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Moderate physical training counterbalances harmful effects of low-protein diet on heart: metabolic, oxidative and morphological parameters

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Abstract — Aims: Maternal low-protein diet induces several impairments on cardiac system. Conversely, moderate exercise has been widely recommended to health improvement due to its effects on heart function. Thus, we investigated whether the moderate physical training is capable to offset the lasting injuries of a maternal protein restriction on the hearts of male adult rats. Methods: Pregnant rats were divided into two groups: Control (C=17% casein) and undernutrition (U=8% casein). Offspring from the undernutrition group, at 60 days of life, were subdivided into undernutrition (U) and undernutrition+exercise (UT) groups. Treadmill exercise was performed: (8 weeks, 5 days/week, 60 min/day at 70% of VO2máx). 48 hours after last exercise session, tissues were collected for morphological and biochemical analysis. Results: Despite the deleterious effect induced by low-protein diet, physical training was able to restore morphological parameters to similar levels to the control group. Additionally, oxidative stress index was also improved in UT group, due to the increase in antioxidant enzymatic defense. In metabolic enzymes, maternal low-protein diet induced a change in metabolism, and moderate physical training improved oxidative metabolism. Conclusion: We demonstrated that moderate physical training can offset the cardiac metabolism in adult rats that were exposed to a maternal low-protein diet.

Keywords: maternal low-protein diet, moderate exercise, cardiac morphology, oxidative stress; cardiac metabolism.

Introduction

It is well known that the energy requirements vary among the tissues. In the heart, the low amounts of adenosine triphosphate (ATP) and the high energy demands impose to this organ a fast and continuous ATP turnover. In normal conditions, adult heart obtains ATP mainly through fatty acid oxidation, however, in some pathological conditions, the ATP supplied to this organ can mostly be from glucose and lactate.

Studies have demonstrated that the onset of cardiac impairments can be related to the intrauterine life, wherein external factors, such as malnutrition, can induce long-lasting biochemistry and structural changes to the organism. In this context, Hales, et al. and Barker, Winter, Osmond, Margetts, Simmonds analyzing people with an average of 64 years, who were born in Hertforshire between 1920 and 1930, concluded that death from cardiac ischemia was more frequent in people born with low weight those with than normal weight. Further studies show that malnutrition during the critical period of development can induce metabolic syndrome indicating a strong correlation between perinatal undernutrition and the development of metabolic and cardiovascular diseases later in life. Previous work has shown that protein restriction during the critical period of development changes cardiac metabolism. In addition, studies from our laboratory also have shown that protein undernutrition during the developmental period induces oxidative stress in hearts and brainstems of adult offspring.

Exercise training, on the other hand, is well recognized as a reducer in the propensity to cardiovascular disease by the reduction of risk factors such as obesity, hypertension and oxidative stress. It is proposed that the exercise upregulates the antioxidant defenses which leads to an improvement in the cellular resistance to oxidative stress. In addition, moderate physical training has also been related with an improvement in cardiac function, likely via modification in ventricular structure, including left ventricular, wall thickness, end-diastolic diameter, among others.

Previous reports demonstrated that protein restriction during the critical period of development modulates morphological heart parameters as well as induces deleterious effects on the oxidative status in rat hearts. Since the moderate physical exercise is known as an important non-pharmacological strategy to combat cardiac diseases, the present study was performed to investigate whether the moderate physical training in adult rats is capable to compensate the deleterious effects of a maternal low protein diet on cardiac metabolism, ventricular morphology and oxidative stress.
METHODS

Experimental Approach to the Problem

In the present study we examined whether the moderate physical training can counterbalance the injuries on the hearts from adult offspring that suffered nutritional insult during the critical period of development, on the cardiac morphology, oxidative stress parameters and metabolic enzymes. In order to investigate the effects of moderate physical training on the cardiac parameters of undernourished rats, we used male adult rats that had their mothers fed either with normoprotein (17% casein) or low-protein (8% casein) diet throughout gestation and lactation period. A moderate physical training based on the 70% of VO2 max capacity was used daily (5 days week−1) during 8 weeks. 48 hours after the last session of training, rats were killed by decapitation and had their hearts collected to morphological and to biochemical analysis.

Animals

All experiments were carried out with offspring from an out-bred colony strain of Wistar rats obtained from the Nutrition Department of the Federal University of Pernambuco. The experimental design was performed in accordance with the guidelines of the Institutional Ethics Committee for Animal Research (approval protocol no. 23076.016335/2012-11), complying with the “Principles of Laboratory Animal Care” (National Institutes of Health, Bethesda, USA).

Procedures

Females with age between 80-90 days were mated in a ratio of 1 male to 2 females. They were daily checked and the day when spermatozoa were found in vaginal smear was considered as the day of conception and the pregnant rats were divided into two groups. The pregnant rats were fed with different diets, normoprotein (17% casein, n=4) or low-protein (8% casein, n=8), throughout the perinatal period (i.e gestation and lactation). Both diets had the same energetic value and were prepared at the Laboratory of Dietetic Technic at Academic Center of Vitoria – Federal University of Pernambuco-UFPE according to research previously published\(^{12}\).

Twenty-four hours after delivery, the litters were standardized to 8 pups each and the female pups maintained only to fill the litters, no adoption was used in this study. To avoid litter influence, at weaning, three male animals from each mother were randomly chosen to continue in the study and started to receive laboratory chow (LABINA, Purina Brasil\(^{18}\)) until ending the experiment.

Heart preparation for biochemical analysis

Rat hearts were collect as previous described\(^{12}\). The atriums were removed and the ventricles completely homogenized in and untrained groups. After this new division three groups were formed: control (C) n=12, undernutrition (U) n=12 and undernutrition trained (UT) n=12. The moderate physical training was performed in accordance to the parameters previously standardized in animals at 60 days of life\(^{18}\). Briefly, a treadmill adapted for rats (EP-131, Insight Equipments, SP, Brazil) and the protocol was conducted during 8 weeks (5 days week−1, 60 min day−1), each day-session was divided into four stages: (1) warm-up (5 min); (2) intermediary (20 min); (3) day target speed (30 min); and (4) cool down (5 min). The percentage of VO2\(_{\text{max}}\) during the training sessions was kept around 65–70%.

Rats from the sedentary groups were placed on the treadmill just as the exercised animals, but the treadmill remained off. After 48 hours of the last exercise session, the rats had their hearts collected to morphological and to biochemical analysis. All animals completed the whole experiment protocol.

Morphological analysis

The following parameters were measured: left ventricle (LV) cross-section, left ventricle thickness and left ventricle cavity.

1. Processing of the material for histological study

Heart processing was conducted as recommended by Toscano, et al.\(^{17}\). Briefly, after anesthesia, the atrium were removed and ventricles were fixed in 10% formalin followed by a cross-cut at the midpoint between the atrioventricular groove and the apex of the heart at the ventricular level, dividing the heart in two. After fixation, the fragments were conventionally processed for light microscopy being dehydrated in increasing ethanol concentrations (80 to 100%), cleared in xylene baths (20-minute), and immersed in paraffin. After inclusion, histological cross sections of the heart were made serially. The ventricular cavities were sectioned into thicknesses of 4 mm each, using a micromote Leica (RM 2125RT). Then they were stained with hematoxylin-eosin (HE), mounted on slides using entellan, photographed and observed under a light microscope.

2. Measurement of LV thickness, cross-sectional area and LV cavity area

The images of histological cross-sections of the heart were captured on graph paper using a scanner (HP brand, model C4700) coupled to a computer. To measure the LV thickness, LV cross-sectional area and LV cavity, Image J software (version 1.47t for Windows) was used.
cold buffer containing 50 mM-TRIS and 1mM-EDTA (pH 7.4), with the addition of 1 mM-sodium orthovanadate and 1.1 mM phenylmethanesulfonyl fluoride (PMSF) using a sampling homogenizer (Tecnal, Sao Paulo, Brazil). Ventricular homogenates were centrifuged at 4000 RPM for 10 min at 4°C. Protein concentration was determined in the supernatant using the Bradford protocol and this supernatant was used for the following biochemical analyses.

**Evaluation of Malondialdehyde production**

A total of 0.2 mg/ml heart homogenate was used to measure malondialdehyde production (MDA) following reaction with thiobarbituric acid (TBA) at 100 °C according to the method of Draper, Squires, Mahmoodi, Wu, Agarval, Hadley19. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances react to produce a pink pigment with a maximum absorption at 535 nm. The reaction was developed by the sequential addition to the sample of 30% trichloroacetic acid and Tris-HCl (3mM) followed by thorough mixing and centrifugation at 2 500 g for 10 min. This supernatant was transferred to another tube and 0.8% TBA (v/v) was added before mixing and boiling for 30 min. After cooling, the absorbance of the organic phase was read at 535 nm in a spectrophotometer. Results were expressed as nmol per mg of protein12.

**Superoxide dismutase (SOD) assay**

The determination of total superoxide dismutase enzyme activity (t-SOD) was performed according to the method of Misra, Fridovich20. Supernatants (0.1 mg/ml) collected from homogenized hearts following centrifugation were incubated with 80 µl sodium carbonate (0.05%, pH 10.2, 0.1 mM EDTA) at 37°C before development of the reaction by the addition of 30 mM epinephrine (in 0.05% acetic acid) and determined by measuring the kinetic of the inhibition of adrenaline auto-oxidation at 480 nm expressed as U/mg protein.

**Catalase (CAT) assay**

CAT activity was measured according to the method described by Aebi21. The principle of the assay is based on the determination of the rate constant (k) of H2O2 decomposition, which under our conditions of temperature and pH, was defined as 4.6 x10^7. The assay mix was compounded by 50 mM-phosphate buffer (pH 7.0), 300 mM H2O2, and sample (0.3 mg/ml). The rate constant of the enzyme was determined by measuring change in absorbance (at 240 nm) per minute over a 4-minute period at 30 °C and the CAT activity was expressed as U/mg protein.

**Phosphofructokinase (PFK) assay**

PFK was determined on the homogenate by a modification method developed by Opie, Newsholme22. The enzymatic reaction system consisted of 50 mM Imidazole (pH 7.4), 6 mM MgCl2, 0.4 mM NADH, 5 mM ATP, 5mM glucose-phosphate-dehydrogenase, 5 mM triose phosphate isomerase, 5 mM glycerophosphate, 5 mM aldolase and 0.3 mg/ml of sample. The reaction started with 20 mM fructose-6-phosphate at 37°C and the oxidation of NADH was followed at 340 nm to measure PFK activity.

**β-hydroxyacyl-CoA dehydrogenase (β-HAD) assay**

β-HAD was determined as previously published by Ito, et al.23. The enzymatic reaction system consisted of 50 mM Tris-HCL (pH 7.4), 50 mM Imidazole, 12 mM EDTA, 0.018 mM NADH and 0.3 mg/ml of sample. The reaction started with 0.1 mM acetocetil-CoA at 37°C and absorbance of 340 nm. β-HAD was measured from oxidation of NADH.

**Citrate synthase (CS) assay**

CS activity was determined as described previously24. For citrate synthase activity, 0.3 mg/ml of sample was incubated in a reaction mixture containing (in mM) 100 Tris-HCL (pH 8.1), 1 MgCl2, 1 EDTA, 0.2 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) (ε=13.6 µmol·ml⁻¹·cm⁻¹), 0.1 acetyl-CoA, and 0.5 oxaloacetate at 25°C. The citrate synthase activity was measured by assessing the rate of change in absorbance at 412 nm over 3 min (30-s intervals).

Obs: All drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sovereign (Sao Paulo, Brazil).

**Statistical analyses**

Analyses were performed using GraphPad Prism (version 6.0). The analysis of the enzyme activity was performed according to the specific reference for each enzyme. For nonparametric data, the Kruskal-Wallis test followed by Dunn’s post-test was used; for parametric data, the ANOVA One-Way test and followed by Tukey post-test were used. The values were expressed as mean ± standard error of mean (SEM). Data were considered as statistically significant for p≤0.05.

**Results**

In the present study we aimed to investigate the effect of moderate physical training on adult rats that had suffered nutritional insult during the critical period of development. Evaluating cardiac morphology, our data demonstrates that perinatal protein restriction significantly interferes in cardiac morphology decreasing left ventricle thickness and cross-section. On the other hand, moderate physical training restored left ventricle thickness and the cross-section to control values (figure 1A-B). Related to the left ventricle cavity, we observed that undernourished trained rats showed an increase compared to control or undernourished LV (figure 1C).
Figure 1: Effect of maternal low-protein diet and moderate physical training on cardiac morphology. Left ventricle thickness (A), cross-section (B) and cavity area (C) in male offspring at 120 days of life. C= Control group; U= undernourished group; U+T= undernourished plus 8 weeks of moderate physical training group. Data are shown as mean + SEM for 4-6 animals per group. *p<0.05; ** p<0.01.

Figure 2: Effect of maternal low-protein diet and moderate physical training on cardiac oxidative stress. Lipid peroxidation evaluated by MDA levels (A) Antioxidant enzymatic defense, Superoxide dismutase activity (B) and catalase activity (C) in male offspring at 120 days of life. C= Control group; U= undernourished group; U+T= undernourished plus 8 weeks of moderate physical training group. Data are shown as mean + SEM for 4-6 animals per group. *p<0.05; **p<0.01; *** p<0.001.
After the observation of cardiac morphology alteration induced by perinatal protein restriction, we next evaluated oxidative status and metabolic enzymes. One important biomarker for oxidative stress is the MDA levels. As demonstrated in Figure 2A, protein restriction increases significantly MDA levels in the cardiac tissue, although moderate physical training restored MDA product to control levels (Control: 3.85 ± 0.51 nmol/mg protein; U: 7.81 ± 0.49 nmol/mg protein; U+T: 3.47 ± 0.08 nmol/mg protein). Associated to the levels of pro-oxidant agents, there are the antioxidant enzymes that combat and decrease the action of the oxidant agents. Therefore in the evaluation of the first antioxidant enzyme, superoxide dismutase-SOD, we observed that in undernourished group this enzyme was significantly reduced, otherwise moderate physical training raised SOD activity to control values (Control: 3.23 ± 0.17 U/mg protein; U: 1.39 ± 0.21 U/mg protein; U+T: 3.26 ± 0.21 U/mg protein, figure 2B). The function of SOD is convert anion superoxide to hydrogen peroxide, and additional enzyme, catalase-CAT, is capable to convert hydrogen peroxide in oxygen and water. Our observation of CAT activity showed that undernutrition during the critical period also decreased the activity of this enzyme, but physical training significantly improved the catalase activity (Control: 0.89 ± 0.09 U/mg protein; U: 0.53 ± 0.08 U/mg protein; U+T: 1.30 ± 0.05 U/mg protein, figure 2C).

Either glucose or fatty acids can feed the Krebs cycle. After the glucose is internalized, a series of reactions occur to transform the glucose into pyruvate, wherein Phosphofructokinase-PFK catalyzes the critical step in the glycolytic pathway, converting fructose-6-phosphate to fructose-1,6-bisphosphate. This enzyme was not modulated in any experimental condition used (figure 3A). Regarding the Krebs cycle maintaining from β-oxidation, we further quantified the activity of β-hydroxyacyl-CoA dehydrogenase-β-HAD, one key enzyme responsible for β-oxidation of fatty acid. Our data demonstrate that undernourished rats had a significant decrease in β-HAD activity; on the other hand, moderate physical training overcompensated the enzymatic activity impairment (Control: 3.90 ± 0.39 U/mg protein; U: 1.18 ± 0.25 U/mg protein; U+T: 5.79 ± 0.15 U/mg protein, figure 3B).

Then, we analyzed the citrate synthase-CS activity, this enzyme being responsible for catalysis of the first reaction in the Krebs cycle, condensing acetyl-CoA and oxaloacetate to citrate, and used as a marker for mitochondrial oxidative capacity. Our data demonstrates that perinatal protein restriction significantly decreases the activity of this enzyme, but physical training restored the CS activity (Control: 3.29 ± 0.54 U/mg protein; U: 1.20 ± 0.15 U/mg protein; U+T: 2.61 ± 0.16 U/mg protein, figure 3C).

Figure 3: Effect of maternal low-protein diet and moderate physical training on cardiac metabolism. Enzymatic activities of Phosphofructokinase-PFK (A); 3-hydroxybytyryl-CoA dehydrogenase-β-HAD (B) and Citrate synthase (C) in male offspring at 120 days of life. C= Control group; U= undernourished group; U+T= undernourished plus 8 weeks of moderate physical training group. Data are shown as mean ± SEM for 4-6 animals per group. *p<0.05; **p<0.01; ***p<0.001.
Discussion

In the present study we examined the effects of moderate physical training, on adult offspring that suffered nutritional insult during the critical period of development, on the cardiac morphology, oxidative stress parameters and metabolic enzymes. Our data, for the first time in the literature, brings the combination of a detrimental situation induced by deficient diet with a non-expensive treatment, as the physical exercise to combat cardiac damage induced by a situation still present in our society, which is the undernutrition. There are epidemiological and clinical data supporting the hypothesis that poor quality of diet during developmental period increases the risk for several diseases including cardiovascular diseases. In the opposite direction, hefty evi
dences have demonstrated that physical exercise counteracts the deleterious effect of several metabolic and cardiovascular diseases.

The Developmental Origins of Health and Disease hypothesis considers that nutritional insult between development and early ages induces fetus or newborn adaptation in response to the signals in the intraterotum environment, resulting in adjustments to support immediate survival and improve success in an adverse postnatal environment. However, inappropriate interpretations of prenatal signals and alterations in the postnatal environment may result in a mismatch between prenatal projections and postnatal reality.

It is suggested that these adaptive responses may eventually be detrimental in postnatal life, leading to an increase in the risk of chronic diseases in adulthood, wherein the early undernutrition causes loss of cardiac muscle as well as alterations in cardiac morphology. Recent studies demonstrated that physical training acts as a treatment by ameliorate cell function in different tissues. Our data demonstrate that moderate physical training also attenuates the deleterious effect of nutritional insult in heart tissue, corroborating with previous studies that demonstrate which physical exercise improves cardiac function both in humans and experimental models.

Our data suggest that protein restriction may induce alteration in cardiac morphology due to the increase in oxidative stress associated with decrease in antioxidant defense, which corroborates with previous findings. Tarry-Adkins, et al., demonstrated that maternal low-protein diet induces oxidative stress in the heart and suggest that this effect may contribute to the development of disease in adulthood. Corroborating with these previous observations, the study from our laboratory recently demonstrated that adult offsprings that suffered nutritional deprivation during the critical period of development have an impairment in the mitochondrial function (i.e decrease in mitochondrial oxygen consumption and increase in ROS-production) associated with a marked oxidative stress in hearts. Additionally our data demonstrates that moderate physical training can act as a positive stimulus against the deleterious effect induced by undernutrition, increasing antioxidant defense and diminishing oxidative stress, corroborating with previous studies that demonstrate that exercise is a positive health modulator, wherein the antioxidant improvement seem to be upregulated via NF-kB signaling pathway.

The adult heart prefers fatty acid oxidation as the main substrate to obtain energy supply. During the development of heart failure, the main energy substrate often switches from fatty acid oxidation to carbohydrate metabolism, with a declining regulation of enzymes involved in fatty acid oxidation. Studies suggest that initially the substrate switch improves the efficiency of the pathological heart, since the amount of ATP produced per oxygen consumed is higher in glucose oxidation than fatty acid oxidation. In agreement with this reasoning, Tappia, Guzman, Dunn, Arouitounova, demonstrated that a maternal low-protein diet can increases the gene expression and protein levels of key components of glucose metabolism in the neonatal rat heart, which may be related to accelerated energy supply, demand and utilization for ventricular remodeling due to compromised contractile performance during early life, however, at the current study, no statistic changes were observed in cardiac PFK activity. The decrease in the β-HAD activity, nevertheless, suggests that protein undernutrition decreases the lipid oxidation capacity in the heart, being the moderate physical training a feasible strategy to offset it. Taken together, our data and literature data suggest that undernutrition during the developmental period induces an adaptive response that modulates cardiac metabolism that can lead to cardiac diseases in adulthood, similar to alterations observed in other cardiac diseases such as hypertrophy and heart failure.

In relation to citrate synathase activity, a marker for mitochondrial oxidative capacity, our data show that undernourished rats had a significant decrease in citrate synathase activity, suggesting that nutritional insult during the critical period of development significantly decreases oxidative capacity in cardiac tissue. Moderate physical training, however, attenuated the enzymatic impairment observed in the undernourished group. Corroborating our data, Laker, Włodek, Wadley, Gallo, Meikle, McConnell, using an experimental model of intrauterine growth restriction (IUGR), showed that treadmill exercise training increases skeletal muscle β-HAD and citrate synathase enzymatic activities in male offspring. In addition, Horowitz, Leone, Feng, Kelly, Klein demonstrated that endurance training increases the skeletal muscle expression of TCA cycle enzyme, citrate synathase, with concomitant increase in the mitochondrial oxidative capacity. These data suggest that moderate physical training induces an energy metabolic recovery in the heart reflecting an increased capacity for mitochondrial fatty acid oxidation and improvement in oxidative metabolism, minimizing the pathological effect of an early nutritional insult to the heart.

Here, we demonstrated that maternal low protein diet induces deleterious effects on cardiac morphology, antioxidant enzymatic capacity and oxidative metabolism. Notwithstanding, moderate physical training is capable to offset both morphologic as well as metabolic parameters. Taken together, our results demonstrated the benefits of moderate physical training, which in only 8 weeks was capable of restoring ventricular remodeling, cardiac metabolism and oxidative stress in animals that suffered maternal protein restriction.
References


