Sueli Moreno Senna

Efeito do Treinamento Físico Moderado sobre Linfócitos do Sangue e Baço de Ratos Adultos Submetidos à Desnutrição Perinatal

Recife, 2014

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Tese apresentada ao Programa de Pós-Graduação em Nutrição do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Nutrição.

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Recife

2014

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Resumo

A desnutrição infantil é um problema de saúde pública que afeta principalmente países em desenvolvimento. Dentre as mortes associadas à desnutrição, 68% são de causa infecciosa. Isso ocorre pois, nas fases de desenvolvimento, a desnutrição leva a respostas imunológicas distorcidas frente a um desafio infeccioso. Entretanto, a plasticidade fenotípica confere ao organismo constante adaptação ao interagir com as demandas do ambiente. Dentre vários fatores ambientais, o exercício físico pode ser benéfico para o sistema imunológico. Portanto, investigamos o efeito do treinamento físico moderado sobre parâmetros imunológicos de animais adultos submetidos à desnutrição proteica materna perinatal. Ratas Wistar virgens foram acasaladas e, após a constatação da cópula, foram divididas em dois grupos conforme a dieta oferecida: controle (C, n=10, proteína a 17%); e desnutrido (LP – low protein, n=10, proteína a 8%). Após o desmame das ninhadas os filhotes machos permaneceram no experimento recebendo dieta padrão de laboratório. Aos 60 dias de vida os filhotes foram submetidos a treinamento físico moderado (70% VO_{2max}, 60 minutos/dia, 5 dias/semana, 8 semanas) e subdivididos da seguinte forma: controle (C, n=17); treinado (T, n=19); desnutrido (low protein) (LP, n=19) e desnutrido treinado (LP+T, n=17). Após 24 horas do término do treinamento os ratos receberam injeção intraperitoneal de lipopolissacarídeo (LPS, 1mg/ml/kg de peso corporal) para simular um estado séptico. Permaneceram então oito grupos: controle (C, n=8); controle endotoxêmico (C+LPS, n=9); treinado (T, n=10); treinado endotoxêmico (T+LPS, n=9); desnutrido (LP, n=9); desnutrido endotoxêmico (LP+LPS, n=10), desnutrido treinado (LP+T, n=8) e desnutrido treinado endotoxêmico (LP+T+LPS, n=9). Após 24 horas da injeção os animais foram decapitados para coleta de sangue e baço. Os seguintes parâmetros imunológicos foram avaliados: distribuição de linfócitos T, B e Nk no baço e no sangue; taxa de apoptose de linfócitos esplênicos e concentração sérica de TNF-α. Ratos LP apresentaram maior porcentagem de linfócitos NK esplênicos que ratos controle (LP+LPS = 3,3±0,3%; C+LPS = 1,9±0,3%), situação que foi revertida pelo treinamento físico (LP+T+LPS = 1,6±0,3%, $p<0,001$). Adicionalmente, linfócitos do baço de ratos LP apresentaram maiores taxas de despolarização mitocondrial (MTD) e externalização de fosfatidilserina (PSE) que ratos controle (*MTD*: LP+LPS = 18,0±1,9% vs C+LPS = 6,1±1,9%, $p<0,001$; *PSE*: LP+LPS = 51,0±3,7% vs C+LPS = 26,5±2,8%, $p<0,001$). Entretanto, ratos LP+T+LPS apresentaram menor taxa de apoptose que ratos LP+LPS (*MTD*: LP+T+LPS = 9,0±1,8%, $p<0,01$; *PSE*: LP+T+LPS = 30,7±3,4%, $p<0,001$). A desnutrição proteica perinatal também alterou a distribuição de linfócitos T circulantes. Ratos LP+LPS apresentaram menores porcentagens que ratos C+LPS (39.6±2.3% vs 72,9±1,8%, $p<0,001$). Em ratos LP+T+LPS, essa resposta foi revertida (LP+T+LPS = 58,1±2,8%, $p<0,001$). Avaliamos ainda a concentração sérica de TNF-α, importante citocina inflamatória. Os ratos LP apresentaram elevada concentração sérica de TNF-α, independentemente do treinamento físico (LP+LPS = 26,7±3,8 vs C+LPS = 7,9±3,4 pg/mL, $p<0,001$; LP+T+LPS = 23,9±3,3 vs T+LPS = 4,7±2,9 pg/mL, $p<0,001$). Em resumo, a desnutrição perinatal induziu aumento da taxa de apoptose de linfócitos acompanhada de alteração do perfil linfocítico do baço e sangue durante evento endotoxêmico na prole adulta. O treinamento físico em intensidade moderada foi capaz de reverter esse quadro.

Palavras-chave: Nutrição. Exercício físico. Toxemia. Morte celular. Linfócitos.

Abstract

Early life undernutrition is a public health problem of developing countries. Among the deaths related to undernutrition, 68% were caused by infectious diseases. When undernutrition took place at developmental period, it can lead to impaired responses after an immune challenge. However, phenotypic plasticity keeps the organism in constant adaptation because of interactions with the environment through lifetime. Among several environmental factors the physical exercise can be positive for the immune system. Thus, we investigated the moderate physical training effect on immune parameters from adult rats submitted to maternal perinatal protein undernutrition. Female virgin Wistar rats were mated and, after the copulation confirmation, they were divided in two groups: control (C, n=10, 17% casein-based diet); and low protein (LP, n=10, 8% casein-based diet). At weaning only the male pups remained in the experiment receiving standard laboratory chow. At 60^{th} days of life the offspring started a moderate physical training (70% VO_{2max}, 60 minutes/day, 5 days/week, 8 weeks) e divided as follows: control (C, n=17); trained (T, n=19); low protein (LP, n=19) and low protein trained (LP+T, n=17). Twenty-four hours after the end of the training protocol the rats were injected with lipopolysaccharide (LPS, 1mg/ml/kg b.w.) to simulate a septic condition. There were eight groups then: control (C, n=8); endotoxemic control (C+LPS, n=9); trained (T, n=10); endotoxemic trained (T+LPS, n=9); low protein (LP, n=9); endotoxemic low protein (LP+LPS, n=10), low protein trained (LP+T, n=8) e endotoxemic low protein trained (LP+T+LPS, n=9). Rats were decapitated after 24 h from the injection, and blood and spleen were collected. The following immune parameters were evaluated: T, B and Nk spleen and blood lymphocytes distribution; splenic lymphocytes apoptosis rate and TNF-α serum concentration. LP rats presented higher percentage of splenic NK lymphocytes than control rats (LP+LPS = 3,3±0,3%; C+LPS = 1,9±0,3%), finding that was reverted by physical training (LP+T+LPS = 1,6±0,3%, $p<0,001$). Additionally, splenic lymphocytes from LP rats presented higher rates of mitochondrial depolarization (MTD) and phosphatidylserine externalization (PSE) than control rats (MTD: LP+LPS = 18,0±1,9% vs C+LPS = 6,0±1,9%, $p<0,001$; PSE: LP+LPS = 51,0±3,7% vs C+LPS = 26,5±2,8%, $p<0,001$). However, rats LP+T+LPS presented lower rates of apoptosis events than LP+LPS rats (MTD: LP+T+LPS = 9,0±1,8%, $p<0,01$; PSE: LP+T+LPS = 30,7±3,4%, $p<0,001$). Perinatal protein undernutrition also affected the circulating T lymphocytes distribution. LP+LPS rats presented lower percentages than C+LPS rats (39,6±2,8% vs 72,9±1,8%, $p<0,001$). In LP+T+LPS rats, this outcome was reverted (LP+T+LPS = 58,10±2,83%, $p<0,001$). We still evaluated TNF-α serum concentration, an important proinflammatory cytokine. LP rats presented higher TNF-α concentration, a physical training independent result (LP+LPS = 26,7±3,8 vs C+LPS = 7,9±3,4 pg/mL, $p<0,001$; LP+T+LPS = 23,9±3,3 vs T+LPS = 4,7±2,9 pg/mL, $p<0.001$). In conclusion, perinatal protein undernutrition increased apoptosis rate of lymphocytes and changed blood and spleen lymphocytes distribution during endotoxemic challenge in the adult offspring. Moderate physical training reverted these outcomes.

Key-words: Nutrition. Physical exercise. Toxemia. Cell death. Lymphocytes.

Lista de Tabelas

Sumário

1. Apresentação

Atualmente, a mortalidade infantil atinge cerca de 8,7 milhões de crianças menores de cinco anos de idade em países em desenvolvimento. Dentre essas, 68% estão associadas a doenças infectocontagiosas como pneumonia, diarreia e malária. Além das más condições sanitárias relacionadas a esse quadro, a desnutrição tem um papel fundamental no processo de redução das defesas do organismo, levando a infecções recorrentes.

Nas fases iniciais da vida, ainda intraútero, o sistema imunológico de mamíferos inicia sua formação com o surgimento das células primitivas no saco vitelínico e na aorta-gônada-mesonéfron. Essas células migram para o fígado fetal, órgão que oferece condições de proliferação até que os nichos linfoides definitivos possam receber as células progenitoras linfoides, mieloides e as células hematopoiéticas pluripotentes. Após a migração para os sítios definitivos de hematopoiese, o sistema imunológico continua em processo de maturação até o início da vida adulta.

Suporte nutricional adequado é fundamental para o processo de desenvolvimento do organismo. Esse período inicial da vida (gestação, amamentação/lactação, infância) é marcado por intensas demandas energéticas e metabólicas voltadas para o crescimento rápido, proliferação celular e maturação do organismo. Nesse período de plasticidade, as interações que ocorrem entre o organismo em formação e o meio ambiente nutricional podem provocar alterações nos tecidos e órgãos permanecendo ao longo da vida.

Os organismos vivos permanecem em constante interação com o ambiente onde estão inseridos. O conceito de plasticidade fenotípica se refere à habilidade de adaptação dos organismos aos estímulos ambientais ao longo da vida. Possui características ativas e adaptativas e é resultado da interação da influência do ambiente (variação do fenótipo) e dos genes (genoma individual). Essas adaptações fenotípicas visam à sobrevivência, garantia de reprodução para perpetuação da espécie, e longevidade.

Os processos metabólicos são então redirecionados para esse fim. Ocorre uma redistribuição de energia onde o organismo tende a beneficiar determinados órgãos e sistemas em detrimento de outros. Estudos indicam que o principal órgão preservado em indivíduos que sofreram desnutrição nas fases iniciais da vida é o cérebro. Os demais órgãos e sistemas apresentam redução de tamanho, celularidade e perda mais acentuada das funções, como o sistema imunológico.

Diversos estudos vêm demonstrando que insultos ocorridos nas fases de desenvolvimento de órgãos e tecidos provocam alterações que permanecem até a vida adulta. Nesse contexto, a desnutrição nas fases iniciais da vida está associada ao aparecimento de doenças crônicas não degenerativas, como diabetes, coronariopatias, obesidade e síndrome metabólica. Essas doenças possuem duas características fundamentais: induzem alterações inflamatórias basais ou respostas imunológicas aberrantes; e são afetadas por mudanças nos hábitos de vida.

De fato, a desnutrição proteica nas fases iniciais da vida leva à produção aumentada de citocinas pro-inflamatórias como TNF-α e IL-6, prejuízo nos processo de migração de neutrófilos, burst respiratório de macrófagos e neutrófilos, bem como na fagocitose e atividade microbicida dos fagócitos. A imunidade adaptativa também é afetada, como por exemplo, através da redução da produção de imunoglobulinas e da modificação do perfil de linfócitos T CD4+, CD8+. Dessa forma, a resposta imunológica em situações sépticas ou de estresse ocorre de forma irregular e ineficaz nesses indivíduos. Taxas elevadas de indicadores de apoptose nessas células e alterações hormonais de cortisol e leptina são alguns dos mecanismos associados.

Dentre as alterações no estilo de vida que apresentam maior impacto sobre a prevenção e progressão de doenças crônicas não transmissíveis, o exercício físico se destaca. O resultado dessa intervenção depende da duração, tipo, frequência e intensidade do exercício. Estudos demonstram que o treinamento em intensidade moderada (50 a 80% do $VO_{2máx}$) pode trazer benefícios através da modulação da resposta imunológica. Vários estudos com humanos em diversas condições de saúde, como câncer, doenças autoimunes, processos alérgicos, diabetes e obesidade tem demonstrado que o treinamento físico em intensidade moderada exerce influência benéfica sobre o estado inflamatório e a resposta imunológica desses pacientes.

Diante do exposto, levantamos a hipótese que o treinamento físico de intensidade moderada reverte os efeitos negativos da desnutrição proteica perinatal sobre alguns parâmetros do sistema imunológico, especificamente a taxa de apoptose de linfócitos do baço de ratos adultos endotoxêmicos. Neste sentido, o objetivo geral deste estudo foi identificar os efeitos do treinamento físico moderado sobre alguns indicadores imunológicos de ratos adultos endotoxêmicos cujas mães foram submetidas à desnutrição proteica nos períodos de gestação e lactação.

Para tanto, foram utilizados ratos Wistar, cujas mães foram submetidas a dietas à base de proteína (17% para o grupo controle e 8% para o grupo desnutrido) durante a gestação e

lactação. Dos 21 aos 120 dias de vida os ratos receberam dieta comercial de laboratório. Aos 60 dias de vida os ratos foram divididos em quatro grupos experimentais: controle (C, n = 17), treinado (T, n = 19), desnutrido (LP, n = 19) e desnutrido treinado (LP+T, n = 17). Os ratos treinados foram submetidos a um protocolo de treinamento físico de intensidade moderada em esteira (70% $VO_{2máx}$, 60 minutos/dia, 5 dias/semana, 8 semanas). Após 24h do final do treinamento, os grupos foram novamente divididos conforme a injeção intraperitoneal de LPS (1mg/mL/Kg de peso corporal): controle (C, n = 8), controle endotoxêmico (C+LPS, n = 9), treinado (T, n = 10), treinado endotoxêmico (T+LPS, n = 9), desnutrido (LP, n = 9), desnutrido endotoxêmico (LP+LPS, n = 10), desnutrido treinado (LP+T, n = 8) e desnutrido treinado endotoxêmico (LP+T+LPS, n = 9). Após 24h da injeção de LPS, os animais foram sacrificados por decapitação para coleta de sangue. O baço foi removido cirurgicamente, pesado e fragmentado para posterior análise. Foram avaliados os seguintes parâmetros imunológicos: distribuição de linfócitos TCD4+, TCD8+, B e NK no sangue; distribuição de linfócitos T, B e NK no baço; taxa de apoptose de linfócitos do baço e concentração sérica de TNF-α.

O presente trabalho deu origem a dois artigos: um artigo de revisão intitulado *"Neuroimmunomodulation of the perinatal malnutrition and the protective role of the physical training"*, submetido à revista Neuroimmunemodulation; e um artigo original intitulado *"Moderate physical training attenuates perinatal low-protein-induced spleen lymphocyte apoptosis in endotoxemic adult offspring rats"* submetido à revista European Journal of Nutrition.

2. Revisão da Literatura

Title: Neuroimmunomodulation of the perinatal malnutrition and the protective role of the physical training

Short-title: Nutrition, physical activity and neuroimmunomodulation

Abstract:

Developing organisms have the ability to cope with environmental demands through physiologic and morphologic alterations. Early life malnutrition has been recognized as an environmental stimulus that is related with down-regulation of some immune responses. Here, we discuss some of the short and long-term effect of perinatal malnutrition (undernutrition and overnutrition) that occurs in offspring during development through adult life. Some of these effects are explained by the epigenetics and the programming of hormones and cytokines impairing the modulation of the immune cells in response to environmental stimuli. Recently, it has been demonstrated that these effects are not deterministic and current environment, such as physical activity, can positively influence the immune system. Moreover, these effects can also attenuate the long-last effects of early malnutrition by a mechanism of neuroimmunomodulation. Here, we discuss the effects of perinatal malnutrition on the immune system and how it can be modulated by physical training. The mechanism includes the normalization of some hormones concentrations related to growth and metabolism such as leptin, IGF-1 and glucocorticoids.

Key words: Maternal undernutrition, physical activity, phenotypic plasticity, immune response, developmental plasticity, hormone.

Introduction

The development of the immune system occurs in the beginning of gestation period in both human and animals [1]. During the development, stem immune cells migrate from primitive site of differentiation as the liver and endothelium through bone marrow in order to advance the process of maturation [2]. The interaction between mother and fetus via placenta is important in order to keep fetus under a strict condition of development. This interaction includes hormonal environment, blood exchange, and oxygen and nutrients availability [3]. In addition, the critical period of development of the immune system also occurs during lactation and the first infancy [4]. Glucose and amino acids are the main nutrients for the normal development of fetus during pregnancy. During lactation, fatty acids assume a similar importance for normal development of suckling offspring [5].

Adverse nutritional availability during perinatal life can increase the individual's susceptibility to adulthood metabolic disease [6]. Metabolic diseases are classified as inflammatory disorders because they are accompanied by elevated concentrations of proinflammatory cytokines such as IL-1, IL-6, and TNF-α, as well as increased concentrations of glucocorticoids [7]. Recently, it has been recognized that mother's nutrition from conception through lactation may program the structure and/or function of the immune system by permanently altering specific cell populations with a lasting impact on the development of immune response and high susceptibility to infection and allergy [8-10]. The mechanisms by which maternal malnutrition may exert an influence on the emerging immune system include the phenotypic plasticity that explain how environmental stimuli influence the expression of a phenotype characteristic from a single genotype [11]. In addition, the crosstalk between the immune system and the neuroendocrine system, epigenetic alterations as DNA methylation, histone acetylation and microRNA expression of immune mediators and hormones [12].

The postnatal environment such as lifestyle (diet and exercise) as well as genetics plays a large role in programming the offspring's susceptibility to disease [13-15]. It has been well established that regular physical training enhances the cell-mediated immunity, phagocytosis, migration of neutrophils to the infection, cytokine production and increased lymphocyte function [16-18]. For example, moderate physical exercise (75% VO_2max, 5 times week, during 8 weeks) increased the percentage of TCD4 lymphocytes in blood and thymus and attenuated the rate of lymphocytes apoptosis in adult rats submitted to acute restraint stress [19].

It reasonable to consider that this kind of stimuli can induce positive adaptations on immune system even though the system was programmed to develop early disease or more susceptible immunity. Indeed, our previous studies showed that moderate physical training attenuated the effects of perinatal low-protein diet on the secretion of leptin by visceral adipose tissue, the phenotype of skeletal muscle fiber and the morphology of the spleen in adult offspring submitted to perinatal low-protein diet [13-15]. The underlying mechanism includes the normalization of some hormones concentration such as leptin, IGF-1 and glucocorticoids that were programmed by perinatal malnutrition [7,20].

The present paper reviews the effects of perinatal malnutrition with emphasis on the imprinting factors and mechanisms acting during gestation and lactation that can predispose to deregulations the integration of the neuro-endocrine-immune system. Furthermore, we discuss about the effects of physical training by attenuation or restoring the long-last effects of early life adverse nutrition. Finally, we highlight the probable underlying mechanisms including neuroimmunomodulation.

The development of the immune system in response to nutritional stimuli

Malnutrition (undernutrition or overnutrition) has been now recognized as an environmental stimulus that is related with down-regulation of some immune responses [21]. In low and middle income countries, children mortality reached more than 8.7 million of deaths of children under 5 years old, where 68% were due to infectious diseases like pneumonia, diarrhea and malaria [22]. This infant population suffers with infection re-incidence because of early state of undernutrition [23]. On the other hand, maternal overnutrition was associated with high inflammatory state in children under 5 years old [24,25].

In rats, previous studies have shown that maternal low-protein diet (9.5% casein during gestation and lactation) is related to peritoneal macrophages impaired spreading, phagocytosis and microbicide functions [21]. Maternal free-protein diet is also responsible for inhibited leukocyte bone marrow mobilization and migration of neutrophils under stimulation of *Carrageenan* in offspring at 60^{th} day of life [26,27]. In addition, maternal overnutrition (high-fat diet during gestation and lactation) was related to up-regulation of proinflammatory pathway, especially of genes related to inflammatory response and cytokine signaling in rat offspring on 12 months of age [28]. In humans, early life undernutrition is related to the high rate of permanent infection in children under 5 years old [29]. Table 1 shows the list of studies that evaluated the association between maternal malnutrition and the consequences for the immune system of the offspring.

The underlying mechanisms for the short and long-term effects of malnutrition on the immune system can be explained by the phenotypic plasticity. This biological phenomenon was firstly used to explain how environmental stimuli influence the expression of a phenotype characteristic from a single genotype [11]. In addition, epigenetic alterations as DNA methylation, histone acetylation and microRNA expression can explain how an organism can adapt to environmental stimulus during the critical period of development and the association

with consequences during the lifespan [12]. For example, perinatal undernutrition is related to down-regulation of leptin gene expression in adult mice, and leptin participates in the effector T lymphocytes activation [30,31]. The maternal overnutrition is associated with the methylation of offspring genes that express IL-8, B-lymphocyte receptor signaling and glucocorticoids receptor signaling pathways [32]. The methylation of some genes from the IL-8 pathway is related to the plasma C-reactive protein expression [32].

Malnutrition during the critical period of development can alter the development of immune system with long-lasting consequences by a mechanism that includes epigenetic adaptations [30]. However, these effects are not deterministic and current environmental stimuli can also induce phenotypic plasticity. For example, regular physical activity has been associated with positive effects to immune system [16,33,34]. It plausible to consider that this kind of stimuli can induce positive adaptations on immune system even though the system was programmed to develop early disease or more susceptible immunity.

Immunological adaptations to the physical training

It has been well known that regular physical exercise can induce immune adaptations, but these effects are dependent on the magnitude of the effort [35]. Physical exercise can be classified according to intensity (light, moderate or intense), frequency (number of session per week), type (anaerobic or aerobic) and duration (short or long) [36]. According to the American College of Sports Medicine (2011), a regular (at least three times a week), moderate physical exercise (50 – 75% VO_2max) is associated with benefits for health [37]. For the immune system, moderate physical training enhanced macrophage phagocytosis and oxidative burst, neutrophils oxidative burst, high percentage of TCD4 lymphocytes and cytokines production [16-18]. For example, moderate physical exercise (75% VO_2max, 5 times week, during 8 weeks) increased the percentage of TCD4 lymphocytes in blood and thymus and attenuated the rate of lymphocytes apoptosis in adult rats submitted to acute

restraint stress [19]. Table 2 shows some examples of studies that evaluated the immune response to moderate physical training.

The underlying mechanisms can be related to the neuro-endocrine-immune modulation in response to a repeated boat of exercise-induced stress [38]. In response to acute exercise, the neuroendocrine system is activated by both sympathetic nervous system (SNS) and hypothalamus-pituitary-adrenal (HPA) axis. The initial response includes the increase of noradrenalin and dopamine concentration in the central nervous system that activates immediately the release of adrenalin from adrenal medulla. Then, there is an increase of corticotrophin-release-hormone (CRH) from the hypothalamus that activates the release of adrenocorticotrophic hormone (ACTH) from intermediary zone of the pituitary. The ACTH will activate the cells from the adrenal cortex to release glucocorticoids [39-41]. Immune cells present adrenergic receptors (α and β) that are responsive to the increase of blood noradrenalin, adrenalin and beta-endorphins [42,43]. Similarly, immune cells present receptors for glucocorticoids (RG) that are over-expressed in response to stress [44]. Immune cells can also produce and release cytokines that can modulate cells of neuro-endocrine system as a bi-directional fashion. Immune cells can also produce and release hormone like ACTH.

Physical exercise is a model of physical stress that activates both SNS and HPA axis. For example, moderate physical exercise (55% VO_{2max}, 45 min) was associated with an increased expression of alpha-adrenergic receptors in neutrophils [45]. Previous studies have shown that in response to a long duration physical exercise, neutrophils are more responsible to the increase of blood beta-endorphin [46,47]. Receptors for glucocorticoids are responsive for a regular physical exercise and immune cells present a down regulation that can be important for the process of inflammation [48].

Physical training, perinatal malnutrition and Neuroimmunomodulation

Developing organisms have the ability to cope with environmental demands through physiologic and morphologic alterations [49]. The resulting phenotype can be continuously modulated by adaptive mechanisms of some tissues, like adipose tissue and skeletal muscle. Perinatal malnutrition has been showed to affect the synthesis and action of hormones in the receptors. For example, GH-IGF-1 axis are affected by maternal protein restriction and adult offspring from low-protein mothers presented a lower GH mRNA expression and limiting growth by reducing hepatic IGF-1 synthesis [7]. Children born small for gestational-age showed altered GHRH-GHIGF1 axis and GH resistance [50,51].

Recently, it was described that there is an interaction between the IGF system and the inflammatory immune response [52]. For example, pigs with elevated IGF-1 expression presented a less increased expression of TNF-α while pigs with the high expression of IGFBP-3 presented elevated IL-6 expression [52]. It seems that there is an inverse association between the hepatic expression of the IGF system (IGF-1, IGFBP-3, GHR) and certain cytokines (IL-1β, IL-18, TNF-α) and acute-phase proteins [52]. Thus, the long-last effects of maternal malnutrition on the inflammatory response of immune cells can be related to the down-regulation of IGF-1 and GH-IGF1 axis. Physical exercise can modulate hormonal response and the GH/IGF-I system [53] by a mechanism that include inflammatory response and muscular repair [54]. In adult trained men submitted to a resistance exercise followed by cold water immersion, there was a IGF-mediated responses on slower-acting lymphocytes [54]. Our previous study showed that moderate physical training also reverted the profile of skeletal fibers toward oxidative phenotype in adult rats submitted to a perinatal low-protein diet by a mechanism the included high concentration of IGF-1 [14].

Adipose tissue secretes a number of adipocytokines that are important in the metabolism and intrauterine growth. Leptin is one of the most important hormones secreted by adipocytes resembling proinflammatory cytokines (IL-6 and IL-12) [55]. It assumes an important role in regulating immune responses. For example, it has been shown that disease conditions of reduced leptin production are associated with increased infection susceptibility [55]. There is also a physiological role including the mediation of the nutritional status and immune competence [7]. Serum concentration of leptin is altered in adult offspring submitted to perinatal protein-restriction that was associated with leptin resistance, hyperletipnemia, accumulation of adipose tissue and inflammation as described in previous studies [7,56]. Our previous studies have shown that a perinatal low-protein diet induced an increased content of leptin on visceral adipose tissue of adult male rat offspring. These effects were attenuated by moderate physical training (70% VO2max, 60 min/day, 5 days/week, 8 weeks). Thus, an important mechanism related to the immunomodulation of the physical training on adult subjects submitted to perinatal malnutrition is closely associated to the action of leptin.

Perinatal malnutrition during gestation or lactation is a stressful event that can activate the HPA axis by a mechanism that includes permanent the up-regulation of glucocorticoids receptors [20]. Pups (40-day-old) from food restricted mothers during gestation presented higher corticosteronemia and respond less to dexamethasone suppression than the controls [20]. Our previous study showed that a protocol of physical training to adult endotoxemic offspring rats from dams submitted to perinatal low-protein diet [15]. In these studies, moderate physical training reverted morphologic spleen alterations such as reduced number and size of lymphoid follicles and marginal zone area by a mechanism related to plasma corticosterone concentration [15]. Thus, current environmental stimuli, such as physical training, can modulate the neuroendocrine and metabolic status, which have direct impact on the immune system function [57]. It means that early life insults induce short-term adaptations

but it does not means that this is deterministic since organs and physiological systems are constantly responsible to new environmental stimuli responding in terms of phenotypic plasticity [58].

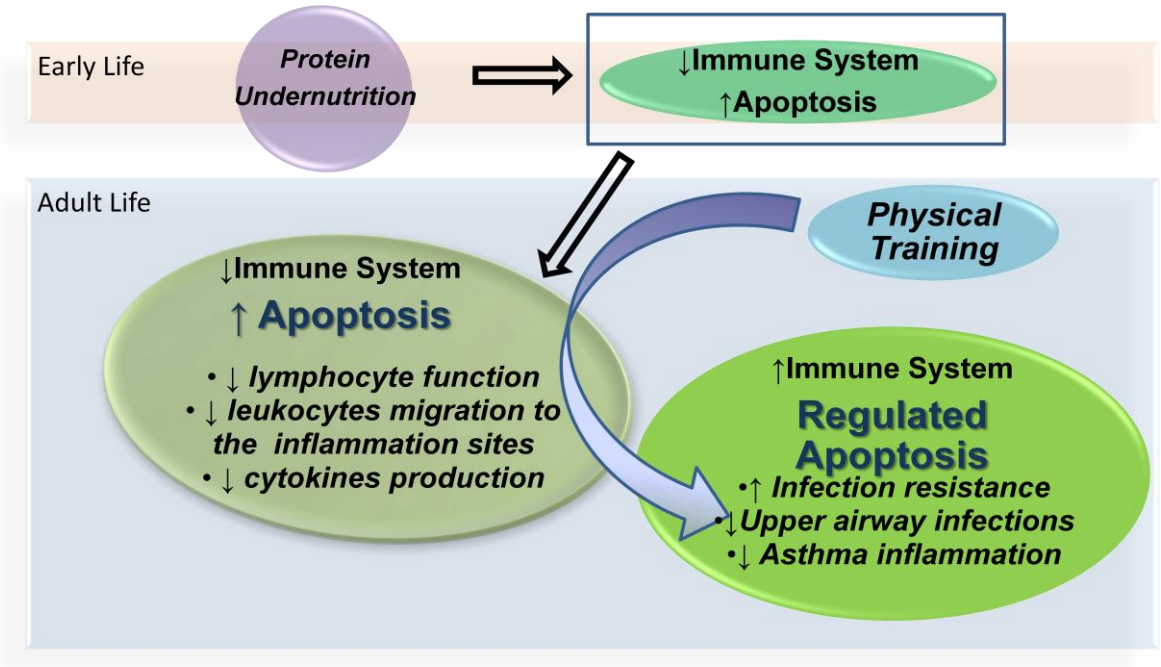

Figure 2. Deleterious effects of protein undernutrition on the developmental phase of life on the immune system, and the physical training recovery.

Conclusions

Perinatal malnutrition has been recognized as an environmental stimulus that is can alter the physiological developmental during a critical period of life, when the tissues still have some plasticity and are in a higher proliferating and differentiating phase. The immune system seems to be susceptible to perinatal malnutrition since it has been seen effects at short and long-term on the inflammatory response, synthesis of cytokines, down regulation of macrophages and monocytes, migration of neutrophils to infection and up-regulation of B-lymphocytes. The underlying mechanism includes the epigenetic influence enabling the animal to adapt to a lower nutrient supply by methylation of DNA and acetylation of histones. However, in terms of phenotypic plasticity, the immune system can also respond as an

adaptive fashion the current environmental stimuli like physical exercise. It has been well established that moderate physical training induces positive effects on immune system by a mechanism of neuroimmunomodulation. It means that the current influence of environment can revert the effects of early life programming on immune system by malnutrition. The mechanism includes the normalization of some hormones concentration related to growth and metabolism such as leptin, IGF-1 and glucocorticoids (Figure 2).

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Table 1. Early-life malnutrition effects on offspring immune cells (2010 to 2014).

Cells	Species	Model of Malnutrition	Period of Malnutrition	Age of evaluation (offspring)	Effects	References
Bone marrow macrophages	Sprague-Dawley rats	50% restriction diet	Gestation	160 days	↑Serum TNF-α and IL-1β; ↑Production of IL-6, IL-1β and IL-10 after LPS (supernatant); TNF-α wasn't altered; ↑M1 phenotype marker CD11c, and ↓M2 phenotype marker PPAR-γ	[59]
Thymocytes and Splenocytes	Wistar rats	8% protein diet vs 22% protein diet	Gestation and lactation	30 days	Thymocytes:↓Double positive cells; ↑CD4+; ↑CD8+; ↑ObR protein expression; ↓apoptosis (AnnexinV); Proliferation: N/A ↑Bcl2; ↓Bax; ↑Nuclear NF-kB p65; ↓IkB; Splenocytes: N/A surface markers	[60]
Thymus and Spleen Lymphocytes	CD1 Mice	6% protein diet vs 24% protein diet	Gestation and lactation	2, 7, 14 and 21 days	↑Serum eotaxin Thymus: ↓total cell number Spleen: ↓total cell number ↓Spleen mass ↑CD4+/CD8+ ↑CD8+	[61]
Peripheral blood mononuclear cells (PBMC)	German Landrace Pigs	6.5% protein diet vs 12.1% protein diet (adequate) vs 30% protein diet	Gestation	1, 27, 80 and 180 days	6.5%: Serum: ↓IgA (day 1) ↑IL-10 (day 47) ↑IL-6 (day 47) Before vs after weaning: ↓Lymphocytes proliferation 30%: Serum: ↓imunoglobulins (IgG, IgM and IgA) (day 1) Before vs after weaning: ↑CD4+ ↑CD4/CD8 ↓Lymphocytes proliferation	[62]
Bronchoalveolar lavage (BAL) lymphocytes, eosinophils and neutrophils	Wistar rats	50% restriction diet	Gestation	60 days	BAL: ↓total lymphocytes counts ↓CD4+ ↓eosinophils and neutrophils migration	[63]

					↑TNF-α ↓IL-6 <u>Lung Tissue:</u> ↑IFN-γ ↓IL-4	
Spleen and mesenteric T cells	C57BL/6 J Mice	0.6% low protein diet vs control	19 to 33 days of age	22 and 33 day (third and 14th day of experimental diet)	↓Serum IL-10	[64]
T lymphocytes	Sprague-Dawley rats	50% restriction diet	Gestation and lactation	8-9 weeks	↓WBC; ↓Lymphocytes; ↓CD4+; ↓CD8+; ↑CD4/CD8; <u>Serum:</u> ↓IL-2; IL-7. ↓Actin polimerization; ↓Proliferation; <u>T cells:</u> ↓IL-2; IFN-γ.	[65]
Blood neutrophils	Wistar rats	Protein-free diet vs 22% protein diet	First 10 days of lactation	50 to 60 days	↓Leukocyte migration ↓Leukocyte blood pool ↑Superoxide production ↑Nitric oxide production ↑iNOS expression ↑NF-kB ↓IkB ↑TNF-α (serum)	[27]
Thymus	C57/B16 Mice	8% protein diet vs 20% protein diet	Gestation or lactation	21 days or 12 weeks	Gestation: ↑PCNA (21 days) ↓PCNA (12 weeks) ↑SIRT1 (21 days) ↑p53 (both ages) ↑IL-7 expression (21 days) ↑IL-7R expression (21 days) Lactation: ↑Thymus relative weight (12 weeks) ↑PCNA (both ages) ↑ SIRT1 (both ages)	[66]
Splenocytes	C57BL/6 J Mice	29% lard (High fat) diet vs control	Gestation and lactation; gestation; or lactation	20 weeks	Lactation: ↓ Thymus and Spleen relative weight ↓IgG Gestation: ↓Thymus cortex thickness ↓Splenocytes total number ↑Serum TNF-α ↓IgG ↑IgE	[67]
Kidney Macrophages	Sprague-Dawley Rats	45% fat (High fat) diet + 10% fructose	Gestation and lactation;	17 weeks	↑TGF-β ↑CD68+ on kidney tissue	[68]

		drinking water vs control	gestation; or lactation			
Peritoneal macrophages; Splenocytes and Colon tissue	BALB/c and C57BL/6 Mice	Western diet - WD (40% fat) or control diet (10% fat)	Gestation and lactation	5 to 6 weeks	After skin infection, WD offspring developed larger abcesses with higher bacteria number; Skin: ↓IL1-β; ↓TLR2; ↓IL17A; ↓IL-10; ↓β-defensin 4 Colon: ↑IL-6; ↑IL-1β; ↑IL17; ↓TRegs. Spleen: ↓TNF-α; ↓IL-6; ↓TReg Macrophage: ↓TLR4; ↓LBP	[69]

TNF-α - tumor necrosis factor alpha; IL-1β - interleukin-1 beta; IL-6 – interleukin-6; IL-10 – interleukin-10; LPS – lipopolysaccharide; CD11c – cluster differentiation 11c; PPAR-γ - peroxisome proliferator-activated receptor-γ; CD4 – cluster differentiation 4; CD8 – cluster differentiation 8; ObR – obesity receptor; Bcl2 – B cell lymphoma-2; Bax – BcL2 associated protein; NF-kB - nuclear factor-kB; IkB – inhibitor of NF-kB; IL-10 – interleukin-10; IgA – immunoglobulin A; IL-6 – interleukin-6; IFN-γ - interferon-γ; IL-4 – interleukin-4; WBC – white blood cells; IL-7 – interleukin-7; iNOS – inducible nitric oxide synthase PCNA – proliferating-cell nuclear antigen; SIRT1 – silent information regulator 1; LBP – lipid binding protein; IL-7R – interleukin-7 receptor; IgG – immunoglobulin G; IgE – immunoglobulin E; TGF-β – tumor growth factor-β; CD68 – cluster differentiation 68; TLR2 – toll-like receptor 2; TRegs – regulatory T cells; TLR4 – toll-like receptor 4; LBP – LPS binding protein.

Table 2. Some effects of moderate physical training* on immune system (2010 to 2014).

Cells/tissues	Model	Type of training	Effect	Reference
T cell CD4+ (TCD4)	Human (HIV positive men)	60-79% heart rate 45-60 min/d; 3 times/week; 8 weeks	↑TCD4+; Positive correlation between TCD4+ and $VO_{2\ max}$	[70]
Serum interleukins	Human (Systemic Lupus Erythematosus women)	Heart rate correspondent to the interval between the VAT and 10% below the rcp; 30-50 min/d; twice a week; 12 weeks	↓IL-10 Trend to ↓ TNF-α; ↓IL-6; ↓sTNFR1 and ↓sTNFR2	[71]
Blood CD4+ lymphocytes	Human (sedentary health man)	60% VO2max; 30 min/d; 5 days/week; 5 weeks	↓Active caspase-3; ↓Phosphatidylserine externalization (apoptotic markers); ↓beclin-1; ↓Atg-1; ↓Lamp-2 (autophagic markers)	[72]
Blood lymphocytes; Neuronal and intestinal tissue	Swiss mice (*Trypanosoma cruzi* infected)	Light to mild effort; 30-45 min/d; 5 days/week; 8 weeks.	↓Total parasitemia; ↑Neuronal survival and hypertrophia; ↑Total thickness of intestinal wall; ↑Intraepithelial lymphocytes number; ↓Formation of inflammatory foci; ↑Serum TNF-α	[73,74]
T and B lymphocytes	BALB/C	70% VO_{2max}; 60 min; 5 days/week; 11 weeks	↓Serum IL-4; ↓TNF-a; ↑B lymphocytes; ↑TCD4+;	[75]
T and B lymphocytes	Wistar rats	60% VO2max; 1h/d; 5 days/week; 8 weeks	↑Proliferative capacity of T and B lymphocytes; ↑IL-2; ↑IL-4; ↑TNFR; ↑IL-2R; ↑IgG	[76]
	Wistar rats (Diabetes-induced)	60% VO2max; 30 min/d; 6 days/week; 3 weeks	Serum: ↓TNF-α; ↓IL-6; ↓IL-1β; ↓CINC 2α/β; ↓C-reactive protein	[77]

* In order to characterize moderate intensity effort, the selected studies presented here must describe the intensity parameter of the physical training applied. HIV – human immunodeficiency virus; $VO_{2\ max}$ – maximal oxygen consumption; IL-6- interleukin 6; IL-10 – interleukin-10; TNF-α – tumor necrosis factor alpha; sTNFR1 – soluble tumor necrosis factor α receptor 1; sTNFR2 – soluble tumor necrosis factor 2;

VAT – ventilator anaerobic threshold; RCP – respiratory compensation point; Atg-1 – autophagy related 1; Lamp-2 - Lysosome-associated membrane protein 2; IL-2 – interleukin-2; IL-4 – interleukin-4; IL-1β – interleukin 1 beta; CINC 2α/β - cytokine-induced neutrophil chemotactic factor 2alpha/beta.

3. Pergunta Condutora

O treinamento físico moderado pode reverter os efeitos da desnutrição proteica perinatal sobre o sistema imunológico de ratos adultos endotoxêmicos?

4. Hipótese

O treinamento físico moderado reverte os efeitos da desnutrição proteica perinatal sobre a distribuição de linfócitos do sangue e do baço, bem como a taxa de apoptose de linfócitos de ratos adultos endotoxêmicos.

5. Objetivos

5.1. Objetivo Principal

Avaliar os efeitos do treinamento físico moderado sobre a distribuição dos subgrupos de linfócitos do sangue e baço, bem como a taxa de apoptose de linfócitos em ratos adultos endotoxêmicos submetidos à desnutrição proteica perinatal.

5.2. Objetivos Secundários

-Identificar as proporções das subpopulações de linfócitos T, B, e Nk do sangue e do baço de ratos adultos submetidos à desnutrição proteica perinatal, endotoxêmicos ou não;

-Identificar as alterações provocadas pelo treinamento físico em intensidade moderada nas populações de linfócitos T, B, e Nk do baço e do sangue de ratos adultos, endotoxêmicos ou não;

-Verificar o efeito do treinamento físico em intensidade moderada em ratos adultos submetidos à desnutrição proteica perinatal sobre as populações de linfócitos T, B, e Nk do sangue e do baço desses animais, endotoxêmicos ou não;

-Identificar indicadores de apoptose em linfócitos do baço de ratos adultos submetidos à desnutrição proteica perinatal, endotoxêmicos ou não;

-Identificar indicadores de apoptose em linfócitos do baço de ratos adultos submetidos a treinamento físico em intensidade moderada, endotoxêmicos ou não;

-Verificar o efeito do treinamento físico em intensidade moderada em ratos adultos submetidos à desnutrição proteica perinatal sobre os indicadores de apoptose em linfócitos do baço desses animais, endotoxêmicos ou não;

6. Métodos

6.1 Animais e Dieta

Foram utilizadas 20 ratas Wistar, provenientes da colônia do Departamento de Nutrição da UFPE. Os animais foram mantidos em biotério de experimentação com temperatura de 22°C ± 2, ciclo claro-escuro de 12/12 horas (luzes das 18 às 06 h) com livre acesso a água e ração. Depois de detectada a gestação, as ratas foram divididas em dois grupos: dieta controle (C, n = 9, proteína a 17%) e desnutridas (LP (low-protein diet), n = 9, proteína a 8%) (REEVES; NIELSEN; FAHEY, 1993).

Tabela 3. Composição das dietas experimentais (à base de proteína 17% e 8%).

Ingredientes	**LP**	**Controle**
Proteína, g	79.3	179.3
Mix vitamínico*, g	10.0	10.0
Mix mineral**, g	35.0	35.0
Celulose, g	50.0	50.0
Bitartarato de colina, g	2.5	2.5
D-Metionina, g	3.0	3.0
Óleo de soja, mL	70.0	70.0
Amido de milho, g	750.2	650.2
Sacarose, g	100.0	100.0
TBHT, g	0,014	0,014
Total	1000 g	1000 g

Fonte: REEVES, 1993.
*Conteúdo da mistura de Vitaminas (mg/kg de dieta): retinol, 12; colecalciferol, 0.125; tiamina, 40; riboflavina, 30; ácido pantotênico, 140; piridoxina, 20; inositol, 300; cianocobalamina, 0.1; menadiona, 80; ácido nicotínico, 200; colina, 2720; ácido fólico, 10; p-ácido aminobenzóico, 100; biotina, 0.6.
** Conteúdo da mistura mineral (mg/kg de dieta): CaHPO4, 17200; KCI, 4000; NaCl, 4000; MgO, 420; MgSO4, 2000; Fe2O2, 120; FeSO4·7H2O, 200; elementos traços, 400 (MnSO4·H2O, 98; CuSO4·5H2O, 20; ZnSO4·7H2O, 80; CoSO4·7H2O, 0.16; KI, 0.32; amido suficiente par 40g [per kg de dieta]).
TBHT – hidroxitolueno butilado.

Durante a lactação as ratas continuaram recebendo dieta experimental e a ninhada foi ajustada para 8 filhotes. No desmame (21 dias de idade) somente os filhotes machos (C, n = 36 e LP, n = 36) permaneceram no experimento e receberam dieta equilibrada (74,5% de

carboidratos, 23% de proteínas e 2,5% de lipídeos) (Labina, Purina do Brasil-Agribrands, Paulínia, São Paulo).

Tabela 4: Ingredientes da dieta LABINA (Purina Brasil) utilizada após o desmame

Ingredientes*	Porcentagem
Proteína	23%
Fibras	5%
Gordura	4%
Minerais	12%

*Composição básica: milho, farelo de trigo, farelo de soja, farinha de carne, farelo de arroz cru, carbonato de cálcio, fosfato de bicálcico, sal, pré-mix.

Aproximadamente aos 60 dias de idade os grupos foram subdivididos de acordo com o protocolo de treinamento físico: controle (C, n = 17), controle + treinamento (T, n = 19), desnutrido (LP, n = 19) e desnutrido treinado (LP+T, n = 17).

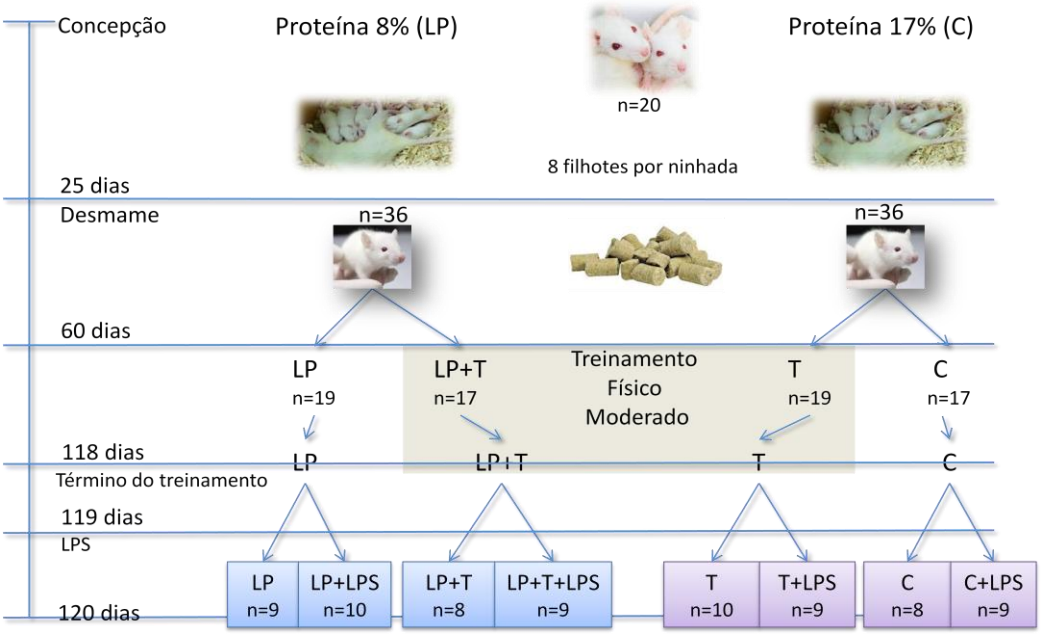

Figura 1: Desenho experimental representando a formação dos diferentes grupos (controle e desnutrido) quanto à manipulação da dieta e o período de treinamento físico, administração de LPS e do sacrifício.

Após o protocolo de treinamento físico, os grupos foram subdivididos de acordo com a administração de LPS (E. coli serotype 055:B5, Sigma-Aldrich, SP, Brasil, 10mg/mL/Kg) ou

água destilada: controle (C, n=6), controle endotoxêmico (C+LPS, n=6), controle treinamento (T, n=6), controle treinado endotoxêmico (T+LPS, n=6), desnutrido (LP, n=6), desnutrido endotoxêmico (LP+LPS, n=6), desnutrido treinado (DT, n=6), desnutrido treinado endotoxêmico (LP+T+LPS, n=6) (Figura1).

6.2 Protocolo de Treinamento Físico

A partir de 60 dias de idade os ratos do grupo treinado foram submetidos a um programa de treinamento físico moderado em esteira motorizada (EP-131/Insight Equipamentos, São Paulo, Brasil). O protocolo experimental de treinamento físico moderado (8 semanas, 5 dias/semana e 60 min/dia até 70% do VO_{2max}) foi realizado de acordo com Leandro et al. (2007) e está descrito na tabela 3 (LEANDRO et al., 2007). O grupo não treinado permaneceu nas gaiolas.

6.3. Administração de Lipopolissacarídeo (LPS)

Após 24 horas do término do programa de treinamento os animais receberam injeção intraperitoneal (i.p.) de LPS (1 mL/Kg de peso corporal de uma solução 1mg/mL) ou água destilada estéril apirogênica (1 mL/Kg), sendo sacrificados 24 horas depois, através de decapitação.

6.4. Coleta das células e tecidos

A coleta das células foi realizada 24 horas após a injeção de LPS ou de água destilada. Os animais foram sacrificados por decapitação em guilhotina, e o sangue foi colhido em tubos secos. Após coagulação por 30 minutos, o sangue foi centrifugado (10 minutos, 213 g, 4ºC) e o soro foi congelado a -20°C para análises posteriores.

Tabela 5. Protocolo de treinamento físico moderado em esteira para ratos Wistar adultos.

Semanas	Velocidade (km/h)	Inclinação (º)	Duração (min)
1ª Semana (Adaptação)	0,3	0	5
	0,4	0	5
	0,5	0	5
	0,3	0	5
2ª Semana	0,4	0	5
	0,5	0	20
	0,6	0	30
	0,4	0	5
3ª Semana	0,5	0	5
	0,6	0	10
	0,8	0	10
	0,9	0	30
	0,5	0	5
4ª Semana	0,5	0	5
	0,8	0	10
	0,9	0	10
	1,1	0	30
	0,5	0	5
5ª Semana	0,5	5	5
	0,8	5	10
	0,9	5	10
	1,1	5	30
	0,5	0	5
6ª Semana	0,5	10	5
	0,8	10	10
	0,9	10	10
	1,1	10	30
	0,5	0	5
7ª Semana	0,5	10	5
	0,8	10	10
	0,9	10	10
	1,1	10	30
	0,5	0	5
8ª Semana	0,5	10	5
	0,8	10	10
	0,9	10	10
	1,1	10	30
	0,5	0	5

O sangue colhido em tubos contendo EDTA foi utilizado para avaliação do percentual de linfócitos por citometria de fluxo. Foram utilizados 100 mL de sangue por amostra, e a marcação foi realizada com os anticorpos do cocktail T/B/Nk (BD Bioscience, San Jose, USA) ou com os anticorpos para marcação de linfócitos T: CD3+ (FITC), CD4 (PE) e CD8 (PerCp) (BD Biosciences, San Jose, USA).

O baço foi removido e pesado em balança de precisão HR-200, 0.1 mg (AND Weighing, San Jose, USA). Posteriormente, o baço foi fragmentado com tesoura em PBS pH 7,4 (solução salina tamponada). Os fragmentos foram comprimidos com o êmbolo da seringa descartável contra um cilindro de malha estéril com poros de 70 µm (Cell Strainer, BD Falcon, San Jose, USA). A seguir, as células foram centrifugadas a 300g por 10 minutos a 4ºC (Centrífuga Beckman Coulter, Inc., Brea, USA). Posteriormente os eritrócitos foram osmoticamente lisados pela adição de tampão de cloreto de amônio (0.1mM EDTA, 0.15M NH_4Cl, 10mM $KHCO_3$, pH 7.3) por 4 minutos em temperatura ambiente. A contagem das células viáveis foi determinada com Trypan Blue (Sigma-Aldrich, São Paulo, Brasil) em câmara de Neubauer (PITHON-CURI et al., 2002). A suspensão celular foi então acrescida de meio de cultura (RPMI 1640 com 1% soro fetal bovino, $NaHCO_3$ (2g/L), glutamina (300 mg/L), gentamicina (50 mg/mL), anfotericina B (2 mg/L) (Vitrocell Embriolife, Campinas, Brasil) e centrifugada novamente. Este protocolo de manutenção de linfócitos foi uma adaptação de Leandro et al., 2006 (LEANDRO et al., 2006). Os linfócitos assim obtidos foram mantidos em gelo e utilizados em futuras análises em citômetro de fluxo.

6.5. Avaliação das concentrações séricas de TNF-α

As concentrações de TNF-α foram quantificadas no soro através do método de ELISA (enzyme-linked immunosorbent assay) com anticorpos monoclonais, seguindo as orientações do fabricante (Rat Ready-Set-Go ELISA kit, R&D Systems, Minneapolis, USA).

6.6. Análise de subpopulações de linfócitos do baço e sangue

As linfócitos T, B e NK foram incubados na quantidade de $1x10^6$ linfócitos por tubo por 5 minutos a 4°C, com anticorpos (1 µg) contra marcadores de superfície CD3, CD4, CD8

e NKR-P1A (CD161a), correspondentes as populações de linfócitos T, T helper, T citotóxico e NK de rato. Linfócitos B foram analisados através da detecção do marcador CD45RA. As células foram incubadas por 30 minutos em recipiente com gelo, na ausência de luz, com ≤ 1 μg de anticorpo em PBS contendo 1% de FCS, adicionado de 0.02% N_3Na (tampão de FACS), dos seguintes anticorpos monoclonais: clone G4.18 (anti-CD3); clone 1F4 (anti-CD3); clone OX-35 (anti-CD4); clone OX-8 (anti-CD8α); clone 10/78 [anti-NKR-P1A (CD161a)] e clone OX-33 (anti-CD45RA). Todos os anticorpos foram obtidos da BD-Biosciences Pharmingen (San Jose, USA). Todas as análises de citometria foram realizadas em FACSCalibur™ utilizando o programa CellQuest™ (Becton Dickinson – BD Biosciences, San Jose, USA). Cinquenta mil eventos foram adquiridos por amostra.

6.7. Análise da integridade da membrana celular

A capacidade da célula em manter uma membrana plasmática íntegra e seletiva foi avaliada. Os linfócitos ($1x10^6$) foram ressuspensos em 550 μL de PBS/iodeto de propídio (PI) (50 μg por mL PBS, BD Biosciences, San Jose, CA). As células foram analisadas em citômetro de fluxo de acordo com a técnica de (NICOLETTI et al., 1991). A fluorescência será mediada no canal FL2 (630/622 nm). Dez mil eventos foram adquiridos por amostra em histogramas.

6.8. Determinação do potencial transmembrânico da mitocôndria

A rodamina 123 (corante fluorescente catiônico) é excitável por laser de argônio (480 nm) e emite fluorescência na faixa de 515-530 nm (FL1). É permeável à membrana celular e é rapidamente sequestrada pela mitocôndria. Células com potencial mitocondrial transmembrânico inalterado captam a rodamina e emitem alta fluorescência quando atingidas pelo laser. Alterações no potencial mitocondrial transmembrânico impedem a captação de rodamina para dentro da mitocôndria, gerando eventos que emitirão menor fluorescência (despolarização) ou maior fluorescência (hiperpolarização). Os linfócitos ($2x10^6$) foram ressuspensos em 1 mL de solução salina com rodamina 123 (5mg/mL em etanol, Rhodamine 123, Sigma-Aldrich, São Paulo, Brasil) e incubados por 10 minutos a 37^{o}C segundo a técnica

de Pithon-Curi et al. (2002) (PITHON-CURI et al., 2002). As células foram lavadas com PBS por duas vezes, ressuspensas em 0,5 mL de PBS e incubadas por 30 minutos a 37^{o}C.

6.9. Análise da fragmentação de DNA por citometria de fluxo

Os linfócitos (1x10^{6}) foram ressuspensos em 200 μL do tampão de lise contendo PI (0,1 % citrato de sódio, 0,1% Triton X-100, PI 2μg/mL) de acordo com a técnica de Nicoletti et al (1991) (NICOLETTI et al., 1991). A leitura no citômetro de fluxo foi realizada a 560-580 nm (FL2). A membrana plasmática foi permeabilizada pelo tampão de lise e os núcleos foram impregnados com PI, que se liga ao DNA e as células contendo núcleos íntegros emitem alta fluorescência. A condensação de cromatina e a fragmentação de DNA podem ser observadas pela ocorrência de eventos com baixa fluorescência.

6.10. Externalização de fosfatidilserina por citometria de fluxo

A fosfatidilserina é um fosfolipídio da membrana plasmática que se localiza na face interna da mesma. Um dos sinais precoces de apoptose, juntamente com a alteração do potencial de membrana mitocondrial, é a migração desse fosfolipídio para a face externa da membrana plasmática. Dessa forma, a célula apoptótica sinaliza sua condição ao meio externo, o que possibilita a interação com fagócitos (VERMES et al., 1995). A Anexina V é uma proteína que possui afinidade pela fosfatidilserina que foi exposta na superfície celular. Quando conjugada a um fluorocromo pode ser detectada por citometria de fluxo (VERMES et al., 1995). Os linfócitos (2x10^{6}) foram ressuspensas em 200 μL do tampão de ensaio fornecido pelo kit comercial, 3 μL de 7-AAD (7-actinomicina D, BD Biosciences) e 3 μL de anexina V-FITC (Annexin V-FITC, BD Biosciences) e incubadas no escuro à temperatura ambiente, por 15 minutos. No final desta incubação, as células foram analisadas por citometria de fluxo. Anexina V-FITC foi avaliada no canal de fluorescência verde (FL-1) e o 7-AAD no canal de fluorescência vermelho (FL-3).

6.11. Análise Estatística

A análise estatística foi realizada utilizando o teste ANOVA two-way, onde os fatores foram a dieta materna e o treinamento físico, seguidos por pós-teste de Bonferroni. A diferença entre o estado basal (água destilada) e séptico (LPS) foi avaliada por Teste T de Student, com $p<0.05$. Os dados foram expressos em média ± erro padrão da média. O programa estatístico utilizado foi GraphPad Prisma 5.

7. Resultados

Title: Moderate physical training attenuates perinatal low-protein-induced spleen lymphocyte apoptosis in endotoxemic adult offspring rats

Running Title: Physical training attenuates splenic lymphocytes apoptosis in malnourished rats

Abstract

Purpose: To evaluate the effects of a moderate physical training on the lymphocytes subsets in blood and the rate of apoptosis and subpopulation of lymphocytes in adult offspring submitted to perinatal low-protein diet. **Methods**: Male Wistar rats were divided into two groups according to their mother's diet during gestation and lactation, control (C, 17% casein) and, undernourished (Low-protein diet, LP, 8% casein). At the 60^{th} day post-natal, animals were submitted to moderate physical training (8 wk, 5 $d.wk^{-1}$, 60 $min.d^{-1}$, at 70% of VO_{2max}). After physical training period, half of each group received an injection of either lipopolysaccharide (LPS) or distilled water. Blood and splenic T lymphocytes (CD4+, CD8+), B-lymphocytes and NK cells were analyzed by flow cytometry. Spleen lymphocytes apoptosis was evaluated by cell viability, DNA fragmentation, phosphatidylserine externalization and mitochondrial transmembrane depolarization using a flow cytometer. **Results:** In LP + LPS pups, there was reduced blood concentrations of TCD3 and TCD4/CD8 ratio and high concentrations of B and TCD8 lymphocytes. Moderate physical training did not revert these effects. LP + LPS pups showed high percentage of splenic lymphocytes with mitochondrial depolarization and phosphatidylserine externalization. There is no difference when LP rats were submitted to training. **Conclusion**, moderate physical training was able to attenuate the effects of perinatal low protein-induced splenic lymphocytes apoptosis.

Key-words: Protein malnutrition; developmental plasticity; rats; physical exercise; splenic lymphocytes; cell death, LPS.

INTRODUCTION

Maternal and childhood malnutrition (or protein-energy restriction) is considered one of the most important risk factor for common disease and death, affecting pregnant women and young children in poor country [1-4]. In response to malnutrition, there is a reduction in the production of proteins of complement system, phagocytic function of macrophages, antibody production, lymphopenia and low expression of proteins that activate T lymphocytes during an infection [5-7]. Neonatal undernourishment (multideficient diet with 7% of protein) induced decrease *in vitro* nitric oxide release by alveolar macrophage of adult rats when compared to their control whose mothers fed a normal diet (23% of protein) [8]. Previous studies have shown that dietary deficiencies of specific nutrients, for example protein, severely alter the immune responses with significant changes in the immunocompetence as reduced number of T lymphocytes with a memory phenotype and deficits in cell-mediated immunity, involution of lymphoid tissues (thymus and spleen), and suppression of antibody responses to vaccination [5, 7, 9-11].

The spleen is the site of haemopoiesis from the third month until birth and remains a potential site for it throughout life [12]. The white pulp of spleen is the better place to accumulate lymphoid tissue and 30-50% of the bodies circulating granulocytes are stored in the spleen [5]. In the periarteriolar area, there are around 25% of TCD3 lymphocytes while 10% of B-lymphocytes are found in the germinal centers in the white pulp. The marginal zone containing B-lymphocytes and phagocytes positioned along the marginal sinus, and the white pulp composed of nodules containing lymphoid follicles (rich in B-lymphocytes) [13]. During the immune response, TCD8 lymphocytes act in association with macrophages to stimulate phagocytosis of blood-borne bacteria that is an important mechanism for protection against viral infection and parasites such as plasmodium [12]. Because splenic development mainly occurs *in utero* and in early postnatal life, a nutritional insult at a critical stage in splenic development may lead to a permanent impairment in T-lymphocyte immunity, splenic involution and atrophy, circulation of immature lymphocytes, and high splenic lymphocytes apoptosis [5, 9, 12, 14, 15]. Malnourished rats (litters with 16 pups) showed a reduction in absolute and relative numbers of splenic lymphocyte subpopulations [14]. Thus, early nutritional insults to the spleen may have long-term consequences for splenic activity and immunocompetence that is characterized by morphologic and functional changes.

Recently, it has been considered that regular physical activity is associated with positive effects to immune system [16-18]. According to the American College of Sports Medicine, a regular (at least three times a week), moderate physical exercise (50 – 75% VO_{2max}) is

associated with benefits effects for health [19]. Moderate physical training enhanced macrophage phagocytosis and oxidative burst, neutrophils oxidative burst, high percentage of TCD4 lymphocytes and cytokines production [18, 20, 21]. For example, moderate physical exercise (75% VO_2max, 5 times week, during 8 weeks) increased the percentage of TCD4 lymphocytes in blood and thymus and attenuated the rate of lymphocytes apoptosis in adult rats submitted to acute restraint stress [22]. It reasonable to consider that this kind of stimuli can induce positive adaptations on immune system even though the system was programmed to develop early disease or more susceptible immunity.

In our previous study [13], we demonstrated that moderate physical training attenuated the long-last effects of a perinatal low-protein diet on the blood lymphocyte subsets and the morphology of the spleen. Therefore, we set out to investigate the hypothesis that a defect in the underlying kinetics and distribution of lymphocytes (T, B and NK) and the high rate of apoptosis of splenic lymphocytes may explain observations of lymphopenia, low number of the splenic lymphoid follicles and a reduced area of the marginal zone in those pups submitted to protein deprivation. Thus, the main goal of the present study is to evaluate the effects of a moderate physical training on the lymphocytes subsets in blood and the rate of apoptosis and subpopulation of lymphocytes in adult offspring submitted to perinatal low-protein diet.

MATERIAL AND METHODS

The experimental protocol was approved by the Ethics Committee of the Biological Sciences Center (protocol no. 23076.021093/2011-99), Federal University of Pernambuco, Recife, PE, Brazil, and followed the Guidelines for the Care and Use of Laboratory Animals [23].

Animals and Diet

Virgin female albino Wistar rats (*Rattus norvegicus*) and males of the same strain were obtained from the Department of Nutrition, Federal University of Pernambuco. The female rats were 90-120 days old, body weight 220-280g and male (70-90 days old, body weight 260-290g) when they mated. The day on which spermatozoa were present in a vaginal smear was designated as the day of conception, day 0 of pregnancy. Pregnant rats were then transferred to individual cages and maintained at a room temperature of 22 ± 1ºC with a controlled light–dark cycle (light 06.00–18.00 h). Pregnant rats were randomly divided in two groups (n=9/each): control fed a 17% casein diet and low-protein diet fed an 8% casein diet. On postnatal day 1, litters were reduced to 8 pups per mother, ensuring only males per litter when possible. During the suckling period, male pups were randomly distributed into two nutritional groups according to their mother's diet during gestation and lactation: a well-

nourished group (C, n=26) and a low-protein group (LP, n=26). The offspring were kept in litters of eight pups. At weaning (25 d old), male offspring (2 – 3 from each mother) remained in the experiment and received standard chow for rodents Labina® (Purina Brazil) until the end of the experiment, when they were killed by decapitation. Female pups were used in another experiment. At the 63th day after birth, animals were divided into four groups according to physical training: control (C, n=17), low-protein diet (LP, n=19), control and submitted to training (T, n=19), and low-protein diet and submitted to training (LP+T, n=17). Trained rats run in a treadmill over a period of 8 weeks (5 days.wk^{-1}, 60 min.day^{-1}, at 70% VO_{2max}) [24]. After 24 h of the last exercise session, half of the number of rats in each group received an injection of either lipopolysaccharide (LPS) (1mg/mL/kg *i.p.*; *E. coli* serotype 0111:B4, Sigma-Aldrich, São Paulo, Brazil) or distillated water (LPS^{-}, 1ml/kg, *i.p.*). Four more groups were then formed: control (C, n = 8), control toxemic (C + LPS, n = 9), low-protein (LP, n = 9), low-protein toxemic (LP + LPS, n = 10), control and trained (T, n = 10), control and trained and toxemic (T + LPS, n = 9), low-protein and trained (LP + T, n = 8), and low-protein and trained and toxemic (LP + T + LPS, n = 9). The body weight of pups was weekly recorded throughout the experiment with a Marte Scale, AS-1000, approaching 0.01 g. After 24h, rats were decapitated and the blood was collected. The spleen was removed and weighed by using an AND Scale, HR-200, 0.1 mg accuracy (AND Weighing, San Jose, USA).

Protocol of Moderate Physical Training Protocol

The protocol of physical training was performed according to previous study [24]. Briefly, rats ran in a treadmill (EP-131®, Insight Equipments, SP, Brazil) during 8 weeks (5 days.wk^{-1}, 60 min.day^{-1}). The protocol was divided into four progressive stages in each session: (i) warm-up (5 minutes); (ii) intermediary (10 minutes); (iii) training (30 minutes), and (iv) cool-down (5 minutes) periods. The percentage of VO_{2max} during the sessions of training was kept around 65 – 70%; the exercise was classified as aerobic with moderate intensity of effort. The non-trained group remained in their cages. The animals were not submitted to any kind of reinforcement during exercise.

Blood Sampling and Spleen Processing

Blood was collected in dry tubes and tubes containing EDTA. Serum was collected after a period of 30 minutes to allow coagulation followed by a 300 *g* centrifugation, 10 minutes (Beckman Coulter, Inc., Brea, USA) [25]. Serum samples were kept at -20^{o} C. The anticoagulated blood samples were used as described later.

The spleens were removed, dissected, weighed and kept in cold phosphate buffered saline (PBS), pH 7.4. Half of the spleen from each animal was used and kept in cold PBS right after the organ extraction. The tissue was minced by scissors and pressed with a cell strainer (70μm) using the syringe plunger. Spleen lymphocytes were washed with 50 mL of PBS and hemolysed for 1-2 times with hemolysis solution (0.1mM EDTA, 0.15M NH_4Cl, 10mM $KHCO_3$, pH 7.3) during 4 minutes at room temperature. The cells were then washed again with culture medium (50 mL), [RPMI-1640 with $NaHCO_3$ (2g/L), glutamine (300 mg/L), gentamicin (50 mg/mL), amphotericin B (2 mg/L) and 1% fetal calf serum (FCS) (Vitrocell Embriolife, Campinas, Brazil)]. The remaining cells were suspended in 5 mL of culture medium, counted in Neubauer's chamber and used in the experiments [26].

Blood and Spleen Lymphocytes Subsets

The blood lymphocytes subsets were also evaluated: T lymphocytes (CD4+, CD8+), B lymphocytes and NK cells. 100 μL-blood samples with EDTA were incubated with antibodies in the dark at 40°C for 30 minutes. Besides the T/B/NK kit, the following clones were used: clone G4.18 anti-CD3 FITC-labeled (490/525 nm), clone OX-35 anti-CD4 PE-labeled (348/395 nm), and clone OX-8 anti-CD8α PerCP-labeled (490/675 nm). The blood was then hemolysed (BD FACS Lysing Solution, BD Biosciences, San Jose, USA) and the lymphocytes were evaluated by flow cytometry. All the antibodies were purchased from BD Biosciences, San Jose, USA.

The spleen lymphocytes ($2x10^6$ cells per sample) were washed 2 times with cytometry buffer (PBS added 1% FCS and 0.02% N_3Na) to remove the culture medium. Then, the suspension was incubated for 30 minutes with a antibody cocktail for T/B/NK cells identification (Rat T/B/NK Cell Cocktail, BD Biosciences, San Jose, USA), protected from light at 4° C. The cell suspensions were washed 2 more times with cytometry buffer and then analyzed by flow citometry (FACSCalibur, BD Biosciences, San Jose, USA) [27].

Spleen Lymphocyte Apoptosis

Spleen lymphocytes apoptosis evaluation included cell viability, DNA fragmentation, phosphatidylserine externalization (PSE) and mitochondrial transmembrane depolarization (MTD). The viability of spleen lymphocytes was assessed by using a flow cytometer FACSCalibur (Becton Dickinson Systems, San Jose, CA). The percentage of viable cells in each sample was determined using propidium iodide (PI) staining (BD Biosciences, San Jose, CA) to identify dead cells. PI fluorescence detected in 630/622-nm wavelength [28].

PI was also used to measure DNA fragmentation. It binds to DNA by intercalating between the DNA bases [28]. Spleen lymphocytes (1x106) were incubated for 30 minutes in the dark,

at room temperature, in a solution containing 0.1% citrate, 0.1% Triton X-100, and 50 g/mL PI. Cells with PI fluorescence were then evaluated by flow cytometry, after acquisition of 10.000 events per sample [29].

In apoptotic cells phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. This change is part of the intercellular communication which is signaling programmed cell death by apoptosis. Annexin V is a phospholipid-binding protein that has high affinity for PS [30]. Therefore, PSE can be detected by annexin V fluorochrome-conjugated. Spleen lymphocytes ($2x10^6$) were incubated for 15 min in a dark room following the instructions of the Annexin-V kit (Annexin V: FITC Apoptosis Detection Kit II, BD Biosciences, San Jose, CA). Cells with fluorescent annexin V were then evaluated by flow cytometry [31].

Another apoptosis parameter is MTD. Spleen lymphocytes ($2x10^6$) were incubated for 30 min in a dark room in the presence of a solution containing the fluorochrome rhodamine 123 (2.6M) in PBS [32]. Rhodamine 123 is a cell permeant, cationic, fluorescent dye that is sequestered by active mitochondria without inducing cytotoxic effects [33]. Cells with fluorescent rhodamine were then evaluated by flow cytometry (511/534 nm).

TNF-α Serum Levels

To examine the TNF-α level in the serum from rats, enzyme-linked immunosorbent assay – ELISA was performed. A commercially available kit was used following the manufacturer's instructions (Rat TNF alpha ELISA Ready-SET-Go!®, eBioscience, San Diego, USA).

Statistical Analysis

Results are presented as means ± standard error of the mean (SEM). For statistical analysis, data were analyzed by two-way repeated- measures ANOVA, with maternal diet (C, LP) and physical training (T, T + LP) as factors. Bonferroni's post hoc test was used. The difference between distillated water and LPS was evaluated using Student's T Test. Significance was set at $p<0.05$. The statistical program used was GraphPad Prisma 5 (GraphPad Software, Inc., La Jolla, USA).

RESULTS

From weaning to 60^{th} d, rats from LP mothers showed a lower body weight than control (Figure 1). During the period of physical training, T rats showed a similar body weight when compared to C rats. LP rats remained with reduced body weight while LP + T rats showed no difference when compared to T rats (Figure 1).

After the last session of exercise (24h), rats were divided according to the peritoneal injection of either distillated water (basal condition) or LPS. At basal condition, the relative weight of the spleen was not different among groups. After LPS injection, all groups showed an increase in the relative weight of the spleen that was more pronounced in trained group (T + LPS vs C + LPS, $p < 0.05$) (Figure 2). T rats showed a higher relative weight of spleen than their pair C while there is no difference in rats that was submitted to perinatal low-protein diet.

The effect of moderate physical training and maternal low protein diet was evaluated on circulating lymphocytes subtypes. At basal condition, rats from LP mothers did not show differences in circulating lymphocytes subsets compared to control rats (Figure 3A, 3B and 3C). Trained rats showed an increase in T and NK lymphocyte counts, and decrease in B lymphocyte counts compared to non-trained rats (*T lymphocyte*: Trained vs Non-trained, $p < 0.001$; *NK lymphocyte*: Trained vs Non-trained, $p < 0.001$; *B lymphocyte*: Trained vs Non-trained, $p < 0.001$). Trained rats from LP mothers presented higher NK and lower B lymphocytes counts than LP rats (*NK lymphocyte*: LP + T vs LP, $p < 0.001$; *B lymphocyte*: LP + T vs LP, $p < 0.001$) (Figure 3A, 3B and 3C).

After LPS injection, rats from LP mothers presented lower counts of circulating T lymphocytes and higher counts of B lymphocytes than control rats (*T lymphocyte*: LP vs C, $p < 0.001$; *B lymphocyte*: LP vs C, $p < 0.001$) (Figure 3A and 3B). Trained rats showed a similar pattern of lymphocytes counts than basal condition: higher T and NK lymphocytes counts, and lower B lymphocyte counts than non-trained rats (*T lymphocyte*: Trained vs Non-trained, $p < 0.001$; *NK lymphocyte*: Trained vs Non-trained, $p < 0.001$; *B lymphocyte*: Trained vs Non-trained, $p < 0.05$) (Figure 3A, 3B and 3C). Trained rats from LP mother presented higher T lymphocytes counts than non-trained LP rats (LP + T + LPS vs LP + LPS, $p < 0.001$) (Figure 3A).

Differential T lymphocytes subsets (CD4+ and CD8+) were assessed in blood. At basal condition, rats from LP mothers presented lower percentage of CD4+ and CD4+/CD8+ ratio and higher percentage of CD8+ than control rats (CD4+: LP vs C, $p < 0.05$; *CD4+/CD8+*: LP vs C, $p < 0.05$; *CD8+*: LP + T vs C, $p < 0.01$) (Figure 4A, 4B and 4C). After LPS injection, rats from LP mothers presented higher CD4+, CD4+/CD8+ ratio, and lower CD8+ counts than control rats (*CD4+*: LP + LPS vs C + LPS, $p < 0.01$; *CD4+/CD8+*: LP + LPS vs C + LPS, $p < 0.001$; *CD8+*: LP + LPS vs C + LPS, $p<0.01$). Physical training did not attenuate the effects of maternal low-protein diet in the counting of CD4 and CD8 lymphocytes (Figure 4A, 4B and 4C).

At basal conditions, rats from LP mothers did not show difference in spleen lymphocyte counts while trained rats presented higher percentage of B lymphocytes than non-trained rats ($p < 0.05$) (Figure 5B). Trained rats from LP mothers showed increased T lymphocytes counts and decreased B and NK lymphocytes counts when compared to control trained rats (*T lymphocyte*: LP + T vs T, $p < 0.01$ and LP, $p < 0.05$; *B lymphocyte*: LP + T vs T, $p < 0.01$; *NK lymphocyte*: LP + T vs T, $p < 0.01$, and LP, $p < 0.001$) (Figure 5A, 5B and 5C).

After the LPS injection, rats from LP mothers showed increased NK lymphocyte counts in the spleen when compared to control rats (LP + LPS vs C + LPS, $p<0.01$). Trained rats did not present differences in T, B and NK lymphocytes counts when compared to non-trained rats (Figure 5A, 5B and 5C). Trained rats from LP mothers showed reduced NK lymphocyte counts when compared to LP non-trained rats (LP + LPS vs LP + T + LPS , $p < 0.001$) (Figure 5C).

Apoptosis of spleen lymphocytes was assessed by measuring cell viability, DNA fragmentation, PSE and MTD. All groups presented the spleen with more than 92% of viable lymphocytes and with no differences in DNA fragmentation (data not shown). At basal condition, rats from LP mothers and trained rats did not present differences in all the apoptosis indicators (Figure 6A and 6B). Trained rats from LP mothers presented lymphocytes with increased percentage of phosphatidylserine externalization (PSE) than control trained rats (LP + T vs T, $p < 0.05$) (Figure 6A and 6B).

After LPS injection, rats from LP mothers showed spleen lymphocytes with higher percentage of mitochondrial depolarization and phosphatidylserine externalization than control rats (*MTD*: LP + LPS vs C + LPS, $p < 0,001$; *PSE*: LP + LPS vs C + LPS, $p < 0,001$). Trained rats showed lymphocytes with lower phosphatidylserine externalization levels than non-trained rats (Trained vs Non-trained), but no differences in mitochondrial depolarization. Trained rats from LP mothers presented lymphocytes with reduced percentage of mitochondrial depolarization and phosphatidylserine externalization than LP rats (*MTD*: LP + T + LPS vs LP + LPS, $p < 0.01$; *PSE*: LP + T + LPS vs LP + LPS, $p < 0,001$) (Figure 6A and 6B).

Blood TNF-α was evaluated on blood of adult offspring. At basal condition, there was no difference among groups. After LPS injection, rats from LP mothers showed higher values of TNF-α than control. Similarly, trained and LP rats showed higher values of TNF-α than trained rats (LP + LPS vs C + LPS, $p < 0.001$; LP + T + LPS vs T + LPS, $p < 0.001$) (Figure 7).

DISCUSSION

It has been well described that early malnutrition is an important cause of immune suppression and increases host susceptibility to infectious diseases later in life [6, 7, 34]. The present study showed that early malnutrition is associated with reduction of circulating splenic lymphocytes, and loss of lymphoid cells in the spleen by enhanced apoptosis rate. Regardless of the period of the recovery, maternal low-protein diet largely affected the spleen as aligned with previous study [13]. However, these effects seems to be reversible and we tested the hypothesis that moderate physical training attenuates the long-term effects of a perinatal low-protein diet on the process of immune defense by maintaining the counting of lymphocyte in the blood and spleen and reducing the rate of apoptosis of splenic lymphocytes of endotoxemic rats. Conversely, blood T lymphocytes count was altered in response to LPS in perinatal low-protein rats and there are no effects of moderate physical training to revert these effects. However, splenic lymphocytes derived from perinatal low-protein diet rats showed higher spontaneous apoptosis than the rate observed in splenic lymphocytes from control rats. Moderate physical training was able to revert these effects of perinatal undernutrition-induced splenocytes apoptosis in terms of annexin-V assays and mitochondrial membrane depolarization.

In the present study, low-protein offspring presented growth retarded throughout life and there was no catch-up growth even when fed the control diet *ad libitum* from weaning. In contrast, previous studies have shown that when diet is recovered after perinatal undernutriton, offspring showed a fast body weight gain in order to align body weight with control [35-37]. However, exposure to a low-protein diet (8% casein) during gestation followed by the consumption of a normoproteic diet throughout the life-course was associated with catch up growth but when maternal protein restriction is continued during lactation, there is long-lasting growth restriction even when the offspring are recovered [38]. Supported by previous studies, moderate physical training recovered the body weight of low-protein offspring by a mechanism that probable includes an increase of lean-mass [39, 40].

The perinatal period is a time of critical immunologic susceptibility to nutritional compromise when lymphoid organs and the lymphocytes repertoire are being established [11]. In addition, protein restriction at this time may negatively affect at long-term the establishment of clonal diversity and the homeostasis of lymphocytes [11]. Consistent with previous findings [7, 41], the present study found reduced blood concentrations of TCD3 and TCD4/CD8 ratio and high concentrations of B and TCD8 lymphocytes in LP offspring submitted to LPS injection. The

reduction in T cells in the peripheral blood of LP offspring was a preferential loss of helper/inducer (T4) T cell subsets in parallel with increased TCD8 cell-mediated immunity and consequent reduced CD4/CD8 ratio. There was also a high concentration of B-lymphocytes that may be related to the high interferon-gamma production or defects in the subsets of dendritic cells or both [41]. Anyhow, the imbalance in the number, trafficking and function of lymphocytes subsets is related to high susceptibly to autoimmune and infectious diseases and changes in the allergic response by increased TCD8 and B-lymphocytes as seen in malnourished human [15].

Moderate physical training increased T-lymphocyte and NK cells and reduced B-lymphocyte but it was unable to recovery the blood concentration of lymphocytes subsets in undernourished endotoxemic rats except for TCD3 lymphocytes counting. It is possible that low-protein profoundly alter cell-counting immune responses as thymic atrophy and the production of thymic hormones critical for the differentiation of T lymphocytes [9, 15, 41]. Herein, we propose that the mechanism may be related to high concentration of TNF−α as seen in the LP offspring and T + LP offspring. TNFα stimulate the acute phase reaction and increase in response to sepsis promoting the apoptosis and inflammatory response. Thus, the long-last effects of maternal low-protein diet can be associated to increased peripheral blood lymphocytes apoptosis mediated by increased TNF-α production. These observations agree with previous reports involving malnourished children and increased rate of apoptosis of peripheral blood lymphocytes [7, 11].

The critical period of development of the spleen begins during gestation and acquires the mature morphology and physiology early in the fetal period [5]. Maternal nutritional deficits have been demonstrated to induce apoptosis in various cell types including thymus and spleen [5, 9]. In the present study, there was an expected increase in the relative spleen weight, in the number of NK lymphocytes per spleen observed and a high rate of apoptosis in endotoxemic LP rats. This finding agrees with previous observations which determined that splenic lymphocytes depletion is a consequence of both acute and chronic experimental protein malnutrition [7, 9, 14, 15, 34]. Because splenic atrophy and lymphopenia were presented in LP offspring, it seemed probable that apoptosis is associated with the interference of lymphopoiesis in these LP offspring. It shows that malnutrition during the lactation period is associated with a loss of immune response. The underlying mechanism can be associated with the elevated production of glucocorticoids, which initiate apoptosis in splenic lymphocytes [42]. In addition, our previous results show that adult offspring from LP mothers presented spleen structural alterations such as decreased splenic follicle count and decreased marginal

zone area [13]. The concentration of plasma corticosterone was increased in LP rats then it could be suggested that these animals are the most susceptible to glucocorticoids-induced apoptosis [13].

Moderate physical training restored the count of NK lymphocytes and reduced the rate of splenic lymphocytes apoptosis in endotoxemic LP offspring rats. Physical training before a stressful stimulus can be associated with more efficient proliferative activity of lymphocytes from spleen and thymus [22]. In our previous study using the same experimental design of perinatal low-protein diet (8% casein) and moderate physical training (70% VO_{2max}, 1 hour/day, 5 days/week, 8 weeks), we demonstrated that LP + T rats showed a less pronounced reduction of the number of the splenic lymphoid follicles and the area of the marginal zone when compared to sedentary LP rats. The present study confirmed the hypothesis that moderate physical training attenuates these maternal low-protein diet-induced morphological changes by a mechanism that includes modulation of the rate of apoptosis of splenic lymphocytes. Previous study has shown that voluntary training in mice reduced splenic lymphocyte apoptosis by increasing Bcl-2 and reducing caspase 3 levels relative to control mice [43]. In addition, our results showed that LP + T offspring did not alter the plasma concentration of TNF-α that can be related to the reduced rate of splenic lymphocyte apoptosis. Our previous study also showed that LP + T offspring normalized the corticosterone concentration after LPS injection. Taken together, we can purpose some mechanisms for the effects of moderate physical training on rate of splenic lymphocyte apoptosis of LP offspring that include change in the expression of protein related to apoptosis, concentration of TNF-α and corticosterone levels.

CONCLUSION

The present study showed that at basal state, lymphocyte populations remains largely unaffected in rats exposed to perinatal malnutrition. Our results suggest that proliferation and death rates within these lymphocyte subsets is dependent on immune challenger as we used LPS. Although subtle changes suggested that moderate physical training can alter lymphocytes subsets, the significance of these changes were not seen in LP offspring. However, these results are consistent with a robust maintenance of splenic lymphocytes by reducing apoptosis in trained offspring, despite exposure to adverse perinatal nutritional and immunologic challenges throughout life. Thus, moderate physical training was able to revert these effects of perinatal undernutrition-induced splenic lymphocytes apoptosis.

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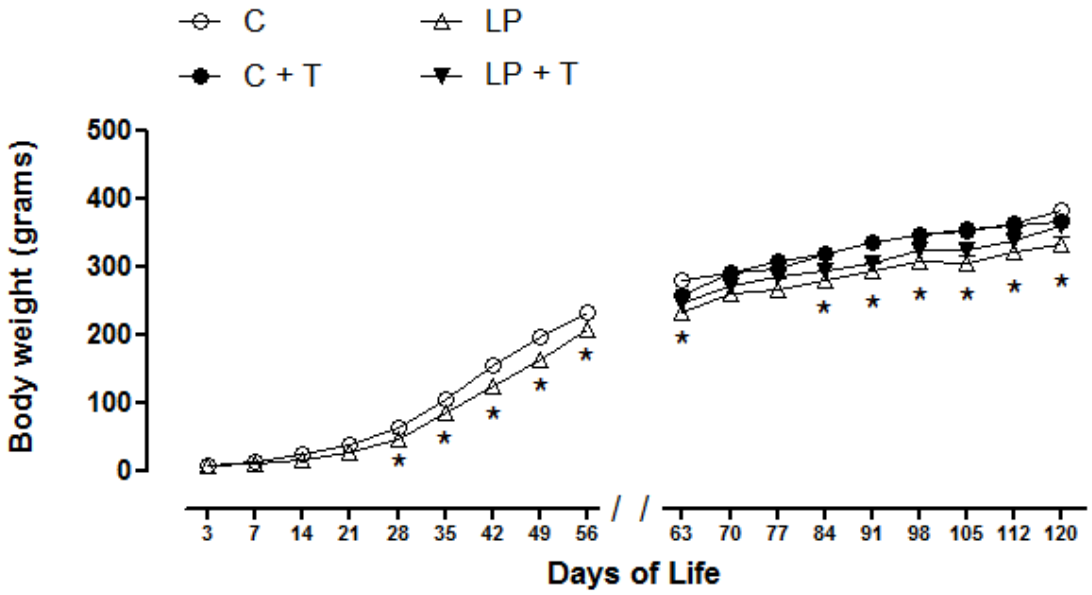


Figure 1. Body weight measurements in pups. During gestation and lactation, the dams were fed either a control or low protein diet. The pups were evaluated at 3rd, 7th day and then weekly until 120th day of life. Around 60th d the pups were divided in two more groups according to moderate physical training program or not. Groups until 60th d: control (C, n=22); low protein diet (LP, n=22). Groups after 60th d: control (C, n=9); low protein diet (LP, n=7); control trained (C + T, n=13); and low protein diet trained (LP + T, n=15).

*p<0.05 vs C. One-way ANOVA followed by Bonferroni´s post hoc test.

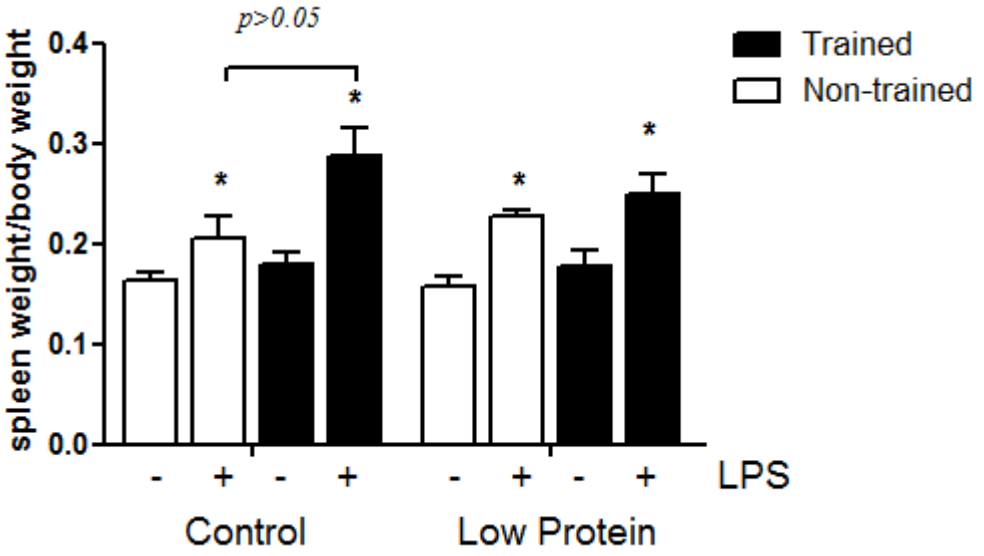
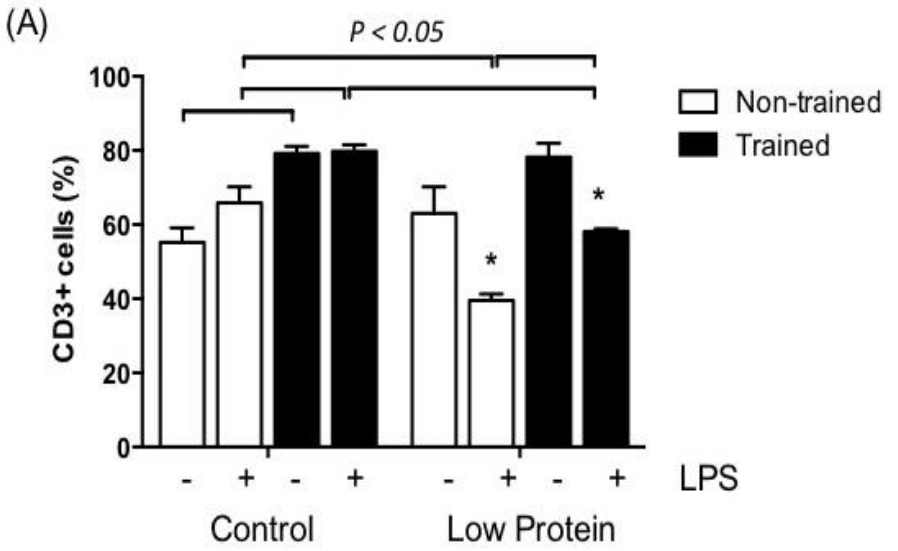
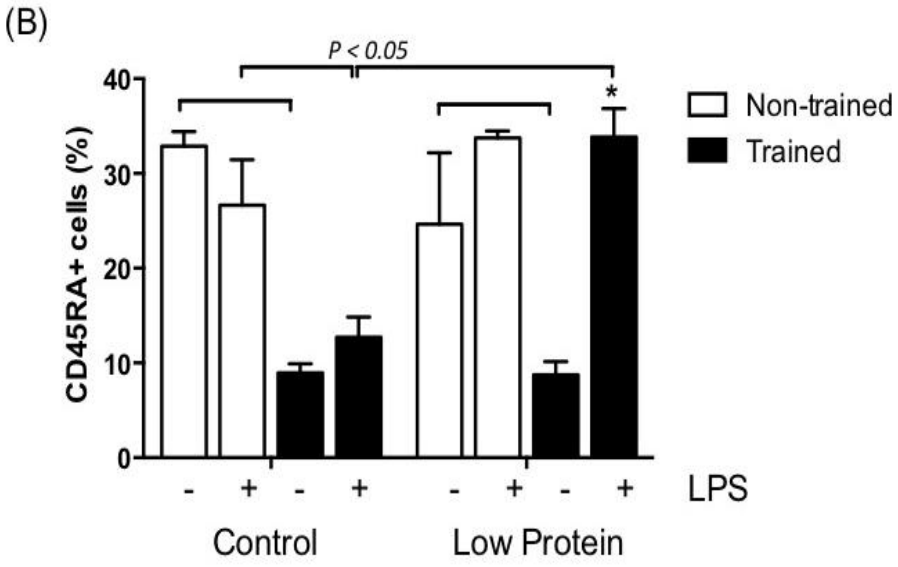


Figure 2. Spleen relative weight in pups. Groups: control (C, n=7); control LPS (C + LPS, n=7); trained (T, n= 10); trained LPS (T + LPS, n= 6); low protein diet (LP, n= 9); low protein diet LPS (LP + LPS, n= 6); low protein diet trained (LP + T, n= 7); and low protein diet trained LPS (LP + T + LPS, n= 8). Data are presented as means ± SEM.

Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test (T + LPS vs C + LPS, $p< 0.05$). *$p<0.05$ LPS vs pair LPS-. Student's T Test.


(A)
P < 0.05
100
80
60
40
20
0
CD3+ cells (%)
Non-trained
Trained
*
*
- + - + - + - +
LPS
Control
Low Protein


(B)
P < 0.05
40
30
20
10
0
CD45RA+ cells (%)
Non-trained
Trained
*
- + - + - + - +
LPS
Control
Low Protein

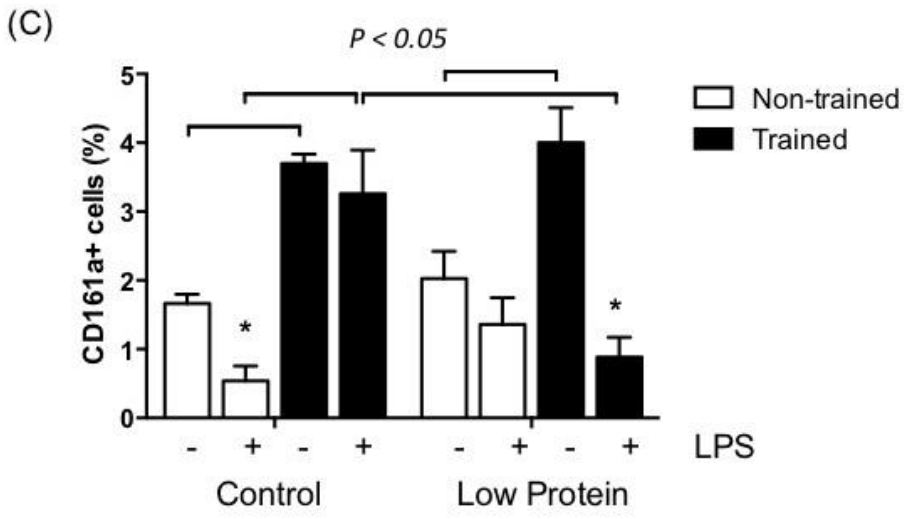
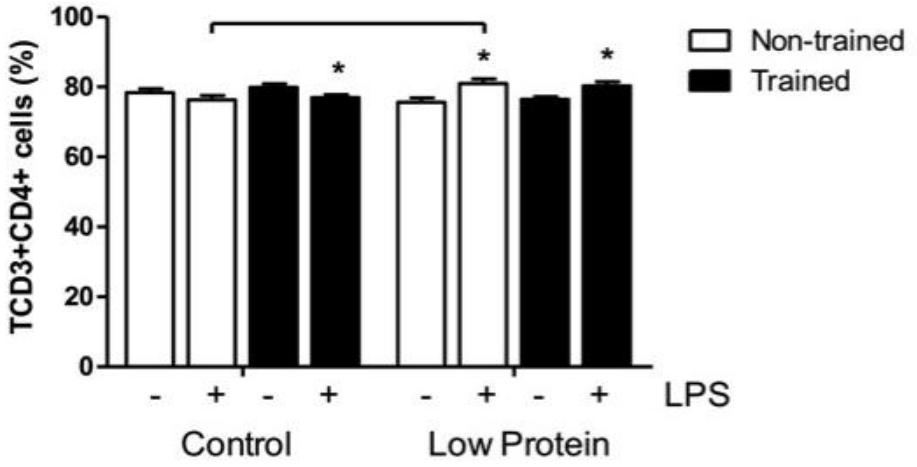
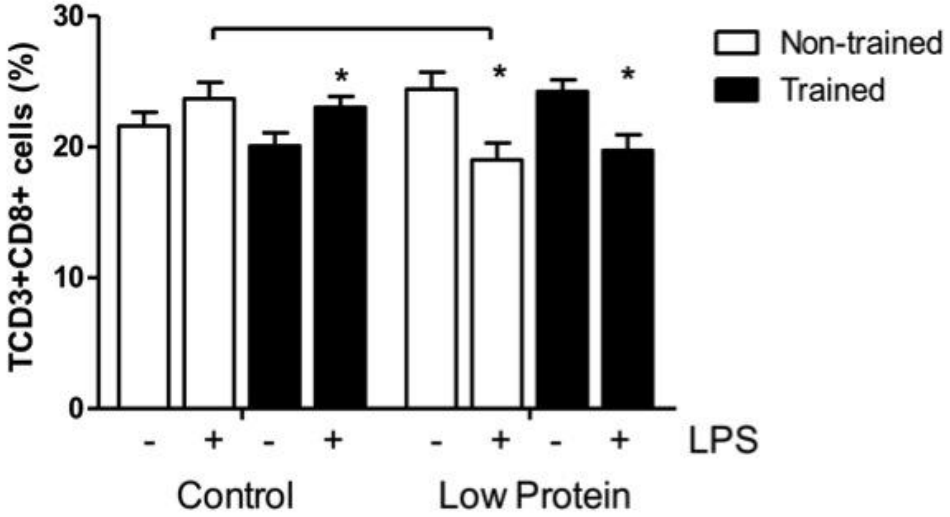


Figure 3. Percentage of blood lymphocytes subsets. (A) T lymphocytes CD3+; (B) B lymphocytes CD45R+; (C) NK lymphocytes CD161+. Groups: control (C, n= 6); control LPS (C + LPS, n= 6); trained (T, n= 5); trained LPS (T + LPS, n= 6); low protein diet (LP, n= 5); low protein diet LPS (LP + LPS, n= 5); low protein diet trained (LP + T, n= 6); and low protein diet trained LPS (LP + T + LPS, n= 5). Data are presented as means ± SEM.
Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test
*p<0.05 LPS+ vs pair LPS-. Student's T Test.

(A)


TCD3+CD4+ cells (%)
100
80
60
40
20
0
*
*
*
Non-trained
Trained
- + - + - + - + LPS
Control
Low Protein

(B)


TCD3+CD8+ cells (%)
30
20
10
0
*
*
*
Non-trained
Trained
- + - + - + - + LPS
Control
Low Protein

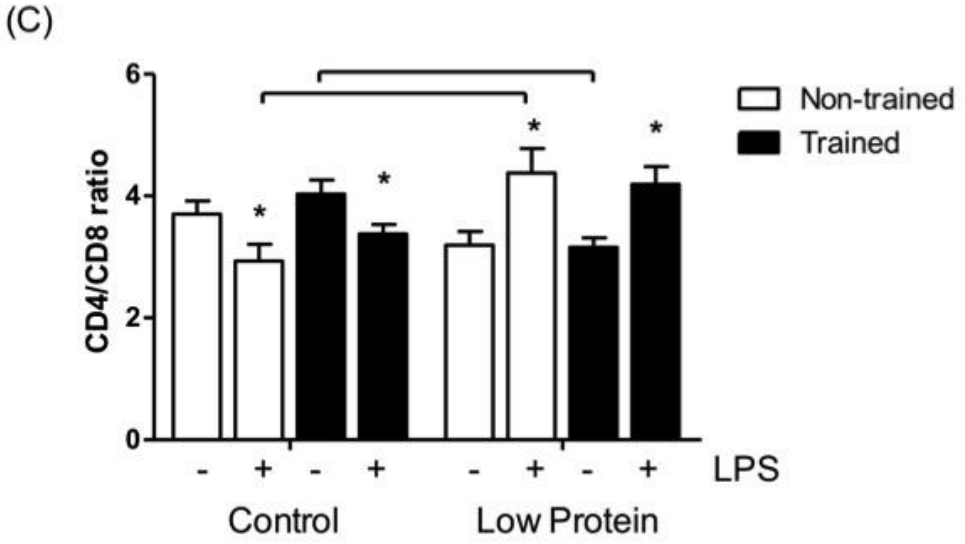
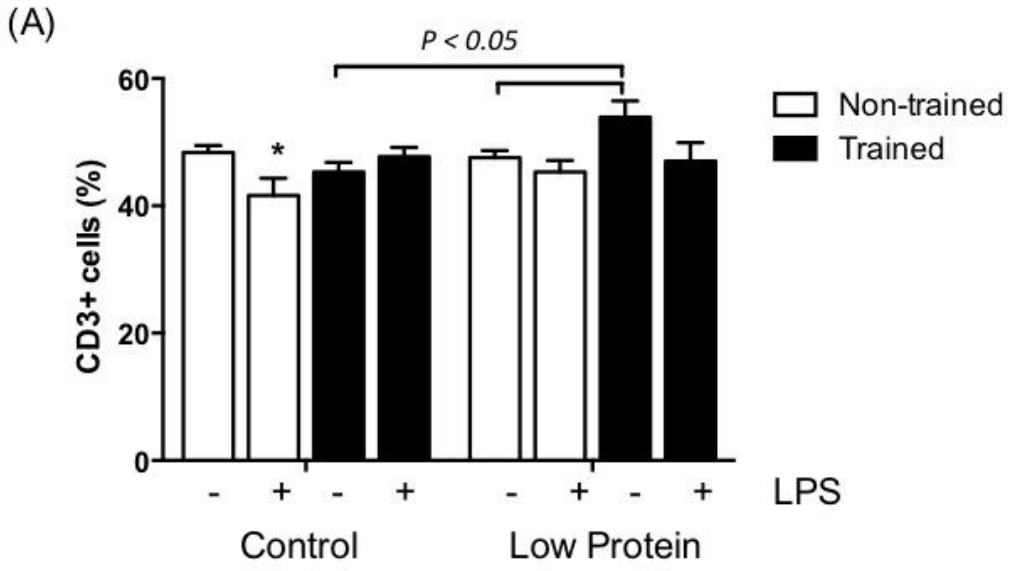
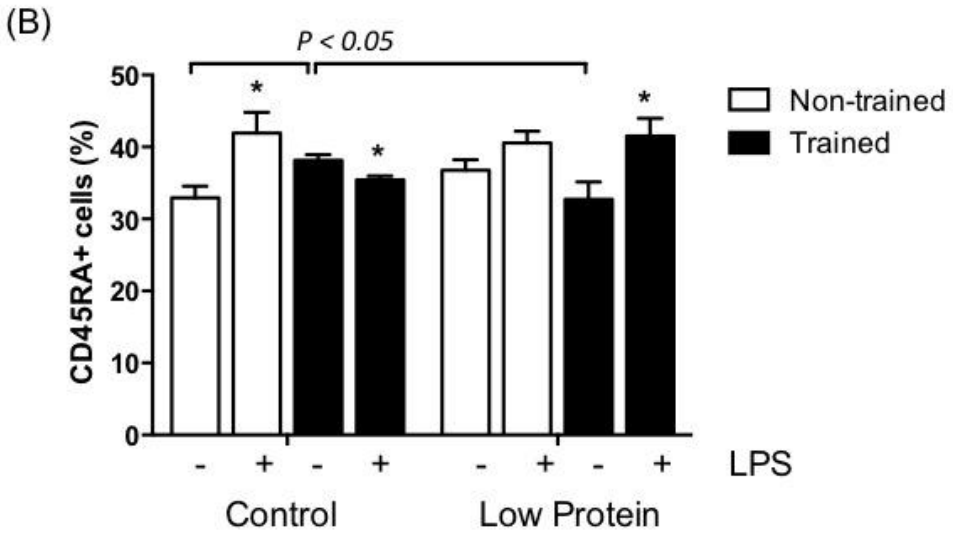


Figure 4. Blood T lymphocytes subsets and CD4/CD8 ratio. (A) T lymphocytes CD4+; (B) T lymphocytes CD8+; (C) CD4/CD8 ratio. Groups: control (C, n= 8); control LPS (C + LPS, n= 5); trained (T, n= 6); trained LPS (T + LPS, n= 8); low protein diet (LP, n= 9); low protein diet LPS (LP + LPS, n= 10); low protein diet trained (LP + T, n= 5); and low protein diet trained LPS (LP + T + LPS, n= 8). Data are presented as means ± SEM.
Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test


(A)
P < 0.05
CD3+ cells (%)
60
40
20
0
*
Non-trained
Trained
- + - + - + - +
LPS
Control
Low Protein


(B)
P < 0.05
CD45RA+ cells (%)
50
40
30
20
10
0
*
*
*
Non-trained
Trained
- + - + - + - +
LPS
Control
Low Protein

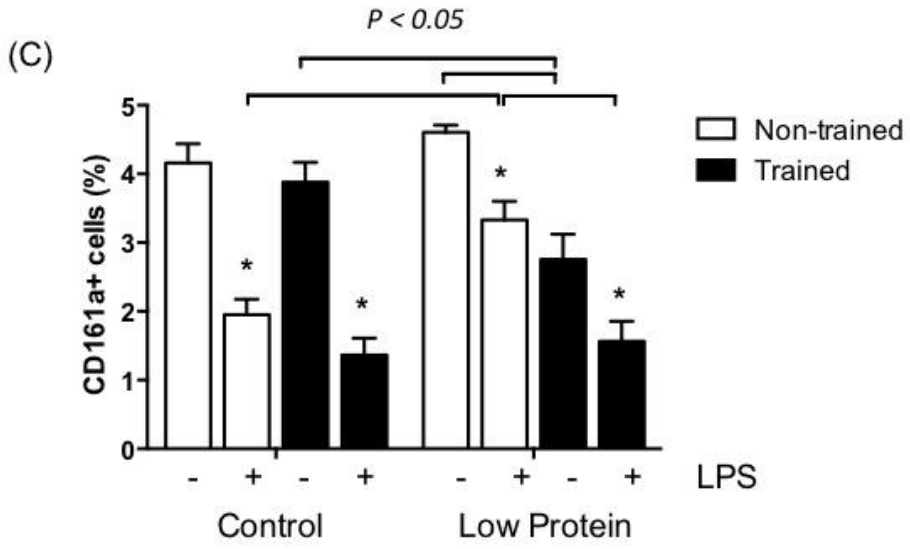
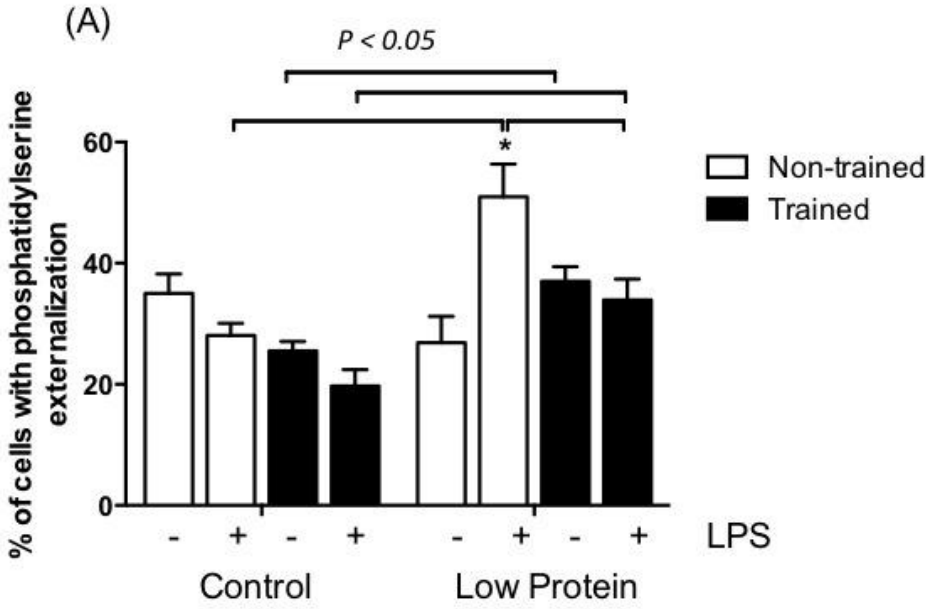
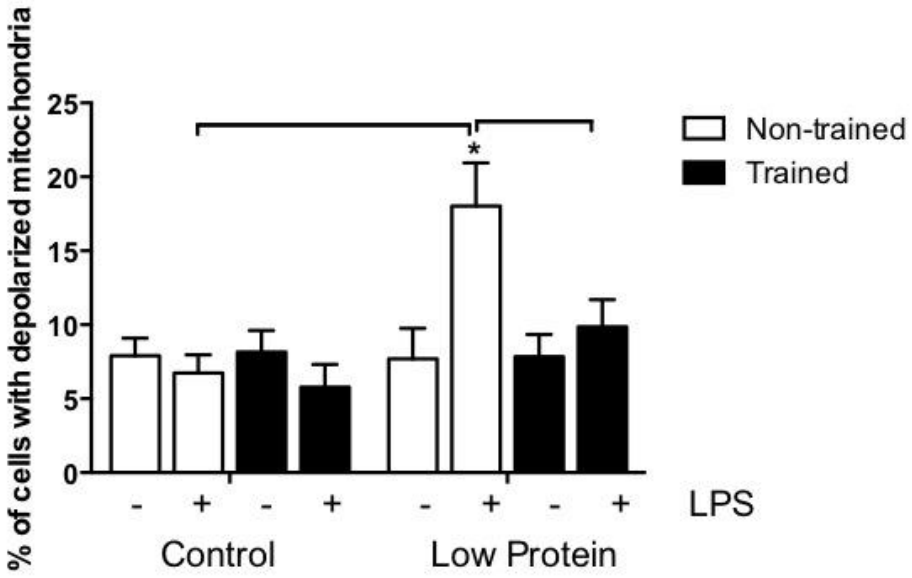


Figure 5. Percentage of spleen lymphocytes subsets. (A) T lymphocytes CD3+; (B) B lymphocytes CD45R+; (C) NK lymphocytes CD161+. Groups: control (C, n=7); control LPS (C + LPS, n=7); trained (T, n= 8); trained LPS (T + LPS, n= 7); low protein diet (LP, n= 7); low protein diet LPS (LP + LPS, n= 7); low protein diet trained (LP + T, n= 8); and low protein diet trained LPS (LP + T + LPS, n= 9). Data are presented as means ± SEM.
Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test.


(A)
P < 0.05
% of cells with phosphatidylserine externalization
60
40
20
0
*
Non-trained
Trained
- + - + - + - +
LPS
Control
Low Protein


% of cells with depolarized mitochondria
25
20
15
10
5
0
*
Non-trained
Trained
- + - + - + - +
LPS
Control
Low Protein

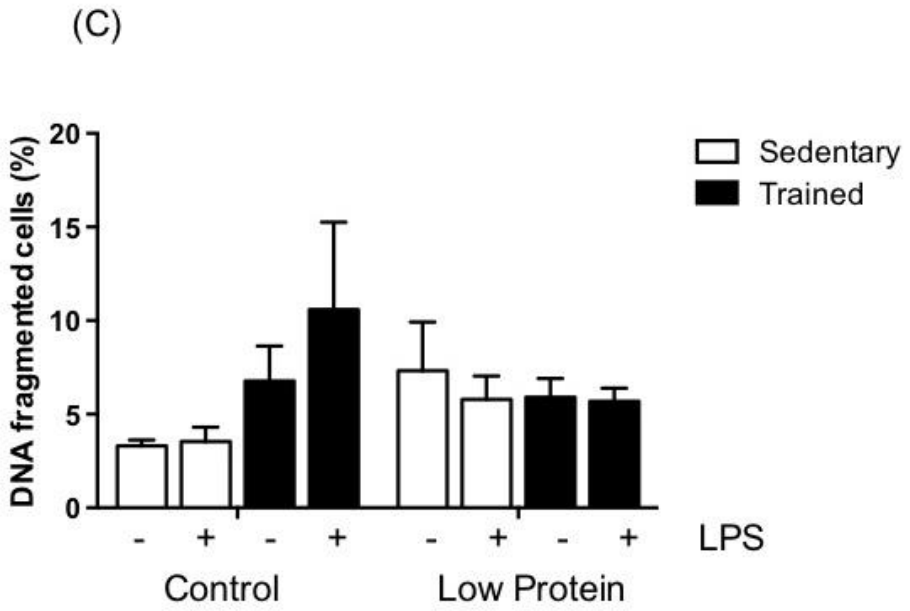


Figure 6. Apoptosis indicators in spleen lymphocytes. (A) Phosphatidylserine externalization (PSE); (B) Mitochondrial transmembrane potential depolarization (MTD); (C) DNA fragmentation (DNAF). Groups: control (C, n=8); control LPS (C + LPS, n=9); trained (T, n= 7); trained LPS (T + LPS, n= 9); low protein diet (LP, n= 6); low protein diet LPS (LP + LPS, n= 7); low protein diet trained (LP + T, n= 7); and low protein diet trained LPS (LP + T + LPS, n= 8). Data are presented as means ± SEM.

Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test

*$p<0.05$ LPS+ vs pair LPS-. Student's T Test.

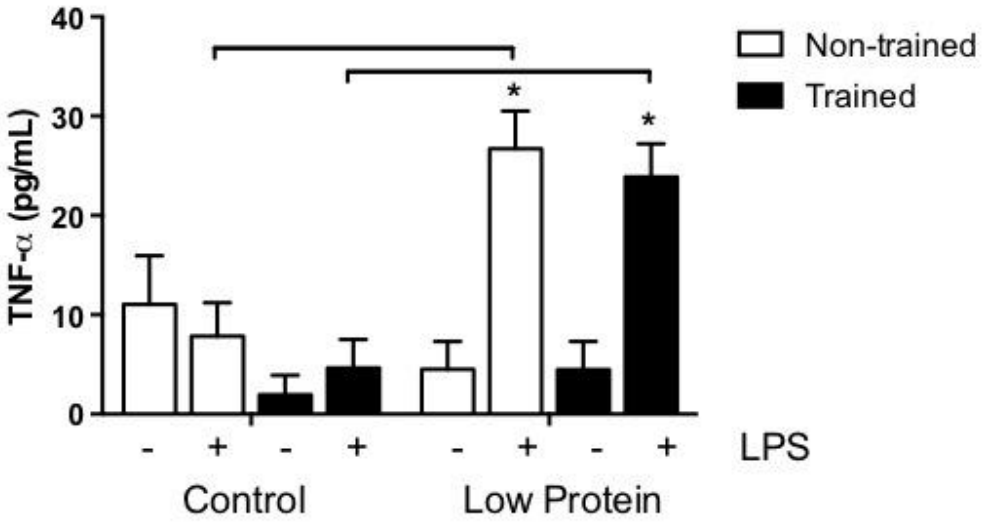


Figure 7. TNF-alpha concentration in adult offspring blood. Groups: control (C); control LPS (C + LPS); trained (T); trained LPS (T + LPS); low protein diet (LP); low protein diet LPS (LP + LPS); low protein diet trained (LP + T); and low protein diet trained LPS (LP + T + LPS). n=5. Data are presented as means ± SEM.

Bar indicates two-way ANOVA followed by Bonferroni´s post hoc test

*$p<0.05$ LPS vs pair LPS-. Student's T Test.

8. Considerações finais

A desnutrição proteica materna durante os períodos de gestação e lactação induz adaptações imunológicas nos filhotes, e essas adaptações apresentam reflexos na vida adulta. Observamos que o treinamento físico de intensidade moderada atua como fator ambiental capaz de reverter algumas dessas alterações frente a uma situação séptica.

O baço é um importante órgão linfoide na organização da resposta imunológica adaptativa. É um sítio de reconhecimento de antígenos pelos linfócitos, desencadeando a proliferação de linfócitos T e B em sua maioria. No estado basal, os linfócitos de animais adultos submetidos à desnutrição proteica perinatal praticamente não sofreram alterações. Por outro lado, verificamos que, frente a um desafio imunológico, a porcentagem de linfócitos Nk no baço de animais desnutridos foi maior que no baço de animais controle. O grupo desnutrido treinado apresentou porcentagem de linfócitos Nk similar ao grupo controle. Esse acúmulo de células Nk no baço sugere algumas situações: que linfócitos Nk são as células mais afetadas pelo processo apoptótico e por isso permanecem no baço; que essas células perderam sua capacidade migratória como reação a quimiocinas, ou que houve algum prejuízo no mecanismo de moléculas de adesão pela desnutrição perinatal. Outros trabalhos relatam a diminuição da capacidade migratória de neutrófilos e macrófagos para o sítio de infecção, mas estudos mais específicos são necessários para responder a esses questionamentos.

Neste trabalho demonstramos também que a desnutrição perinatal altera a resposta de linfócitos do baço ao estímulo apoptótico tanto da via intrínseca (dependente da mitocôndria) quanto da via extrínseca (iniciado por outros sítios que não mitocondriais, como por exemplo, a ativação de receptores de morte da membrana plasmática). Os processos iniciais indicativos de apoptose se apresentaram elevados em animais desnutridos endotoxêmicos, e o exercício físico foi capaz de prevenir seu disparo, em uma resposta similar aquela dos ratos do grupo controle. Mas essa resposta parece ser independente dos níveis séricos de TNF-α, uma vez que os ratos desnutridos não alteraram a elevada concentração sérica dessa após o treinamento físico moderado.

No sangue desses animais também observamos que a desnutrição proteica perinatal induz à redução de linfócitos T durante o desafio endotoxêmico. Essa redução foi prevenida pelo treinamento fisco moderado. Outros estudos devem ser realizados para identificar a

funcionalidade dessas células. Neste estudo demonstramos que a desnutrição perinatal causa alterações na distribuição de linfócitos no sangue e baço de ratos adultos endotoxêmicos, mas talvez os linfócitos remanescentes sejam eficientes e consigam debelar a infecção presente. Acreditamos que as funções imunológicas de linfócitos e de outras células como macrófagos e neutrófilos estejam diminuídas nos animais desnutridos, mas outros estudos são necessários para abordar essa problemática.

O treinamento físico moderado mostrou ser um eficiente fator ambiental na recuperação de algumas características imunológicas dos animais submetidos à desnutrição materna. O treinamento físico moderado foi capaz de reverter os efeitos da desnutrição proteica perinatal sobre a apoptose de linfócitos do baço. Parece que o treinamento físico prepara o sistema imunológico para suportar situações de estresse. Nesse contexto, outros estudos são necessários para explorar melhor as respostas de linfócitos em 24 horas e em outros períodos de tempo, como após seis a sete dias do insulto endotoxêmico. Além disso, o efeito do treinamento pode estar atrelado ao condicionamento físico e ser transitório, ou causar modificações permanentes para futuras situações sépticas.

Em resumo, o treinamento físico em intensidade moderada é um fator ambiental positivo que deve ser explorado como modulador do sistema imunológico. Essa modulação ocorre mesmo em situações onde o efeito deletério ocorreu nos períodos de desenvolvimento do organismo, e essas alterações apresentam reflexos na vida adulta. Dessa forma, a desnutrição proteica perinatal induziu alterações imunológicas nos filhotes que permanecem até a vida adulta. O treinamento físico moderado foi capaz de modular essas alterações.

9. Referências

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VERMES, I. et al. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods, v. 184, n. 1, p. 39-51, Jul 17 1995.

APÊNDICE A – Parecer do Comitê de Ética em Pesquisa Animal

Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 19 de agosto de 2011.

Ofício nº 389/11

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profa. Carol Virgínia Góis Leandro**
Núcleo de Educação Física – Centro Acadêmico de Vitória
Universidade Federal de Pernambuco
Processo nº 23076.021093/2011-99

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Desnutrição perinatal e sistema imunológico: papel modulador do treinamento físico moderado em ratos adultos endotoxêmicos".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério; Animais: Ratos; Linhagem: Wistar; Sexo: Machos e Fêmeas; Idade: Serão utilizadas ratas adultas gestantes e seus filhotes que serão estudados na vida adulta; Peso: 220-250g (ratas) e 5-7g (filhotes);
Número de animais previsto no protocolo: 10 ratas gestantes/lactantes e 48 filhotes machos.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

CCB: Integrar para desenvolver

ANEXO B – Documentação de encaminhamento do artigo de revisão à revista

Imprimir

De: **Carol Leandro** (carolleandro22@yahoo.com.br) Enviada:segunda-feira, 17 de novembro de 2014 02:42:48 Para:Sueli Moreno Senna (susenna@hotmail.com)

On Monday, November 17, 2014 1:36 AM, "nim@karger.com" <nim@karger.com> wrote:

Dear Dr. Carol Leandro:

If you have any queries please send an email to: nim@karger.com.

With kind regards,

Editorial Office

https://bay168.mail.live.com/ol/mail.mvc/PrintMessages?mkt=pt-br

Thank you for submitting your manuscript entitled "Neuroimmunomodulation of the perinatal malnutrition and the protective role of the physical training " to "Neuroimmunomodulation"; the submission number is: 1730. Your submission will now be checked by the editorial office, and you will receive a confirmation mail. This step will also activate your personal user-id and password, enabling you to login to the system to check the status of your manuscript.

ANEXO C – Documentação de encaminhamento do artigo original à revista

Imprimir

From: **Editorial Office (EJON)** <em@editorialmanager.com> Date: Thu, Nov 13, 2014 at 1:34 PM Subject: EJON: Submission Confirmation for Moderate physical training attenuates perinatal low-protein-induced spleen lymphocyte apoptosis in endotoxemic adult offspring rats To: Carol Gois Leandro <carolleandro22@gmail.com>

Dear Dr Carol Leandro,

The URL is http://ejon.edmgr.com/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards, Springer Journals Editorial Office European Journal of Nutrition

https://bay168.mail.live.com/ol/mail.mvc/PrintMessages?mkt=pt-br

Your submission entitled "Moderate physical training attenuates perinatal low-protein-induced spleen lymphocyte apoptosis in endotoxemic adult offspring rats" has been received by journal European Journal of Nutrition

--Dr Carol Góis Leandro, PhD Postgraduate Program Nutrition, Physical Activity and Phenotypic Plasticity (www.ufpe.br/ppgnafpf)

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1 of 217/11/2014 02:33

Department of Physical Education and Sport Science Federal University of Pernambuco, Brazil Phone/Fax 00 55 81 35233351

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