to increase adiponectin concentration itself through the injection of adiponectin which presents some methodological problems due to its very high concentration in blood and the high molecular weight of the active form (Pajvani et al. 2004). Other option is the use of adiponectin receptors (AdipoR1 and AdipoR2) agonists that mimic adiponectin effects. Adiponectin administration in mice models has shown positive effects on insulin sensitivity (Yamauchi et al. 2001, Combs et al. 2004) as well as using adiponectin agonists (AdipoRon) in high-fat diet obese mice (Okada-Iwabu et al. 2013). However, little is known about the effects of AdipoRon administration during gestation, especially in those pregnancies affected by GDM.

Several studies have shown impaired placental transport of docosahexaenoic acid (22:6 n-3, DHA) by GDM (Herrera & Ortega-Senovilla 2010, Leveille et al. 2018), likely as a consequence of alterations in fatty acids (FA) transport proteins related to phospholipids transfer such as the major facilitator superfamily domain-containing 2a (MFSD2a) and FA transport protein 4 (FATP-4) (Prieto-Sanchez et al. 2017, Segura et al. 2017). Insufficient supply of long-chain polyunsaturated FA (LC-PUFA) to the foetus might compromise adequate growth and later on visual and cognitive development (Innis 2007).

The aim of the present study was to investigate the effects of AdipoRon administration during gestation on glycaemia and lipid profile in both mothers and adult offspring using a rat model of GDM.

Materials and methods

Animals and study design

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Murcia (Murcia, Spain) and conformed to the ARRIVE guidelines for animal research (Kilkenny et al. 2010). Animals received humane treatment in accordance with the European Union guidelines for the care and use of laboratory animals. Female adult Sprague Dawley rats (12 weeks of age) were supplied by the Animal Laboratory Service of the University of Murcia. Average weight of rats was 225 ± 5 g. Animals were housed individually with access to food and water ad libitum in a humidity and temperature-controlled (22 ± 1°C) room on a 12 h light:12 h darkness cycle.

Female rats were mated (1:1), and the first day of gestation was estimated by the presence of a mating plug in the cage. They were randomly assigned to three groups of 15 animals each: diabetic rats (GDM), diabetic rats treated with adiponectin agonist (AdipoRon, Biorybt, UK) (GDM + ADI), and healthy controls without diabetes (control). Diabetes was induced on day 12 of gestation by a single injection of streptozotocin (STZ) (50 mg/kg i.p.) (Sigma–Aldrich) dissolved in citrate buffer (10 mM, pH 4.5), control animals received vehicle only. Once the diabetes was established, GDM + ADI animals received AdipoRon (50 mg/kg/day by gavage) freshly dissolved in ethanol for days 14–20 of gestation. All diabetic rats (GDM and GDM + ADI) were given exogenous neutral protamine Hagedorn insulin (Novo Nordisk) every morning in order to maintain glycaemia values of mothers between 100 and 400 mg/mL. The units of insulin administered to pregnant rats varied depending on daily glucose values but were similar in both GDM and GDM + ADI groups (GDM: 3.44 ± 0.30 IU/d, GDM + ADI: 3.63 ± 0.33 IU/d, P = 0.653).

On day 20 of gestation, ten pregnant rats per group were anesthetized with a mixture (per 100 g) of 5 mg ketamine hydrochloride, 0.25 mg chlorobutanol, and 1 mg xylazine. Maternal blood was extracted by heart puncture in EDTA-coated tubes and centrifugated at 1400 g for 10 min at 4°C.
to obtain plasma. Foetal plasma was not possible to collect due to methodological difficulties related to the small size of the foetus at delivery. Placentas, whole foetus, and foetal brain were also collected (four foetus and placentas were pooled per rat), frozen in liquid nitrogen and stored at −80°C until analysis.

Three to four pregnant rats per group were allowed to breed their pups until weaning (21 days). Sex-matched offspring animals were maintained on a standard chow diet until adult state (12 weeks of age) when they were sacrificed, and plasma and brain samples collected (GDM \( n = 16 \) (8 males/8 females), GDM + ADI \( n = 14 \) (6 males/8 females), control \( n = 11 \) (6 males/5 females)). Rats were allocated during 4 days in individual metabolic cages for the collection of urine samples. Oral glucose tolerance test was performed in the offspring at 3 months of age (2 g/kg), blood was collected by a tail puncture at basal, 30, 60, and 90 min postchallenge.

Biochemical analysis

Plasma biochemical parameters (glucose, total cholesterol, HDL cholesterol, and triglycerides) were quantified by an automatic analyser (Roche–Hitachi Modular PyD Autoanalyzer, Mannheim, Germany). Daily monitoring of glucose in diabetic mothers was performed with Ascensia Breeze 2 blood glucose monitoring system (Bayer AG). HbA1c was measured with A1C Now+ Multi-test System (Bayer AG).

Fatty acids analyses

Total lipids were extracted from 125 µL plasma and tissues (150 mg placenta, 600 mg whole foetus, and 80 mg brain) into chloroform:methanol (2:1 v/v) according to Folch et al. method (Folch et al. 1957). Previous to the extraction, 0.05 mg pentadecanoic acid was added to the samples as internal standard. FA methyl esters were produced according to Stoffel et al. (Stoffel et al. 1959) by adding 1 mL of 3 N methanolic HCl (Supelco, Sigma–Aldrich) and heating at 90°C for 1 h. The derivatives were extracted into hexane and stored at −20°C until gas chromographic analysis.

FA methyl esters were analysed by gas chromatography using a SP-2560 capillary column (100 m × 0.25 mm i.d. × 20 µm) (Supelco, Sigma–Aldrich) in a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies) equipped with a flame ionization detector (Larque et al. 2003). The temperature of the detector and the injector was 240°C. The oven temperature was programmed at 175°C for 30 min and increased at 2°C/min to 230°C and held at this temperature for 17 min. Helium was used as the carrier gas at a pressure of 45 psi. Peaks were identified by comparison of their retention times with appropriate FA methyl esters standards (Sigma–Aldrich), and FA concentrations were determined in relation to the peak area of internal standard.

Foetal adiposity was estimated by dividing the whole foetus total FA content by the foetal weight.

Protein extracts for Western blotting

Placental tissue of 30 mg was homogenized in 0.3 mL ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/mL leupeptin) from Cell Signaling Technology. Phenylmethanesulfonyl fluoride solution of 1 mM was added to the lysis buffer before homogenization (Ruiz-Alcaraz et al. 2005). Samples were homogenized using a Tissue Lyser LT device (Qiagen Iberia SL). Protein lysates were obtained from the supernatant after 15 min centrifugation at 10,000 × g 4°C. Protein was quantified by Bradford assay (Bradford 1976), and samples were stored at −80°C until Western blot analysis.

Western blot analysis

The primary antibodies used were rabbit polyclonal antibody against the orphan transporter called MFS2a (Abcam), rabbit monoclonal against FA translocase (FAT) (Abcam), rabbit monoclonal against FATP-4 (Abcam), rabbit polyclonal antibody against adipocyte FA binding protein (A-FABP) (Sigma–Aldrich), rabbit monoclonal against phosphorylated AKT (Abcam), rabbit polyclonal against extracellular signal-regulated kinase (ERK) (Proteintech, Manchester, UK) and mouse monoclonal anti-beta-actin (Sigma–Aldrich). Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology. Protein extracts (15 µg protein) diluted in sample buffer were resolved on 10% polyacrylamide gels and transferred onto PVDF membranes (Merck Millipore). Membranes were then blocked in phosphate saline buffer with 0.05% Tween 20 (PBS-T) containing 2% BSA for 1 h at room temperature. Thereafter, membranes were incubated with primary antibodies overnight at 4°C. Blots were then washed with PBS-T and probed for 1 h at room temperature with the correspondent secondary antibodies conjugated with horseradish peroxidase. Finally, membranes were stripped with Tris/HCl buffer pH 2.3 containing
beta-mercaptoethanol 0.1 M and re-probed with anti-beta-actin to perform loading controls. Proteins were detected using a chemiluminescence kit according to the manufacturer’s instruction (Pierce ECL 2 Western Blotting Substrate; Thermo Fisher Scientific) (Prieto-Sanchez et al. 2017). Density of all bands was determined by densitometry using Image Quant LAS 500 software (GE Healthcare). Relative protein expression data were normalized for beta-actin expression.

Levels of inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), and nitrated proteins

Renal cortex was homogenized in ice-cold 50 mmol/L Tris-HCl buffer pH 7.4 containing 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L EDTA, and 10% protease inhibitor cocktail (Sigma-Aldrich). Kidney homogenates were then centrifuged (10,000 g for 30 min) and stored at −80°C until analysis.

Concentrations of IL-1β, IL-6, TNF-a in renal cortex homogenates from each group were determined by using the methodology described by Taylor et al. (2008). In brief, urine samples were centrifuged at 1400 g for 5 min at room temperature. The supernatant (4 mL) was mixed with ultra-pure water (4 mL) and internal standard (PGE2-d4 50 ng in methanol). The sample pH was adjusted to 6.0 ± 0.05. Cartridges were preconditioned with 4 mL of methanol containing 2% formic acid (v/v) and subsequently equilibrated with 4 mL water. The diluted urine sample (8 mL) was then loaded onto the cartridge. The cartridge was washed with 4 mL water, followed by 8 mL methanol 25% (in water, v/v) and 4 mL methanol 50% (in water, v/v). Elution was subsequently performed with 3 mL of methanol containing 2% formic acid (v/v), 3 mL of methanol, and followed by 2 mL of methanol containing 50% acetonitrile (v/v). Cartridges were then dried under nitrogen gas and reconstituted in 50 μL methanol containing 0.1% formic acid (v/v) and injected onto the UHPLC-MS.

Chromatographic separation was carried out using a UPLC 1290 Infinity Series system (Agilent Technologies) equipped with a triple quadrupole mass spectrometer (6460 Jet Stream Series, Agilent Technologies) based on the methodology described by Le Faouder et al. (2013). An Excel C18 column (100 mm × 3.0 mm i.d., 1.7 μm) (ACE, Aberdeen, UK) was used. Limits of quantification and of the ABTS cation radical (ABTS+). ABTS+ was generated in media containing 240 μL phosphate buffer (50 mM pH 7.5), ABTS 2 mM, H2O2 60 μM, and horseradish peroxidase 0.25 μM. At stable ABTS+ solutions, 10 μL of plasma or protein homogenate (kidney) were added, measuring the quenching of the absorbance at 730 nm after 6 min of reaction. Antioxidant activity was calculated by comparing the values of the sample with a standard curve of Trolox and expressed as Trolox millimoles equivalents by grams of albumin/protein (eq. Trolox millimoles/grams albumin or protein).

Eicosanoids analysis in urine samples

n-6 and n-3 LC-PUFA metabolites 8-iso-prostaglandin F2α (8-iso-PGF2α), PGD2, PGE2, PGF2, PGF3, Lipoxin A4 (LxA4), 15R-LxA4, Lx-B4, 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 12-hydroxyicosatetraenoic acid (12-HETE), 15-hydroxyicosatetraenoic acid (15-HETE), and the deuterated internal standard PGE2-d4 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). All solvents and reagents used were HPLC grade.

Solid-phase extraction procedure of eicosanoids using Strata™ X-AW cartridges 200 mg/3mL (Phenomenex, Torrance, CA, USA) was performed in urine samples as previously described by Taylor et al. (2008). Cartridges were preconditioned with 4 mL of methanol containing 2% formic acid (v/v) and subsequently equilibrated with 4 mL water. The diluted urine sample (8 mL) was then loaded onto the cartridge. The cartridge was washed with 4 mL water, followed by 8 mL methanol 25% (in water, v/v) and 4 mL methanol 50% (in water, v/v). Elution was subsequently performed with 3 mL of methanol containing 2% formic acid (v/v), 3 mL of methanol, and followed by 2 mL of methanol containing 50% acetonitrile (v/v). Cartridges were then dried under nitrogen gas and reconstituted in 50 μL methanol containing 0.1% formic acid (v/v) and injected onto the UHPLC-MS.
detection were also previously reported (Le Faouder et al. 2013). The relative standard deviation for peak area was in the range of 0.5–4.7% in the intraday test and 1.3–3.5% in the case of the inter-day test.

Standard curves were constructed at five concentrations for all compounds (0.025–0.5 µg/mL). The metabolite concentrations in urine were corrected for creatinine, which was measured using a commercial kit (CREATININE-J, Spinreact, Girona, Spain).

**Statistical analysis**

Sample size was estimated based on HbAlc percentages in diabetic mice treated with AdipoRon published by Choi et al. (2018). Type I error was set at α = 0.05 and type II error β = 0.2 (power 80%), obtaining a minimum sample size of 11 animals per group. The software used for this estimation was nQuery 7.0 (Statsols HQ, Cork, Ireland).

The three experimental groups were compared using one-way ANOVA followed by a post hoc Bonferroni test. P < 0.05 was considered statistically significant. The results are expressed as the mean ± s.e.m. Statistical analysis was performed using SPSS, version 24.0 (IBM Corp.).

**Results**

Pregnant diabetic rats (GDM) had significantly higher glycaemic levels than controls for both glucose (Fig. 1A) and HbAlc (Table 1). Adiponectin agonist (AdipoRon) administration tended to reduce glycaemia in diabetes-treated mothers compared to non-treated dams (Fig. 1A), following HbAlc the same non-significant trend (P = 0.073 GDM vs GDM + ADI). Maternal serum lipids (total cholesterol, HDL and LDL cholesterol, and TG) were similar in the three groups of animals (Table 1). Foetus from diabetic dams (GDM and GDM + ADI) presented intrauterine growth restriction compared to controls (Table 1). However, foetal fat content/foetal weight ratio indicated higher foetal adiposity in offspring of diabetic mothers, and AdipoRon tried to counteract this foetal fat accumulation (Fig. 1B).

No major differences were observed in maternal FA plasma, placenta, or foetal tissues (Table 2). In maternal plasma, eicosapentaenoic acid (20:5 n-3, EPA) was reduced in GDM mothers compared to control while DHA did not change significantly (Table 2). In placenta, DHA tended to increase by GDM (P = 0.107) while EPA again decreased significantly. However, no differences were observed in foetal brain (Table 2). Placental MFSD2a expression did not show differences between groups (GDM: 0.78 ± 0.12; GDM + ADI: 0.73 ± 0.10; control: 0.89 ± 0.15 arbitrary units, P = 0.636). Likewise, other FA transport proteins (FAT, FATP-4, and A-FABP) or proteins involved in insulin signalling (p-AKT and ERK) analysed in the placenta showed similar results between groups (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

At the adult state, offspring of diabetic and control mothers showed similar fasting glucose levels (Table 1). GDM offspring showed lower glucose concentration than GDM + ADI ones after 30 min of an oral glucose overload test (Fig. 2A). However, GDM offspring had significantly higher glucose increase between basal and 90 min
post-challenge compared to controls (P = 0.009) while a positive effect of AdipoRon was observed in reducing this basal-90 min glucose increase in the offspring (Fig. 2B). Both GDM+ADI and control were returning to basal values of glycaemia after 90 min while GDM still presented elevated values of serum glucose (Fig. 2A). Serum LDL cholesterol was also higher in the offspring of GDM mothers compared to the control group while no differences were found in LDL cholesterol or triglycerides level (Table 1). GDM+ADI showed lower serum albumin than GDM or control in adult offspring (Table 1).

Concerning to FA profile in the adult offspring, DHA of the adiponectin agonist on glucose control in the maternal, foetal, and adult offspring parameters. Table 1. Maternal, foetal, and adult offspring parameters.

|                                | GDM (n = 8)  | GDM+ADI (n = 9) | Control (n = 10) | P      |
|--------------------------------|--------------|-----------------|-----------------|--------|
| Maternal parameters (day 20 of gestation) |              |                 |                 |        |
| Weight (g)                     | 298.50 ± 7.74| 316.40 ± 7.18   | 317.90 ± 12.82  | 0.350  |
| HbA1c (%)                      | 5.03 ± 0.12a | 4.68 ± 0.13a    | 4.21 ± 0.11b    | 0.001  |
| Cholesterol (mg/dL)            | 90.50 ± 7.47 | 101.78 ± 6.51   | 94.89 ± 6.83    | 0.481  |
| HDL cholesterol (mg/dL)        | 31.50 ± 5.98 | 29.67 ± 6.10    | 19.56 ± 3.61    | 0.209  |
| LDL cholesterol (mg/dL)        | 61.63 ± 11.02| 67.87 ± 10.57   | 75.33 ± 8.33    | 0.598  |
| TG (mg/dL)                     | 488.88 ± 81.84| 458.00 ± 61.63  | 493.22 ± 78.33  | 0.893  |
| Albumin (g/dL)                 | 2.45 ± 0.18  | 2.62 ± 0.10     | 2.38 ± 0.23     | 0.570  |
| TAA (eq. Trolox mM/g albumin)  | 1.04 ± 0.06  | 0.89 ± 0.10     | 1.22 ± 0.34     | 0.567  |
| Placental weight (g)           | 0.45 ± 0.01a | 0.51 ± 0.02b    | 0.49 ± 0.02ab   | 0.010  |
| Foetal parameters              |              |                 |                 |        |
| Weight (g)                     | 1.95 ± 0.06a | 2.10 ± 0.05a    | 2.84 ± 0.27b    | 0.002  |
| Foetus number                  | 14.75 ± 0.59 | 13.60 ± 0.78    | 14.20 ± 0.84    | 0.590  |
| Total fat (mg/g)               | 6.83 ± 0.33  | 6.43 ± 0.24     | 6.02 ± 0.21     | 0.102  |
| Adult offspring (3 months old) |              |                 |                 |        |
| Weight (g)                     | 300.44 ± 16.35| 304.29 ± 16.87  | 293.55 ± 16.95  | 0.600  |
| Glucose (mg/dL)                | 77.45 ± 3.25 | 80.23 ± 4.01    | 75.21 ± 1.96    | 0.777  |
| Cholesterol (mg/dL)            | 82.55 ± 1.67 | 80.50 ± 4.02    | 69.13 ± 2.37    | 0.291  |
| HDL Cholesterol (mg/dL)        | 57.18 ± 2.10 | 58.93 ± 3.58    | 48.31 ± 2.3    | 0.685  |
| LDL Cholesterol (mg/dL)        | 12.64 ± 1.24a| 9.50 ± 1.01ab   | 8.00 ± 0.92b    | 0.026  |
| TG (mg/dL)                     | 62.45 ± 5.11 | 60.07 ± 2.55    | 64.44 ± 4.72    | 0.846  |
| Albumin (g/dL)                 | 3.75 ± 0.11a | 2.88 ± 0.24b    | 3.80 ± 0.06a    | 0.002  |

Results are expressed as means ± S.E.M.
Different superscript letters indicate significant differences between experimental groups (P < 0.05) (bold face).
GDM, gestational diabetes mellitus; GDM+ADI, animals with gestational diabetes mellitus treated with AdipoRon; TAA, total antioxidant activity.

Discussion

Here we report the effects of the orally active adiponectin agonist termed AdipoRon on glycaemia and lipid profile in both mother and adult offspring, after its administration during gestation to diabetic rats. As expected, diabetic animals presented higher glucose and HbAlc than non-diabetic control animals. However, AdipoRon treatment tended to reduce glycaemia, indicating a positive effect of the adiponectin agonist on glucose control in the mothers and offspring. It is worth to mention that diabetic animals received daily exogenous insulin which might have affected the results observed. Okada-Iwabu et al. reported a significant reduction in fasting plasma glucose and insulin levels of high-fat diet-induced obese mice after the administration of AdipoRon for 10 days (Okada-Iwabu et al. 2013). They found increased exercise endurance and increased expression of genes involved in FA oxidation in muscle (e.g. medium-chain acyl-CoA dehydrogenase...
Table 2  Maternal plasma, placenta, total foetus, and foetal brain fatty acid profile at day 20 of gestation.

| Fatty acids (g/100 g fatty acids) | GDM (n = 8) | GDM+Adi (n = 9) | Control (n = 10) | P      |
|----------------------------------|-------------|----------------|-----------------|--------|
| Maternal plasma                  |             |                |                 |        |
|                                | 16:0        | 18:0           | 18:1 n-9        | 18:2 n-6 | 20:4 n-6 | 20:5 n-3 (EPA) | 22:6 n-3 (DHA) | SFA | MUFA | PUFA | n-6/n-3 | LC-PUFA n-6 | LC-PUFA n-3 |
|                                | 21.07 ± 0.91| 8.73 ± 0.77    | 14.26 ± 1.21    | 21.32 ± 1.19 | 15.04 ± 0.85 | 0.15 ± 0.05<sup>a</sup> | 6.70 ± 0.71 | 31.72 ± 1.63 | 18.55 ± 1.44 | 46.95 ± 1.49 | 5.41 ± 0.51 | 19.91 ± 0.97 | 7.81 ± 0.80 |
|                                | 20.29 ± 0.40| 9.24 ± 0.61    | 15.35 ± 0.75    | 20.26 ± 0.76 | 16.59 ± 0.81 | 0.27 ± 0.06<sup>ab</sup> | 5.84 ± 0.35 | 31.50 ± 0.58 | 20.13 ± 0.81 | 48.21 ± 0.81 | 6.00 ± 0.30 | 20.34 ± 0.73 | 6.88 ± 0.38 |
|                                |             |                |                 |          |          | 0.39 ± 0.05<sup>a</sup> |          |            |                |          | 49.34 ± 1.01 |          |          | 21.13 ± 1.04 |
|                                |             |                |                 |          |          | 5.60 ± 0.46 |          |            |                |          | 0.576        |          |          | 0.615        |
|                                |             |                |                 |          |          | 0.268       |          |            |                |          | 0.336        |          |          | 0.355        |
| Placenta                        |             |                |                 |          |          | 0.020       |          |            |                |          | 0.006        |          |          | 0.053        |
|                                | 16:0        | 18:0           | 18:1 n-9        | 18:2 n-6   | 20:4 n-6  | 20:5 n-3 (EPA) | 22:6 n-3 (DHA) | SFA | MUFA | PUFA | n-6/n-3 | LC-PUFA n-6 | LC-PUFA n-3 |
|                                | 21.49 ± 0.28<sup>ab</sup> | 16.87 ± 0.23    | 9.85 ± 0.54     | 14.27 ± 0.52 | 16.50 ± 0.56<sup>a</sup> | 0.08 ± 0.01<sup>a</sup> | 4.55 ± 0.20 | 41.63 ± 0.19 | 14.11 ± 0.63 | 44.14 ± 0.62 | 7.21 ± 0.26 | 23.16 ± 0.94 | 5.20 ± 0.21 |
|                                |             |                |                 |          |          | 17.99 ± 0.35<sup>b</sup> |          |            | 13.70 ± 0.41 |          | 44.96 ± 0.37 |          |          | 15.74 ± 0.49 |
|                                |             |                |                 |          |          | 17.85 ± 0.35<sup>ab</sup> |          |            | 13.74 ± 0.21 |          | 44.37 ± 0.35 |          |          | 0.735        |
|                                |             |                |                 |          |          | 0.028       |          |            |                |          | 0.390        |          |          | 0.075        |
| Total foetus                    |             |                |                 |          |          | 0.020       |          |            |                |          | 0.020        |          |          | 0.236        |
|                                | 16:0        | 18:0           | 18:1 n-9        | 18:2 n-6   | 20:4 n-6  | 20:5 n-3 (EPA) | 22:6 n-3 (DHA) | SFA | MUFA | PUFA | n-6/n-3 | LC-PUFA n-6 | LC-PUFA n-3 |
|                                | 23.73 ± 0.43| 12.10 ± 0.18   | 14.82 ± 0.21    | 8.91 ± 0.49 | 14.83 ± 0.22 | 0.11 ± 0.01<sup>ab</sup> | 6.12 ± 0.13 | 38.25 ± 0.47 | 24.00 ± 0.30 | 37.53 ± 0.40 | 4.44 ± 0.12 | 21.30 ± 0.30 | 6.64 ± 0.13 |
|                                |             |                |                 |          |          | 14.76 ± 0.22 |          |            | 38.16 ± 0.30 |          | 37.69 ± 0.50 |          |          | 25.16 ± 0.29 |
|                                |             |                |                 |          |          | 1.70 ± 0.25 |          |            | 39.51 ± 0.58 |          | 36.49 ± 0.68 |          |          | 0.572        |
|                                |             |                |                 |          |          | 0.069       |          |            | 23.77 ± 0.37 |          | 25.44 ± 0.12 |          |          | 0.225        |
| Foetal brain                   |             |                |                 |          |          | 0.020       |          |            |                |          | 0.020        |          |          | 0.237        |
|                                | 16:0        | 18:0           | 18:1 n-9        | 18:2 n-6   | 20:4 n-6  | 20:5 n-3 (EPA) | 22:6 n-3 (DHA) | SFA | MUFA | PUFA | n-6/n-3 | LC-PUFA n-6 | LC-PUFA n-3 |
|                                | 29.97 ± 0.51| 15.08 ± 0.37   | 12.45 ± 0.44    | 1.33 ± 0.16 | 11.85 ± 1.82 | 0.09 ± 0.10 | 9.87 ± 0.35 | 45.77 ± 0.23 | 22.89 ± 1.94 | 31.11 ± 1.95 | 2.08 ± 0.24 | 19.34 ± 2.22 | 10.06 ± 0.42 |
|                                |             |                |                 |          |          | 14.62 ± 0.13 |          |            | 46.38 ± 0.47 |          | 30.04 ± 0.48 |          |          | 10.45 ± 0.30 |
|                                |             |                |                 |          |          | 11.92 ± 0.16 |          |            | 47.20 ± 0.71 |          | 30.01 ± 0.85 |          |          | 10.07 ± 0.41 |
|                                |             |                |                 |          |          | 0.124       |          |            | 14.80 ± 0.37 |          | 0.477        |          |          | 0.786        |

Results are expressed as means ± s.e.m. Only selected fatty acids are listed but all fatty acids analysed were considered for the calculation of percentages. Different superscript letters indicate significant differences between experimental groups (P < 0.05) (bold face).

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GDM, gestational diabetes mellitus; GDM + Adi, animals with gestational diabetes mellitus treated with AdipoRon; LC-PUFA, long-chain polyunsaturated fatty acids (>18 carbons); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
and lower activation of gluconeogenesis pathways in the liver (e.g. glucose-6-phosphatase) of animals treated with AdipoRon (Okada-Iwabu et al. 2013). Low levels of serum adiponectin are negatively and independently associated with metabolic syndrome-related disorders such as insulin resistance, diabetes, and dyslipidaemia (Kishida et al. 2012). Thus, administration of AdipoRon during gestation may be a useful strategy to improve glycaemic control in GDM subjects, reducing the risk of later glucose intolerance and insulin resistance in the offspring (Scholtens et al. 2019).

Diabetes can result in intrauterine growth restriction in animals. STZ-induced diabetes in rats can be associated with mild diabetes and macrosomia or with insulin-deficient diabetes and foetal growth restriction, the latter being the more common situation (Eriksson et al. 1984, Canavan & Goldspink 1988, Holmans et al. 1997, Ergaz et al. 2005). Despite intrauterine growth restriction, GDM foetus showed higher adiposity than controls, which tried to be mitigated by AdipoRon treatment. As no differences were detected in FA carriers expression in placenta, changes in foetal adiposity could be driven by maternal glucose transfer. There are some evidences of increased glucose transporters expression in diabetes-complicated pregnancies (Stanirowski et al. 2018). The independent-insulin glucose transporter 1 (GLUT-1) may be responsible...
for excessive glucose transfer and acceleration of foetal adiposity in the GDM foetus. Aye and colleagues reported reduced insulin receptors activity (IR-β and IRS-1), p-AKT, and mTORC1 activity in the placenta of obese pregnant mice after adiponectin administration while no differences in ERK expression (Aye et al. 2015). mTORC1 is a placental nutrient sensor activated by insulin that promotes nutrient transport across the placenta (especially amino acids) contributing to foetal growth (Jansson et al. 2012, Dimasuay et al. 2016). We were not able to quantify placental mTORC1 because of technical problems, but we did not find differences in p-AKT or ERK expression (Supplementary Fig. 1). Thus, AdipoRon may serve as a therapeutic treatment against negative foetal outcomes of GDM related to foetal adiposity. Growth-restricted foetus (GDM and GDM+ADI) showed higher levels of serum LDL cholesterol in adulthood (3 months old) while similar concentrations were observed for total and HDL cholesterol. Tenhola et al. (2000) reported negative associations between birthweight of small for gestational age infants and later total cholesterol concentration in blood during childhood, supporting the relevance of foetal nutrition to adult disease (Ornoy 2011).

Our group previously reported impaired placental transfer of 13C-DHA in GDM women compared to healthy women (Pagan et al. 2013). MFSD2a is postulated as one of the major transporters of Lyso-DHA across the blood–brain barrier (Nguyen et al. 2014) and also in the placenta (Prieto-Sanchez et al. 2017). In the present study, no differences were observed in MFSD2a expression in the placenta or DHA in the total foetus. However, DHA percentage was estimated in the whole foetus and not in cord blood, which could have mask differences in FA foetal plasma profile.

We demonstrated for the first time that GDM+ADI offspring presented an improved response to glucose overload than GDM offspring, indicating the effectiveness of the maternal treatment with AdipoRon on the long-term glucose tolerance in the next generation. However, it was surprising that GDM offspring showed better glucose tolerance than GDM+ADI offspring at 30 min post-challenge, which may be related to an alteration in gastric emptying (Goyal et al. 2019, Wu et al. 2020). Impaired insulin response and altered pancreatic β-cell function are thought to be responsible for the transgenerational transmission of GDM (Aerts & van Assche 2006). The use of AdipoRon in the treatment of GDM may lead to a lower

| Fatty acids (g/100 g fatty acids) | GDM (n = 16) | GDM+ADI (n = 14) | Control (n = 11) | P       |
|----------------------------------|-------------|------------------|-----------------|---------|
| 16:0                             | 22.74 ± 0.56 | 22.58 ± 0.65     | 20.75 ± 0.77    | 0.383   |
| 18:0                             | 13.77 ± 0.63 | 15.54 ± 0.78     | 15.82 ± 0.79    | 0.528   |
| 18:1 n-9                         | 9.16 ± 0.43  | 5.95 ± 0.33      | 10.63 ± 0.53    | <0.001  |
| 18:2 n-6                         | 15.36 ± 0.77 | 12.02 ± 0.71     | 11.80 ± 1.01    | 0.184   |
| 20:4 n-6                         | 29.87 ± 1.02 | 29.81 ± 0.70     | 27.47 ± 1.33    | 0.045   |
| SFA                              | 38.60 ± 0.52 | 43.04 ± 0.82     | 39.93 ± 0.66    | 0.007   |
| MUFA                             | 12.47 ± 0.66 | 9.38 ± 0.47      | 15.45 ± 0.86    | <0.001  |
| PUFA                             | 48.92 ± 0.60 | 47.53 ± 0.62     | 44.61 ± 1.45    | 0.001   |
| LC-PUFA n-6                      | 30.00 ± 0.98 | 30.97 ± 0.71     | 28.09 ± 1.25    | 0.038   |
| LC-PUFA n-3                      | 3.20 ± 0.19  | 3.67 ± 0.18      | 4.27 ± 0.39     | 0.110   |
| Brain                            |             |                  |                 |         |
| 16:0                             | 17.69 ± 0.14 | 17.91 ± 0.11     | 17.79 ± 0.12    | 0.464   |
| 18:0                             | 19.38 ± 0.08 | 19.87 ± 0.17     | 19.66 ± 0.10    | 0.150   |
| 18:1 n-9                         | 16.04 ± 0.17 | 15.79 ± 0.12     | 16.34 ± 0.10    | 0.119   |
| 18:2 n-6                         | 0.72 ± 0.02  | 0.70 ± 0.02      | 0.72 ± 0.02     | 0.819   |
| 20:4 n-6                         | 10.63 ± 0.11 | 10.56 ± 0.07     | 10.56 ± 0.11    | 0.973   |
| 22:6 n-3 (DHA)                   | 17.18 ± 0.17 | 17.36 ± 0.15     | 17.15 ± 0.16    | 0.694   |
| SFA                              | 40.27 ± 0.13 | 41.17 ± 0.23     | 40.70 ± 0.12    | 0.037   |
| MUFA                             | 25.21 ± 0.31 | 24.22 ± 0.24     | 24.79 ± 0.31    | 0.120   |
| PUFA                             | 34.42 ± 0.22 | 34.55 ± 0.17     | 34.36 ± 0.20    | 0.596   |
| n-6/n-3                          | 0.90 ± 0.01  | 0.87 ± 0.01      | 0.90 ± 0.01     | 0.337   |
| LC-PUFA n-6                      | 15.50 ± 0.14 | 15.38 ± 0.11     | 15.50 ± 0.14    | 0.820   |
| LC-PUFA n-3                      | 17.47 ± 0.16 | 17.67 ± 0.15     | 17.44 ± 0.16    | 0.623   |

Results are expressed as means ± s.e.m. 3 months old offspring from 3 to 4 pregnant rats per group. Only selected fatty acids are listed but all fatty acids analysed were considered for the calculation of percentages. GDM n = 16 (8 males/8 females); GDM + ADI n = 14 (6 males/8 females); Control n = 11 (6 males/5 females).

Different superscript letters indicate significant differences between experimental groups (P < 0.05) (bold face).

DHA, docosahexaenoic acid; GDM, gestational diabetes mellitus; GDM + ADI, animals with gestational diabetes mellitus treated with AdipoRon; LC-PUFA, long-chain polyunsaturated fatty acids (>18 carbons); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
incidence of metabolic syndrome and diabetes in future generations. Our results in pregnant rats agree with those recently published by Scholten et al. (2019) which showed, across the maternal glucose spectrum, associations between the exposure to hyperglycaemia in utero and elevated glucose values and insulin resistance during childhood (HAPO follow-up cohort). In summary, both studies corroborate the long-term health consequences of GDM on the FA in the offspring. AdipoRon administration (Okada-Iwabu et al. 2013). Oxidative stress status in kidneys of adult offspring was also reduced by AdipoRon treatment (higher TAA and lower nitrotyrosine). Nevertheless, no significant differences were observed in plasma TAA or urinary eicosanoids. Several studies have recently reported a reduction in renal oxidative stress, apoptosis, lipotoxicity, and inflammation after AdipoRon administration in animal models, which could be especially beneficial in the pathophysiology of diabetic nephropathy and other related disorders (Choi et al. 2018, Xiong et al. 2020, Qiu et al. 2021). This is probably mediated by activating the AMPK pathway (Kim et al. 2018, Gu et al. 2020). Thus, AdipoRon seems to promote positive effects on antioxidant and immune systems that could protect against the renal injury associated to diabetes and other pathologies in long term.

DHA was strongly reduced in plasma of GDM and GDM + ADI adult offspring. Reduced DHA status has been extensively described in the cord blood of GDM women of GDM + ADI but was effective to reduce the unbalanced
n-6/n-3 PUFA ratio observed in GDM offspring. In the present study, we cannot discard that this situation of lower DHA in GDM offspring was likely established already at delivery since cord blood samples were not available. Zhao et al. showed associations between low circulating DHA levels and compromised foetal insulin sensitivity, and this may be involved in the early programming of the susceptibility to type 2 diabetes in offspring of GDM women (Zhao et al. 2014). The AdipoRon administration during pregnancy may also have additional effects in the offspring under different metabolic challenges (e.g. high-fat diet) and this should be explored in future studies.

The strengths of the present study are the administration of the first time of the novel adiponectin agonist AdipoRon in pregnant diabetic animals and the evaluation of its effect for the offspring at both birth and adult state. Our study also presents some limitations since the animal model of diabetes induced by STZ used has some physiopathological differences with human GDM that may include insulin treatment requirement, absence of increased insulin resistance, or foetal growth restriction among others. We cannot exclude that these physiological differences in the rat model could have biased the results reported and the extrapolation to the human GDM condition.

Conclusions

In conclusion, AdipoRon administration to pregnant diabetic rats led to improved maternal glycaemia and reduced foetal adiposity associated with GDM. At the adult state, GDM offspring presented impaired glucose response compared to control, and it was counteracted by AdipoRon treatment. In addition, AdipoRon improved the plasma n-6/n-3 ratio in the offspring and showed some positive effects on antioxidant and inflammatory status. Adiporon seems to be a promising therapeutic drug for the control of glycaemia in the diabetic mother and the offspring in long term.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0617.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

The datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Author contribution statement

A G, F R, and E L designed and conducted the study, performed experiments, and wrote the manuscript. A G, F R, M S-C, L E M-G, and M D A-O performed laboratory analyses. P S-G and M B A acquired data. A G, F R, M S-C, M B A, and E L interpreted data. All authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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