Cardiac and placental mitochondrial characterization in a rabbit model of intrauterine growth restriction

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Running head: Mitochondrial and cardiovascular hallmarks of IUGR

ABSTRACT

Background: Intrauterine growth restriction (IUGR) is associated with cardiovascular remodeling persisting into adulthood. Mitochondrial bioenergetics, essential for embryonic development and cardiovascular function, are regulated by nuclear effectors as sirtuins. A rabbit model of IUGR and cardiovascular remodeling was generated, in which heart mitochondrial alterations were observed by microscopic and transcriptomic analysis. We aimed to evaluate if such alterations are translated at a functional mitochondrial level to establish the ethiopathology and potential therapeutic targets for this obstetric complication.

Methods: Hearts and placentas from 16 IUGR-offspring and 14 controls were included to characterize mitochondrial function.

Results: Enzymatic activities of complexes II, IV and II+III in IUGR-hearts (-11.96±3.16%; -15.58±5.32%; -14.73±4.37%; $p<0.05$) and II and II+III in IUGR-placentas (-17.22±3.46%; $p<0.005$ and -29.64±4.43%; $p<0.001$) significantly decreased. This was accompanied by a not significant reduction in CI-stimulated oxygen consumption and significantly decreased complex II SDHB subunit expression in placenta (-44.12±5.88%; $p<0.001$). Levels of mitochondrial content, Coenzyme Q and cellular ATP were conserved. Lipid peroxidation significantly decreased in IUGR-hearts (-39.02±4.35%; $p<0.001$), but not significantly increased in IUGR-placentas. Sirtuin3 protein expression significantly increased in IUGR-hearts (84.21±31.58%; $p<0.05$) despite conserved anti-oxidant SOD2 protein expression and activity in both tissues.

Conclusions: IUGR is associated with cardiac and placental mitochondrial CII dysfunction. Up-regulated expression of Sirtuin3 may explain attenuation of cardiac oxidative damage and preserved ATP levels under CII deficiency.

General significance: These findings may allow the design of dietary interventions to modulate Sirtuin3 expression and consequent regulation of mitochondrial imbalance associated with IUGR and derived cardiovascular remodeling.

KEYWORDS

Cardiovascular function, mitochondrial dysfunction, sirtuin3, rabbit animal model, fetal growth, mitochondrial complex II.

1. INTRODUCTION

Intrauterine growth restriction (IUGR) refers to fetuses that do not reach their predefined genetic potential weight and is usually defined as fetuses with an estimated fetal and birth weight under the 10th percentile for gestational age (1, 2). IUGR is a major cause of perinatal mortality and long-term morbidity (3). The etiopathology of this complication remains unknown, although there is evidence suggesting that placental dysfunction followed by hypoxia could lead to IUGR by interference with molecular and bioenergetic processes (3, 4). Placenta is crucial in fetal programming in that, for example, changes in the pattern of the substrates or oxygen transported to the fetus can ultimately lead to cardiovascular or metabolic disease (5-7). Indeed, it is well known that IUGR is associated with cardiovascular remodeling and dysfunction, persisting into adulthood (8-11). Establishing the molecular mechanisms of cardiovascular remodeling in IUGR newborns is hampered by the difficulty of accessing the heart as a target tissue. For this reason, our group developed a rabbit model to study IUGR in which cardiovascular remodeling with altered spatial arrangement of intracellular energetic units was evidenced by microscopy in the offspring (12, 13), also reflecting the hemodynamic alterations found in newborns of IUGR pregnancies. The global gene expression profile of the hearts of offspring in this IUGR-rabbit model showed alterations in different pathways, all of which converged to mitochondria, including oxygen homeostasis, mitochondrial respiratory chain (MRC) complex I, oxidative phosphorylation and NADH dehydrogenase activity (10, 14).

Alternative animal models of IUGR have been developed consisting in carunclectomy, uterine artery ligation, uterine space restriction, caloric restriction or hypoxic conditions, among other procedures, in different species of animals (mainly sheep, pigs or rats models) (15-26). Heart development is different among species (in structure, timing of maturation, morphology, function, etc.). However, cardiac remodeling have been demonstrated in most of these models (16, 18-20, 22, 27), as well as mitochondrial alterations (24, 25, 28).

It is important to note that mitochondrial alterations consisting of suboptimal oxygen consumption (29), as well as altered transcriptomic profile of genes involved in mitochondrial function, oxidative phosphorylation and complexes of MRC, and thus energy production and metabolism (30), have been reported in IUGR patients. However, these findings have never been demonstrated in IUGR-hearts, in which depth characterization of mitochondrial function has not been performed to date.

Alterations in mitochondrial and metabolic pathways are critical in fetal programming within the setting of placental insufficiency (31). Proteins regulating these crucial processes, such as sirtuins, may finally modulate the development of obstetric manifestations and associated complications. Sirtuins are a family of cellular sensors exerting a deacetylase function which regulates cell bioenergetics by synchronizing nuclear and mitochondrial activities (32-35). Despite evidence of deregulation of sirtuins in skeletal muscle of pigs with spontaneous IUGR (36), little is known about their implication in this obstetric complication. Interestingly, growing evidence supports sirtuins involvement in cardiac disease, independent of IUGR, either by modulation of bioenergetic shift and cardiomyocyte survival (37).

The development of this study in heart and placenta of a validated IUGR animal model facilitated the evaluation of potential disturbances in tissues responsible of cardiovascular remodeling and placental insufficiency. Our hypothesis is that IUGR could be associated with mitochondrial alterations potentially leading to dysfunction of cardiomyocytes and placental metabolic adaptations to hypoxia. We have therefore analyzed whether the transcriptomic and ultrastructural alterations previously reported in cardiomyocytes from offspring of the rabbit model of IUGR with associated cardiovascular remodeling are translated to mitochondrial dysfunction in the same cell type. Second, we evaluated whether heart alterations were also present in the placental tissue, limiting oxygen and nutrient supply. Finally, we aimed to determine the molecular mechanisms responsible for downstream cell adaptations to mitochondrial imbalance, including sirtuins, in order to establish potential therapeutic targets to prevent or revert the development of this obstetric complication and associated cardiovascular remodeling.

2. MATERIALS AND METHODS

2.1 Animal model

Six New Zealand white pregnant rabbits were used to obtain 16 IUGR and 14 control offspring by reproducing a model of IUGR previously reported (see TableS1) (12, 13). This model was based on the selective ligature of uteroplacental vessels to reduce 40 to 50% of oxygen and nutrient supply into the fetuses in development. The ligation was performed only in one of the two uterine horns in order to obtain, in the same pregnancy, the IUGR (from the manipulated horn) and control (from the non-manipulated horn) offspring. Concretely, pregnant animals were fed with standard diet and water ad libitum, with 12h/12h of light cycle. At day 25 of gestation, in each pregnant rabbit, the selective ligature of uteroplacental vessels in only one of the two uterine horns was performed. Briefly, tocolysis (progesterone 0.9mg/kg im) and antiobiotic prophylaxis (Penicillin G 300.000 UI iv) were administered before uteroplacental vessel surgery. Ketamine (35 mg/kg) and xylazine (5 mg/kg) were given intramuscularly for anesthesia induction. Inhaled anesthesia was maintained with a mixture of 1-5% isoflurane and 1-1.5 l/min oxygen. After a midline laparotomy, both uterine horns were exteriorized but only one was ligated to reproduce IUGR. At day 30 (full-term pregnancy), a cesarean section was proceeded to obtain both IUGR and control offspring from the same pregnancy (all six pregnant rabbits with the same gestational age) (12, 13). After the procedure, the abdomen was closed and animals received intramuscular meloxicam 0.4 mg·kg_1·24 h_1·48 h, as postoperative analgesia. Offspring (from the uterine horn experimentally modified) weighting under the 10th percentile of birth weight were considered IUGR (cutoff less than 60 grams) and never weighting higher than any control from the not modified uterine horn and from the same nest. All newborn rabbits were sacrificed by decapitation and the hearts of newborn rabbits were removed from the chest cavity and were then weighed and preserved with Biops Medium (2.77mM CaK_2EGTA, 7.23mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_20$, 20mM Taurine, 15mM Na_2Phosphocreatine, 20mM Imidazole, 0.5mM Dithiothreitol and 50mM MES, pH 7.1) on ice. Likewise, the placentas of these newborn rabbits were identified, weighed and preserved with Biops Medium (prepared as mentioned previously), on ice.

All biometric parameters were measured once, following standardized protocols (12, 13).

Animal handling and all the procedures were performed in accordance to the prevailing regulations and guidelines (38-40) and with the approval of the Animal Experimental Ethics Committee of the University of Barcelona (Barcelona, Spain).

2.2 Sample processing

Left ventricle was used for mitochondrial studies because is the target tissue in which previous transcriptomic and ultrastructural alterations were observed. Additionally, is the tissue were cardiomyopathies are preferentially manifested. Each left ventricle and placenta tissue were processed as follows: a piece of each tissue was maintained in fresh conditions with Biops Medium to assess mitochondrial oxygen consumption, and the remaining tissue was cryopreserved at -80ºC and further homogenized (Caframo technologies, Ontario, Canada) at 5% (w/v) in mannitol buffer for mitochondrial analysis. The protein content was quantified in left ventricle heart and placental homogenates using the bicinchoninic acid colorimetric assay (Thermo Scientific assay kit Prod #23225, Waltham, MA, USA) to normalize experimental measures.

2.3 MRC activity and mitochondrial content in heart and placenta

In order to study MRC function, the enzymatic activities of mitochondrial complex I, II, IV, I+III and II+III (CI, CII, CIV, CI+III and CII+III) in both heart and placental homogenates were spectrophotometrically measured at 37ºC, as reported elsewhere (41).

Citrate synthase activity was also spectrophotometrically determined in heart and placenta at 37ºC (41, 42), as it is considered a reliable marker of mitochondrial content (43). Mitochondrial content was further confirmed by alternative methods (see 2.8.2 section).

All enzymatic assays consisted of national standardized methods run in parallel with internal quality controls (41) and all the enzymatic assays, measured once per sample, simultaneously included both cases and controls .

Absorbance changes of the enzymatic activities along time were monitored in a HITACHI U2900 spectrophotometer using the UV-Solution software version 2.2 and were expressed as nanomoles of consumed substrate or generated product per minute and milligram of protein (nmol/minute·mg protein).

2.4 Mitochondrial oxygen consumption in heart and placenta

To determine oxygen consumption of heart and placental tissue from IUGR and control offspring, 3-5 miligrams of each tissue were permeabilized on ice with 5% (w/v) saponine for 30 minutes in Biops Medium. This permeabilized tissue was washed with cold respiration medium (Mir05: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 0.1% (w/v) bovine serum albumin, pH 7.1). High-resolution respirometry was performed at 37°C by polarographic oxygen sensors in a two-chamber Oxygraph-2k system according to the manufacturer's instructions (OROBOROS Instruments, Innsbruck, Austria). Manual titration of substrates and inhibitors was performed using Hamilton syringes (Hamilton Company, Reno, NV, USA). Data was recorded using the DatLab software v5.1.1.9 (Oroboros Instruments, Innsbruck, Austria).

Glutamate and malate oxidation (GM Oxidation), corresponding to electron donation of both substrates to mitochondrial CI, was quantified once in all samples. Specific oxygen uptake rates sensitive to antimycin a (which specifically inhibits mitochondrial oxygen consumtion) were obtained following the manufacture's recommendations (44).

Oxygen consumption was normalized for the milligrams of dry tissue, thus, results were expressed as picomoles of oxygen consumed per second and milligram of tissue (pmol O2/s·mg).

2.5 Mitochondrial coenzyme Q (CoQ) content in heart and placenta

Tissue levels of CoQ9 or CoQ10 (mobile electron transfer located within CI, CII and CIII in the MRC) were assessed in duplicates in the heart and placental homogenates from both cases and controls by high pressure liquid chromatography (HPLC in reverse form) with electrochemical detection of the reduced and oxidized molecule, as described previously (45). Values were expressed as micromoles per liter (μmol/L).

2.6 Total cellular ATP levels in heart and placenta

Cellular ATP levels were quantified in duplicates in the heart and placental homogenates from both cases and controls using the Luminescent ATP Detection Assay Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. The results were normalized for protein content and expressed as picomolar of ATP per milligram of protein (pmol ATP/mg protein).

2.7 Lipid peroxidation in heart and placenta

Lipid peroxidation was measured in duplicates as an indicator of oxidative damage to lipid membranes of hearts and placentas using the BIOXYTECH® LPO-586™ assay by spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) levels (both peroxides derived from fatty acid oxidation), according to the manufacturer's instructions (Oxis International Inc., CA, USA). The results were normalized for protein content and expressed as micromolar of MDA and HAE per milligram of protein (μM MDA+HAE/mg protein).

2.8 Western Blot analysis

Twenty to forty μg of total protein homogenate of heart and placenta from both cases and controls were separated using 7/13% SDS-PAGE and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, Life Technologies, Waltham, MA, USA). The membranes were hybridized with specific antibodies overnight at 4ºC. The expression of all studied proteins was measured once and normalized to β-actin protein (47kDa; 1:30.000; Sigma-Aldrich, St.Louis, MO, USA) which was used as a loading control. Inter-blot control samples were used to evaluate membrane variability. The ImageQuantLD program was used to quantify chemiluminiscence.

2.8.1Expression of subunits of the MRC complexes in heart and placenta

To determine the levels of protein expression of the subunits of the MRC complexes the cleared lysates were subjected to SDS-PAGE and electroblotted. Proteins were visualized by immunostaining with anti-SDHA and anti-SDHB (both for CII; 70kDa and 30kDa respectively; 1:1000; Invitrogen, Paisley, UK) and also with anti-COX5A (for CIV; 16kDa; 1:1000; MitoSciences, Oregon, USA). Results were expressed as SDHA/β-actin, SDHB/β-actin and COX5A /β-actin ratios.

2.8.2 Expression of mitochondrial import receptor subunit TOM20 (Tom20) in heart and placenta

As a mitochondrial content marker, anti-Tom20 (20kDa; 1:1000; Santa Cruz Biotechnology, Dallas, USA) was hybridized with membranes. Results were expressed as the Tom20/β-actin ratio.

2.8.3 Expression of superoxid dismutase 2 (SOD2) in heart and placenta

SOD2 is a mitochondrial anti-oxidant enzyme pivotal in ROS release during oxidative stress. Membranes were hybridized with anti-SOD2 (24kDa; 1:1000; ThermoFisher Scientific, Waltham, MA, USA). Results were expressed as the SOD2/β-actin ratio.

2.8.4 Expression of the acetylated form of SOD2 in heart and placenta

In order to determine the activity of SOD2 enzyme, we evaluate its acetylated form (indicating less activity) by hybridizing the membranes with anti-SOD2/MnSOD (24kDa; 1:1000; Abcam, Cambridge, UK). Results were expressed as the acetylated SOD2/β-actin or acetylated/total SOD2 ratio.

2.8.5 Expression of Sirtuin3 in heart

The protein content of Sirtuin3, which is a sensor of mitochondrial and metabolic balance, was determined by hybridizing the membrane with anti-Sirtuin3 (29 KDa; 1:500; Abcam, Cambridge, UK). Results were expressed as the Sirtuin3/β-actin ratio.

2.9 Statistical analysis

Statistical analysis was performed with the 'IBM SPSS Statistics 20' and STATA software. Biometric and experimental results were expressed as means and standard error of the mean (SEM) or as a percentage of increase/decrease of IUGR-offspring compared to control offspring after filtering for outliers. Case-control differences were sought by non-parametric statistical analysis (Mann–Whitney independent sample test) and, in case of difference, significance was adjusted by maternal influence (Random Effect regression model). Additionally, different correlations were obtained between biometric features and experimental data using the Spearman test in order to assess dependence of biometric measures in mitochondrial function or vice versa. Differences were considered significant with a p value <0.05.

3. RESULTS

3.1 Biometric offspring data

The biometric results of both the IUGR and control offspring of the rabbit model are shown in Figure 1 and Table S2. Birth weight, heart weight, left and right ventricle weight and placental weight were significantly decreased in IUGR-offspring compared to controls (-30.35±2.99%, $p<0.001$; -29.73±2.70%, $p<0.001$; -30.00±0.00%, $p<0.005$; -36.36±9.09%, $p<0.001$; -21.49±4.85%, $p<0.001$, respectively). When cardiac or placental weights were normalized to body weight, no significant differences were evidenced between IUGR and control offspring.

3.2 MRC activity, MRC expression and mitochondrial content

A significant decrease of CII, CIV and CII+III enzymatic activities (-11.96±3.16%, -15.58±5.32% and -14.73±4.37%, respectively; $p<0.05$ in all cases) was found in heart of IUGR-offspring compared to controls, while other complexes (CI and CI+III) also showed a decrease, although not significant (Fig.2A). The same pattern was observed in placenta, although the decrease in CIV did not reach statistical significance in IUGR-offspring (CII: -17.22±3.46%, $p<0.005$; CIV: -24.03±8.26%, p=NS; CII+III: -29.64±4.43%, $p<0.001$; Fig.2A). MRC enzymatic activities relative to citrate synthase activity confirmed absolute MRC enzymatic activities reduction. All of the raw data are presented in Table S3.

MRC CII subunits SDHA and SDHB and CIV subunit COX5A were conserved in cardiac tissue of IUGR-offspring compared to controls. Interestingly, regardless maintained expression of CII SDHA and CIV COX5A subunits in placental tissue, MRC CII SDHB subunit was significantly decreased (-44.12±5.88%; $p<0.001$) in IUGR-offspring compared to controls (Fig.3 and Table S3).

Citrate synthase activity in both heart and placental tissues showed preserved mitochondrial content in IUGR-offspring compared to controls (Fig.2A and Table S3). These results were confirmed by conserved Tom20 expression in these samples (Figure S1and Table S3).

3.3 Mitochondrial oxygen consumption

We found a not significant decrease of oxygen consumption on CI stimulation with glutamate and malate (GM oxidation) in heart and placenta from IUGR-offspring compared to controls (-5.56±6.46% and -25.64±18.97%, respectively, both p=NS, Fig.2B and Table S3).

3.4 Mitochondrial CoQ content

No differences were observed in CoQ9 or CoQ10 content in either cardiac or placental tissue of IUGR-offspring with respect to control individuals (Table S3).

3.5 Total cellular ATP levels

No remarkable differences were observed in total content of cellular ATP in either heart or placental tissues in IUGR and control offspring (Fig.2D and Table S3).

3.6 Oxidative damage

Oxidative damage, estimated by the rate of lipid peroxidation, showed a significant decrease of 39.02±4.35% in hearts of IUGR-offspring compared to controls ($p<0.001$; Fig.2C). Contrarily, lipid peroxidation was 10.65±7.33% increased, albeit not significantly, in placenta from IUGR-offspring compared to the control group (p=NS; Fig.2C and Table S3).

3.7 Expression and activity of SOD2

No significant differences were observed in the protein content and activity of the anti-oxidant SOD2 enzyme between cases and controls in none of the studied tissues (Figures S2 and S3 and Table S3; either total SOD2/β-actin content or acetylated SOD2/β-actin). Only acetylated/total SOD2 ratio showed trends to increase in IUGR-offspring, both in heart and placental tissues (Table S3).

3.8 Expression of Sirtuin3

In heart tissue of IUGR-offspring, Sirtuin3/β-actin protein levels showed a significant increase of 84.21±31.58% ($p<0.05$) compared to hearts from controls (Fig.4 and Table S3).

3.9 Associations between biometric features and experimental results

Table 1 describes all the significant associations between the biometric data and experimental results in the cohorts of IUGR and control offspring from the rabbit model. The most remarkable associations are described below and showed in Fig.5.

Firstly, birth weight was positively and significantly correlated with heart and left ventricle weight and also with placental weight ($R^2=0.610$, $R^2=0.446$ and $R^2=0.557$, respectively, $p<0.001$ in all cases; Fig.5A

and B). Similarly, heart weight was correlated with left ventricle weight and placental weight (R^2=0.322, $p<0.005$; R^2=0.346, $p<0.001$). See Table 1 for other biometric associations.

Secondly, birth weight was positively and significantly correlated with enzymatic activities of CI, CII and CII+III in the heart of the both offspring (R^2=0.157, $p<0.05$; R^2=0.117, $p<0.05$; R^2=0.289, $p\leq0.005$; respectively; Fig.5C). The weight of the other tissues was also positively and significantly correlated with the enzymatic activity of some MRC complexes (Table 1). Similarly, body, heart, left ventricle and placental weights were significantly and positively correlated with CII SDHB protein expression in placenta (R^2=0.304, $p\leq0.001$; R^2=0.349, $p<0.001$; R^2=0.215, $p<0.005$; R^2=0.364, $p<0.05$, respectively; Fig.5D). Interestingly, we found CII, CII+III and CIV enzymatic activity positively and significantly correlated with CII SDHB subunit expression in placenta (R^2=0.145, $p<0.005$; R^2=0.192, $p<0.005$; R^2=0.241, $p<0.001$, respectively). On the other hand, birth weight and heart weight showed a significant positive correlation with lipid peroxidation in heart (R^2=0.325, $p<0.005$; R^2=0.498, $p<0.001$; respectively; Fig.5E). Moreover, oxidative damage in heart was positively and significantly correlated with the enzymatic activities of MRC CII and CII+III (R^2=0.136 and R^2=0.173, $p<0.05$ in both cases).

Finally, significant negative correlations were found between both birth and heart weight and Sirtuin3/β-actin levels (R^2=0.153, $p<0.05$ and R^2=0.266, $p<0.05$, respectively; Fig.5F).

4. DISCUSSION

A wide range of clinical manifestations has been associated with IUGR, including metabolic, neurological and cardiovascular disorders (46-51). Among these, fetal cardiovascular remodeling has recently been demonstrated in IUGR (8, 11, 14, 16, 18-20, 22, 27). However, disease modeling and investigation in cardiac or placental target tissues are lacking.

It is important to note that in the present study, offspring with induced IUGR showed altered biometric measures by significant decrease in birth weight, accompanied by a reduction in heart and placenta, as previously reported (14). Thus, organ to body weight measures were preserved due to global and proportioned organ and body mass reduction. Cardiac hypertrophy and consequent increase in heart to body weight is not present in IUGR rabbits, resembling human conditions, in which globular and elongated hearts are usual, and hypertrophy occurs only in 17% of cases of IUGR (13, 47, 52-58).

Cardiac metabolic and mitochondrial impairment has been previously demonstrated in the present animal model at a transcriptional and ultrastructural level (14). However, the bioenergetic functional imbalance has not yet been demonstrated in the target tissue of cardiovascular remodeling (heart) or in the tissue responsible for oxygen and nutrient supply (placenta), and neither have the potential causal agents or downstream cell consequences for this imbalance been described.

The present study provides evidence of mitochondrial deficiency in the cardiac tissue of IUGR-rabbits, with a significant decrease of enzymatic activities of MRC CII, CIV and CII+III. The same pattern was observed in IUGR-placentas regarding CII and CII+III. Interestingly, CII SDHB protein expression showed a significant decrease in placental tissue in front of preserved mitochondrial content, CoQ levels and ATP content. Lipid peroxidation was found to be significantly decreased in cardiomyocytes but showed a not significant increase in placental tissue. All these mitochondrial functional deficiencies validate previous transcriptomic and ultrastructural findings (14).

Interestingly, birth weight, placental and left ventricle weight were significantly and positively correlated with the enzymatic MRC activities (including CI, CII, CII+III and CIV), thereby strengthening the relevance of the need for adequate bioenergetic mitochondrial status to reach potential body, heart and placental weight.

In this sense, mitochondrial functional alterations in IUGR-offspring are not confined to the target tissue of cardiovascular remodeling (heart), but rather are also present in placenta. Mitochondrial dysfunction in placenta (and probably also in heart and other tissues) might be caused by the *de novo* rearrangement adaptations imposed by the experimental hypoxia induced by the ligature of uteroplacental vessels in this animal model (6). Similar hypoxic conditions are also present in patients with IUGR due to placental insufficiency. These are undoubtedly examples of the fetal programing, first described by Barker (5), which establishes that physiologic adaptations to the fetal environment may condition organ development and consequent disease in adulthood.

Both heart and placenta from IUGR-offspring showed a common enzymatic alteration in MRC CII dysfunction. CII, also known as succinate dehydrogenase, is the molecular link between the Krebs cycle and the MRC. The Krebs cycle is a central pathway of the mitochondrial energy metabolism, which is responsible for the oxidative degradation of the different dietetic supplies, feeding the MRC and, subsequently, activating ATP synthesis. In order to further investigate the molecular basis of CII dysfunction in heart and placenta of IUGR-offspring, the expression of CII SDHA and SDHB proteins were assessed, together with CoQ levels, the electron donor for CII. Interestingly, in front of preserved CoQ levels, SDHB subunit was found significantly decreased in placenta and positively and significantly correlated with CII, CII+III and CIV enzymatic activities. These findings suggest that MRC enzymatic dysfunction may be due, at least partially, to down-regulation of MRC subunits expression. Additionally, the significant and positive correlation between CII SDHB levels and all biometric parameters from IUGR and control offspring (body, placental and heart weights), highlighted the relevance of SDHB and CII in this obstetric complication. As CII is the only complex of the MRC exclusively encoded by the nuclear DNA (59), its deficiency may be more likely associated with alterations in the oxidative metabolism events controlled by the nucleus, rather than a regulation associated with mitochondrial genome.

Sirtuin3 is the most important mitochondrial deacetylase encoded in the nucleus and plays a role in the regulation of mitochondrial function and oxidative phosphorylation (60-62). Interestingly, since mitochondrial CII is one of the targets of Sirtuin3 (60, 63), this protein is able to simultaneously interfere with MRC and the Krebs cycle. In our study, the significant increase of cardiac Sirtuin3 observed in IUGR-offspring was associated with a significant decrease of CII enzymatic activity in both heart and placental tissues. This up-regulation in Sirtuin3 expression may be the compensatory response to

imbalanced bioenergetics status. In accordance with these findings obtained in IUGR-offspring with placental hypoxia, environmental hypoxia has also been associated with alterations in the Krebs cycle (64), which may also lead to neurological or cardiac clinical consequences (65).

Additionally, Sirtuin3 has been reported to modulate the induction of hypertrophy in heart (34) and it has been proposed that this protein has a role in antioxidation, promoting the maintenance of ATP levels, especially in hypoxic conditions (66, 67). Interestingly, in heart from IUGR-offspring, oxidative stress was found to be significantly decreased in the setting of conserved cellular ATP levels, suggesting that increased Sirtuin3 levels may protect cardiomyocytes from oxidative stress insults in placental hypoxia. Sirtuin3 would exert a compensatory role in this phenotype by modulating mitochondrial lesion in cardiomyocytes. Moreover, the presence of cardiac oxidative damage increased according to body weight, heart weight and proper MRC activity, suggesting that the antioxidant protection of Sirtuin3 may be more necessary in MRC dysfunction and abnormal fetal development. This hypothesis seems to be confirmed by the significant negative correlation of body and heart weight with cardiac Sirtuin3 levels. In parallel, cellular ATP levels were also maintained in placental tissue, albeit with a not significant increased lipid peroxidation. This juxtaposed oxidative phenotype between heart and placenta may also be explained by the poor antioxidant defenses characteristic of the placenta (68, 69). Interestingly, CoQ levels and SOD2 expression, that take part in the antioxidant defense system, did not seem to play a role in the mitochondrial and oxidative phenotype observed in either cardiac or placental tissue. Actually, trends to higher inactivation of SOD2 (acetylated vs. total SOD2 ratio) were noticed in IUGR-offspring that, together with up-regulated expression of Sirtuin 3, have been previously associated with aging (35). Nonetheless, further research is needed to address this topic and deeper in the potential association between increased Sirtuin 3 expression and observed dysfunctional mitochondrial phenotype.

The present study has some limitations and technical considerations that should be mentioned. IUGR is considered a multifactorial disease involving many pathways and etiologies which finally lead to a single phenotype. Further studies are required to elucidate the functional consequences of the loss of some enzymatic MRC complexes in order to know whether CII dysfunction is a consequence or the cause of this obstetric complication and associated cardiovascular remodeling. It is also important to keep in mind that the bioenergetic findings of the present approach may become more evident by analysis under hypoxic conditions, other than the normoxic environment of experimental mitochondrial measures. Additionally, the reduced sample size of the present study may limit statistical findings which should be

interpreted as a proof of concept However, further studies including a larger sample size are needed, where additional measures may ideally be collected (brain sparing, sex distribution, timing of development and maturation of the heart, cardiac severity markers, etc) to explore underlying mechanistic pathways. Additionally, all rising evidences that support mitochondrial dysfunctional phenotype in other obstetric complications associated to placental insufficiency (for instance small for gestational age, preeclampsia, miscarriage or stillbirth) may strengthen the relevance of mitochondrial role in proper fetal development.

In summary, the present study shows that mitochondrial impairment is not only associated with IUGR but could also ultimately be the source of cardiac dysfunction and consequent cardiovascular remodeling. A potential mechanistic explanation for MRC CII dysfunction and decreased oxidative damage in heart of IUGR-offspring could be that Sirtuin3 regulates this unbalanced mitochondrial function by interfering MRC and Krebs cycle activity, empowering antioxidant defenses in an attempt to recover mitochondrial function in cardiomyocytes in the setting of placental insufficiency and derived hypoxia (Fig.6).

Since sirtuins and cardiac function can be modulated through dietary interventions (37), the potential use of such non-invasive approaches in human pregnancies is a strategy that should be further investigated to reduce the risk of obstetric complications and associated diseases in adulthood.

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ABBREVIATIONS

IUGR: intrauterine growth restriction

MRC: mitochondrial respiratory chain

EGTA: ethylene glycol tetraacetic acid

ATP: adenosine triphosphate

$MgCl_2$: magnesium chloride

MES: 2-(N-morpholino)ethanesulfonic acid

CI: complex I

CII: complex II

CIV: complex IV

CI+III: complex I+III

CII+III: complex II+III

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

GM Oxidation: glutamate malate oxidation

MDA: malondialdehyde

HAE: hydroxyalkenal

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDHA: Succinate dehydrogenase complex, subunit A

SDHB: Succinate dehydrogenase complex, subunit B

COX5A: Cytochrome c oxidase subunit 5a

Tom20: Mitochondrial import receptor subunit TOM20

SOD2: Superoxid dismutase 2

SEM: standard error of the mean

FIGURE LEGENDS

Fig.1 Biometric results of heart and placenta from rabbit offspring with intrauterine growth restriction (IUGR). IUGR- offspring are presented as column bars demonstrating percentage of increase or decrease compared to controls (represented as the baseline 0). There was a significant decrease in birth weight in IUGR-offspring (control N=14 and IUGR N=16), accompanied by a reduction in heart (control N=13 and IUGR N=15), left (control N=11 and IUGR N=14) and right ventricle (control N=10 and IUGR N=13) and placental weight (control N=14 and IUGR N=16). Heart body weight relative to body weight (control N=13 and IUGR N=15) and also placental weight corrected by body weight (control N=14 and IUGR N=16) did not show significant differences between cases and controls. The results are expressed as a percentage of decrease compared to controls. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted by maternal influence.. **: $p<0.001$;; ##: $p<0.005$; LV: left ventricle; RV+S: right ventricle and septum.

Fig.2 Mitochondrial impairment of heart and placenta from rabbit offspring with intrauterine growth restriction (IUGR). A) Enzymatic activities of the mitochondrial respiratory chain (MRC) of heart (left) and placenta (right) from IUGR-offspring compared to controls (represented as the baseline). A significant decrease of CII, CIV and CII+III enzymatic activities was observed in heart of IUGR-offspring, while CI and CI+III showed a not significant decrease (control N=10 and IUGR N=14 for all). The same pattern was observed in placenta, although the CIV decrease did not reach statistical significance in IUGR-offspring (CI: control N=14 and IUGR N=14; CII and CIV: control N=14 and IUGR N=16; CI+III: control N=13 and IUGR N=15; CII+III: control N=13 and IUGR N=16). Citrate synthase (CS) activity (mitochondrial content) was conserved in both heart (control N=10 and IUGR N=14) and placental (control N=14 and IUGR N=16) tissues from IUGR-offspring. **B) Mitochondrial oxygen consumption** stimulated with substrates of complex I (glutamate and malate) in heart (left; control N=11 and IUGR N=14) and placenta (right; control N=13 and IUGR N=13) from IUGR-offspring (striped bars) compared to controls (empty bars). Oxygen consumption showed a not significant decrease on CI stimulation in heart and placenta from IUGR-offspring. **C) Lipid peroxidation** in heart (left; control N=10 and IUGR N=14) and placenta (right; control N=13 and IUGR N=16) from IUGR-offspring (stripped bars) compared to controls (empty bars). Lipid peroxidation showed a significant decrease in hearts while showing a not significant increase in placenta from IUGR-offspring. **D) Total cellular ATP levels** in heart (left; control N=10 and IUGR N=14) and placenta (right; control N=14 and IUGR N=16)

from IUGR-offspring (striped bars) compared to controls (empty bars). No remarkable differences were observed in the total cellular ATP content in either heart or placenta tissue. The results are expressed as a percentage of decrease compared to controls (**A**) and as means and standard error of the mean (SEM) compared to controls (**B-D**). Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted by maternal influence. #: $p<0.05$; ##: $p<0.005$; **: $p<0.001$; CI, CII, CIV, CI+III, CII+III: MRC complex I, II, IV, I+III, II+III; CS: Citrate synthase.

Fig.3 Expression of the mitochondrial respiratory chain (MRC) subunits SDHA and SDHB (from complex II) and COX5A (from complex IV) in IUGR-offspring and controls. A) This column bar graph represents protein SHDB subunit levels in placenta from IUGR-offspring (striped bars; N=16) compared to placenta from controls (empty bars; N=14). SHDB/β-actin expression is significantly decreased in placenta from IUGR-offspring. The results are expressed as mean and standard error of the mean (SEM) compared to controls. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted by maternal influence. **: $p<0.001$. **B)** A representative Western Blot of SDHA, SDHB and COX5A protein expression in placenta is shown in which β-actin was used as the loading control. **C)** A representative Western Blot of SDHA, SDHB and COX5A protein expression in heart is shown in which β-actin was used as the loading control. IUGR: Intrauterine growth restriction; SDHA: Succinate dehydrogenase complex, subunit A; SDHB: Succinate dehydrogenase complex, subunit B; COX5A: Cytochrome c oxidase subunit 5a.

Fig.4 Mitochondrial levels of protein Sirtuin3 (Sirt3/β-actin ratio) in heart of rabbit offspring with intrauterine growth restriction (IUGR). A) This column bar graph represents protein Sirtuin3 levels in hearts from IUGR-offspring (striped bars; N=13) compared to hearts from controls (empty bars; N=10). Sirtuin3/β-actin levels in heart tissue of IUGR-offspring showed a significant increase. The results are expressed as mean and standard error of the mean (SEM) compared to controls. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted by maternal influence.. #: $p<0.05$. **B)** A representative Western Blot of Sirt3 protein expression in hearts is shown in which β-actin is used as the loading control.

Fig5 Associations between birth weight of intrauterine growth restriction (IUGR) and control offspring and some biometric data or experimental results. Herein, there are correlations demonstrating the positive association between birth weight and mitochondrial parameters and also the

negative association with Sirtuin3 expression, probably up-regulated as a homeostatic intent to revert mitochondrial lesion.

Fig.6 Association between metabolic and mitochondrial impairment and compensation according to Sirtuin3 levels in a rabbit model of intrauterine growth restriction (IUGR) and associated cardiovascular remodeling. In the context of IUGR there is a situation of placental insufficiency that creates a hypoxic environment during fetal development leading to metabolic and mitochondrial rearrangements and cardiovascular remodeling. In these settings Sirtuin3 levels increase, probably in an attempt to regulate this adverse situation and revert the metabolic and mitochondrial imbalance that may finally lead to the development of IUGR and cardiovascular remodeling. MRC: Mitochondrial respiratory chain; CI-IV; Complex I-IV; AC: Acetylation; O_2^-: superoxide anion; H_2O_2: Hydrogen peroxide; OH^-: Hydroxil anion.

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Table

Table 1. Associations between biometric data and experimental results in the IUGR and control offspring.

Parameter	With respect to	Correlation coefficient	p	R^2
Birth weight (g)	Heart weight (g)	0.783	<0.001	0.610
	Left ventricle (g)	0.676	<0.001	0.446
	Right ventricle + septum (g)	0.735	<0.001	0.459
	Placental weight (g)	0.758	<0.001	0.557
	CI enzymatic activity[a] in heart	0.429	<0.05	0.157
	CII enzymatic activity[a] in heart	0.448	<0.05	0.117
	CII+III enzymatic activity[a] in heart	0.543	≤0.005	0.289
	Citrate synthase activity[a] in heart	0.517	<0.01	0.125
	Lipid peroxidation[b] in heart	0.591	<0.005	0.325
	Sirtuin3/β-actin ratio (AU) in heart	-0.498	<0.05	0.153
	CI+III enzymatic activity[a] in placenta	0.380	<0.05	0.172
	CII+III enzymatic activity[a] in placenta	0.574	≤0.001	0.347
	Citrate synthase activity[a] in placenta	0.371	<0.05	0.217
	SDHB/β-actin ratio (AU) in placenta	0.570	≤0.001	0.304
Heart weight (g)	Left ventricle (g)	0.511	<0.005	0.322
	Right ventricle + Septum (g)	0.843	<0.001	0.658
	Heart/body weight x 100 (g)	0.469	≤0.005	0.106
	Placental weight (g)	0.575	<0.001	0.346
	Lipid peroxidation[b] in heart	0.710	<0.001	0.498
	Sirtuin3/β-actin ratio (AU) in heart	-0.505	<0.05	0.266
	CII enzymatic activity[a] in placenta	0.450	<0.05	0.146
	CI+III enzymatic activity[a] in placenta	0.594	≤0.001	0.277
	CII+III enzymatic activity[a] in placenta	0.515	<0.005	0.234
	Citrate synthase activity[a] in placenta	0.402	<0.05	0.183
	SDHB/β-actin ratio (AU) in placenta	0.500	<0.01	0.349
Left ventricle (g)	Placental weight (g)	0.563	≤0.001	0.336
	CII enzymatic activity[a] in heart	0.480	<0.05	0.130
	CII+III enzymatic activity[a] in heart	0.512	<0.01	0.209
	Citrate synthase activity[a] in heart	0.594	<0.005	0.338
	CII enzymatic activity[a] in placenta	0.461	<0.05	0.230
	CIV enzymatic activity[a] in placenta	0.476	<0.05	0.412
	CII+III enzymatic activity[a] in placenta	0.507	<0.01	0.270

	Citrate synthase activity[a] in placenta	0.606	≤0.001	0.332
	SDHB/β-actin ratio (AU) in placenta	0.544	<0.005	0.251
Right ventricle + Septum (g)	Heart/body weight x 100 (g)	0.467	<0.05	0.048
	Sirtuin3/β-actin ratio (AU) in heart	-0.593	<0.005	0.293
	CI+III enzymatic activity[a] in placenta	0.459	<0.05	0.228
	CII+III enzymatic activity[a] in placenta	0.442	<0.05	0.181
Heart/body weight x 100	CI+III enzymatic activity[a] in placenta	0.424	<0.05	0.018
	Sirtuin3/α-tubulin ratio (AU) in heart	-0.551	≤0.01	0.053
Placental weight (g)	Placenta/body weight x 100 (g)	0.436	<0.005	0.301
	CIV enzymatic activity[a] in placenta	0.436	<0.05	0.245
	CII+III enzymatic activity[a] in placenta	0.548	≤0.001	0.267
	SDHB/β-actin ratio (AU) in placenta	0.514	<0.005	0.364
Placenta/body weight x 100	Sirtuin3/β-actin ratio (AU) in heart	0.732	<0.001	0.599
Lipid peroxidation in heart	CII enzymatic activity[a] in heart	0.436	<0.05	0.136
	CII+III enzymatic activity[a] in heart	0.432	<0.05	0.173
SDHB/β-actin ratio (AU) in heart	Total ATP levels[c] in heart	0.537	<0.01	0.226
SOD2/β-actin ratio (AU) in heart	Citrate synthase activity[a] in heart	-0.418	<0.05	0.112
SDHB/β-actin ratio (AU) in placenta	CII enzymatic activity[a] in placenta	0.497	<0.005	0.145
	CIV enzymatic activity[a] in placenta	0.617	<0.001	0.241
	CII+III enzymatic activity[a] in placenta	0.521	<0.005	0.192
	Citrate synthase activity[a] in placenta	0.613	<0.001	0.261
	SOD2/β-actin ratio (AU) in placenta	0.402	<0.05	0.314
COX5A/β-actin ratio (AU) in placenta	CIV enzymatic activity[a] in placenta	0.409	<0.05	0.149
	Citrate synthase activity[a] in placenta	0.406	<0.05	0.179
Tom20/β-actin ratio (AU) in placenta	CIV enzymatic activity[a] in placenta	0.429	<0.05	0.164
SOD2/β-actin ratio (AU) in placenta	CIV enzymatic activity[a] in placenta	0.513	<0.005	0.249
	CII+III enzymatic activity[a] in placenta	0.482	<0.01	0.216

CI: Complex I; CII: Complex II; CI+III: Complex I+III; CII+III: Complex II+III; CIV: Complex IV; SDHA: Succinate dehydrogenase complex, subunit A; SDHB: Succinate dehydrogenase complex, subunit B; COX5A: Cytochrome c oxidase subunit 5a; Tom20: Mitochondrial import receptor subunit TOM20; SOD2: Superoxid dismutase 2; AU: Arbitrary units; g: grams; [a]: nmol/minute· mg protein; [b]: μM MDA+HAE/mg protein; [c]: pmol ATP/mg protein.

Figure

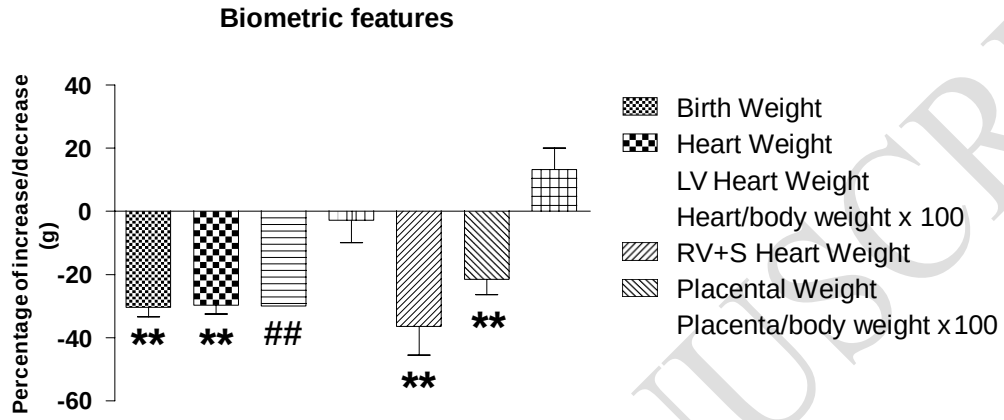

Fig.1

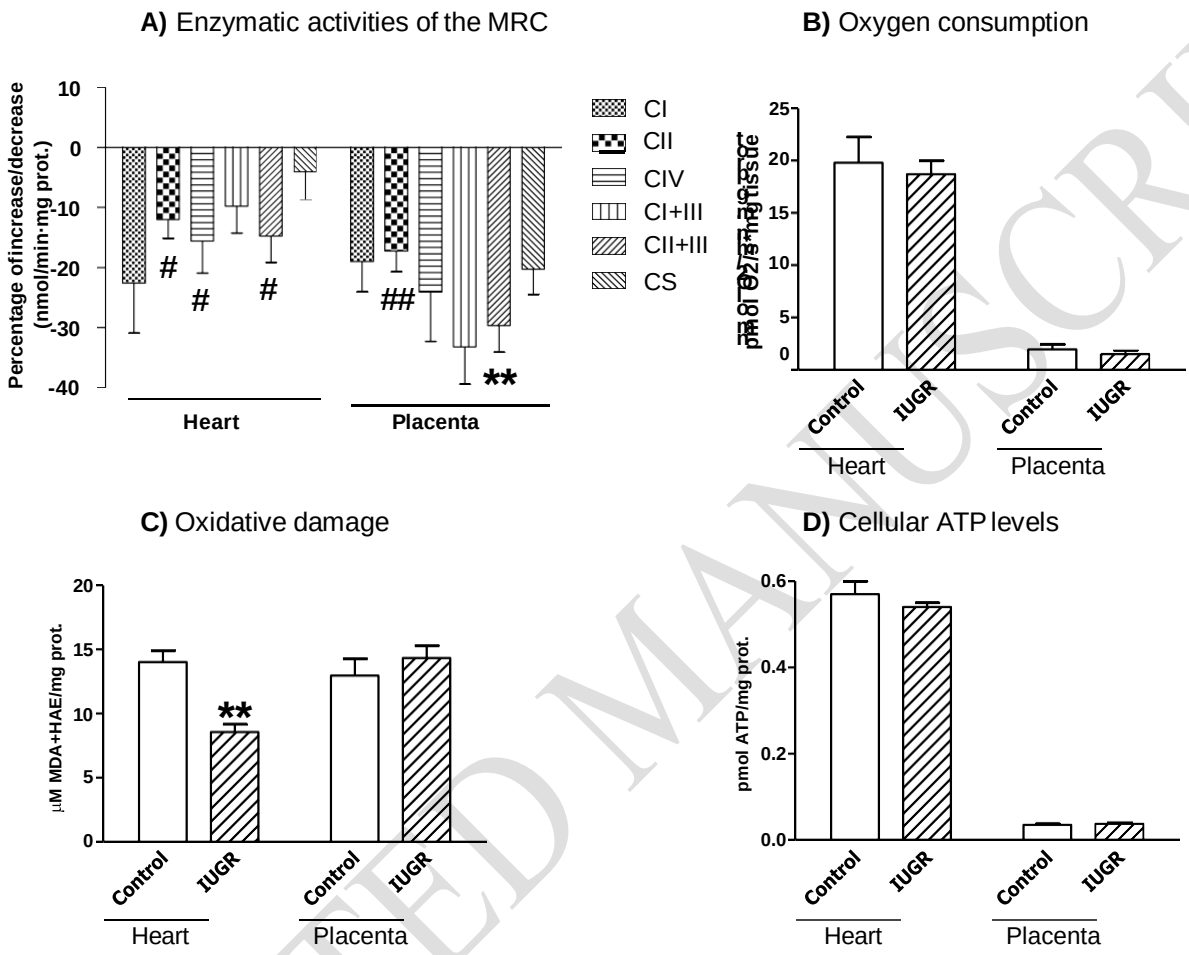

Fig.2

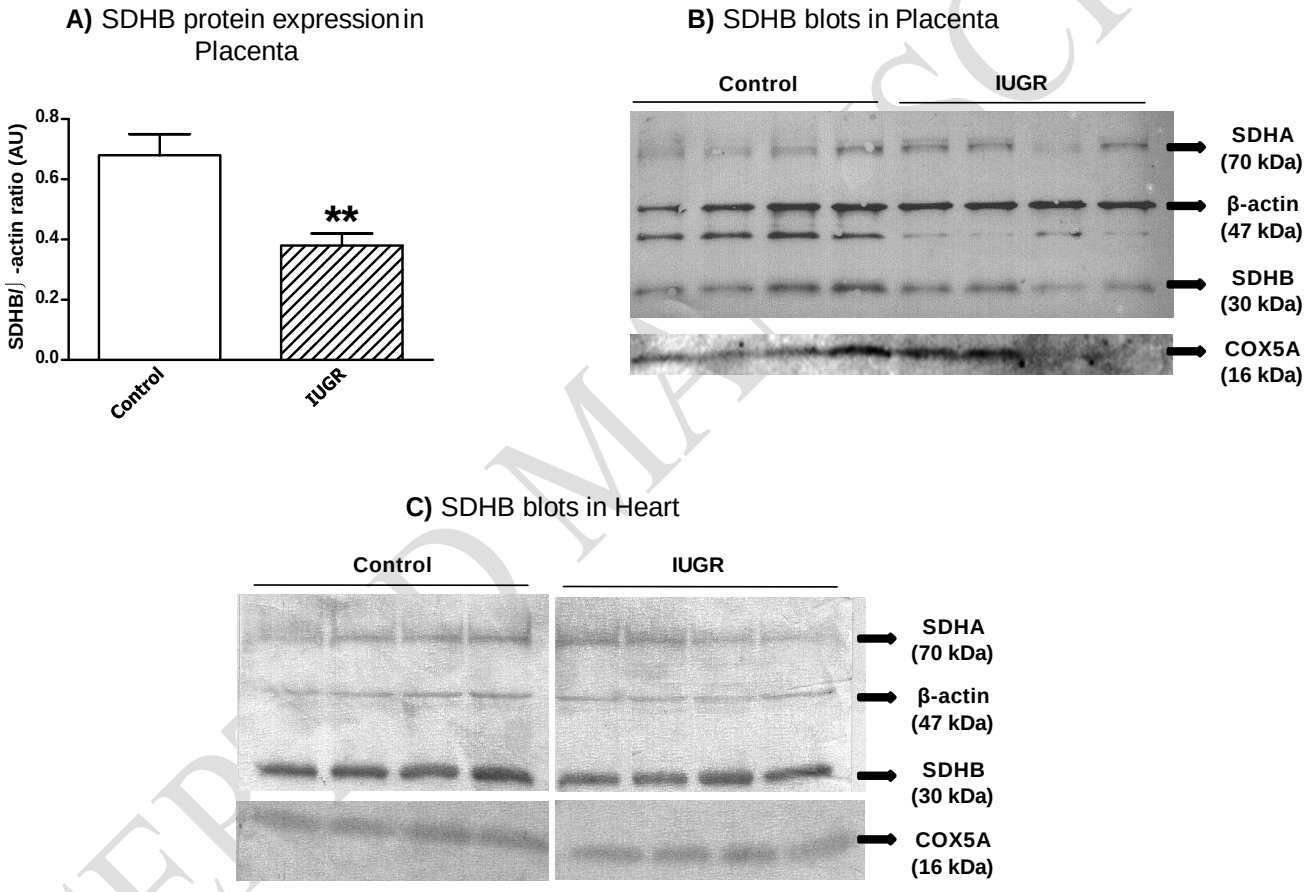

Fig.3

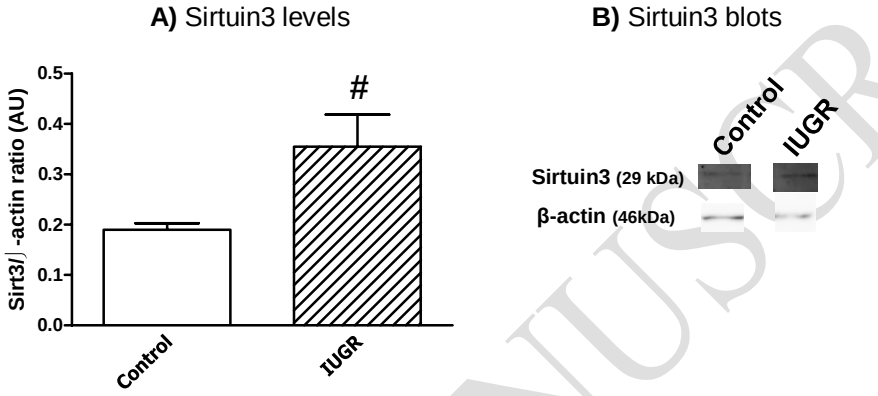

Fig.4

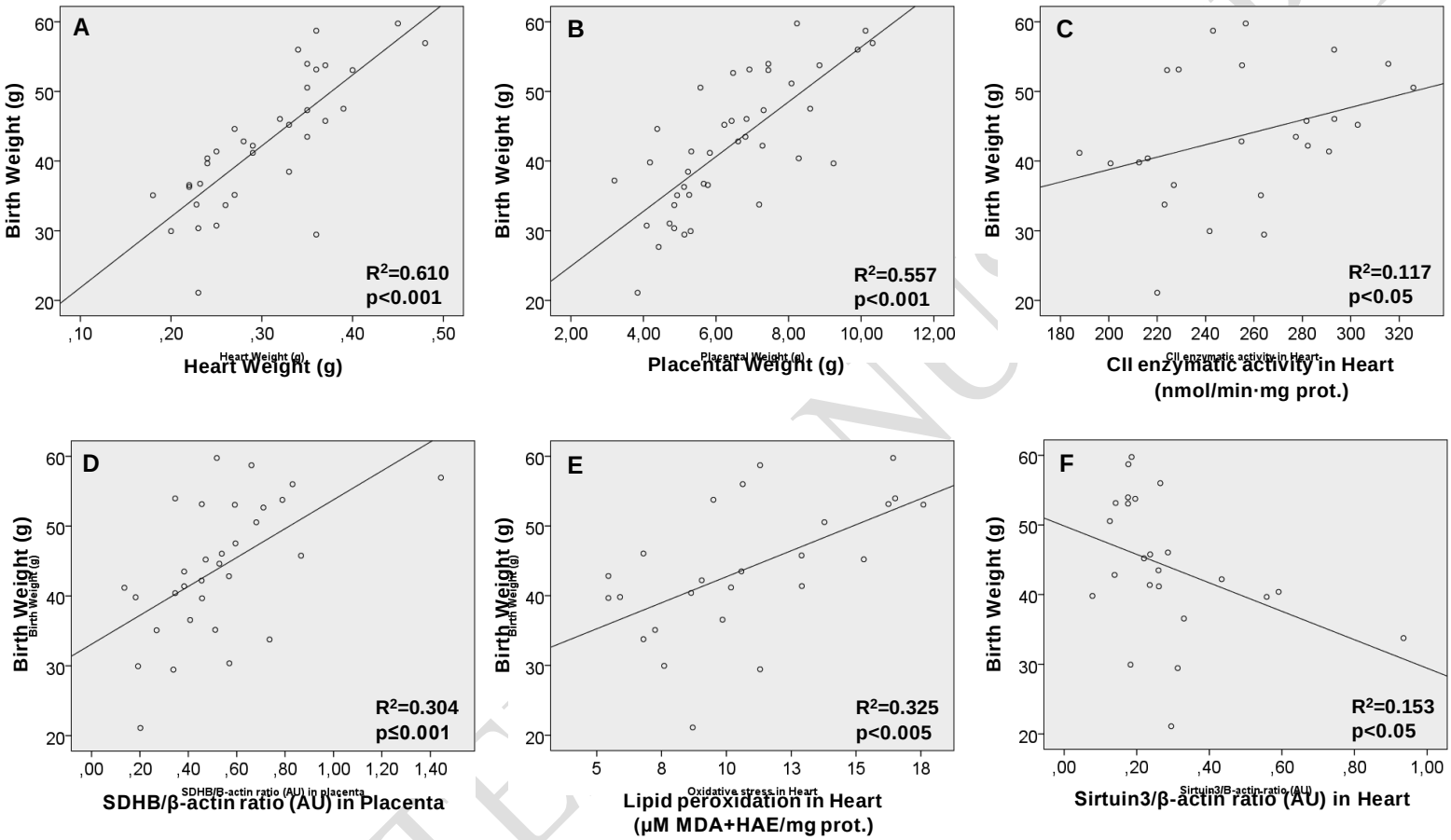

Fig.5

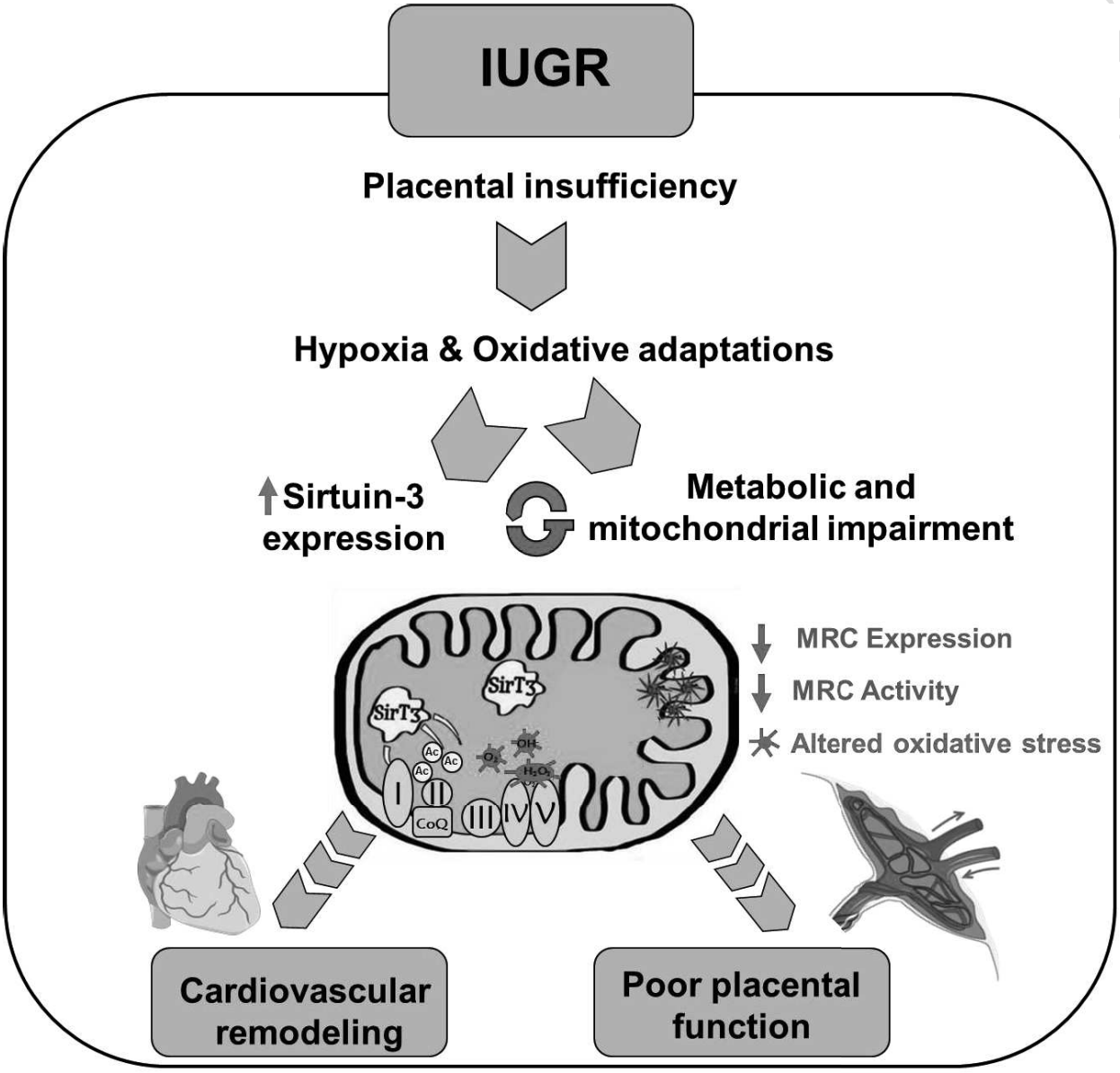
IUGR
Placental insufficiency
Hypoxia & Oxidative adaptations
Sirtuin-3 expression
Metabolic and mitochondrial impairment
SirT3
SirT3
Ac
Ac
Ac
OH
O_2
H_2O_2
I
II
CoQ
III
IV
V
MRC Expression
MRC Activity
Altered oxidative stress
Cardiovascular remodeling
Poor placental function

Fig.S1 Representative Blot for the expression of mitochondrial import receptor subunit TOM20 (Tom20) in heart and placental tissue from IUGR-offspring and controls. No remarkable differences were observed in heart (control N=4 and IUGR N=8) and placenta (control N=15 and IUGR N=15) between cases and controls. β-actin was used as loading control. IUGR: Intrauterine growth restriction

Fig.S2 Representative Blot for the expression of superoxid dismutase 2 (SOD2) anti-oxidant enzyme in heart and placental tissue from IUGR-offspring and controls. No remarkable differences were observed in heart (control N=10 and IUGR N=14) and placenta (control N=15 and IUGR N=15) between cases and controls. β-actin was used as loading control. IUGR: Intrauterine growth restriction

Fig.S3 Representative Blot for the expression of the acetylated form of superoxid dismutase 2 (SOD2) anti-oxidant enzyme in heart and placental tissue from IUGR-offspring and controls. No remarkable differences were observed in heart (control N=8 and IUGR N=13) and placenta (control N=X and IUGR N=X) between cases and controls. β-actin was used as loading control. IUGR: Intrauterine growth restriction

Supplementary Material (for online publication)

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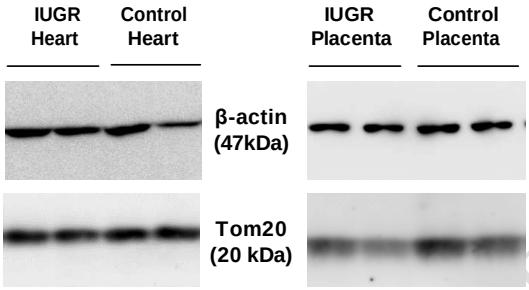

Fig.S1

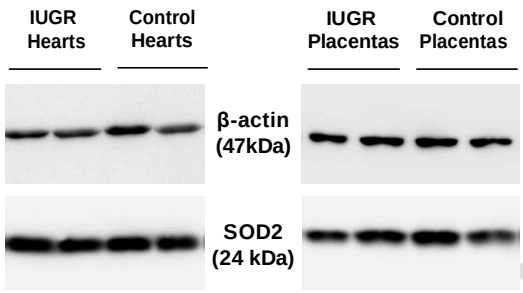

Fig.S2

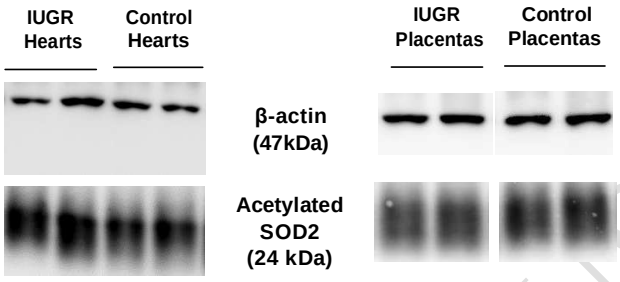

Fig.S3

SUPPLEMENTARY MATERIAL

TableS1. Sample size included for each pregnant rabbit according to 10th percentile of birth weight.

	N Offspring	
Pregnant rabbit	***N Control***	***N IUGR***
1	1	1
2	4	4
3	4	4
4	2	2
5	1	1
6	2	4
N total	**14**	**16**

N: number of sample; IUGR: intrauterine growth restriction

Table S2. Biometric data of experimental groups. Whole body, cardiac and placental weight are reduced in IUGR-offspring compared to control-offspring

	Control	***IUGR***	***% of increased (+) or decreased (-)***	***P value***
Birth weight (g)	52.26±1.32	36.40±1.56	-30.35±2.99	<0.001 [a]
Heart weight (g)	0.37±0.01	0.26±0.01	-29.73±2.70	<0.001 [a]
Left ventricle heart weight (g)	0.10±0.01	0.07±0.00	-30.00±0.00	<0.005 [a, †]
Heart/body weight x 100	0.71±0.02	0.73±0.05	+2.82±7.04	NS
Right Ventricle + Septum heart weight (g)	0.11±0.01	0.07±0.01	-36.36±9.09	<0.001 [a]
Placental weight (g)	7.63±0.48	5.99±0.37	-21.49±4.85	<0.001 [a, †]
Placenta/body weight x 100	0.15±0.01	0.17±0.01	+13.33±6.67	NS

Values are expressed as mean ± standard error of the mean. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted ([a]) by maternal influence (†). IUGR: Intrauterine Growth Restriction.

Table S3. Raw data of absolute and relative enzymatic activities of complex I, II, IV, I+III and II+III of the mitochondrial respiratory chain, MRC subunits expression (SDHA, SDHB and COX5A), citrate synthase (CS) activity, Tom20 expression, Complex I-stimulated oxygen consumption (GM Oxidation), CoQ9 and CoQ10 levels, cellular ATP levels, lipid peroxidation, SOD2 expression and Sirtuin3/β-actin levels in the experimental groups.

HEART	*Control*	*IUGR*	*% of increased (+) or decreased (-)*	*P value*
Complex I (nmol/minute· mg protein)	147.21±18.57	114.02±12.31	-22.55±8.36	NS
Complex I relative to CS activity (nmol/minute· mg protein)	0.33±0.04	0.28±0.03	-15.15±9.09	NS
Complex II (nmol/minute· mg protein)	272.68±11.45	240.08±8.63	-11.96±3.16	<0.05 [a]
Complex II relative to CS activity (nmol/minute· mg protein)	0.63±0.03	0.59±0.03	-6.35±4.76	NS
Complex IV (nmol/minute· mg protein)	576.95±23.93	487.06±30.71	-15.58±5.32	<0.05 [a]
Complex IV relative to CS activity (nmol/minute· mg protein)	1.34±0.08	1.17±0.07	-12.69±5.22	NS
Complex I+III (nmol/minute· mg protein)	111.62±4.99	100.71±4.99	-9.77±4.47	NS
Complex I+III relative to CS activity (nmol/minute· mg protein)	0.23±0.03	0.24±0.01	+4.35±4.35	NS
Complex II+III	182.55±5.76	155.66±7.98	-14.73±4.37	<0.05 [a]

(nmol/minute· mg protein)				
Complex II+III relative to CS activity (nmol/minute· mg protein)	0.43±0.02	0.37±0.01	-13.95±2.33	NS
SDHA/β-actin (AU)	2.60±0.19	2.85±0.27	+9.62±10.38	NS
SDHB/β-actin (AU)	1.02±0.11	1.37±0.13	+34.31±12.75	NS
COX5A/β-actin (AU)	2.50±0.61	2.29±0.64	-8.40±25.60	NS
Citrate Synthase (nmol/minute· mg protein)	436.96±21.72	419.33±20.56	-4.03±4.71	NS
Tom20/β-actin (AU)	4.09±0.18	6.60±1.30	+61.37±31.78	NS
GM Oxidation (pmol O2/s*mg)	19.80±2.46	18.70±1.28	-5.56±6.46	NS
CoQ9 levels (µmol/L)	68.99±6.31	61.03±4.47	-11.54±6.48	NS
CoQ10 levels (µmol/L)	479.29±15.74	435.66±32.27	-9.10±6.73	NS
ATP levels (pmol ATP/mg protein)	0.57±0.03	0.54±0.01	-5.56±1.75	NS
Lipid peroxidation (µM MDA+HAE/mg protein)	14.02±0.89	8.55±0.61	-39.02±4.35	<0.001 [a]
SOD2/β-actin (AU)	6.05±1.02	4.41±0.76	-27.11±12.56	NS
SOD2 acetylation/β-actin (AU)	7.84±0.77	10.84±1.37	+38.27±17.47	NS
Ratio SOD2 acetylation/SOD2 expression (AU)	~~1.78~~1.71±0.~~17~~07	~~1.87~~2.29±0.30	~~5.06~~+33.92±~~16.29~~17.54	NS

Sirtuin3/β-actin (AU)	0.19±0.01	0.35±0.06	+84.21±31.58	<0.05 [a]
PLACENTA	***Control***	***IUGR***	***% of increased or decreased***	***P value***
Complex I (nmol/minute· mg protein)	10.28±1.37	8.33±0.52	-18.97±5.06	NS
Complex I relative to CS activity (nmol/minute· mg protein)	0.25±0.04	0.24±0.01	-4.00±4.00	NS
Complex II (nmol/minute· mg protein)	17.94±0.99	14.85±0.62	-17.22±3.46	<0.005 [a]
Complex II relative to CS activity (nmol/minute· mg protein)	0.41±0.02	0.42±0.02	+2.44±4.88	NS
Complex IV (nmol/minute· mg protein)	57.27±6.53	43.51±4.73	-24.03±8.26	NS
Complex IV relative to CS activity (nmol/minute· mg protein)	1.27±0.12	1.18±0.09	-7.09±7.09	NS
Complex I+III (nmol/minute· mg protein)	9.52±1.51	6.36±0.59	-33.19±6.20	NS
Complex I+III relative to CS activity (nmol/minute· mg protein)	0.21±0.02	0.18±0.02	-14.29±9.52	NS
Complex II+III (nmol/minute· mg protein)	28.44±2.24	20.01±1.26	-29.64±4.43	<0.001 [a]
Complex II+III relative to CS	0.61±0.03	0.55±0.02	-9.84±3.28	NS

activity (nmol/minute· mg protein)				
SDHA/β-actin (AU)	0.68±0.15	0.54±0.07	-20.59±10.29	NS
SDHB/β-actin (AU)	0.68±0.07	0.38±0.04	+44.12±5.88	<0.001 [a, †]
COX5A/β-actin (AU)	0.47±0.07	0.33±0.04	-29.79±8.51	NS
Citrate Synthase (nmol/minute· mg protein)	45.64±3.26	36.39±1.91	-20.27±4.18	NS
Tom20/β-actin (AU)	2.78±0.40	2.91±0.34	+4.68±12.23	NS
GM Oxidation (pmol O2/s*mg)	1.95±0.47	1.45±0.37	-25.64±18.97	NS
CoQ9 levels (µmol/L)	33.19±0.00	34.63±1.44	+4.16±4.16	NS
CoQ10 levels (µmol/L)	55.99±6.14	54.89±8.61	-1.96±15.38	NS
ATP levels (pmol ATP/mg protein)	0.04±0.00	0.04±0.00	+5.71±8.57	NS
Lipid peroxidation (µM MDA+HAE/mg protein)	12.96±1.31	14.34±0.95	+10.65±7.33	NS
SOD2/β-actin (AU)	2.29±0.35	1.92±0.22	-16.16±9.61	NS
SOD2 acetylation/β-actin (AU)	5.21±0.60	5.64±0.48	+8.25±9.21	NS
Ratio SOD2 acetylation/SOD2 expression (AU)	~~2.55~~3.03±0.~~48~~46	~~2.27~~3.94±0.~~30~~53	- +~~10.98~~30.03±~~11.76~~17.49	NS

Values are mean ± standard error of the mean. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted ([a]) by maternal influence (†). IUGR: Intrauterine Growth Restriction; SDHA: Succinate dehydrogenase complex, subunit A; SDHB:

Succinate dehydrogenase complex, subunit B; COX5A: Cytochrome c oxidase subunit 5a; Tom20: Mitochondrial import receptor subunit TOM20; GM Oxidation: Glutamate and malate oxidation; CoQ9 and 10: Coenzyme Q9 and Q10; MDA: Malondialdehyde; HAE: Hydroxyalkenal; SOD2: Superoxid dismutase 2; AU: Arbitrary units; NS: not significant.

SUPPLEMENTARY MATERIAL

TableS1. Sample size included for each pregnant rabbit according to 10th percentile of birth weight.

	N Offspring	
Pregnant rabbit	***N Control***	***N IUGR***
1	1	1
2	4	4
3	4	4
4	2	2
5	1	1
6	2	4
N total	**14**	**16**

N: number of sample; IUGR: intrauterine growth restriction

Table S2. Biometric data of experimental groups. Whole body, cardiac and placental weight are reduced in IUGR-offspring compared to control-offspring

	Control	***IUGR***	***% of increased (+) or decreased (-)***	***P value***
Birth weight (g)	52.26±1.32	36.40±1.56	-30.35±2.99	<0.001 [a]
Heart weight (g)	0.37±0.01	0.26±0.01	-29.73±2.70	<0.001 [a]
Left ventricle heart weight (g)	0.10±0.01	0.07±0.00	-30.00±0.00	<0.005 [a, †]
Heart/body weight x 100	0.71±0.02	0.73±0.05	+2.82±7.04	NS
Right Ventricle + Septum heart weight (g)	0.11±0.01	0.07±0.01	-36.36±9.09	<0.001 [a]
Placental weight (g)	7.63±0.48	5.99±0.37	-21.49±4.85	<0.001 [a, †]
Placenta/body weight x 100	0.15±0.01	0.17±0.01	+13.33±6.67	NS

Values are expressed as mean ± standard error of the mean. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted ([a]) by maternal influence (†). IUGR: Intrauterine Growth Restriction.

Table S3. Raw data of absolute and relative enzymatic activities of complex I, II, IV, I+III and II+III of the mitochondrial respiratory chain, MRC subunits expression (SDHA, SDHB and COX5A), citrate synthase (CS) activity, Tom20 expression, Complex I-stimulated oxygen consumption (GM Oxidation), CoQ9 and CoQ10 levels, cellular ATP levels, lipid peroxidation, SOD2 expression and Sirtuin3/β-actin levels in the experimental groups.

HEART	***Control***	***IUGR***	***% of increased (+) or decreased (-)***	***P value***
Complex I (nmol/minute· mg protein)	147.21±18.57	114.02±12.31	-22.55±8.36	NS
Complex I relative to CS activity (nmol/minute· mg protein)	0.33±0.04	0.28±0.03	-15.15±9.09	NS
Complex II (nmol/minute· mg protein)	272.68±11.45	240.08±8.63	-11.96±3.16	<0.05 [a]
Complex II relative to CS activity (nmol/minute· mg protein)	0.63±0.03	0.59±0.03	-6.35±4.76	NS
Complex IV (nmol/minute· mg protein)	576.95±23.93	487.06±30.71	-15.58±5.32	<0.05 [a]
Complex IV relative to CS activity (nmol/minute· mg protein)	1.34±0.08	1.17±0.07	-12.69±5.22	NS
Complex I+III (nmol/minute· mg protein)	111.62±4.99	100.71±4.99	-9.77±4.47	NS
Complex I+III relative to CS activity (nmol/minute· mg protein)	0.23±0.03	0.24±0.01	+4.35±4.35	NS
Complex II+III (nmol/minute· mg	182.55±5.76	155.66±7.98	-14.73±4.37	<0.05 [a]

protein)				
Complex II+III relative to CS activity (nmol/minute· mg protein)	0.43±0.02	0.37±0.01	-13.95±2.33	NS
SDHA/β-actin (AU)	2.60±0.19	2.85±0.27	+9.62±10.38	NS
SDHB/β-actin (AU)	1.02±0.11	1.37±0.13	+34.31±12.75	NS
COX5A/β-actin (AU)	2.50±0.61	2.29±0.64	-8.40±25.60	NS
Citrate Synthase (nmol/minute· mg protein)	436.96±21.72	419.33±20.56	-4.03±4.71	NS
Tom20/β-actin (AU)	4.09±0.18	6.60±1.30	+61.37±31.78	NS
GM Oxidation (pmol O2/s*mg)	19.80±2.46	18.70±1.28	-5.56±6.46	NS
CoQ9 levels (µmol/L)	68.99±6.31	61.03±4.47	-11.54±6.48	NS
CoQ10 levels (µmol/L)	479.29±15.74	435.66±32.27	-9.10±6.73	NS
ATP levels (pmol ATP/mg protein)	0.57±0.03	0.54±0.01	-5.56±1.75	NS
Lipid peroxidation (µM MDA+HAE/mg protein)	14.02±0.89	8.55±0.61	-39.02±4.35	<0.001 [a]
SOD2/β-actin (AU)	6.05±1.02	4.41±0.76	-27.11±12.56	NS
SOD2 acetylation/β-actin (AU)	7.84±0.77	10.84±1.37	+38.27±17.47	NS
Ratio SOD2 acetylation/SOD2 expression (AU)	1.71±0.07	2.29±0.30	+33.92±17.54	NS
Sirtuin3/β-actin (AU)	0.19±0.01	0.35±0.06	+84.21±31.58	<0.05 [a]

PLACENTA	*Control*	*IUGR*	*% of increased or decreased*	*P value*
Complex I (nmol/minute· mg protein)	10.28±1.37	8.33±0.52	-18.97±5.06	NS
Complex I relative to CS activity (nmol/minute· mg protein)	0.25±0.04	0.24±0.01	-4.00±4.00	NS
Complex II (nmol/minute· mg protein)	17.94±0.99	14.85±0.62	-17.22±3.46	<0.005 [a]
Complex II relative to CS activity (nmol/minute· mg protein)	0.41±0.02	0.42±0.02	+2.44±4.88	NS
Complex IV (nmol/minute· mg protein)	57.27±6.53	43.51±4.73	-24.03±8.26	NS
Complex IV relative to CS activity (nmol/minute· mg protein)	1.27±0.12	1.18±0.09	-7.09±7.09	NS
Complex I+III (nmol/minute· mg protein)	9.52±1.51	6.36±0.59	-33.19±6.20	NS
Complex I+III relative to CS activity (nmol/minute· mg protein)	0.21±0.02	0.18±0.02	-14.29±9.52	NS
Complex II+III (nmol/minute· mg protein)	28.44±2.24	20.01±1.26	-29.64±4.43	<0.001 [a]
Complex II+III relative to CS activity (nmol/minute· mg protein)	0.61±0.03	0.55±0.02	-9.84±3.28	NS

SDHA/β-actin (AU)	0.68±0.15	0.54±0.07	-20.59±10.29	NS
SDHB/β-actin (AU)	0.68±0.07	0.38±0.04	+44.12±5.88	<0.001 [a, †]
COX5A/β-actin (AU)	0.47±0.07	0.33±0.04	-29.79±8.51	NS
Citrate Synthase (nmol/minute· mg protein)	45.64±3.26	36.39±1.91	-20.27±4.18	NS
Tom20/β-actin (AU)	2.78±0.40	2.91±0.34	+4.68±12.23	NS
GM Oxidation (pmol O2/s*mg)	1.95±0.47	1.45±0.37	-25.64±18.97	NS
CoQ9 levels (µmol/L)	33.19±0.00	34.63±1.44	+4.16±4.16	NS
CoQ10 levels (µmol/L)	55.99±6.14	54.89±8.61	-1.96±15.38	NS
ATP levels (pmol ATP/mg protein)	0.04±0.00	0.04±0.00	+5.71±8.57	NS
Lipid peroxidation (µM MDA+HAE/mg protein)	12.96±1.31	14.34±0.95	+10.65±7.33	NS
SOD2/β-actin (AU)	2.29±0.35	1.92±0.22	-16.16±9.61	NS
SOD2 acetylation/β-actin (AU)	5.21±0.60	5.64±0.48	+8.25±9.21	NS
Ratio SOD2 acetylation/SOD2 expression (AU)	3.03±0.46	3.94±0.53	+30.03±17.49	NS

Values are mean ± standard error of the mean. Case-control differences were sought by non-parametric statistical analysis and, in case of difference, significance was adjusted ([a]) by maternal influence (†). IUGR: Intrauterine Growth Restriction; SDHA: Succinate dehydrogenase complex, subunit A; SDHB: Succinate dehydrogenase complex, subunit B; COX5A: Cytochrome c oxidase subunit 5a; Tom20: Mitochondrial import receptor subunit TOM20; GM Oxidation: Glutamate and malate oxidation; CoQ9 and 10: Coenzyme Q9 and Q10; MDA: Malondialdehyde; HAE: Hydroxyalkenal; SOD2: Superoxid dismutase 2; AU: Arbitrary units; NS: not significant.