

Effect of Exercise Training on Enos Expression, NO Production and Oxygen Metabolism in Human Placenta

Robinson Ramírez-Vélez^{1*}, Juanita Bustamante², Analia Czerniczyniec², Ana C. Aguilar de Plata³, Silvia Lores-Arnaiz²

1 Facultad de Cultura Física, Deporte y Recreación, Universidad Santo Tomás, Bogotá, D.C., Colombia, **2** Departamento de Ciencias Fisiológicas, Facultad de Salud, Universidad del Valle, Cali, Colombia, **3** Instituto de Bioquímica y Medicina Molecular (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

Abstract

Objective: To determine the effects of combined aerobic and resistance exercise training during the second half of pregnancy on endothelial NOS expression (eNOS), nitric oxide (NO) production and oxygen metabolism in human placenta.

Methods: The study included 20 nulliparous in gestational week 16–20, attending prenatal care at three tertiary hospitals in Colombia who were randomly assigned into one of two groups: The exercise group (n = 10) took part in an exercise session three times a week for 12 weeks which consisted of: aerobic exercise at an intensity of 55–75% of their maximum heart rate for 60 min and 25 mins. Resistance exercise included 5 exercise groups circuit training (50 repetitions of each) using barbells (1–3 kg/exercise) and low-to-medium resistance bands. The control group (n = 10) undertook their usual physical activity. Mitochondrial and cytosol fractions were isolated from human placental tissue by differential centrifugation. A spectrophotometric assay was used to measure NO production in cytosolic samples from placental tissue and Western Blot technique to determine eNOS expression. Mitochondrial superoxide levels and hydrogen peroxide were measured to determine oxygen metabolism.

Results: Combined aerobic and resistance exercise training during pregnancy leads to a 2-fold increase in eNOS expression and 4-fold increase in NO production in placental cytosol (p = 0.05). Mitochondrial superoxide levels and hydrogen peroxide production rate were decreased by 8% and 37% respectively in the placental mitochondria of exercising women (p = 0.05).

Conclusion: Regular exercise training during the second half of pregnancy increases eNOS expression and NO production and decreases reactive oxygen species generation in human placenta. Collectively, these data demonstrate that chronic exercise increases eNOS/NO production, presumably by increasing endothelial shear stress. This adaptation may contribute to the beneficial effects of exercise on the vascular and antioxidant system and in turn reduce the risk of preeclampsia, diabetes or hypertension during pregnancy.

Citation: Ramírez-Vélez R, Bustamante J, Czerniczyniec A, Aguilar de Plata AC, Lores-Arnaiz S (2013) Effect of Exercise Training on Enos Expression, NO Production and Oxygen Metabolism in Human Placenta. PLoS ONE 8(11): e80225. doi:10.1371/journal.pone.0080225

Editor: Prasun K. Datta, Temple University, United States of America

Received: February 13, 2013; **Accepted:** October 1, 2013; **Published:** November 14, 2013

Copyright: © 2013 Ramírez-Vélez et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded by Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología “Francisco José de Caldas” COLCIENCIAS (Grant N° 1106-45921540). Robinson Ramírez-Vélez received a grant from Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología “Francisco José de Caldas”) to do a doctorate (Grant Colciencias/Icetex N° 067/2002). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors declare that they have no competing interests.

* E-mail: robin640@hotmail.com

Introduction

Pregnancy is a physiological state characterized by hemodynamic changes, such as an increase in heart rate at rest, increased cardiac output and diminished peripheral vascular resistance [1]. These adaptations appear to be mediated to a great extent by an increased endothelium-dependent vasodilation. The endothelial release of nitric oxide (NO) has been proposed as the main factor responsible for the decreased systemic vascular resistance observed during pregnancy [1]. In addition, a decreased production or an increased inactivation of NO has been linked to the endothelial dysfunction that characterizes pre-eclampsia, gestational diabetes and hypertension [2]. Moreover, while pregnancy is referred to as “a state of oxidative stress” [3], certain pathological conditions,

such as pre-eclampsia further increase placental oxidative stress and may result in early pregnancy loss, intrauterine growth restriction (IUGR) and impaired placentation [4]. Placental mitochondria are one of the a major source of oxidative stress in pre-eclampsia [3,4]

In recent years, evidence has accumulated to support the popular belief that physical activity is associated with better psychological health during pregnancy [5,6]. The American College of Obstetricians and Gynecologists (ACOG) [7] recommend regular exercise for pregnant women, including those who are sedentary, for its overall benefits on physical and psychological health. Physical activity during pregnancy appears to be beneficial to the maternal-foetal unit [8] and may prevent the occurrence of maternal disorders, such as preeclampsia [9], hypertension [10]

and gestational diabetes [11]. Other studies have demonstrated that either beginning or continuing regular weight-bearing exercise throughout pregnancy improves placental growth [12,13]. Bergmann et al. [14] reported that regular weight-bearing forms of exercise influence placental growth and anatomic indices of functional capacity. Our previous work, showing that 12 weeks of exercise enhances endothelium-dependent brachial artery dilation in pregnant women suggested that exercise-induced increases both blood flow and eNOS expression [15]. The exact mechanism for this remains unclear, but it is proposed that the exercise-induced intermittent fluctuations in substrate and oxygen delivery produce a recurrent stimulus which evokes an overall growth response [8–10].

It is currently unknown, however if exercise produces these effects in human placenta. The aim of this study was to determine the effects of exercise training during the second half of pregnancy effect on endothelial NOS expression (eNOS), nitric oxide (NO) production and oxygen metabolism in human placenta.

Methods

Subjects

Twenty nulliparous women with a gestational age ranging 16–20 weeks of healthy pregnancy and a live fetus at the routine ultrasound scan were included in the study. Participants were originally deemed eligible for this investigation if they met all of the following criteria in the first trimester of pregnancy: *i*) gravida with singleton and uncomplicated gestation; *ii*) not at high risk for preterm delivery (p.e: alcohol use, drug use, history of smokers, placenta previa, incompetent cervix, polyhydramnios, oligohydramnios, or miscarriage in the last twelve months); *iii*) 18–25 years of age; *iv*) being sedentary before gestation (exercising <20 min on <3 days/week); *v*) being under medical follow-up throughout the entire pregnancy period (and planning to give birth) in the same obstetrics hospital department (Red de Salud Ladera, Cali-Colombia); and *vi*) having no absolute or relative contraindication to exercise participation during pregnancy (such as, among others, haemodynamically significant heart disease, restrictive lung disease, pregnancy induced hypertension, severe anaemia, maternal cardiac arrhythmia, chronic bronchitis, type I diabetes or extreme morbid obesity (body mass index ≥ 40 kg/m²)). Maternal and fetal characteristics are presented in (Table 1.)

The Ethics Committees of Universidad del Valle and Red de Salud Ladera (Resolution-017/08-UV and SCAH/0408-A/08) approved the trial. All participants provided written informed consent before entering the study. Participants and their legal representatives received information sheets and all provided

written informed consent and approval to take part in the study. The protocol was in accordance with the latest revision of the Declaration of Helsinki.

Experimental design

This study involved a subset of subjects enrolled in a randomized controlled trial to evaluate the effect of regular exercise on maternal endothelium-dependent vasodilatation (A detailed account of the methods used in this study has been published elsewhere [15], from March 2010 to January 2011.

Interventions and training program

The pregnant women who were assigned to the exercise group participated in a 60 min supervised aerobic and resistance exercise session, three times a week [15,16]. An average of 32 training sessions per subject was planned for each participant, in consideration that delivery could occur before 40 weeks' gestation. All women had their heart rate monitored during the training sessions to ensure that the exercise was of a moderate-to vigorous intensity. A physiotherapist and a physical educator were present during the exercise sessions. Each session included 30-min of aerobic circuit training accompanied by an audio recording of music and instructions which guided the participants to exercise at each station for approximately 1 min and then move to the next station in three circuits of 10 stations. Participants wore a heart rate monitor (Polar Pacer, USA) in every exercise session which were checked at rest after 15 and 30 mins to ensure that they exercised in the prescribed target heart rate zone. Each session was preceded and followed by a gradual warm-up and cool down period which were both of 10 min duration and consisted of walking and light, static stretching (avoiding muscle pain) of major muscle groups (upper and lower limbs, neck and trunk muscles). The cool down period also included relaxation and stretching exercises.

Resistance exercises were performed through the full range of motion normally associated with correct technique for each exercise and engaged the major muscle groups (abdominal, dorsal, shoulder, upper and lower limb muscles). They included 5 exercise groups circuit training (50 repetitions of each) using barbells (1–3 kg/exercise) or low-to-medium resistance bands (Therabands). Each type of exercise on the back was performed for 2 min. As a general rule and to reduce potential risks, we excluded activities that promoted that Valsalva maneuver, ballistic or plyometric exercises and positions of extreme muscular tension.

The control group received no intervention; that is, participants neither attended the exercise classes nor took part in an exercise program at home. Both groups continued with their normal prenatal care and physical activity. Each woman met with the study dietician for nutrition assessment and counselling, and an individualized nutrition intervention plan was developed from the baseline food intake assessment, participant preferences, and the meal plan [16,17]. On a weekly basis, each participant all participants received a light breakfast/meal 45 min before the exercise session which typically consisted of a meal designed to promote weight and recommended during pregnancy (approximately (400 kcal) [carbohydrate to 40–55%, fat 30% and 20–30% to protein of total energy] [17]. The food records were analyzed for nutritional content and caloric intake using the ESHA Food Processor SQL (version 9.8; Canadian Nutrient File database). The participants received support for public transport to attend the exercise classes (US10 per session).

Table 1. Characteristics of the population: maternal age, BMI, gestational age at birth, fetal and placental weight and fetal gender in pregnancies.

Characteristics	Control	Exercised
Maternal age (yr)	19.5±3.4	19.2±2.6
Body mass index (BMI)	24.1±4.5	22.9±3.8
Gestational age at birth (yr)	39.8±2.0	38.7±1.0
Fetal weight (g)	3013.2±493.8	3133.3±406.5
Placental weight (g)	430.4±28.9	390.0±20.4
Fetal gender (female/male)	6/4	5/5

Data reported are mean ± SD.

doi:10.1371/journal.pone.0080225.t001

Tissue preparation and sampling

Placentas were collected within 15 min of delivery. The maternal decidua was removed and the central portion between the maternal and fetal surfaces used in the preparation. Maternal villous tissue (100 g) was chopped into small pieces, washed with NaCl 0.9 per cent to remove blood and filtered through gauze.

Isolation of human placental mitochondria

Human placental mitochondria were obtained with the modified protocol of differential centrifugation, previously described by Martinez et al. [18,19]. Briefly, several placental cotyledons were removed from the maternal side of the placenta and placed immediately into an ice-cold medium containing MSHE buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EDTA, pH 7.4). All steps were carried out at 4°C. Soft villous tissue (4–7 g) was freed from connective tissue and minced into small pieces with scissors. The tissue was washed with the same solution and then filtered three times through a thin surgical gauze layer. The tissue was re-suspended in the same medium and homogenized with a Potter-Elvehjem homogenizer (7 up-and-down strokes). The homogenate was centrifuged at 1,500 g for 10 min in a refrigerated centrifuge. The supernatant was recovered and centrifuged at 4,000 g for 15 min to pellet large mitochondria (predominantly cytotrophoblast mitochondria) was resuspended in a minimal volume of respiratory medium containing MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) adjusted to pH 7.4. A part of the isolated mitochondrial fraction was frozen at –80°C until the enzyme assays were performed. This method has previously been shown to yield mitochondria free from contaminants from other parts of the cell. This method yields a total mitochondrial protein content estimated between 1–2 mg/g of wet placental tissue.

Endothelial NOS expression

The frozen placental tissue were thawed and minced into small pieces and homogenized in cell lysis buffer containing 100 mM Tris, 50 mM NaCl, 10 mM EDTA, 1% TritonX-100. Insoluble placenta tissues were removed by centrifugation at 3,000 g, 4°C, for 10 min. Samples were loaded and subjected to SDS-PAGE in 7.5% polyacrylamide gels. After electrophoresis, proteins were electro-transferred to nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ). Equal loading of samples (80 µg) and even transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% nonfat dry milk, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and then probed with a rabbit polyclonal antibody against the endothelial isoform of NOS (amino terminus, H-299, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:500) at room temperature. The nitrocellulose membrane was subsequently incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (dilution 1:1000), and revealed by chemiluminescence with ECL reagent (Amersham Biosciences; Piscataway, NJ). Densitometric analysis of the eNOS bands was performed using the NIH Image 1.54 software. β-actin expression levels were used to normalize the results [20].

NOS activity

Nitric oxide production was measured in cytosolic fractions using a spectrophotometric method by following the oxidation of oxyhemoglobin to methemoglobin at 37°C, in a reaction medium containing 50 mM phosphate buffer (pH 5.8 for mitochondrial

preparations and pH 7.4 for the cytosolic fractions), 1 mM CaCl₂, 50 µM L-arginine, 100 µM NADPH, 10 µM dithiothreitol (DTT), 4 µM Cu-Zn superoxide dismutase (SOD) (to avoid interference by O₂^{•-}), 0.1 µM catalase (to avoid oxyhemoglobin oxidation by H₂O₂), 0.5–1.0 mg submitochondrial protein/ml and 25 µM oxyhemoglobin (expressed per heme group). Kinetics were followed at 577–591 nm ($\epsilon = 11.2 \text{ mM}^{-1} \text{ cm}^{-1}$) in a double-beam double wave length spectrophotometer (Beckman-Coulter Series DU) [21].

Oxygen metabolism

Superoxide anion (O₂^{•-}) was detected using the fluorogenic dye MitoSOX (C₄₃H₃₄N₃IP) a membrane permeant and rapidly targeted mitochondrial O₂^{•-} indicator for living cells, with excitation/emission maxim of 510/580 nm. Based on the ability of the positively charged TPP⁺ moiety of MitoSox Red to accumulate in the mitochondrial matrix, and also due to its ability to intercalate into mtDNA, a sufficient amount of this probe is retained in the matrix. Both MitoSox Red free and DNA-intercalated forms are sensitive to O₂^{•-}. We developed a special protocol by flow cytometry in order to detect superoxide anion not consumed by the MnSOD in isolated mitochondria. Isolated mitochondria from placental tissue were loaded with 2.5 µM MitoSOX, during 20 min at 37°C, in the same MSH buffer supplemented with malate plus glutamate. Antimycin A (0.5 µM), an inhibitor of the ubiquinone–cytochrome c reductase, was used as a positive control. Auto fluorescence was evaluated in samples without probe. Hydrogen peroxide (H₂O₂) generation was determined in intact isolated human placental mitochondria by the scopoletin-HRP method following the decrease in fluorescence intensity at 365–450 nm ($\lambda \text{ exc} - \lambda \text{ em}$) at 37°C [21]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), 0.8 µM HRP, 1 µM scopoletin, 0.3 µM SOD to ensure that all O₂^{•-} was converted to H₂O₂; 6 mM succinate plus glutamate were used as substrates. Calibration was made using H₂O₂ (0.05–0.35 µM) as standard to express the fluorescence changes as nmol H₂O₂/min.mg protein. H₂O₂ production was highly sensitive to catalase addition (3,500 U/ml).

Maternal and Conceptus morphometrics

Study investigators were informed when any study participant was admitted for delivery, and were present to monitor labor and delivery and to collect data. Gestational age at the time of delivery (in weeks and days) was recorded from hospital perinatal records. Anthropometric measurements of newborns: birth weight and placental weight (SECA scale ±10 g), were taken one hour after delivery by standard methods [13,14]. Placental efficiency was defined as the fetal weight/placental weight, as described previously [13,14]

Protein assays

Determination of mitochondrial protein was determined by the Lowry assay [22] using bovine serum albumin as the standard (Bio-Rad Protein Assay). All experiments were performed in triplicate.

Statistical analysis

All the data were subjected to statistical analyses using the SAS program. Maternal and fetal characteristics presented were expressed as means ± SE. Values in figures are the mean ± SEM. Statistical comparisons of superoxide anion level and NO production by groups were performed with two-way ANOVA. Intra-group and patient comparisons of L-NAME or Antimycin

are tested with Wilcoxon test. The eNOS activity, H_2O_2 production, placental weight and placental efficiency were compared by Wilcoxon test. A difference was considered to be statistically significant when $p < 0.05$.

Results

Compliance with the trial method

From enrollment to at least 28–32 wk gestation, the mean participation of the active subjects was 28.9 out of 36 (SD 3.2) sessions over the 12 weeks. No participant experienced adverse events during or after the exercise.

eNOS expression and NO production

Figure 1 shows that in pregnant women 12 weeks of exercise training resulted in a 4-fold increase in eNOS/ β -actin expression in cytosolic samples ($p < 0.05$). A 2-fold increase in NO production was also observed in their cytosolic samples ($p < 0.05$) (Fig 2).

Oxygen metabolism

Within the mitochondria, the electron transport chain is the main source of reactive oxygen species, O_2^- being the primary molecule generated by the mitochondrial respiratory chain. The mitochondria were loaded with MitoSOX, a fluorescence probe that typically increases its emission in the presence of an excessive generation of O_2^- escaping from the action of the MnSOD.

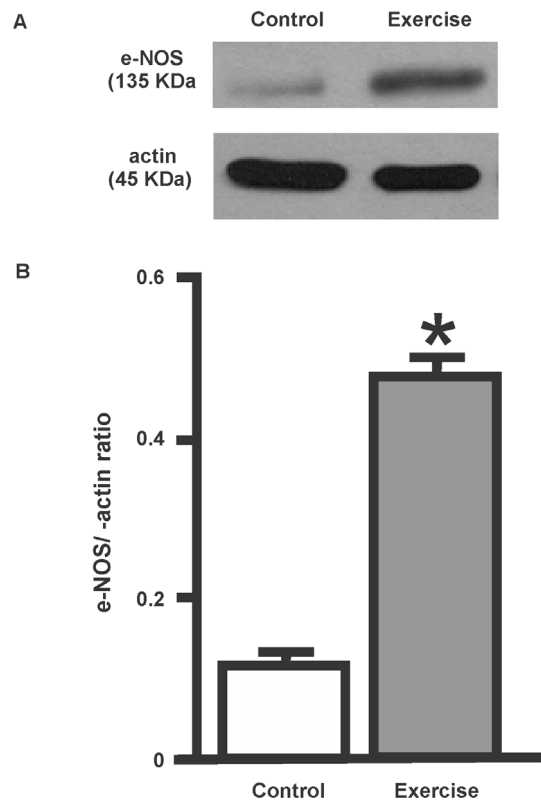


Figure 1. Effects of physical exercise on eNOS protein expression. **A.** Typical examples of Western blots for placental cytosolic fractions for each experimental group. Each blot was normalized to expression of β -actin from the same gel. **B.** Bars represent cytosolic eNOS/ β -actin ratios \pm SEM obtained after densitometric analysis. (* $p < 0.05$, as compared with control value). The results shown are representative of three independent studies. doi:10.1371/journal.pone.0080225.g001

Quantification of the superoxide anion levels as percentage values of MitoSox r. f. i is shown in Fig. 3. Mitosox fluorescence intensity was 8% lower in exercised mitochondria than in control samples. As expected, both mitochondrial samples exposed to antimycin showed higher FL-2 fluorescence values as compared with basal levels.

H_2O_2 production was measured in intact isolated human placental mitochondria from control and exercised women, using succinate-glutamate as mitochondrial substrates. Hydrogen peroxide rates obtained from human placenta mitochondria were within the range of H_2O_2 production rates previously observed in our laboratory for other tissues e.g. rodent heart, liver and brain [19,23]. Mitochondrial H_2O_2 production rate was approximately 0.51 ± 0.06 nmol/min.mg protein for placental mitochondria isolated from the control women. A 37% decrease of H_2O_2 production rate was observed in the placental mitochondria of exercised women, compared with control subjects ($p < 0.05$) (Fig. 4).

Maternal and Conceptus morphometrics

Fetal weight (3013.2 ± 493.8 vs. 3133.3 ± 406.5 g) was not altered in the exercise trained compared to the non-exercise group. In contrast, placental (Figure 5A; $p < 0.05$) weight decreased in the exercise trained compared to the non-exercised. Furthermore, placental efficiency was increased (Figure 5B; $p < 0.05$) in the exercise trained. There was no difference in the number of cotyledons (12.5 ± 0.5 vs. 12 ± 1.0) between the exercise and non-exercise groups.

Discussion

The present study reveals several interesting and novel findings regarding maternal and placental adaptations to chronic exercise training during pregnancy. Exercise training during pregnancy led to a 2-fold increase in eNOS expression and a 4-fold increase in NO production in placental cytosol. Exercised women also showed 6% decreases in mitochondrial O_2^- levels and 26% decreases in H_2O_2 production rate in placental mitochondria. NO generated by NOS has been demonstrated to contribute to the regulation of vascular tone by counteracting the actions of vasoconstrictors [24]. NOS in the placental villous vasculature also corresponds to the type III calcium-calmodulin-dependent endothelial isoform, and no such NOS was found in the cytotrophoblast layer or in small fetal blood capillaries of term placenta [25]. According to the present findings, a combined resistance and aerobic exercise training program leads to an increased eNOS expression and NO production in human placental tissue. Potentially, the underlying mechanisms may relate to an exercise induced increase in shear stress on the endothelial monolayer, in turn enhancing the activity of enzymes such as eNOS and extracellular superoxide dismutase and shifting the reactive oxygen balance toward the beneficial NO [26]. However, other vasoactive substances such as prostacyclin, or endothelium-derived hyperpolarizing factor (EDHF) could also be involved [1,2]. It has been reported that exercise produces a state of hyperemia and an increase of blood flow and shear stress with a consequent increase in the production and bioactivity of NO [15,16]. However, parallel or subsequent events are less clear, and whether the improvement in endothelium function induced by exercise during pregnancy observed in the present study has a beneficial effect in the prevention of these disorders (i.e., reduced risk of preeclampsia, diabetes or hypertension), is yet to be determined.

The placenta generates reactive oxygen species which may contribute to the oxidative stress seen even in normal pregnancy, but this is increased in pregnancies complicated by pre-eclampsia,

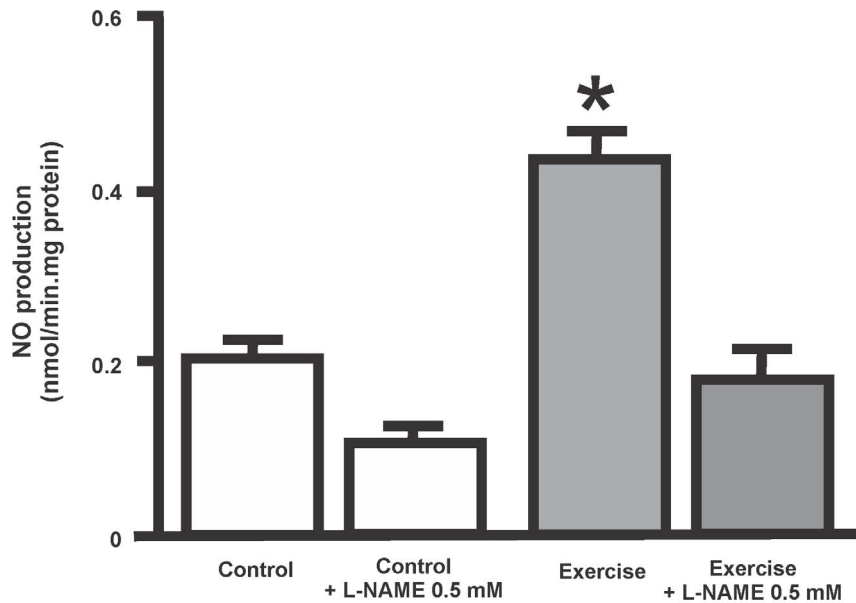


Figure 2. Effects of physical exercise on NO production in cytosolic samples from placental tissue. The specificity of the assay was evaluated using 0.5 mM L-NAME. Data are expressed as mean \pm SEM. (* $p < 0.05$, as compared with control value). doi:10.1371/journal.pone.0080225.g002

IUGR and gestational diabetes where elevated oxidative and nitrative stress have been clearly documented [27]. Nitrative stress is the covalent modification of proteins and DNA by H_2O_2 formed by the interaction of O_2^- and NO [28]. In the present study, reactive oxygen species O_2^- and H_2O_2 were significantly diminished in human placental mitochondria from exercised women as compared with control subjects, suggesting that chronic exercise training results in a lower level of oxidative stress. Studies by Bo et al. [29] showed that during prolonged exercise, UCP2 mRNA expression and activity in rat heart can be

upregulated, thereby reducing cross-membrane $\Delta\psi$ and reactive oxygen species production. One of the important effects of increased UCP2 expression is to decrease the generation of oxygen free radicals in the mitochondria. These results were in agreement with studies by Venditti et al. [30] who found that succinate-supported H_2O_2 release was higher in skeletal muscle from trained rats in both State 4 and State 3, while training did not affect mitochondrial oxygen consumption with both complex-I- and complex II-linked substrates.

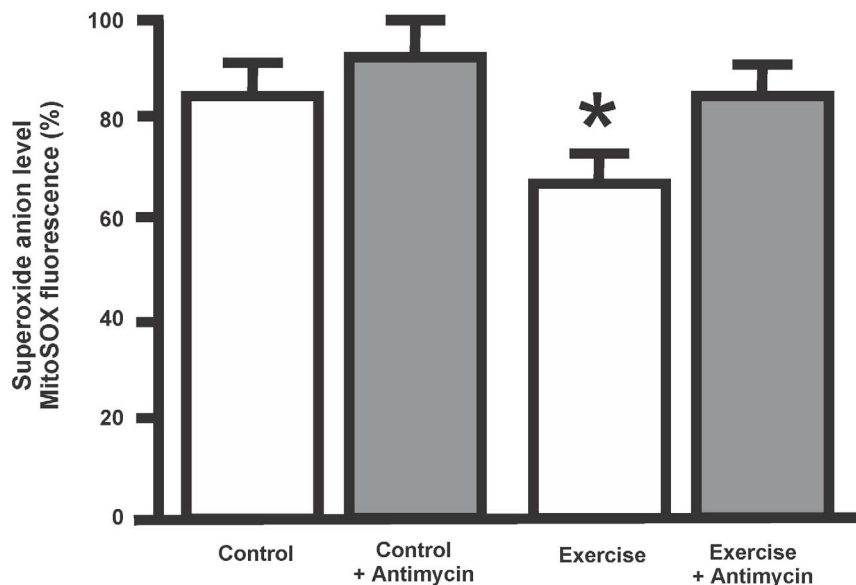


Figure 3. Determination of superoxide anion level. Mitochondrial samples were loaded with the superoxide anion sensor MitoSox during 20 min at 37°C. Antimycin A (0.5 μ M) was used as a positive control. Bar graph quantification of MitoSox fluorescence expressed as percentage values of human placental mitochondria from control and exercised women. Antimycin effect is also shown for both samples. Data are expressed as mean \pm SEM, from three independent experiments. (* $p < 0.05$, as compared with control value). doi:10.1371/journal.pone.0080225.g003

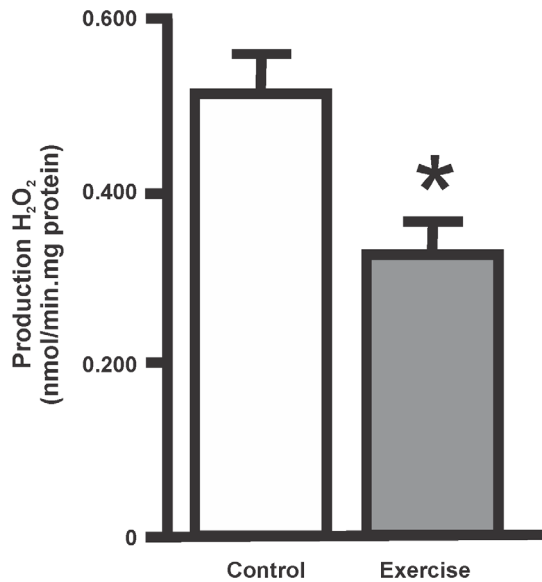


Figure 4. Bar scheme of H₂O₂ production rate of human placental mitochondria from exercised or control subjects. Data are expressed as mean \pm SEM of 4–6 mitochondrial samples for each condition. (* $p < 0.05$, as compared with control value). doi:10.1371/journal.pone.0080225.g004

We also report that exercise training during pregnancy was associated with a decreased placental weight, but an increased placental efficiency (fetal weight: placental weight). Previous studies have reported a wide range of effects of chronic exercise before and during pregnancy on conceptus weight in both humans [13,14] and rats [31]. The underlying differences between these observations remain unclear but the wide variation in the amounts and types of exercise are likely contributing factors. Further studies are needed to determine if other indices of placenta function such as transport of amino acids and vascular density are improved by exercise training.

More laboratory based studies and clinical trials are needed to confirm and elaborate the effects of aerobic and resistance exercises as the small sample size, warrants caution in the interpretation of the results. Nevertheless, these results may offer a plausible explanation for previous reports showing a decreased incidence of preeclampsia in woman participating in exercise training [32] and are consistent with findings from previous studies in other tissues [33].

As a general conclusion, regular exercise training during the second half of pregnancy increases eNOS expression and NO production and decreases reactive oxygen species generation in the mitochondrial respiratory chain in placental mitochondria. This finding provides a pathophysiologic framework for the elucidation of the positive effects of exercise on placental human and demonstrates the therapeutic potential of exercise training to improve fetal oxygenation and in turn potentially reduce risk of gestational disorders associated with impaired endothelial function [34].

References

- Sierra-Laguado J, Garcia RG, Lopez-Jaramillo P (2006) Flow mediated dilatation of the brachial artery in pregnancy. *Int J Gynecol Obstet* 93: 60–61.
- Brown CM, Garovic VD (2011) Mechanisms and management of hypertension in pregnant women. *Curr Hypertens Rep* 13: 338–346.

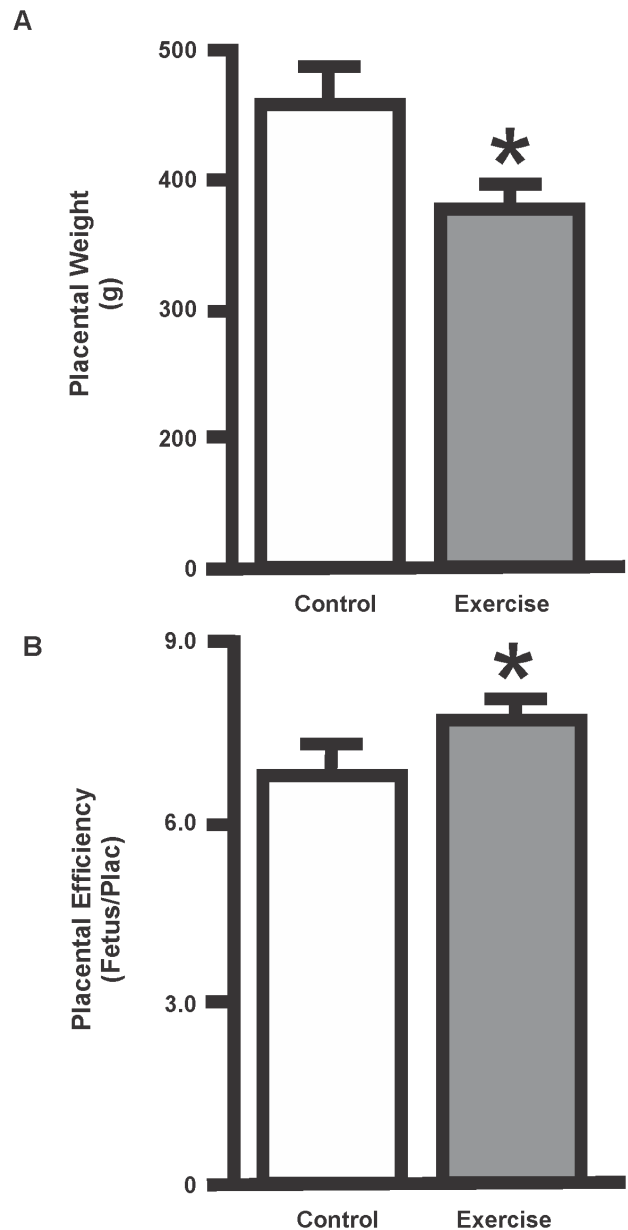


Figure 5. Effects of exercise on placental weight and placental efficiency. **A.** Placental weight was decreased in the exercise trained compared to the non-exercised. **B.** The placental efficiency was increased in the exercise group compared to the control group. Data are expressed as mean \pm SEM. (* $p < 0.05$, as compared with control value). doi:10.1371/journal.pone.0080225.g005

Author Contributions

Conceived and designed the experiments: ACAdP JB RRV. Performed the experiments: SLA RRV JB. Analyzed the data: RRV AC. Contributed reagents/materials/analysis tools: SLA RRV AC JB. Wrote the paper: SLA RRV.

- Wisdom SJ, Wilson R, McKillop JH, Walker JJ (1991) Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. *Am J Obstet Gynecol* 165 (6 Pt 1): 1701–1704.
- Myatt L, Cui X (2004) Oxidative stress in the placenta. *Histochem Cell Biol* 122:369–382.

5. Oostdam N, van Poppel MN, Eekhoff EM, Wouters MG, van Mechelen W (2009) Design of FitFor2 study: the effects of an exercise program on insulin sensitivity and plasma glucose levels in pregnant women at high risk for gestational diabetes. *BMC Pregnancy Childbirth* 5:9:1.
6. Hegaard HK, Pedersen BK, Nielsen BB, Damm P (2007) Leisure time physical activity during pregnancy and impact on gestational diabetes mellitus, preeclampsia, preterm delivery and birth weight: a review. *Acta Obstet Gynecol Scand* 86: 1290–1296.
7. ACOG Committee Opinion No. 267 (2002) Exercise during pregnancy and the postpartum period American college of obstetricians and gynecologists. *Obstet Gynecol* 99: 171–173.
8. Jackson MRP, Gott SJ, Ritchie JW, Clapp III JF (1995) The effects of maternal aerobic exercise on human placental development: placental volumetric composition and surface areas. *Placenta* 16: 179–191.
9. Genest DS, Falcao S, Gutkowska J, Lavoie JL (2012) Impact of exercise training on preeclampsia: potential preventive mechanisms. *Hypertension* 60: 1104–1109.
10. Fortner RT, Pekow PS, Whitcomb BW, Sievert LL, Markenson G, et al. (2011) Physical activity and hypertensive disorders of pregnancy among Hispanic women. *Med Sci Sports Exerc* 43: 639–646.
11. Han S, Middleton P, Crowther CA (2012) Exercise for pregnant women for preventing gestational diabetes mellitus. *Cochrane Database Syst Rev* 7:CD009021.
12. Clapp III JF (2006) Influence of endurance exercise and diet on human placental development and fetal growth. *Placenta* 27:527–534.
13. Clapp III JF, Kim H, Burciu B, Lopez B (2000) Beginning regular exercise in early pregnancy: effect on fetoplacental growth. *Am J Obstet Gynecol* 183: 1484–1488.
14. Bergmann A, Zygmunt M, Clapp III JF (2004) Running throughout pregnancy: effect on placental villous vascular volume and cell proliferation. *Placenta* 25: 694–698.
15. Ramírez-Vélez R, Aguilar de Plata AC, Mosquera-Escudero M, Echeverri I, Ortega JG, et al. (2011) Influence of regular aerobic exercise on Endothelium-Dependent Vasodilation and Cardio-respiratory Fitness in pregnant women. *J Obstet Gynaecol Res* 37: 1601–1608.
16. Ramírez-Vélez R, Aguilar AC, Mosquera M, García RG, Reyes LM, et al. (2009) Clinical trial to assess the effect of physical exercise on endothelial function and insulin resistance in pregnant women. *Trials* 10:104.
17. Institute of Medicine of the National Academies Committee on the Scientific Evaluation of Dietary Reference Intakes (2006). *Dietary Reference Intakes: The Essential Reference for Dietary Planning and Assessment*. Washington (DC): The National Academies Press. 87–94 p.
18. Martínez F, Meaney A, Espinosa-García MT, Pardo JP, Uribe A, et al. (1996) Characterization of the F1F0-ATPase and the tightly-bound ATPase activities in submitochondrial particles from human term placenta. *Placenta* 17: 345–350.
19. Martínez F, Espinosa-García T, Flores-Herrera O, Pardo JP (1993) Respiratory control induced by ATP in human term placental mitochondria. *Placenta* 14:321–331.
20. Boveris A, D'Amico G, Lores-Arnaiz S, Costa LE (2003) Enalapril increases mitochondrial nitric oxide synthase activity in heart and liver. *Antioxid Redox Signal* 5: 691–697.
21. Boveris A (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods Enzymol* 105:429–435.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.
23. Lores-Arnaiz S, D'Amico G, Czerniczyniec A, Bustamante J, Boveris A (2004) Brain mitochondrial nitric oxide synthase: in vitro and in vivo inhibition by chlorpromazine. *Arch Biochem Biophys* 430:170–177.
24. Rossmanith WG, Hoffmeister U, Wolfahrt S, Kleine B, et al. (1999) Expression and functional analysis of endothelial nitric oxide synthase (eNOS) in human placenta. *Mol. Hum. Reprod* 5: 487–494.
25. Myatt L, Brockman DE, Eis AL, Pollock JS (1993) Immunohistochemical localization of nitric oxide synthase in the human placenta. *Placenta* 14: 487–495.
26. Laufs U, Werner N, Link A, Endres M, Wassmann S, et al. (2004) Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 109: 220–226.
27. Wang Y, Walsh SW (2001) Increased superoxide generation is associated with decreased superoxide dismutase activity and mRNA expression in placental trophoblast cells in pre-eclampsia. *Placenta* 22: 206–212.
28. Myatt L (2010) Review: Reactive oxygen and nitrogen species and functional adaptation of the placenta. *Placenta* 31(Suppl): S66–S69.
29. Bo H, Jiang N, Ma G, Qu J, Zhang G, et al. (2008) Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2. *Free Radic Biol Med* 44: 1373–1381.
30. Venditti P, Masullo P, Di Meo S (1999) Effect of training on H₂O₂ release by mitochondria from rat skeletal muscle. *Arch Biochem Biophys* 372: 315–320.
31. Gilbert JS, Banek CT, Bauer AJ, Gingery A, Dreyer HC (2012) Placental and vascular adaptations to exercise training before and during pregnancy in the rat. *Am J Physiol Regul Integr Comp Physiol* 303: R520–R526.
32. Yeo S, Davidge S, Ronis DL, Antonakos CL, Hayashi R, O'Leary S (2008) A comparison of walking versus stretching exercises to reduce the incidence of preeclampsia: a randomized clinical trial. *Hypertens Pregnancy* 27: 113–130.
33. Ramírez-Vélez R (2013) Aerobic exercise training during pregnancy increases antioxidant status in nulliparous women: Secondary analysis of a controlled clinical trial. *Endocrinol Nutr* 60: 279–281.
34. Echeverri JG, Ramírez-Vélez R, Ortega JG, Mosquera M, Mateus JC, et al. (2009) Potencial efecto del ejercicio físico y del consumo de micronutrientes durante la gestación en factores maternos y placentarios asociados a Enfermedades Crónicas No transmisibles (ECNT) del adulto. *Colom Med* 40:448–459.