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ORIGINAL ARTICLE

Intrauterine growth restriction increases circulating mitochondrial DNA and Toll-like receptor 9 expression in adult offspring: could aerobic training counteract these adaptations?

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It has been demonstrated that intrauterine growth restriction (IUGR) can program increase cardiometabolic risk. There are also evidences of the correlation between IUGR with low-grade inflammation and, thus can contribute to development of several *cardiometabolic comorbidities*. Therefore, we investigated the influence of IUGR on circulating mitochondrial DNA (mtDNA)/Toll-like receptor 9 (TLR9) and TNF-α expression in adult offspring. Considering that the aerobic training has anti-inflammatory actions, we also investigated whether aerobic training would improve these inflammatory factors. Pregnant Wistar rats received *ad libitum* or 50% of *ad libitum* diet throughout gestation. At 8 weeks of age, male offspring from both groups were randomly assigned to control, trained control, restricted and trained restricted. Aerobic training protocol was performed on a treadmill and after that, we evaluated circulating mtDNA, cardiac protein expression of TLR9, plasma and cardiac TNF-α levels, and left ventricle (LV) mass. We found that IUGR promoted an increase in the circulating mtDNA, TLR9 expression and plasma TNF-α levels. Further, our results revealed that aerobic training can restore mtDNA/TLR9 content and plasma levels of TNF-α among restricted rats. The cardiac TNF-α content and LV mass were not influenced either by IUGR or aerobic training. In conclusion, IUGR can program mtDNA/TLR9 content, which may lead to high levels of TNF-α. However, aerobic training was able to normalize these alterations. These findings evidenced that the association of IUGR and aerobic training seems to exert an important interaction effect regarding pro-inflammatory condition and, aerobic training may be used as a strategy to reduce deleterious adaptations in IUGR offspring.

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Key words: aerobic training, fetal programming, heart, intrauterine growth restriction

Introduction

Since the first Barker observations, it has been recognized that insults (e.g. maternal manipulation diet, hypoxia and placental complications) occurred in intrauterine environment during critical periods of fetal development can promptly induce intrauterine growth restriction (IUGR) and lead to deleterious adaptations in target organs contributing to increase the risk of developing cardiometabolic diseases in later life.[1–4] Although the exactly mechanisms are not fully understood, it is reported that in both animals and humans, IUGR is able to program immunological disturbances (e.g. reduced maturation and immune suppression), which persist even for several years.[5–8] Furthermore, the role of immune system in hypertension and cardiovascular diseases has been demonstrated in different experimental models, at least in part due to Toll-like receptors (TLRs) pathway.[8–10] TLRs are transmembrane receptors involved in the detection of pathogens or endogenous signals released from damaged tissue, and once activated can produce pro-inflammatory cytokines (e.g. TNF-α).[11] TLRs are expressed in different tissues, and it was found that their expression is modulated by IUGR.[12–14] In fact, IUGR animals showed an increase in TLR9 expression, in ileum and thymus gland, suggesting a potential pro-inflammatory condition.[13,14] Particularly, the TLR9 can be activated by circulating mitochondrial DNA (mtDNA) – a damage-associated molecular pattern.[15] It has been highlighted that increased levels of circulating mtDNA *per se* is an additional risk factor for cardiovascular diseases.[16] Therefore, the development of strategies to reduce serum levels of mtDNA, or its capacity to activate TLR9 in IUGR conditions, is necessary.

Aerobic training, a non-pharmacology approach to reduce the incidence of cardiovascular risk is widely accepted worldwide.[17] Some studies were conducted to investigate the capacity of aerobic training in counteracting the deleterious adaptations IUGR induced.[18–20] In fact, it has been observed that aerobic training is effective to improve cardiovascular parameters in these animals, as blood pressure, and vascular function.[18,20]

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However, the role of aerobic training on circulating mtDNA levels and TLR9 expression in IUGR animals are still to be determined. So, in this study we first investigated the role of IUGR and aerobic training on circulating mtDNA content and TLR9 expression in IUGR offspring. In addition, we also evaluated the circulating and cardiac levels of TNF-α.

Materials and methods

All the procedures below were approved by Ethical Committee for Animal Research (Approval Number: 836880) at Federal University of São Paulo, and agreed to guidelines for Ethical Conduct in the Care and use of Animals established by Brazilian Society of Laboratorial Animal Science (SBCAL/COBEA). The rats used were obtained from colonies maintained at the Institute of Biomedical Sciences of University of São Paulo, were kept in a constant room temperature environment, with a 12:12 h light–dark cycle and free access to standard rat chow and tap water. Female Wistar rats were mated with male Wistar rats (age range 14–16 weeks) overnight. The presence of spermatozoa found in the vaginal smear was considered as the day 1 of conception. Pregnant rats were housed in individual standard cages and randomly divided into two groups: control ($n = 5$), which was fed *ad libitum* with a standard chow laboratory animal diet (Nuvilab CR1 – based on the recommendation of the National Research Council and National Institutes of Health, USA); and restricted group ($n = 6$) submitted to 50% of the typical daily food intake determined by the amount of food consumed by the control group during gestation period.[3] At least one female from the restricted group was unable to complete the gestation period due to miscarriages during pregnancy and/or occurrence of fetal resorption, and was excluded from the current study. Immediately after offspring was born, the pups were weighted, and the mothers from restricted group received *ad libitum* diet. Trying to prevent any alterations in neonatal growth, due to milk availability during suckling, the litter size was standardized to eight pups, and the sex ratio was kept as close as possible to 1:1. In case of more than eight pups per dam those were euthanized by cervical dislocation by a trained technician in accordance with the guidelines for Ethical Conduct in the SBCAL/COBEA. The offspring was nursed by their mothers until weaning at day 21. At 8 weeks of age, only male offspring of control ($n = 19$) and restricted ($n = 18$) groups were randomly assigned into four experimental groups: control (untrained; $n = 9$), trained control ($n = 10$), restricted (untrained; $n = 9$) and trained restricted ($n = 9$). To avoid litter effect we standardized each experimental group with one to two males per litter.

Aerobic training protocol

Beginning at 8 weeks of age, the rats from four experimental groups were initially submitted to a familiarization period to walking/running on a motor treadmill (8–10 sessions at 0.4–0.6 km/h; 0% grade; 10 min). We found that all groups exhibited the same performance level on treadmill (data not shown). After that, the rats were subjected to a maximal exercise test (graded exercise with increments of 0.3 km/h every 3 min, beginning at 0.3 km/h up to the maximal velocity attained for each animal) to establish running velocity. Aerobic training protocol was performed over 10 weeks, 5 days/week, with intensity between 50–60% of the maximal capacity test (low–to-moderate intensity training). The duration of sessions was progressively increased until 1 h/day. At 5 weeks, the maximal capacity test was repeated to adjust the running speed. Only one rat from trained control group was excluded from analysis, as it did not run during training protocol. Therefore, we finalize our study with nine male rats per experimental group. As expected, in both trained groups it was observed an increase in the running velocity in weeks 5 and 10 (data not shown). At 10 weeks, animals were submitted again to the maximal capacity test to verify the performance at the end of protocol. At the end of the protocols, the performance gain was similar between trained groups (data not shown). The untrained rats were also submitted to the same maximal capacity test, handled everyday and subjected once a week to a period of aerobic exercise (0.4–0.6 km/h; 0% grade; 10 min). No differences in the running velocity over 10 weeks of protocol were observed among untrained rats (data not shown). This approach has been used in order of submit untrained animals to the same handled stress; however, it was unable to promote the aerobic training adaptations. After this protocol, only nine males per experimental groups assigned for hemodynamic testing followed by blood and tissue collection.

Surgical procedures to artery cannulation

After 24–30 h of the last training session, rats from four experimental groups were anesthetized *intraperitoneally* with ketamine (80 mg/kg; Fort Dodge, IA, USA) plus xylazine (12 mg/kg; Fort Worth, TX, USA) and a polyethylene catheter was implanted into the left femoral artery. The catheter was exteriorized through the subcutaneous tissue in the back of the neck and sutured. All rats were treated with analgesics (ketoprofen, 2 mg/kg; BioFarm, Brazil) and antibiotic (Veterinary Pentabiotic, 24.000 UI/kg; Fontoura Wyeth, Brazil) administered via subcutaneous injection. After 24 h of this procedure, rats are freely moving in their cages and did not exhibit signs of stress or pain.

Hemodynamic data acquisition

Experiments were started 24 h after cannulation surgery in awake freely moving rats. The arterial catheter was connected to a pressure transducer (Model: CDX III; Cobe Labs, CO, USA) coupled to the amplifier (ML224 Quad Bridge Amp; ADInstruments, New South Wales, Australia) and the recording digital data in the acquisition system (Powerlab; ADInstruments). A period of ~20 min was allowed for cardiovascular parameters stabilization. After this, resting pulsatile blood pressure and mean blood pressure were continuously recorded for 60 min

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and processed using software (LabChart 7; ADInstruments). The inflection points of pressure signal were identified to generate beat-to-beat time series of mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse interval (PI). Heart rate (HR) was calculated as 1/PI.

Blood and tissue collection

After hemodynamic data acquisition, and under anesthesia (Sodium Thiopental – 50 mg/kg, via intraperitoneal), arterial blood from abdominal aorta was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Immediately, blood samples were centrifuged (1500 ***g***, 15 min, 4°C) and plasma was removed and stored in two aliquots at −80°C until TNF-α analysis and DNA isolation procedure. Heart was harvested, cleaned, and the left ventricle (LV) was weighted and maintained at −80°C until analysis of the cardiac TNF-α content.

DNA isolation and circulating mtDNA content

Circulating plasma DNA was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA integrity was checked on a 1% agarose gel. After that, isolated DNA was amplified using the GoTaq® qPCR Master Mix kit (Promega Inc., WI, USA) and Corbett Research system (Corbett Life Sciences, Sydney, Australia). Primers used to amplify mtDNA were as follows: (1) NADH dehydrogenase subunit 5 (ND5) (forward 5'-CAATACCCCACCCCCTTATC-3' and reverse 5'-GAGGCTCATCCCGATCATAG-3'); (2) bacterial 16S ribosomal RNA (rRNA) (forward 5'-AACAGGATTAGA TACCCTGGTAG-3' and reverse 5'-GGTTCTTCGCGTT GCATC-3') used to confirm the absence of bacterial contamination in samples; (3) hypoxanthine guanine phosphoribosyl transferase (HPRT) (forward 5'-GCTGGT

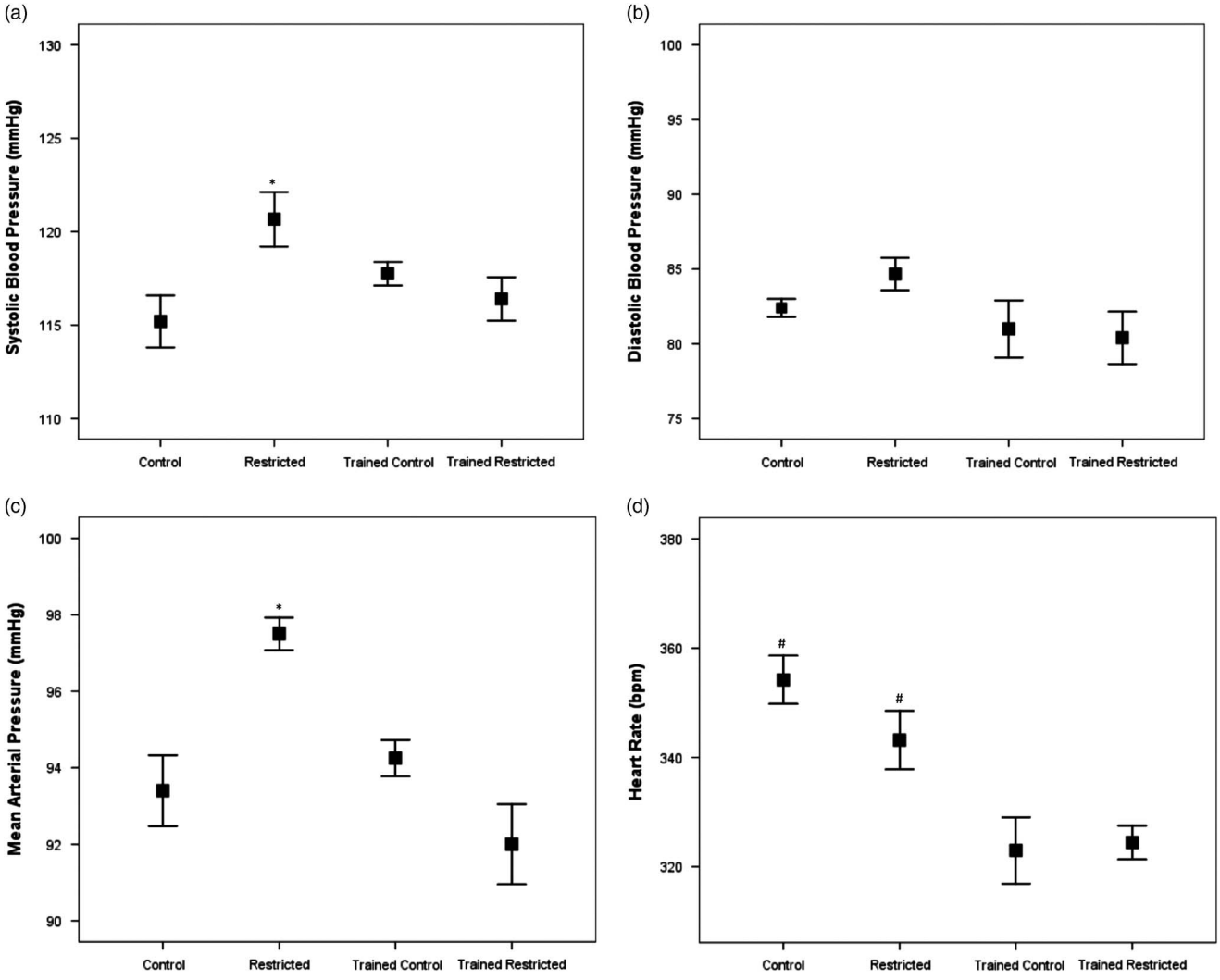

Fig. 1. Comparisons of the hemodynamic parameters between experimental groups: (*a*) systolic blood pressure levels; (*b*) diastolic blood pressure levels; (*c*) mean arterial pressure; (*d*) heart rate. All data were obtained in conscious rats. Data are expressed as mean and error bars represent S.E.M., $n = 6$ for all experimental groups. Two-way analysis of variance followed by the Bonferroni *post-hoc* test. $^{*}P < 0.05$ for restricted rats *v.* the remaining groups. $^{\#}P < 0.05$ for control and restricted groups *v.* their respective trained groups.

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GAAAAGGACCTCT-3' and reverse 5'-CACAGGACTA GAACACCTGC-3') used to confirm the absence of contamination with genomic DNA. All samples were analyzed in duplicate. Expression data were calculated from cycle threshold (Ct) value, using 2^{dCt} for quantification.

Cardiac expression of TLR9

The LV stored at −80°C was used for Western blot analysis. For the analysis of TLR9 expression, the LV was homogenized in liquid nitrogen, and the total tissue lysates were prepared using lysis buffer (50 mM Tris-HCl at pH 7.4, 1 mM EDTA, 10% sucrose, 2 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin and 0.01 mg/ml aprotinin). Homogenates were centrifuged (15,000 ***g***, 30 min, 4°C), and the supernatant was collected. The protein content of the lysates was determined using the BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Proteins from homogenized LV were treated with Laemmli buffer containing 200 mmol/l dithiothreitol and then (50 µg of protein extracts) subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% polyacrylamide gels. Following electrophoresis, proteins were electro transferred onto nitrocellulose membranes (BioRad, Hercules, CA, USA). The membranes were stained with *Ponceau*. After blocking non-specific sites with tris-buffered saline with tween 20 buffer containing 3% bovine serum albumin at room temperature for 2 h, the membranes were incubated overnight at 4°C with primary antibody anti-TLR9 (1:200; Santa Cruz, CA, USA). The primary antibody was detected using peroxidase-conjugated secondary mouse monoclonal antibody (1:5000; Sigma Aldrich, USA). The immune complexes were detected using an enhanced luminal chemiluminescence system (ECL, Plus; GE Healthcare, UK), which was subsequently exposed to a photographic film. The film was developed, and the bands were analyzed using Image J Software (Wayne Rasband; National Institutes of Health, USA). The total expression of TLR9 was normalized to total protein amount, using Ponceau staining content and was expressed as the ratio between the optical density of TLR9 and the Ponceau staining content.[21]

Circulating and cardiac TNF-α content

The LV and plasma, stored at −80°C, were used to determinate TNF-α concentrations. Briefly, the LV was homogenized in liquid nitrogen, and the total tissue lysates were prepared using lysis buffer (100 mM Tris-HCl at pH 7.4, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM NaF, 10 mM EDTA, 2 mM phenyl methyl sulfonyl fluoride, 0.01 mg/ml aprotinin and 1% Triton X-100). Homogenates were centrifuged (15,000 ***g***, 30 min, 4°C) and the supernatant was collected. The protein content of the lysates was determined using the BCA Protein Assay Kit. Following, both cardiac and plasma samples were used for the analysis using Chemokine Magnetic Bead Panel assay plate to measure the TNF-α content's. The kit was performed according to the manufacturer's instructions. Standards and samples were measured in duplicate. The concentration values obtained from each LV sample were normalized using the protein concentrations of each sample. The results were expressed as *picograms* per milligram of protein (pg/mg).

Cardiac hypertrophic index

The index was calculated using the ratio of the LV weight to tibia length (LVW:TL). Values were expressed as milligram per millimeter (mg/mm).

Statistical analysis

Statistical analysis was performed using Statistics software version 7.0. Results are expressed as mean ± S.E.M. Data were analyzed by independent Student's *t*-test or two-way analysis of variance followed by the Bonferroni *post-hoc* test and the significance level was set in $P < 0.05$.

Results

We found that nutrient restriction during pregnancy promotes IUGR in offspring [C: 7.01 ± 0.34 g (variance: 6.3–8.0 g) *v.* RT: 4.9 ± 0.08 g (variance: 4.7–5.7 g); $P < 0.05$]. No significant differences in body weights were observed between control and restricted rats at 8 weeks of age (C: 292.0 ± 12.8 g *v.* RT: 296.7 ± 17.4 g; $P = 0.832$). At 18 weeks of age, we found that male restricted rats have a significant increase in both SBP and MAP without alterations in the DBP and HR. The aerobic training for 10 weeks was effective in promoting reduction of the blood pressure parameters and resting bradycardia in restricted offspring; however, no significant influences were observed in control offspring (Fig. 1a–1d). Significant interaction effect (IUGR × aerobic training) was observed on MAP ($P = 0.042$) and SBP levels ($P = 0.039$), as well as a main effect of the aerobic training for HR ($P < 0.001$).

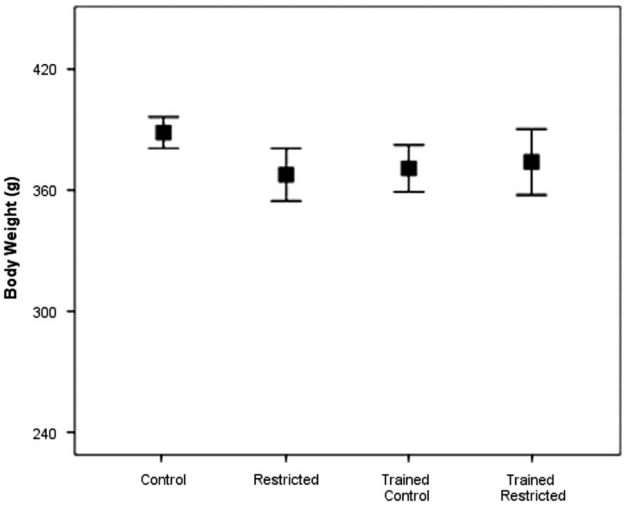

Fig. 2. Comparisons of the body weight between experimental groups at 18 weeks of age. Data are expressed as mean and error bars represent S.E.M., $n = 9$ for all experimental groups. Two-way analysis of variance followed by the Bonferroni *post-hoc* test.

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There were no significant differences in body weights at 18 weeks of age between different groups ($F = 0.536$; $P = 0.661$) (Fig. 2).

Circulating mtDNA gene ND5 was elevated in restricted rats. In addition, no significant differences were observed in the expression of the bacterial 16S rRNA and mtDNA gene HPRT among groups; therefore, no bacterial or DNA genomic contamination was found in our samples. On the other hand, 10 weeks of aerobic training were effective in causing a reduction in the circulating mtDNA gene ND5 in restricted group (Fig. 3a). A significant main effect of the IUGR ($P = 0.022$) and interaction effect (IUGR × aerobic training) ($P = 0.018$) on the mtDNA ND5 were detected. In parallel, we also evaluated the TLR9 expression in the LV mass. Protein expression levels for TLR9 were significantly higher in restricted rats (Fig. 3b). Significant main effect of the aerobic training ($P = 0.031$) and interaction effects (IUGR × aerobic training) ($P = 0.005$) were observed on protein expression levels for TLR9. However, the elevated TLR9 expression was normalized after training in restricted animals (Fig. 3b).

Consistent with the above data, we found that the IUGR was able to promote elevation in circulating TNF-α level, but the aerobic training for 10 weeks caused reduction in this cytokine in restricted rats (Fig. 4a). We found a significant interaction effect (IUGR × aerobic training) for circulating TNF-α levels ($P = 0.026$). Finally, there were no significant differences in the TNF-α content in the LV mass and in the cardiac hypertrophic index (LVW:TL ratio) among groups (Fig. 4b and 4c).

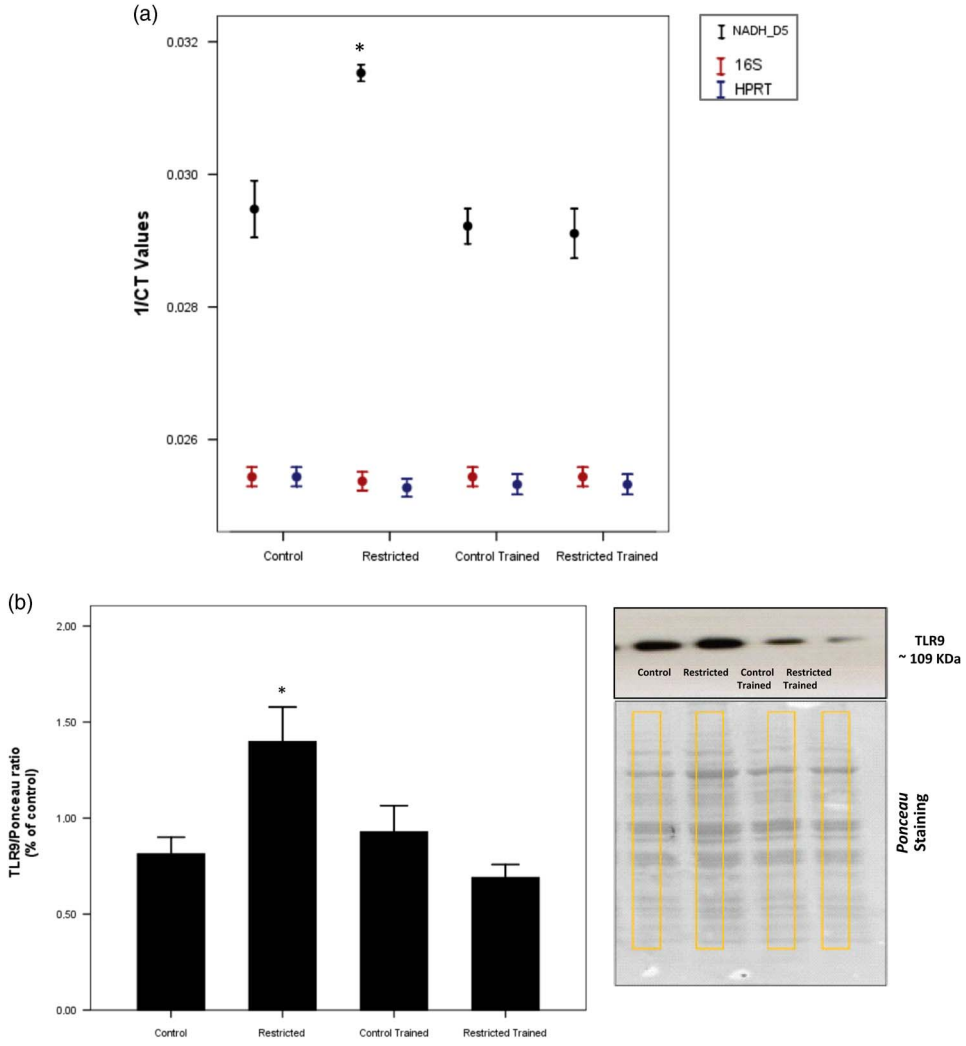

Fig. 3. (*a*) Quantification of circulating mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 5 (ND5) gene. The bacterial 16S ribosomal RNA and mtDNA gene hypoxanthine guanine phosphoribosyl transferase (HPRT) showed no bacterial or DNA genomic contamination in our samples. Data are expressed as mean and error bars represent S.E.M., $n = 6$ for all experimental groups. (*b*) Western blot analyses of Toll-like receptor 9 (TLR9) expression in left ventricle. Representative immunoblots and optical density ratio normalized to total protein amount using *Ponceau* staining content. Data are expressed as mean ± S.E.M., $n = 6$ for all experimental groups. Two-way analysis of variance followed by the Bonferroni *post-hoc* test. $^*P < 0.05$ for restricted rats *v.* the remaining groups.

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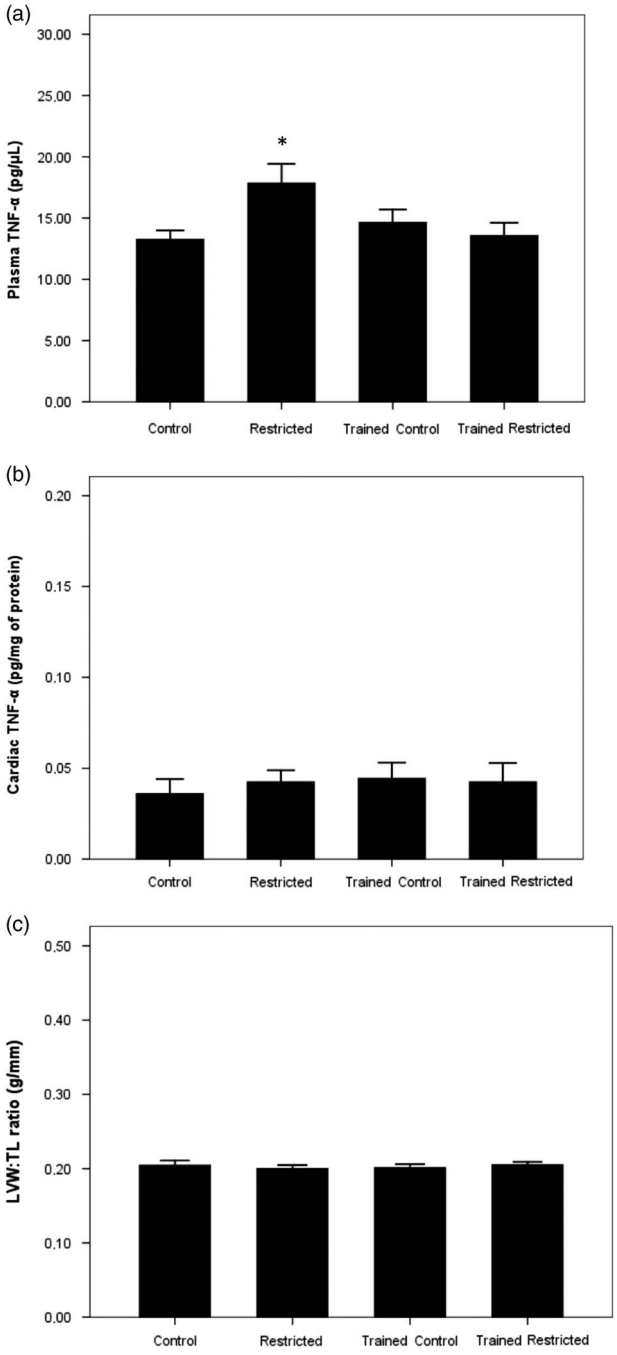

Fig. 4. (*a*) Histogram of the circulating TNF-α level ($n = 8$ for all experimental groups); (*b*) Histogram of the TNF-α content in the left ventricle (LV) mass ($n = 9$ for all experimental groups); (*c*) Histogram of the cardiac hypertrophic index LV weight to tibia length (LVW: TL ratio) ($n = 9$ for all experimental groups). Data are expressed as mean ± S.E.M. Two-way analysis of variance followed by the Bonferroni *post-hoc* test. $^*P < 0.05$ for restricted rats *v.* the remaining groups.

Discussion

There are evidences in literature that implicate IUGR in the development of cardiovascular comorbidities.[1–4] The main finding of our study was the detection of changes in the circulating mtDNA gene ND5 in association with both up-regulation of the TLR9 protein expression in the LV mass and increased circulating TNF-α levels in IUGR rats. On the other hand, 10 weeks of the aerobic training was able to promote improvement in all these parameters. Importantly, we also found that the association of IUGR and aerobic training seemed to exert a synergistic effect on these pro-inflammatory factors, as a significant interaction effect of IUGR and aerobic training was observed on circulating mtDNA, TLR9 protein expression and TNF-α levels.

The association between increased circulating mtDNA levels and cardiometabolic conditions has been reported.[10,22] It is important to emphasize that the mitochondria's and hence mtDNA transmitted to the fetuses comes exclusively from the mother, so considering that metabolic function is its primary function, it is plausible to consider that mitochondrial programming occur to promote fetal survival in a nutrient restricted environment.[23–25] In fact, mitochondria impairment observed in IUGR rats is characterized by changes in mtDNA content, abnormal shape, size and reduced mitochondrial function.[23–25] In addition, it has been postulated that the mtDNA release from mitochondria to plasma occur after mitochondrial disruption due to high levels of mitochondrial oxidative stress.[26] Our present data demonstrated that aerobic training was able to normalize the increased levels of circulating mtDNA in IUGR rats. Despite the mechanisms by which aerobic training exerts its beneficial effects in mtDNA levels are not elucidated, it is plausible to speculate that the anti-oxidative role could be involved. Few studies investigated the effects of exercise training in mtDNA levels. Our data agreed with some studies which reported significantly reduction in circulating mtDNA levels induced by aerobic exercise[27,28], whereas others observed that exercise increased or did not change the mtDNA levels in skeletal muscles and in the heart.[29,30] Nonetheless, further studies are needed to identify potential mechanisms behind the beneficial effects of aerobic training on the mtDNA in IUGR rats.

The circulating mtDNA is an endogenous ligand to TLR9, so our next step was to assess the expression of TLR9 in the LV from IUGR rats. Our results showed upregulation of the TLR9 protein expression in the LV from IUGR rats. Changes in TLRs expression patterns have been already described in IUGR animals;[12–14] however, studies conducted to investigate the effects of IUGR on TLR9 expression are scarce.[13,14] It is known that once activated, TLR9 triggers a signaling cascade by modulating cytokines production, and contributing to possible organ damage occurrence in long term.[15] Hence, the aerobic training could be a crucial tool to reduce TLR9 expression and/or signaling. In fact, it is the first study that illustrates the impact of aerobic training on TLR9 expression in the LV from IUGR animals. On the other hand, the benefic role of aerobic training in reducing the TLRs expression, particularly the TLR4, provides evidence that chronic exercise could, in part, exerts its anti-inflammatory effects via TLRs.[31,32]

TLR9 signaling stimulates pro-inflammatory cytokines production.[15] Here, we demonstrated that the increased

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circulating TNF-α in IUGR rats was reduced to control levels after 10 weeks of aerobic training, whereas the TNF-α content found in the LV was similar among groups. Regardless, we did not observed changes in cardiac TNF-α content, our plasmatic data agreed in part with others that observed an inflammatory condition in different compartments, as lung, adipose tissue and blood.[33,34] We did not assess what are the possible sources of circulating TNF-α, but our results suggested that it is not from the LV. The normal TNF-α levels in LV from IUGR animals suggested that protective mechanisms in the heart of these animals, as anti-inflammatory pathways are not modulated by IUGR. In addition, it is plausible to speculate that the cardiac TFN-α expression may be time dependent in these animals. The anti-inflammatory effects of aerobic training were evidenced in the plasma from IUGR rats in the present study. Other studies have also observed that the decrease in pro-inflammatory cytokines after aerobic training is partially due to reduced TLRs expression and/or signaling.[32,35] In parallel, we investigated the cardiac mass. In this study we did not found differences among groups in cardiac hypertrophic index. Likewise Harvey *et al*.[36] found normal heart weight in male offspring submitted to global nutrient restriction in 60%. On the other hand, in IUGR rats induced by low-protein diet it was observed lower number of cardiomiocytes associated with reduction in heart size, and impairment of cardiac function.[37,38] So, it seems that changes in cardiac morphology depend on IUGR experimental model.

In summary, our data provide evidence that the association of IUGR and aerobic training seems to exert an important interaction effect regarding pro-inflammatory condition. In IUGR rats the aerobic training reduced the high circulating mtDNA, considering that mtDNA is a ligand to TLR9 activation, this beneficial effect of aerobic training could lead to lower TLR9 activation. In addition, the normalized TLR9 expression found in IUGR rats, after aerobic training, could also contribute to reduce cardiovascular complications in long term.

Acknowledgment

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Conflicts of Interest

None.

Ethical Standards

All authors acknowledge that the methods used to obtain the data presented in the current study are in agreement with the ethical standards and guidelines presented by the for Ethical Conduct in the Care and use of Animals established by Brazilian Society of Laboratorial Animal Science (SBCAL/COBEA).

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