UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ - CAMPUS DE CASCAVEL
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM BIOCIÊNCIAS E SAÚDE – NÍVEL MESTRADO

HELOISA DEOLA CONFORTIM

ESTUDO MORFOLÓGICO E MORFOMÉTRICO DAS FIBRAS MUSCULARES E JUNÇÕES NEUROMUSCULARES DO MÚSCULO SÓLEO DE RATOS EM DIFERENTES IDADES SUBMETIDOS À RESTRIÇÃO PROTEICA MATERNA

CASCAVEL-PR
(Fevereiro/2014)

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Dissertação apresentada ao Programa de Pós-Graduação *Stricto Sensu* em Biociências e Saúde – Nível Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Biociências e Saúde.

Área de concentração: Biologia, processo saúde-doença e políticas de saúde**.**

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CASCAVEL-PR

(Fevereiro/2014)

Dados Internacionais de Catalogação-na-Publicação (CIP)
Biblioteca Central do Campus de Cascavel – Unioeste
Ficha catalográfica elaborada por Helena Soterio Bejio CRB-9ª/965

C759e Confortim, Heloisa Deola

Estudo morfológico e morfométrico das fibras musculares e junções neuromusculares do músculo sóleo de ratos em diferentes idades submetidos à restrição proteica materna / Heloisa Deola Confortim.— Cascavel, PR: UNIOESTE, 2014.

110 f. ; 30 cm

Orientadora: Prof.ª Dr.ª Marcia Miranda Torrejais

Co-orientadora: Prof.ª Dr.ª Lígia Aline Centenaro

Dissertação (Mestrado) – Universidade Estadual do Oeste do Paraná.

Programa de Pós-Graduação *Stricto Sensu* em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde.

1. Morfologia. 2. Morfometria. 3. Restrição proteica. 4. Músculo esquelético. 5. Programação fetal. 6. Proteinas. 7. Rato. I. Universidade Estadual do Oeste do Paraná. II. Título.

CDD 21.ed. 590

FOLHA DE APROVAÇÃO

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Esta dissertação foi julgada adequada para a obtenção do título de Mestre em Biociências e Saúde e aprovada em sua forma final pelo Orientador e pela Banca Examinadora.

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CASCAVEL-PR
(fevereiro/2014)

DEDICATÓRIA

"Dedico este trabalho aos meus pais, pelo apoio, incentivo e por continuarem acreditando em mim mesmo nos momentos em que nem eu mesma acreditava. Agradeço enormemente pelo carinho, amor e compreensão dedicados a mim em todos os momentos de minha vida".

AGRADECIMENTOS

Agradecer a todos que ajudaram a construir esta dissertação, não é tarefa fácil. Por isso, precisei selecionar as pessoas mais importantes que estiveram comigo no decorrer dessa trajetória. Sem dúvidas, meu maior agradecimento é dirigido a meus pais, por terem sido o contínuo apoio em todos esses anos. Agradeço, de forma muito carinhosa, a minha mãe. Sua paciência, seu apoio, seu carinho, sua compreensão e sua crença em minha capacidade certamente impulsionaram a conclusão deste trabalho. Agradeço infinitamente ao meu pai pelo apoio, pelas lutas diárias para que eu não parasse os estudos, pelas palavras de conforto e encorajamento e por acreditar em mim acima de tudo. Agradeço também ao André, meu companheiro nesta trajetória, que sempre tentou entender minhas dificuldades, ausências e sempre tentava me animar, agradeço a você, carinhosamente por isto.

Minha seleção, no âmbito acadêmico, deve começar pela minha orientadora, a Prof.ª Dr.ª Márcia Miranda Torrejais, que está comigo desde o primeiro ano de graduação. Nessa trajetória, sempre teve paciência e dedicação em todas as etapas dos trabalhos, sempre disposta a transmitir seus conhecimentos. Minha co-orientadora, Prof.ª Dr.ª Lígia Aline Centenaro, também foi fundamental na realização deste trabalho, pois me auxiliou com as análises estatísticas e com o desenvolvimento dos artigos, sempre muito criteriosa e dedicada. Agradeço às professoras: Dr.ª Maria Lucia Frizon Rizzotto, Dr.ª Rose Meire Costa Brancalhão e Dr.ª Maria Lúcia Bonfleur que participaram das bancas do projeto e da qualificação e auxiliaram com sua experiência quando sugeriam alterações que melhoraram este trabalho. Gostaria de agradecer também à professora Dr.ª Angélica Soares que sugere e dá dicas para o melhor andamento das pesquisas. Ao técnico do laboratório de anatomia, José Carlos Cintra (Zé), que estava sempre pronto a ajudar nos problemas que apareciam no laboratório. Preciso agradecer também aos professores da UNESP de Botucatu. Primeiramente à Prof.ª Dr.ª Patrícia Fernanda Felipe Pinheiro, responsável pela parceria que proporcionou a realização deste trabalho. A Prof.ª Dr.ª Selma Maria Michelin Matheus pelo auxilio com os reagentes utilizados nas análises, por ceder o laboratório para realização de parte dos experimentos em Botucatu e também pelas caronas que ela sempre muito solícita oferecia. Gostaria de agradecer também ao Prof. Dr.º Luiz Justulin do

Departamento de Morfologia, que teve muita paciência para auxiliar na quantificação do colágeno das amostras. Gostaria de agradecer imensamente ao técnico do departamento de anatomia da UNESP, Gelson Rodrigues que sempre me auxiliou em todas as etapas durante a confecção das lâminas histológicas e também pelos momentos de descontração durante esse processo.

Não posso esquecer-me dos meus amigos que, de uma maneira ou outra, também foram muito importantes. Primeiramente, quero agradecer à Lidiane e à Aline, pessoas muito especiais que conheci em Botucatu e que foram muito importantes para mim, pois me auxiliaram com a hospedagem em várias viagens até São Paulo para realização de parte das análises. À minha amiga de graduação, Diandra, pelos momentos de desabafo, desespero e de encorajamento que vivenciamos juntas durante esse período. Ao Wellington, que sempre estava disposto a auxiliar nos problemas com o notebook, pen-drives e afins. Ao Cassiano, por me ensinar a utilizar o programa *Endnote*, que me auxiliou muito com as referências. E às minhas colegas de mestrado e laboratório, Léslie e Bruna. À Léslie, pelo auxílio em várias etapas da pesquisa, principalmente na coleta do material, e à Bruna pela companhia e descontração no laboratório.

RESUMO GERAL

Condições nutricionais maternas inadequadas podem predispor ao aparecimento de alterações neuromusculares na prole, um fenômeno conhecido como programação fetal. Nesse contexto, o presente estudo teve como objetivo avaliar os efeitos da restrição proteica materna, durante os períodos gestacional e de lactação, sobre as fibras musculares e junções neuromusculares (JNMs) do músculo sóleo dos filhotes aos 21 e 365 dias de idade. Ratos *Wistar* machos foram separados em dois grupos experimentais: Controle (GC) - prole de mães alimentadas com ração normoproteica (17%) e restrito (GR) - prole de mães alimentadas com ração hipoproteica (6%). Os filhotes foram mantidos com a mãe durante o período de lactação (21 dias) e, após o desmame, parte dos machos de cada grupo foi eutanasiado para a coleta de amostras do músculo sóleo. Os demais filhotes receberam ração padrão *ad libitum* até os 365 dias, quando também foram eutanasiados. Amostras do músculo sóleo dos animais com 21 dias de idade foram coletadas para análise das fibras musculares (através da coloração em HE e ultraestrutura) e das JNMs (técnica de Esterase-Inespecífica). Nos animais com 365 dias de idade, amostras do músculo sóleo foram obtidas para análise das fibras musculares (através da coloração em HE, reação de NADH-TR e ultraestrutura), quantificação de colágeno intramuscular (coloração Picrosirius-red) e também para a análise das JNMs (técnica de Esterase-Inespecífica). Quanto aos resultados, aos 21 dias de idade as fibras musculares apresentaram-se imaturas, com a presença de miotubos, núcleos centrais, fibras fetais e fibroblastos nos dois grupos experimentais. Foi observado um aumento na quantidade de fibras musculares e de núcleos no GR em comparação ao GC. Fibras musculares com miofibrilas rarefeitas ou frouxamente arranjadas, linha Z desorganizada e núcleos em posição central também foram observadas nos GC e GR. Em relação as JNMs, o GR apresentou redução na área, diâmetro maior e menor quando comparadas ao GC. Aos 365 dias, o GR apresentou redução na área da secção transversal das fibras tipo I e IIa, além de um aumento nas fibras tipo IIb. A porcentagem de colágeno intramuscular foi menor no GR. Também foi observada uma desorganização das miofibrilas e da linha Z em nível ultraestrutural, com a presença de aglomerados de mitocôndrias nos dois grupos estudados. Quanto às JNMs, foi observada uma redução na área e diâmetro menor, além de um aumento no diâmetro maior no GR comparado ao GC. Estes resultados indicam que a restrição proteica materna afeta a morfologia e morfometria das fibras musculares e JNMs. Tais alterações são detectáveis precocemente e perduram ao longo da vida adulta e senescência, parecendo ser irreversíveis.

Palavras-chaves: programação fetal, proteínas, músculo esquelético.

GENERAL ABSTRACT

Inadequate maternal nutritional may predispose to neuromuscular disorders in the offspring, a phenomenon known as fetal programming. In this context, the present study aimed to evaluate the effects of maternal protein restriction (during pregnancy and lactation) on muscle fibers and neuromuscular junctions (NMJs) of the soleus muscle from pups at 21 and 365 days old. Male *Wistar* rats were divided in two experimental groups: Control (CG) - offspring of mothers fed a normal protein diet (17%) and restricted (RG) - offspring of mothers fed a low protein diet (6%). All pups were maintained with their mothers during the lactation period (21 days) and after weaning, one part of males from each group were euthanized to collect samples of the soleus muscle. The remaining rats received standard food *ad libitum* until 365 days, when they were also euthanatized. The samples of the soleus muscle from animals with 21 days old were collected for analysis of muscle fibers (by HE staining and ultrastructure) and NMJs (by Nonspecific Esterase technique). In animals with 365 days of age, soleus muscle samples were obtained for verification of muscle fibers (by HE staining, NADH-TR reaction and ultrastructure), quantification of intramuscular collagen (picrosirius red staining) and also for analysis of NMJs (Nonspecific Esterase technique). Regarding the results, at 21 days muscle fibers was immature and the presence of myotubes, central nuclei, fetal fibres and fibroblasts were observed in both experimental groups. An increase in the number of muscle fiber and nuclei in the RG compared to controls was observed. Muscle fibers with rarefied or loosely arranged myofibrils, Z-line disorganized and nuclei in central position were observed in CG and RG. Regarding the NMJs, the RG showed a decreased in area, larger and smaller diameter compared to the CG. At 365 days, the RG showed a decrease in the cross sectional area in type I and IIa fibers, associated with an increase in type IIb fibers. The percentage of intramuscular collagen was lower in RG. Myofibrils and Z line disorganization was also observed at ultrastructural level, with the presence of mitochondria clusters in both groups studied. A reduction in the area and smaller diameter of NMJs was observed in the GR, along with an increase in the larger diameter of these structures compared to CG. These results indicate that maternal protein restriction affects the morphology and morphometry of the neuromuscular junctions and muscle fibers. Such changes can be detected early and persist throughout adulthood and senescence, seeming irreversible.

Keywords: fetal programming, proteins, skeletal muscle.

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LISTA DE ABREVIATURAS

AIN – Instituto Americano da Nutrição

GC- grupo controle

GR- grupo restrito

HE – hematoxilina e eosina

IBGE – Instituto Brasileiro de geografia e estatística

JNMs – junções neuromusculares

MHC – cadeia pesada da miosina

NADH-TR – nicotinamida adenina dinucleotídeo - *tetrazolium reductase*

OMS – Organização Mundial da Saúde

ONU – Organização das Nações Unidas

PBF – Programa Bolsa Família

POF – Pesquisa de orçamentos familiares

INTRODUÇÃO GERAL

A desnutrição é caracterizada por desequilíbrio e/ou deficiência de nutrientes no organismo (GURMINI *et al*., 2005). Hábitos alimentares inadequados, infecções parasitárias, condições precárias de higiene, baixos níveis de escolaridade e de poder aquisitivo e a deficiência do sistema de saúde são as principais causas desse quadro (COTRAN; KUMAR; ROBBINS, 2005; SOUZA *et al*., 2006).

Há várias décadas, a desnutrição proteica tem preocupado autoridades ligadas aos setores de saúde pública, entre elas a Organização Mundial de Saúde (OMS), visto que essa implica comprometimento funcional de diversos sistemas e órgãos do corpo humano (OSMO, 2007). A desnutrição precoce pode acarretar danos neuronais, com alterações cognitivas e modificações no desenvolvimento e crescimento da criança. Diversos distúrbios eletrolíticos foram identificados em crianças que apresentavam desnutrição proteica. Além da deficiência dietética, infecções, principalmente diarreia e parasitoses, as quais comprometem a nutrição, induzem à má absorção e ao aumento da demanda metabólica para formar uma resposta imune apropriada (PORTO *et al.,* 2010). A desnutrição infantil está relacionada à difícil condição em que vivem as famílias pobres. Afeta não somente as áreas rurais, mas, sobretudo, as periferias das grandes cidades, que são marcadas por elevados níveis de desigualdades sociais (FROTA; BARROSO, 2005).

Os distúrbios nutricionais têm sido mais enfocados na infância, mas, o estudo do tema em idosos tem merecido maior atenção, considerando o aumento da expectativa de vida e os desafios colocados com o avanço da idade. A análise do estado nutricional de idosos torna-se mais complexa em virtude da maior heterogeneidade deste grupo, seja pelas modificações biológicas da idade, pela influência de fatores socioeconômicos ou por hábitos praticados ao longo da vida (REZENDE *et al*., 2010). A má nutrição no idoso pode estar associada também a doenças que diminuem o apetite, à má absorção dos alimentos e à interação com medicamentos. Outras causas que ainda podem ser citadas são o desconhecimento sobre o preparo dos alimentos, problemas dentários, limitações físicas, dificuldade de visão e deambulação, tremores, isolamento social,

depressão, problemas mentais e alcoolismo (MARCHINI; FERRIOLLI; MORIGUTI, 1998; MORIGUTI *et al*., 2001).

Cada vez mais a interdisciplinaridade é citada na literatura como necessária para explicar e compreender problemas complexos como a desnutrição. O crescimento de doenças não infecciosas colocou em crise o modelo explicativo unicausal, que perdurou por muito tempo no âmbito da saúde. Somente a partir da entrada da sociologia e da psicologia nos debates em saúde, foi possível a ampliação desse conceito que não se restringe ao aspecto físico da pessoa, nem pode ser tomado somente como a ausência de doença. Este processo levou à elaboração de uma concepção multicausal do processo saúde-doença, que vem sendo cada vez mais discutida (FERNANDES, 2003).

Tendo em vista esse modelo multicausal, várias são as disciplinas que podem contribuir para o entendimento dessa complexa doença, dentre as quais podemos citar a anatomia, fisiologia, genética, nutrição, enfermagem, psicologia, sociologia e até mesmo a política, pois a partir dela, podemos entender melhor os motivos pelos quais a sociedade enfrenta tantos problemas, muitos dos quais podem agravar os casos de desnutrição.

Com base em estudos prévios, sabe-se que o tecido muscular esquelético é sensível à desnutrição proteica por ser um reservatório de proteína do organismo. Intervenções nutricionais podem causar alterações nas características morfológicas da musculatura estriada esquelética. Assim, quando há um déficit proteico na dieta, o músculo torna-se alvo de depleção, consequentemente ocorrem alterações nas fases de crescimento, funcionamento e diferenciação das fibras musculares (BRAMELD, 2004). Tal comprometimento da musculatura esquelética em ratos desnutridos não ocorre de forma homogênea, pois as musculaturas mais comprometidas são as do tronco e do membro pélvico (SPENCE; HANSEN-SMITH, 1978). Sendo assim, o músculo sóleo foi escolhido, por fazer parte do membro pélvico em ratos e por ter grande importância em humanos. Atua como músculo postural, responsável pelos movimentos de flexão plantar e inversão do pé, esses que auxiliam na marcha (MIRANDA, 2001). Com base nesse contexto, o presente estudo teve como objetivo avaliar os efeitos da restrição proteica materna durante os períodos gestacional e de lactação, sobre as fibras musculares e junções neuromusculares do músculo sóleo dos filhotes machos aos 21 e 365 dias de idade.

REVISÃO GERAL DE LITERATURA

Desnutrição proteica

As proteínas são macromoléculas biológicas abundantes e que representam o principal componente estrutural e funcional de todas as células do organismo. Elas apresentam funções vitais nos processos biológicos tais como catálise enzimática, transporte e estoque de moléculas, contração muscular, proteção imunológica, geração e transmissão do impulso nervoso, regulação hormonal, expressão gênica e função estrutural (na forma de colágeno e elastina) (DE ANGELIS; TIRAPEGUI, 2007).

Quando ocorre um déficit na quantidade de proteínas do organismo, ele sofre vários danos. Uma das patologias advindas desse déficit é a desnutrição proteica, a qual acarreta comprometimento de diversos sistemas e órgãos, dentre eles, destaca-se o sistema muscular (OSMO, 2007). A complexidade do diagnóstico e do tratamento desse tipo de patologia, aliada à falta de preparo dos profissionais de saúde, fez com que a OMS elaborasse um manual que aborda a fisiopatologia e o tratamento da desnutrição energético-proteica, a fim de esclarecer os procedimentos que precisam ser seguidos durante as fases de hospitalização, reabilitação e acompanhamento nas unidades de saúde (OPAS, 1999).

De acordo com Cotran (2005), a desnutrição é muito prevalente nos países em desenvolvimento e é uma das principais causas associadas às altas taxas de mortalidade entre crianças menores de cinco anos de idade. Nas últimas décadas, os índices de desnutrição sofreram redução no Brasil, mas a situação atual, principalmente em relação à desnutrição infantil, ainda exige atenção. Segundo Olinto *et al*. (1993), a desnutrição infantil é considerada um problema multifatorial e sua prevalência apresenta diferenças importantes entre países, entre regiões de um mesmo país, entre populações urbanas e rurais, entre famílias que vivem na mesma comunidade e até mesmo entre crianças de uma mesma família. Sabe-se que hoje o índice de crianças desnutridas em países ricos não chega a 5%, todavia, nos países pobres, esses níveis podem chegar a 50%. Assim, essa doença é caracterizada como a principal causa de mortalidade infantil do mundo (VIANNA *et al.,* 2011).

A desnutrição infantil pode começar precocemente na vida intrauterina ou na infância, em decorrência da interrupção precoce do aleitamento materno e da falta de alimentação complementar adequada nos primeiros dois anos de vida. Pode estar ainda associada à privação alimentar ao longo da vida e à ocorrência de doenças infecciosas (BRASIL, 2005). É expressa pelo comprometimento do crescimento linear e/ou ponderal, e é ainda um dos principais problemas de saúde enfrentados pelos países em desenvolvimento, quer pela elevada prevalência, quer pela carga de morbidade que se associa a esse evento (OLIVEIRA *et al*., 2007).

Diferentes classificações são conhecidas para a desnutrição, segundo a gravidade do quadro. A classificação proposta por Gómez *et al.* (2000) define três níveis de desnutrição, baseado no peso corporal encontrado em relação ao peso corporal esperado para a idade, sendo esses de: 1º Grau ou Leve - o peso corporal encontrado varia de 10 a 24% abaixo do esperado; 2º Grau ou Moderado - o peso corporal varia 25 a 39% abaixo do esperado e o de 3º Grau ou Grave - o peso corporal está 40% abaixo do esperado para a idade.

Outra classificação bastante conhecida e que corresponde a níveis mais graves da doença são o marasmo e o kwashiorkor (DE ANGELIS; TIRAPEGUI, 2007). As principais características do marasmo são o evidente emagrecimento e a perda de massa muscular, peso corporal abaixo de 60% do esperado para a idade. Também são observados retardo no crescimento, gordura cutânea escassa ou ausente e até mesmo caquexia. Diarreia, infecções respiratórias, parasitoses e tuberculose estão frequentemente presentes bem como sinais de carências de micronutrientes, deficiência de vitamina B, anemia ferropriva e outras. O abdome de crianças que apresentam o marasmo pode se mostrar proeminente e essa característica auxilia na identificação da forma clínica da doença (MONTE, 2000). No kwashiorkor, o peso corporal encontra-se de 60 a 80% mais baixo do que o esperado para a idade e edema depressível, essa é uma característica marcante principalmente nas pernas. Outras características incluem o retardo no crescimento, perda de gordura subcutânea e de massa muscular. Podem ocorrer ainda lesões nos cabelos e na pele. Casos de anorexia, diarreia, infecções e deficiências de micronutrientes são frequentes. Encontra-se ainda uma forma clínica intermediária conhecida como kwashiorkor marasmático, onde o peso corporal encontrado está abaixo de 60% do esperado para a idade e também ocorre a presença de edema (MARCONDES, 1979).

A desnutrição infantil é bem conhecida e tornou-se alvo de muitos estudos, principalmente em países onde ela ainda é a causa-morte de milhões de crianças anualmente. Porém, devido às mudanças na economia e consequente aumento da expectativa de vida nos países em desenvolvimento, a deficiência proteica tem chamado atenção também nos idosos (REZENDE *et al*., 2010). Diversos autores têm se preocupado com essa faixa etária da população e atualmente temas sobre o envelhecimento são amplamente estudados (MORIGUTI *et al*., 2001; MASE *et al*., 2006; REZENDE *et al.*, 2010; CHAI *et al.*, 2011); A desnutrição nos idosos é comum, pois com a idade avançada, o consumo alimentar diário diminui. Além disso, os alimentos consumidos são de baixas calorias e contribuem para a deficiência nutricional (DE SOUSA; GUARIENTO, 2009). Quando avaliamos a desnutrição em idosos, o tema torna-se mais complexo devido à heterogeneidade desse grupo. Modificações biológicas da idade como a diminuição na massa óssea e muscular, doenças que diminuem o apetite, má absorção de alimentos e interação com medicamentos precisam ser levadas em consideração (MARCHINI; FERRIOLLI; MORIGUTI, 1998; MORIGUTI *et al*., 2001).

A estratégia fundamental a ser tomada para o controle da desnutrição no Brasil seria o estabelecimento de políticas públicas que melhorem a saúde e a nutrição da sociedade brasileira (FERNANDES, 2003). O Programa Bolsa Família (PBF) é uma das estratégias governamentais desenvolvidas para tentar combater a fome e a pobreza das famílias no Brasil. A expectativa desse programa é que o aumento na renda decorra na melhora do estado nutricional das pessoas beneficiadas (OLIVEIRA *et al*., 2011). O declínio da desnutrição que vem sendo observado na região Nordeste do País já pode ser considerado um mérito das melhorias em escolaridade materna, saneamento, assistência à saúde, antecedentes reprodutivos e, sobretudo, com o aumento do poder aquisitivo familiar em face do PBF (LIMA *et al*., 2010).

Programas sociais como o PBF ou programas assistenciais são de suma importância para ajudar a reduzir a fome e as taxas de desnutrição no Brasil. Entretanto, a reorganização do investimento do dinheiro público aplicado em infraestrutura parece ser a principal solução para a redução da fome e, consequentemente, da desnutrição. Autores sugerem uma estreita relação entre a desnutrição e a situação socioeconômica. No Brasil, existem mais de 55 milhões de

pessoas na linha da pobreza, o que aumentam os índices de desnutrição, principalmente nas regiões Norte e Nordeste (ALVES *et al.*, 2011).

A desnutrição nas crianças brasileiras é considerada crônica. O indicador de estatura para idade reflete o comprometimento nutricional expressivo da nossa população. A Pesquisa de Orçamento Familiar (POF) 2008-2009 do Instituto Brasileiro de Geografia e Estatística (IBGE) descreveu a prevalência de desnutrição medida pelo déficit de altura em crianças menores de cinco anos com um índice de 6,0% nacional, havendo diferenças entre as regiões do País. Na região Norte, foi identificado um índice de 8,5%, enquanto na região Sul, esse índice foi de 3,9%, revelando a forte desigualdade social presente no Brasil. Somente a partir de mudanças nessa desigualdade na distribuição de renda das famílias brasileiras, pode-se erradicar a desnutrição (IBGE, 2010; ALVES *et al.*, 2011).

Efeitos da desnutrição proteica sobre o organismo

Em condições de nutrição normal, a captação e a utilização de nutrientes estão em equilíbrio e asseguram o crescimento, a maturação e a duplicação celular. Se a nutrição está em déficit, o organismo sofre sérios danos, uma vez que a carência de nutrientes importantes influenciará em todas as fases da vida, desde a gestação até a idade adulta (ALVES; DÂMASO; DAL PAI, 2008).

Em animais alimentados com dietas hipoproteicas, o ganho de peso apresenta-se menor quando comparado aos animais alimentados com dietas normoproteicas (NEIVA; GUERINO; MELLO, 1999; NUNES *et al.,* 2002; CHAVES; MELLO, 2003; FRANÇA, 2009). Pode ocorrer também diminuição no peso de órgãos como fígado, baço e pulmões (PEREIRA, 2005). O menor peso corporal é um parâmetro utilizado em muitos estudos para comprovar a desnutrição. Em ratos adultos, a oferta de rações isocalóricas com redução do teor de proteínas leva à estagnação do peso corpóreo e redução das proteínas totais e globulinas (NATALI; NETO; ORSI, 2000). Em modelo experimental de desnutrição crônica, que ocorre desde a gestação até os quatro meses de idade, Berleze (2005) observou diferenças marcantes nos ratos desnutridos, entre elas, destacam-se: diminuição do peso corporal, diminuição no consumo de ração, aumento do peso do fígado,

diminuição na concentração plasmática de albumina e aumento da concentração plasmática de colesterol.

Estudos sobre uma variedade de órgãos demonstram alterações devido à desnutrição proteica. Por exemplo, a desnutrição em roedores altera significativamente a sensibilidade a uma variedade de drogas, atuando em diversos neurotransmissores (SCHWEIGERT; SOUZA; PERRY, 2009). A dieta e a nutrição também desempenham um papel significativo no desenvolvimento dentário e na integridade dos tecidos orais. A deficiência de proteínas durante a fase de desenvolvimento dentário gera atraso na cronologia de erupção dos dentes e defeitos estruturais no esmalte, com aumento do risco da ocorrência de cáries. A desnutrição ainda pode afetar as glândulas salivares, por meio da redução de seu fluxo e da alteração da composição da saliva (COSTA *et al*., 2010).

Em relação ao músculo estriado esquelético, dietas com baixo teor de proteínas (1%) administradas em ratos adultos (21 dias) foram capazes de causar diminuição na área das fibras musculares e na taxa de crescimento do músculo sóleo (TIMSON; DUDENHOEFFER, 1985). Oldfors, Mair e Sourander (1983) demonstraram que uma alimentação hipoproteica administrada, durante a fase adulta, causa alterações no músculo extensor longo dos dedos de ratos. Tais animais desnutridos apresentaram todos os tipos de fibras musculares menores, cuja dieta utilizada foi de 1,5% de proteína no grupo restrito versus 14% de proteína no grupo controle. Estudos feitos por Ihemelandu (1985) evidenciaram que a administração de dietas hipoproteicas (0,5% de proteína versus 18% de proteína no grupo controle) foram capazes de reduzir o peso corporal e o peso do músculo sóleo em ratos. Também foram observadas a perda de fibras musculares e a hipotrofia das fibras restantes. Segundo Voltarelli e Mello (2008), a perda de tecido muscular na desnutrição proteica pode ser considerada um mecanismo homeostático, pois promove uma redistribuição funcional das proteínas musculares para disponibilizar o nitrogênio necessário para a síntese de proteínas teciduais, formação de células vermelhas e também para exercer funções imunes.

Efeitos da restrição proteica fetal sobre os músculos estriados esqueléticos

O ambiente intrauterino é importante na determinação do futuro da saúde individual. Alterações no estado nutricional das mães podem predispor a prole ao

desenvolvimento de doenças na idade adulta (ASSIS; FIDELIS, 2011). Os efeitos da desnutrição intrauterina dependem da fase de desenvolvimento em que está o feto, os quais são mais intensos e permanentes quando ocorrem mais precocemente (GURMINI *et al.,* 2005). A desnutrição que persiste durante a gestação pode causar retardo no desenvolvimento fetal, na próxima fase do crescimento e pode continuar na vida adulta do indivíduo (GOPALAN, 2000).

Alterações na ingestão de proteínas durante a formação dos músculos podem causar alterações na musculatura esquelética, pois ela é considerada o maior reservatório de proteína do corpo. Sob a coordenação do sistema nervoso, os músculos constituem a unidade motora responsável pela locomoção, força, respiração e autonomia funcional do indivíduo bem como o seu desempenho em atividades físicas. A massa muscular esquelética também está relacionada à homeostase metabólica, homeostase glicêmica, fixação tecidual de oxigênio, oxidação de gorduras e ao gasto energético de repouso (NASCIMENTO *et al*., 1990; ROSSI, 2008; PIERINE; NICOLA; OLIVEIRA, 2009).

A musculatura esquelética dos mamíferos é constituída por diferentes tipos de fibras que apresentam características morfológicas e funcionais distintas. As diferentes terminologias usadas para a classificação dessas fibras musculares são resultado de uma grande variedade de procedimentos existentes para sua classificação (MINAMOTO, 2005). Estudos mostram a presença de três tipos básicos de fibras musculares, com diferentes nomenclaturas. O autor que primeiro descreveu os diferentes tipos de fibras musculares foi Ogata (1958), utilizando enzimas oxidativas, classificou as fibras musculares em vermelhas, brancas e intermediárias. Brooke e Kaiser (1970), com base na ATPase miofibrilar (mATPase), em meio de incubação ácido e alcalino, classificaram as fibras em tipos I, IIa e IIb. Peter *et al.* (1972) basearam-se no tempo de contração, capacidade glicolítica e capacidade oxidativa e classificaram as fibras musculares em “*fast twitch glycolytic*” - (FG); “*fast twitch oxydative glycolytic*” - (FOG) e “*slow twitch oxydative*” - (SO). Uma classificação mais atual baseada também na atividade da enzima ATPase miofibrilar (mATPase) foi proposta por Pette e Staron (2000), os quais mostram que as fibras musculares em ratos adultos apresentam quatro fibras puras que são descritas como tipo I, IIA, IID e IIB. Essas fibras musculares expressam quatro isoformas de cadeia pesada da miosina (MHC), enquanto as fibras lentas expressam as isoformas MHCI e as fibras rápidas as

formas MHCIIa, MHCIId e MHCIIb, respectivamente. Segundo os mesmos autores, existem ainda fibras musculares híbridas que apresentam duas ou mais isoformas de miosina.

A administração de dietas hipoproteicas durante os períodos de gestação e lactação podem provocar modificações na estrutura dos diferentes tipos de fibras musculares. Em estudo realizado por Nascimento *et al*. (1990), ratas foram submetidas à restrição proteica durante a gestação com ração a 6,7% de proteínas. Após o desmame, a prole foi alimentada com 3,2% de proteína na ração. As reações histoquímicas realizadas no músculo gastrocnêmio dessa prole revelaram que tanto as fibras do tipo I como as do tipo II foram comprometidas, com maior redução no diâmetro das fibras tipo II. Outro parâmetro marcante neste estudo foi a redução no peso dos animais desnutridos em relação aos do grupo controle, sendo 50% menor nos animais desnutridos. Dwyer e Stickland (1992), estudando o músculo sóleo de cobaias, cujas mães foram submetidas à redução de 40% na ingestão de ração, verificaram diminuição significativa do número de fibras musculares no grupo de filhotes desnutridos.

A desnutrição energético-proteica, somente durante o período de aleitamento, foi estudada por Silvado e Werneck (2006), cujos ratos do grupo desnutrido eram mantidos em jejum (separados da rata nutriz) por 6 horas diárias e os ratos do grupo controle mantidos permanentemente com a rata nutriz. A desnutrição foi provocada tanto pelo período de jejum quanto pelo aumento do número de filhotes por rata nutriz. Este estudo demonstrou que os animais desnutridos apresentaram redução da área da secção transversal das fibras tipo IIa e IIb e retardo na sua diferenciação no músculo gastrocnêmio. Segundo Mallinson *et al.* (2007), a restrição proteica causada pela ingestão de ração a 9% de proteína causou alterações na proporção dos tipos de fibras da prole durante fases especificas da gestação em ratos. No músculo sóleo, foi observado aumento na proporção de fibras do tipo I, quando a restrição foi realizada nas fases iniciais da gestação e redução na proporção das do tipo II, quando a restrição foi na fase intermediária da gestação.

Resultados experimentais em ratos sugerem que, além de alterações no número de fibras, a desnutrição intrauterina e o período de lactação promovem retardo na diferenciação das características morfológicas, metabólicas e contráteis

de todos os tipos de fibras musculares esqueléticas na fase de crescimento (ALVES; DÂMASO; DAL-PAI, 2008).

Efeitos da restrição proteica fetal sobre as junções neuromusculares

As junções neuromusculares (JNMs) são consideradas especializações entre os motoneurônios e as fibras musculares esqueléticas (WU; XIONG; MEI, 2010), e apresentam a função de transmitir os impulsos nervosos (SANES; LICHTMAN, 2001).

Os neurônios motores possuem seus corpos celulares no corno anterior da medula espinal e se projetam até a periferia através de um axônio mielinizado. À medida que se aproximam do músculo, os axônios se ramificam repetidamente para entrar em contato com as várias células musculares e reúnem-se em um grupo funcional conhecido como unidade motora. A arquitetura do terminal nervoso é bastante diferente do restante do axônio. Conforme o terminal atinge a fibra muscular, ele perde a bainha de mielina e é coberto pelas células de Schwann, como mostrado na Figura 1 (MARTYN; FAGERLUND; ERIKSSON, 2009). O potencial de ação do nervo ocasiona a liberação de um transmissor químico, a acetilcolina, que atua na membrana da fibra muscular para iniciar um potencial de ação (WALTON, 1988).

As JNMs de todos os vertebrados têm basicamente a mesma estrutura: A) um terminal axônico contendo acetilcolina; B) células de Schwann e seus prolongamentos citoplasmáticos que envolvem o terminal axonal (exceto na membrana pré-sináptica); C) uma fenda, contendo a enzima acetilcolinesterase e revestida por lâmina basal, chamada goteira sináptica primária; D) uma membrana pós-sináptica, correspondente a uma região especializada da membrana sarcoplasmática, que contém receptores para acetilcolina e, por fim, E) um sarcoplasma juncional, que suporta estrutural e metabolicamente a região pós-sináptica. A forma e o tamanho do terminal axônico, assim como a complexidade das membranas pré e pós-sinápticas, variam de acordo com os diferentes tipos de fibras musculares. As fibras musculares vermelhas possuem JNMs com dimensões pequenas, forma arredondada ou ligeiramente elíptica, ramificação axonal grosseira com botões terminais dilatados, goteira sináptica rasa e dobras juncionais pouco profundas e simples. As fibras musculares brancas apresentam junções com

dimensões maiores, forma elíptica, terminais axônicos finos, longos, ramificados e com botões terminais delicados, goteira sináptica profunda e dobras juncionais profundas e complexas. As fibras intermediárias exibem junções com morfologia característica, evidenciando aspectos estruturais que ficam entre aqueles apresentados pelas fibras brancas e vermelhas (OGATA, 1988).

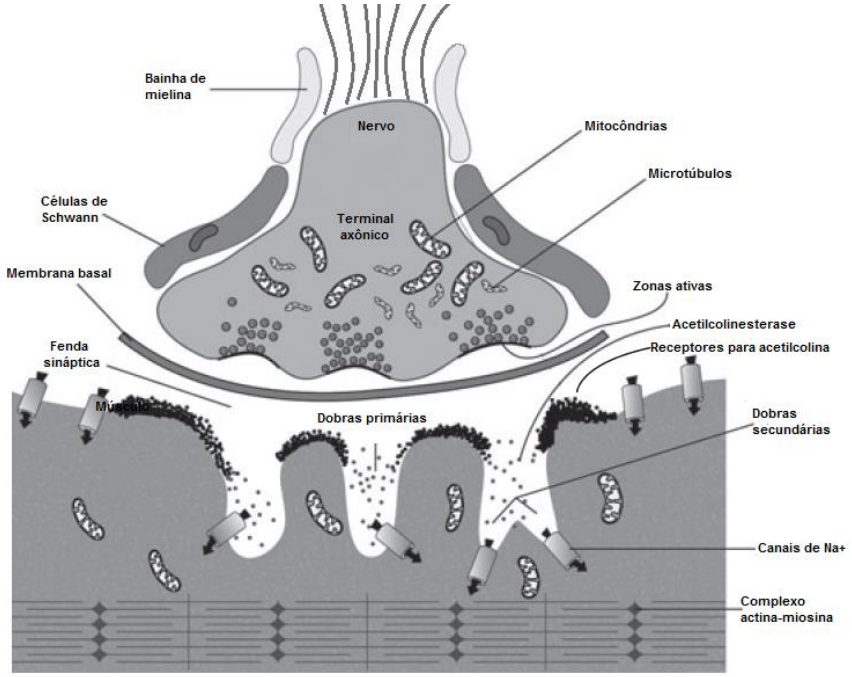

Figura 1. Estrutura de uma junção neuromuscular e seus principais constituintes (Adaptado de MARTYN; FAGERLUND; ERIKSSON, 2009).

As JNMs são funcionais ao nascimento, mas sofrem inúmeras alterações no período pós-natal. Uma vez ocorrida a maturação, elas são mantidas de forma estável em equilíbrio dinâmico, mas pode haver remodelação. As JNMs possuem ainda capacidade de regeneração após lesão do nervo periférico ou músculo (SANES; LICHTMAN, 1999).

Várias condições experimentais podem causar alterações nas JNMs, incluindo secção do nervo periférico (GATTUSO *et al*., 1989), idade (PRZYBYLA *et al.,* 2006) e a restrição proteica (NASCIMENTO *et al*., 1990; CABEÇO; DAL PAI-SILVA; MATHEUS, 2011). Em experimento realizado por Nascimento *et al*. (1990), os ratos foram induzidos à desnutrição pré e pós-natal. A avaliação do músculo gastrocnêmio demonstrou retardo no desenvolvimento, na maturação e hipoplasia nas unidades motoras desse músculo. No estudo realizado por Cabeço (2011),

ratas foram submetidas à restrição proteica durante a gestação pela oferta de ração hipoproteica (6%). A análise das JNMs da prole, aos 30 dias de idade na microscopia eletrônica de transmissão, revelou diminuição nas dobras sinápticas, retículo sarcoplasmático dilatado e presença de figuras de mielina.

Sabe-se que as fibras musculares e as JNMs são formadas durante o período fetal. Portanto, uma dieta adequada da mãe nesse período e nos primeiros estágios de vida bem como o aleitamento materno correto são essenciais para a formação e maturação destas estruturas. O conhecimento sobre as alterações causadas pela restrição de proteínas nessa fase e as consequências destas mudanças, a longo prazo, são de suma importância para o desenvolvimento de terapias que recuperem o tecido lesado e também para alertar os profissionais da saúde e as próprias mães sobre a importância da alimentação no referido período.

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ARTIGO CIENTÍFICO I

(Artigo submetido à Revista Tissue and Cell)

Maternal protein restriction during pregnancy and lactation affects the development of muscle fibers and neuromuscular junctions in rats

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Maternal protein restriction affects muscle fibers

Key words: hypoproteic diet; fetal programming; soleus muscle.

Acknowledgment

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES- Brazil) for the financial support. Also we thank UNIOESTE - Cascavel and UNESP - Botucatu for the great help in all steps of research.

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ABSTRACT

A balanced maternal diet is a determinant factor for the correct development of muscle fibers and neuromuscular junctions (NMJs). The objective of this study was to analyze the morphology of soleus muscle fibers and NJMs in 21-day-old rats born to mothers that had been submitted to protein restriction during pregnancy and lactation. Wistar rats were divided into two groups: control group, offspring of dams fed a normal protein diet (17%); restricted group, offspring of dams fed a low-protein diet (6%). After the experimental period, soleus muscle samples were collected for the analysis of muscle fibers (HE and ultrastructure) and NMJs (nonspecific esterase). Morphological analysis showed characteristics of immature muscle fibers in the two groups. There was an increase of 133% in the number of muscle fibers and of 79% in the number of nuclei in the restricted group. Muscle fibers with sparse myofibrils, a disorganized Z-line and central nuclei were observed in both groups. The NMJs of restricted animals exhibited a reduction in area, major and minor diameter compared to controls. Maternal protein restriction altered the normal development of the neuromuscular system as indicated by changes in soleus muscle fibers and NJMs of the offspring.

1. Introduction

Striated skeletal muscles are involved in locomotion, posture and respiratory movements and are also a reservoir for proteins due to their constitution (Brameld, 2004; Pierine et al., 2009). Skeletal muscle fibers are long cylindrical cells with contractile capacity. These muscle fibers exhibit multiple peripheral nuclei and cross-striated myofibrils in their cytoplasm (Dal Pai Silva and Carvalho, 2007).

Most skeletal muscles originate from cells of the mesoderm (Spielholz, 1982). During the early stages of development, these mesodermal cells are called myoblasts. The expression of

myogenic regulatory factors induces the differentiation of these myoblasts to myocytes, which subsequently fuse to give origin to primary and secondary myotubes (Megeney and Rudnicki, 1995). Primary myotubes contain centrally located nuclei, give origin to type I fibers and provide support for the formation of secondary myotubes which, in turn, differentiate into type II fibers (Ontell and Kozeka, 1984; Dal Pai Silva and Carvalho, 2007).

Neuromuscular junctions (NMJs) are synapses present between motor neurons and skeletal muscle fibers, permitting muscle contraction (Wu et al., 2010) and motor performance (Delbono, 2003). These structures arise concomitantly during muscle formation. Once these structures have come in contact with the newly formed myotube, synaptic transmission is initiated. However, at first, the efficacy of this transmission is extremely low (Sanes and Lichtman, 1999).

The formation of muscle fibers and NMJs occurs during fetal development and nutrient deficiency during this period may therefore alter the characteristics and functions of these structures (Sanes and Lichtman, 1999; Zhu et al., 2004). Previous studies have shown that maternal protein restriction during pregnancy and lactation alters skeletal muscle characteristics in the offspring (Mallinson et al., 2007; Toscano et al., 2008; Da Silva Aragão et al., 2013). However, the influence of this type of restriction on NMJs has not been established. Within this context, the objective of the present study was to evaluate the morphological changes induced by the administration of a maternal low-protein diet during pregnancy and lactation in soleus muscle fibers and NMJs of 21-day-old rats, since there are no studies investigating the influence of this type of diet at this age.

2. Methods

2.1. Animals

The study was approved by the Ethics Committee on Animal Experimentation of the Paulista State University (Universidade Estadual Paulista Júlio de Mesquita Filho - UNESP) (No.

264-CEEA). Male and female Wistar rats obtained from the Animal House of the Department of Anatomy, UNESP, were maintained under standard conditions of light (12-h light/dark cycle) and temperature (23 ± 1ºC). At the beginning of the experiment, two females and one male of reproductive age (12 weeks) were housed in boxes overnight for mating. The male was removed from the box in the following morning and a vaginal smear was obtained from the females. The detection of sperm in the vaginal smear was defined as day 0 of pregnancy, when the females were transferred to individual boxes. On day 0, female rats were divided into two groups: 1) dams fed a normal protein diet (17%) during pregnancy and lactation, and 2) dams fed a low-protein diet (6%) during pregnancy and lactation. The normal protein (17% protein) and low-protein (6% protein) diets were isocaloric and were administered *ad libitum* until the day of weaning of the offspring (Table 1).

On the day of birth, male pups were separated from female pups. Additionally, to guarantee an equal availability of food, eight pups were maintained per dam during lactation (21 days). The male offspring were divided into two groups: a control group (CG) consisting of offspring of dams fed the normal protein diet (17%) during pregnancy and lactation (n=8), and a restricted group (RG) consisting of offspring of dams fed the low-protein diet (6%) during pregnancy and lactation (n=8).

2.2. Collection of soleus muscle samples

At 21 days of age, the animals of the two groups were weighed and then euthanized in a CO_2 chamber, followed by decapitation using a rodent guillotine. The skin of the hind limbs (left antimere) was elevated and the gastrocnemius muscle was removed for exposure and dissection of the soleus muscle. The length (mm) of the soleus muscle from its origin until insertion was measured with a digital caliper (Gigimess®, Brazil) and the muscle was weighed. Next, the soleus muscle was cut into fragments with a stainless steel blade for histological analysis, ultrastructural

analysis by transmission electron microscopy, and histochemical study of NMJs. Fat (epididymal and visceral) and liver samples were also collected and weighed.

2.3. Histological and morphometric analysis of muscle fibers

The soleus muscle samples were fixed in Karnovsky's solution (Karnovsky, 1965) and washed in phosphate-buffered saline (PBS) to remove excess fixative. The muscle samples were embedded in paraplast in the vertical position. Blocks were prepared using an embedding center (Cygni, EasyPath®, Brazil). Next, the muscle fragments were cut into 5-µm thick sections with a microtome (RM2165, Leica®, Germany). The sections were mounted on previously silanized slides, dried for 1 h at 60ºC, and submitted to deparaffinization, hydration and staining with hematoxylin-eosin (HE) (Junqueira and Junqueira, 1983). The stained slides were then immediately dehydrated, cleared, and mounted in Permount (Fisher Scientific®, USA).

For the quantification of nuclei and muscle fibers, 10 images in random fields per animal were examined and captured at 400× magnification. Photomicrographs were obtained at 50× magnification (necessary to comprise the entire diameter of the muscle) for the measurement of total muscle area. These images were merged using appropriate software for subsequent measurement of the muscle area.

2.4. Ultrastructural analysis of muscle fibers

For visualization of the ultrastructure of the soleus muscle fibers, the samples were cut into longitudinal fragments (approximately 1 mm wide) and fixed in 2.5% glutaraldehyde. Next, the samples were washed in PBS 0.1 M pH 7.3, for 15 min and postfixed in 1% osmium tetroxide for 2 h. The samples were then washed in distilled water, incubated in 0.5% uranyl acetate for 2 h, dehydrated in acetone, and embedded in a mixture of resin and 100% acetone for 12 h, for the formation of blocks. The desired fields were selected in semi-thin sections and ultrathin sections

were cut with an ultramicrotome (Ultracut UCT, Leica®, Germany). The ultrathin sections were stained with a saturated solution of uranyl acetate and lead citrate. The material was examined and photographed under a transmission electron microscope (CM100, Philips®, The Netherlands).

2.5. Morphology and morphometry of neuromuscular junctions

For the study of NMJs, the soleus muscle samples were immersed in Karnovsky's fixative (Karnovsky, 1965) at room temperature and cut longitudinally into three or four slices with stainless steel blades. The sections were submitted to nonspecific esterase reaction (Lehrer and Ornstein, 1959). Images of the NMJs were captured at 200× magnification. Measurements of area and major and minor diameter of 50 NMJs per animal were obtained for each group.

2.6. Image analysis

The muscle fibers were observed under a Primo Star microscope (Zeiss®, Germany) coupled to a camera (AxiocamERc5s®) and computer using the Axiovision Rel.4.8® program (Carl Zeiss Microimaging, Inc., Germany). Photodocumentation of the NMJs was performed under an Olympus Bx60® microscope coupled to an Olympus DP71 camera (Japan) using the DP Controller 3.2.1 276 program. All morphometric measurements were made using the Image Pro Plus 6.0® software (Media Cybernetics, USA).

2.7. Statistical analysis

First, the normality of the data was analyzed using the Shapiro-Wilk test. Body weight, relative adipose tissue weight, muscle length, total muscle area, fiber and nucleus count, and major and minor diameter of the NMJs were compared by the Student *t*-test. Relative muscle weight and area of the NMJs were compared by the Mann-Whitney test. The results are expressed as the mean

± standard deviation and a p value <0.05 was considered to be significant. The GraphPad Prism 5.0® program (La Jolla, USA) was used for analysis and construction of the graphs.

3. Results

3.1. Macroscopic parameters

At 21 days of age, the body weight of RG animals was approximately 55% lower than that of CG animals ($p<0.001$). Animals of RG exhibited a reduction of 70% in visceral fat weight ($p<0.001$), of 58% in liver weight ($p<0.001$), of 59% in soleus muscle weight ($p<0.001$), and of 23% in soleus muscle length ($p<0.001$) when compared to CG. The visceral fat weight/body weight ratio was 25% lower ($p<0.01$) and the soleus muscle weight/body weight ratio was 22% lower ($p<0.05$) in RG animals. However, no difference in the liver weight/body weight ratio was observed between groups (Table 2).

3.2. Morphological and morphometric analysis of muscle fibers

Morphological analysis showed a greater interstitial space in RG animals compared to CG (Fig. 1A and 1B). Myotubes, central nuclei, fetal fibers and fibroblasts were observed in regions of the soleus muscle in the two groups (Fig. 1C and 1D). Morphometric analysis revealed an increase of 133% in the number of muscle fibers ($p<0.001$) and of 79% in the number of nuclei ($p<0.001$) in RG animals compared to CG. The total cross-sectional area of the muscle was reduced by 66% in RG ($p<0.001$) (Table 3).

3.3. Ultrastructural analysis of muscle fibers

Muscle fibers containing sparse or loosely arranged myofibrils and a disorganized Z-line were observed in RG animals (Fig. 2B). Central nuclei were also noted in these animals (Fig. 2D). Regions with disorganized or loosely arranged myofibrils and a disorganized Z-line were also seen

in CG (Fig. 2A). Some muscle fibers of this group also exhibited central nuclei (Fig. 2C). These features observed in CG are related to the age of the animals and not to maternal protein restriction.

3.4. Morphological and morphometric analysis of neuromuscular junctions

The morphology of the NMJs analyzed was highly variable. The most common types were round, oval and elliptical NMJs. Animals of RG exhibited smaller junctions with a less defined contour compared to CG animals (Fig. 3A and 3B). Morphometric analysis showed a reduction of 37% in the area ($p<0.001$; Fig. 3C), of 27% in the major diameter ($p<0.001$; Fig. 3D), and of 16% in the minor diameter ($p<0.001$; Fig. 3E) of NMJs in RG.

4. Discussion

The present results show that maternal protein restriction reduced the body weight and soleus muscle weight of restricted animals. The diet consumed by the dams during the period of fetal development directly influenced offspring growth and development. Low-protein diets have been related to low birthweight in animal pups (Bayol et al., 2004) and to developmental delays in humans (Frota et al., 2009). In the present study, a 55% reduction in body weight was observed in RG animals compared to CG. Studies evaluating the effect of protein restriction during pregnancy have shown a reduction in offspring weight irrespective of age (Alves et al., 2008; Toscano et al., 2010; Da Silva Aragão et al., 2013). Animals of RG also exhibited a reduction in visceral fat weight, associated with a reduction of 58% in liver weight, of 59% in soleus muscle weight and of 23% in soleus muscle length when compared to control animals. According to Voltarelli and Mello (2008), the loss of muscle tissue in protein malnutrition can be considered a homeostatic mechanism. The functional redistribution of muscle protein is necessary to supply the nitrogen required for essential functions during the early stages of life, such as the formation of blood cells, tissue protein synthesis and immune functions.

Three types of cells are involved in the development of skeletal muscles: myocytes, adipocytes, and fibroblasts (Du et al., 2010). The large number of fibroblasts, myotubes, central nuclei and fetal fibers observed in regions of the soleus muscle in the two groups confirms the immaturity of part of the muscle fibers at this age. During fetal development, nutrient distribution to organs such as the brain and heart is prioritized. As a consequence, muscles are particularly vulnerable to nutrient deficiency during this period (Zhu et al., 2006). An increase in the number of muscle fibers and nuclei, associated with apparent enlargement of the interstitial space, was also observed in RG when compared to CG. Studies indicate that early malnutrition delays the growth of muscle cells in relation to connective tissue development (Oliveira et al., 1999). This hypothesis would explain the reduction of 66% in total soleus muscle area observed in RG animals, which would be related to an apparent decrease in muscle fiber area.

Ultrastructural analysis of the soleus muscle showed the presence of muscle fibers containing sparse or loosely arranged myofibrils, a disorganized Z-line and central nuclei in animals of CG and RG. The formation of primary myotubes occurs during the intermediate stage of muscle fiber development. These myotubes contain centrally located nuclei and myofibrils at the periphery of the sarcoplasm (Dal Pai Silva and Carvalho, 2007; Spielholz, 1982). According to Stromer *et al.* (1974), it is difficult to determine the exact time when formation of the Z-line occurs during development, but it is known that the Z-line is formed after the sarcomeres. In an experiment conducted by Nascimento *et al.* (1990), the administration of a low-protein diet during pregnancy did not change the peripheral position of the nuclei in muscle fibers of rats at 30 days of age. However, the ultrastructural alterations observed in the present study may be related to the age of the animals and the longer administration of the maternal low-protein diet. As a result of these conditions, part of the muscle fibers may still be in the process of formation, with the organization of sarcomeres and movement of the nuclei to the periphery occurring during a later period than that studied here.

The morphology of NMJs is known to be intimately related to the type of muscle fiber with which they are associated (Ogata and Yamasaki, 1985). According to Nascimento *et al.* (1990), muscle impairment as a result of malnutrition is due to changes that occur throughout the motor unit. In the present study, a reduction of 37% in area, 27% in the major diameter and 16% in the minor diameter of NMJs was observed in RG, a finding that may be directly related to the apparent decrease in muscle fiber size described above. Further studies are needed to elucidate the protein malnutrition-induced changes that occur in NMJs during fetal development.

5. Conclusion

The present results led us to conclude that maternal protein restriction affects the morphology of soleus muscle fibers and adjacent NMJs in 21-day-old rat offspring. Thus, a maternal low-protein diet during pregnancy and lactation seems to interfere with the formation and maturation of these structures, affecting their development in the offspring.

Conflicts of interest

The authors declare no conflicts of interest.

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TABLES TITLES

FIGURE LEGENDS

Fig. 1. Light microscopy photomicrographs of soleus muscle fibers in 21-day-old rats. Cross-section. A and C: control animals; B and D: restricted animals. Peripheral nuclei (thin arrows); central nuclei (thick arrows); myotubes (M); fetal fibers (arrowhead); fibroblasts (star). HE.

Fig. 2. Transmission electron photomicrographs of soleus muscle fibers in 21-day-old rats. Longitudinal section: A: control group (CG), muscle fibers showing a focus of sparse or loosely arranged myofibrils (thick arrows) and a disorganized Z-line (thin arrow); B: restricted group (RG), muscle fibers showing a focus of sparse or loosely arranged myofibrils (thick arrows) and a disorganized Z-line (thin arrow); C: CG, central nucleus (N); D: RG, central nucleus (N).

Fig. 3. Light microscopy photomicrographs of neuromuscular junctions (NMJs) of 21-day-old rats. Longitudinal section (nonspecific esterase). A: control animals; B: restricted animals. Morphometry of NMJs. C: NMJ area; D: major diameter; E: minor diameter. Values are the mean ± standard deviation. ***$p<0.001$ indicates statistical significance (*Student's unpaired t test).*

TABLES

Table 1

Ingredients*	Normal-protein diet (17% protein)	Low-protein diet (6% protein)
Casein (84% protein**)	202.00	71.50
Corn Starch	397.00	480.00
Dextrin	130.50	159.00
Saccharose	100.00	121.00
Soybean oil	70.00	70.00
Fiber (microcelulose)	50.00	50.00
Mineral mix***	35.00	35.00
Vitamin mix ***	10.00	10.00
L – cysteine	3.00	1.00
Choline Chlorine	2.50	2.50

* Diet for gestation stage in rodents - AIN-93G
** Values corrected according to protein content of the casein
*** According to AIN-93G

Table 2

Parameters	CG	RG
Weight body (g)	48.65 ± 6.87	21.91 ± 3.59***
Visceral fat weight (g)	0.54 ± 0.08	0.16 ± 0.04***
Visceral fat weight/body weight (g)	1.04 ± 0.12	0.78 ± 0.11**
Liver weight (g)	2.22 ± 0.17	0.94 ± 0.21***
Liver weight/ body weight (g)	4.31 ± 0.41	4.48 ± 0.84
Soleus muscle weight (g)	0.027 ± 0.006	0.011 ± 0.006 ***
Soleus muscle weight/body weight (g)	0.0545 ± 0.006	0.04229 ± 0.014*
Soleus muscle length (mm)	12.31 ± 1.16	9.42 ± 0.98***

The values are expressed as the mean ± SD. ***p<0.001; **p<0.01; *p<0.05. *Student's unpaired t test.*

Table 3

Parameters	CG	RG
Cross sectional area of muscle (μm^2)	331016 ± 38844	112502 ± 93037***
Fibers muscle number	1565 ± 134	3651 ± 382***
Nuclei number	1722 ± 111	3079 ± 224***

The values are expressed as the mean ± SD. ***p<0.001. *Student's unpaired t test.*

FIGURES

Fig. 1

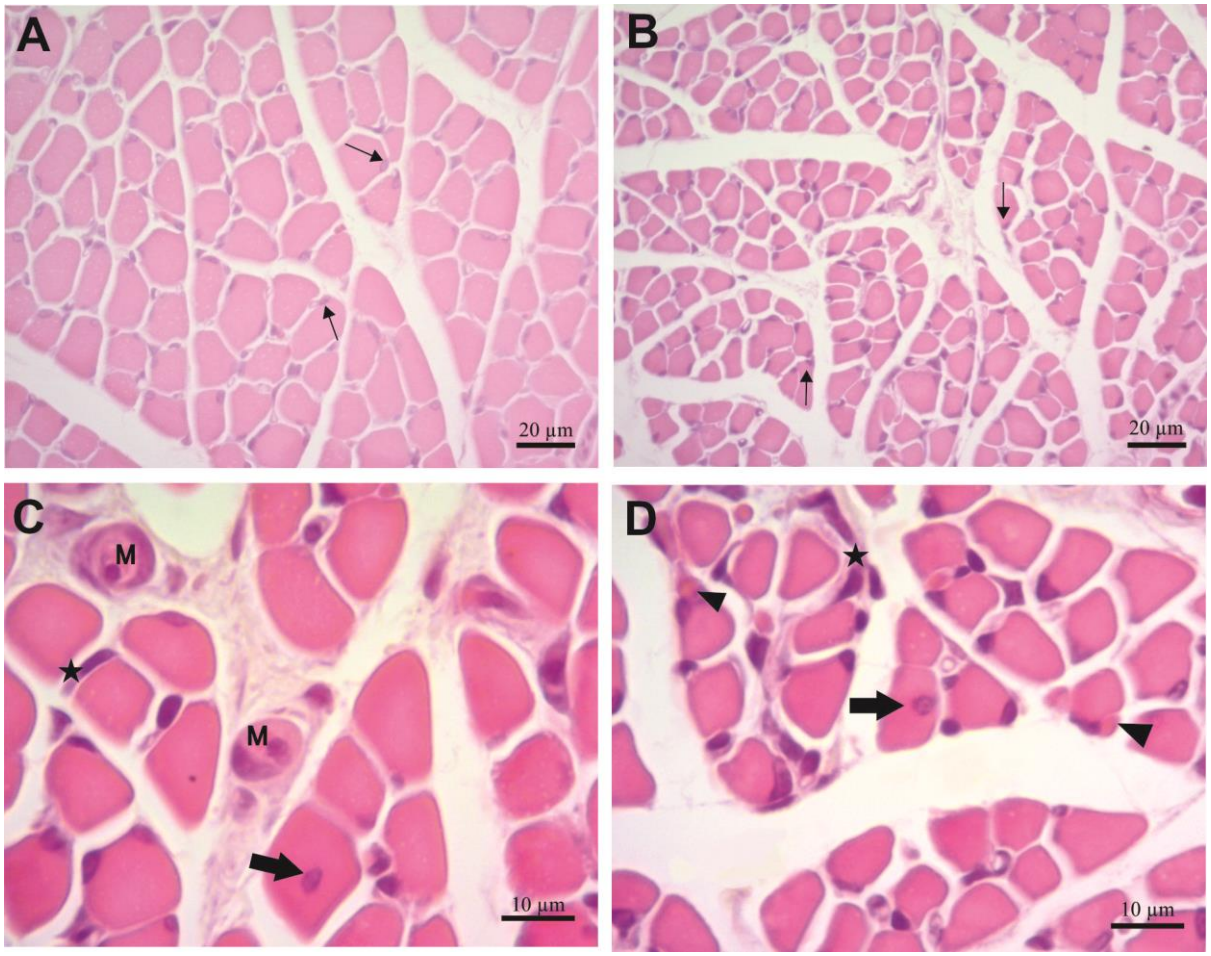


Fig. 2

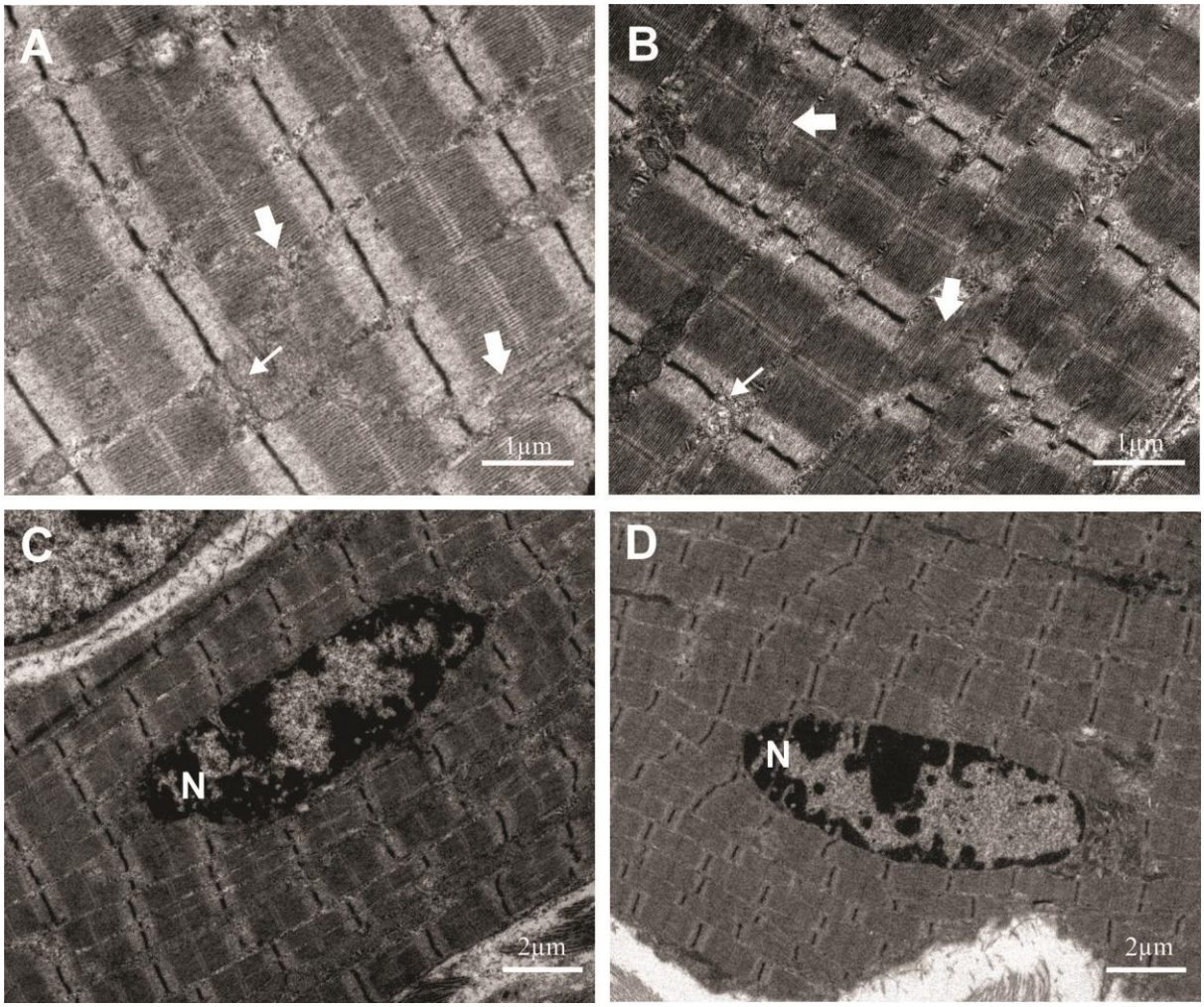
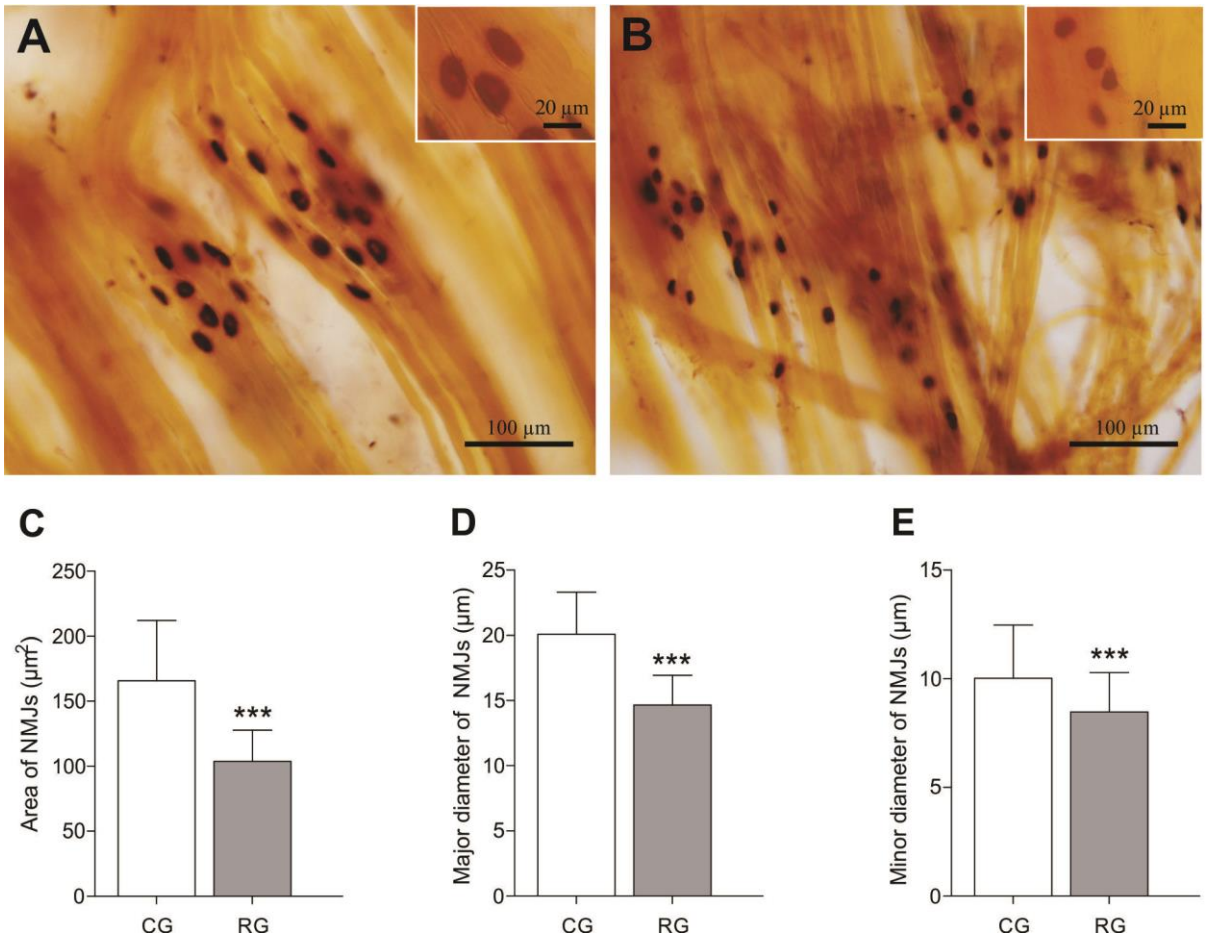


Fig. 3


A
20 µm
100 µm
B
20 µm
100 µm
C
Area of NMJs (µm²)
250
200
150
100
50
0
***
CG
RG
D
Major diameter of NMJs (µm)
25
20
15
10
5
0
***
CG
RG
E
Minor diameter of NMJs (µm)
15
10
5
0
***
CG
RG

ARTIGO CIENTÍFICO II

(Artigo submetido a Revista Histochemistry and Cell Biology)

Effects of aging and maternal protein restriction on the muscle fibers morphology and neuromuscular junctions of rats after nutritional recovery

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ABSTRACT

Changes in the nutritional status of mothers may predispose to long-term neuromuscular disorders in the offspring. This study evaluated the effects of maternal protein restriction during pregnancy and lactation on the muscle fibers and neuromuscular junctions (NMJs) of the soleus muscle in the offspring of rats at 365 days of age that had undergone nutritional recovery. Wistar rats were divided into two groups: Control (CG) - the offspring of mothers fed a normal protein diet (17%) and restricted (RG) - offspring of mothers fed a low protein diet (6%). After lactation, the male pups received standard chow *ad libitum*. At 365 days, samples of soleus muscle were collected for muscle fiber analysis (HE staining, NADH-TR reaction and ultrastructure), intramuscular collagen quantification (picrosirius red staining) and NMJs analysis (Non-Specific Esterase technique). The results show that the RG showed a reduction of 20% in the cross-sectional area of type I fibers and of 5% in type IIa fibers, and an increase of 5% in type IIb fibers compared to the CG. The percentage of intramuscular collagen was 19% lower in the RG. Disorganization of the myofibrils and Z line was observed, with the presence of clusters of mitochondria in both groups. Regarding the NMJs, in the RG there was a reduction of 10% in the area and 17% in the small diameter and an increase of 7% in the large diameter. The results indicate that the effects of maternal protein restriction on muscle fibers and NMJs seem to be long-lasting and irreversible.

KEY-WORDS: soleus muscle, fetal programming, nutrition, low protein diet.

INTRODUCTION

Experimental studies show a close relationship between the conditions offered in the intrauterine environment and the appearance of disease in the offspring, a phenomenon known as fetal programming (Ozanne 1991; Barker et al. 2002; Bellinger and Langley-Evans 2005; Fidalgo et al. 2012). Depending on the stage of fetal development, intrauterine protein restriction causes damage to offspring and is more troubling when it occurs earlier (Patricio et al. 1984). Changes in

the nutritional status of mothers predispose the offspring to cardiovascular (Fowden et al. 2006), urinary (Assis and Fidelis 2011), digestive (Gurmini et al. 2005), respiratory (De Andrade et al. 2012) and muscular changes (Mallinson et al. 2007; Toscano et al. 2008; Cabeço et al. 2012; Dal Pai Silva and Carvalho 2007).

The muscles constitute the body's largest reservoir of protein and, coordinated by the nervous system, they are responsible for the locomotion, respiration and functional autonomy of the individual (Nascimento et al. 1990; Pierine et al. 2009). The neuromuscular junctions (NMJs) are synapses that occur between motoneurons and skeletal muscle fibers (Wu et al. 2010) that allow muscle contraction and the performance of motor functions (Delbono 2003). The NMJs and muscle fibers are formed and their physiological and biochemical properties are determined during the fetal period (Sanes and Lichtman 1999; Nissen et al. 2003 Cabeço et al. 2012). The muscle fibers can be classified into types I, IIa and IIb (Brooke and Kaiser, 1970). Type I fibers have a small diameter and are slow twitching, type IIa fibers have a medium-sized diameter and type IIb have large diameter, and both are fast twitching.

Due to their close relationship with the muscle fibers, according to the type of muscle fibers they innervate, the NMJs have different morphological characteristics. The NMJs of type I muscle fibers are small and rounded or slightly elliptical in shape; those of type IIb muscle fibers are large and elliptical in shape; lastly, the NMJs of type IIa fibers exhibit structural features that are intermediate to those seen in the previously described fibers (Ogata and Yamasaki 1985). The soleus muscle is considered a postural muscle, predominantly composed of type I muscle fibers, which have a higher oxidative metabolism and are slow twitching (Kelly and Rubinstein 2003; Toscano et al. 2010.). The NMJs associated with this muscle are predominantly small and more rounded in shape (Ogata and Yamasaki 1985).

The characteristics of striated skeletal muscles can be altered in the short and long term due to the nutritional conditions (Brito et al. 2006) and aging (Robinson et al. 2012). More specifically,

protein restriction imposed during development can structurally and functionally modify muscles (Mallinson et al. 2007; Toscano et al. 2008; Cabeço et al. 2012). As the NMJs are closely interconnected to the skeletal muscles, it is believed that such structures can also undergo concomitant changes. Recent studies have demonstrated the short-term effect of maternal protein restriction (Mallinson et al. 2007; Toscano et al. 2008; Aragon Da Silva et al. 2013). However, few studies have evaluated the effect of maternal protein restriction on skeletal muscle in aging after nutritional recovery (Bedy et al. 1982; Ozanne et al. 2003).

Economic changes and the consequent increase in life expectancy in developing countries have drawn attention to the effects of aging, and currently a number of topics are being studied in this age group of the population (Moriguti et al. 2001; Chai et al. 2011). In this context, the aim of this study was to evaluate the morphological, morphometric and ultrastructural alterations in the muscle fibers and characterize the NMJs of the soleus muscle of the offspring of rats submitted to protein restriction during pregnancy and lactation, after nutritional recovery and aging.

EXPERIMENTAL METHODS

Animals

The study was approved by the Ethics Committee on Animal Experimentation of the Paulista State University (Universidade Estadual Paulista Júlio de Mesquita Filho - UNESP) (No. 264-CEEA). Male and female Wistar rats obtained from the Animal House of the Department of Anatomy, UNESP, were maintained under standard conditions of light (12-h light/dark cycle) and temperature (23 ± 1ºC). At the beginning of the experiment, two females and one male of reproductive age (12 weeks) were housed in boxes overnight for mating. The male was removed from the box in the following morning and a vaginal smear was obtained from the females. The detection of sperm in the vaginal smear was defined as day 0 of pregnancy, when the females were transferred to individual boxes. On day 0, female rats were divided into two groups: 1) dams fed a

normal protein diet (17%) during pregnancy and lactation, and 2) dams fed a low-protein diet (6%) during pregnancy and lactation. The normal protein (17% protein) and low-protein (6% protein) diets were isocaloric and were administered *ad libitum* until the day of weaning of the offspring (Table 1). After this period, the offspring were provided with standard rodent chow (17% protein).

On the day of birth, male pups were separated from female pups. Additionally, to guarantee an equal availability of food, eight pups were maintained per dam during lactation (21 days). After this period the male pups received a normal protein diet until 365 days of age. Then, the male pups were divided in two experimental groups: the control group (CG) - the pups of mothers fed a normal protein (17%) diet during gestation and lactation (n = 5) and restricted group (RG) – the pups of mothers fed a low protein diet (6%) during pregnancy and lactation (n = 8).

Collecting the soleus muscle

At 365 days of age, the animals of the two groups were weighed and then euthanized in a CO_2 chamber, followed by decapitation using a rodent guillotine. The skin of the hind limbs (left and right antimere) was elevated and the gastrocnemius muscle was removed for exposure and dissection of the soleus muscle. The length (mm) of the soleus muscle from its origin until insertion was measured with a digital caliper (Gigimess®, Brazil) and the muscle was weighed.

Soon after, the soleus muscles were cut into pieces with a stainless steel blade, for the later the histological study, histoenzymological study, ultrastructural examination using an electron microscopy and immunohistochemical study of the NMJs. In addition the liver and fat (epididymal, retroperitoneal and visceral) were collected and weighed.

Histological and morphometric analysis

The soleus muscle samples were fixed in Karnovsky's solution (Karnovsky, 1965) and washed in phosphate-buffered saline (PBS) to remove excess fixative. The muscle samples were

embedded in paraplast in the vertical position. Blocks were prepared using an embedding center (Cygni, EasyPath®, Brazil). Next, the muscle fragments were cut into 5-µm thick sections with a microtome (RM2165, Leica®, Germany). The sections were mounted on previously silanized slides, dried for 1 h at 60ºC, and submitted to deparaffinization, hydration and staining with hematoxylin-eosin (HE) (Junqueira and Junqueira, 1983). The stained slides were then immediately dehydrated, cleared, and mounted in Permount (Fisher Scientific®, USA).

For the quantification of nuclei and muscle fibers and area measurement of these fibers, 10 images in random fields per animal were examined and captured at 400× magnification. Photomicrographs were obtained at 50× magnification (necessary to comprise the entire diameter of the muscle) for the measurement of total muscle area. These images were merged using appropriate software for subsequent measurement of the muscle area.

Collagen quantification

To quantify the percentage of intramuscular collagen, the slides were stained with picrosirius red (Sweat et al. 1964) dehydrated, cleared and mounted with Permount (Fisher Scientific®, New Jersey, USA). Six random images were obtained for each animal (200X magnification). The percentage of collagen/total area was calculated.

Histoenzymological and morphometric analyses of the muscle fiber types

For the histoenzymological study of the muscle fibers, the samples of soleus muscle were kept at room temperature for 30-40 minutes after dissection. Then, the material was covered with talc (to preserve the tissue), frozen in liquid nitrogen and transferred to the cryostat chamber (CM1900, Leica®, Wetzlar, Germany) at -20°C, where they were sectioned (thickness, 7 µm). The sections were subjected to nicotinamide adenine dinucleotide - tetrazolium reductase (NADH-TR)

reaction, according to Pearse (1972) modified by Dubowitz and Brooke (1973). Four fields (200X magnification) were randomly chosen from each animal in order to quantify and measure the areas of the different muscle fiber types (I, IIa and IIb).

Ultrastructural analysis of muscle fibers

For visualization of the ultrastructure of the soleus muscle fibers, the samples were cut into longitudinal fragments (approximately 1 mm wide) and fixed in 2.5% glutaraldehyde. Next, the samples were washed in PBS 0.1 M pH 7.3, for 15 min and postfixed in 1% osmium tetroxide for 2 h. The samples were then washed in distilled water, incubated in 0.5% uranyl acetate for 2 h, dehydrated in acetone, and embedded in a mixture of resin and 100% acetone for 12 h, for the formation of blocks. The desired fields were selected in semi-thin sections and ultrathin sections were cut with an ultramicrotome (Ultracut UCT, Leica®, Germany). The ultrathin sections were stained with a saturated solution of uranyl acetate and lead citrate. The material was examined and photographed under a transmission electron microscope (CM100, Philips®, The Netherlands).

Morphology and morphometry of neuromuscular junctions

For the study of NMJs, the soleus muscle samples were immersed in Karnovsky's fixative (Karnovsky, 1965) at room temperature and cut longitudinally into three or four slices with stainless steel blades. The sections were submitted to nonspecific esterase reaction (Lehrer and Ornstein, 1959). Images of the NMJs were captured at 200× magnification. Measurements of area and major and minor diameter of 50 NMJs per animal were obtained for each group.

Image Analysis

The muscle fibers were observed under a Primo Star microscope (Zeiss®, Germany) coupled to a camera (AxiocamERc5s®) and computer using the Axiovision Rel.4.8® program (Carl Zeiss

Microimaging, Inc., Germany). The amount of collagen was measured in the images captured with a Leica camera DFC 300 FX coupled to Leica DMLB® microscope (Wetzlar, Germany) using Leica QWinV3 software (Leica Microsystems, Wetzlar, Germany). Finally, the photodocumentation of the NMJs was performed under an Olympus Bx60® microscope coupled to an Olympus DP71 camera (Japan) using the DP Controller 3.2.1 276 program. All morphometric measurements were made using the Image Pro Plus 6.0® software (Media Cybernetics, USA).

Statistical analysis

Data were initially analyzed using the Shapiro-Wilk normality test. Body weight, the total and relative weights of the visceral fat, the liver, the soleus muscle, the total cross-sectional area of the soleus muscle, collagen quantification, the nuclei count, the counting and measurements of the areas of the different types muscle fibers were compared using the Student's *t* test. Muscle length, muscle fiber area, area, larger and smaller diameters of the muscle NMJs were analyzed using the Mann-Whitney test. Significance was set at $p < 0.05$, and data were expressed as mean±standard deviation. The analyses were conducted and the graphs constructed with the aid of the GraphPad Prism 5.0® program (La Jolla, USA).

RESULTS

Macroscopic parameters

At 365 days of age, the body weight of the RG animals was approximately 19% lower compared to the CG ($P < 0.001$). The RG presented reductions of 37% in the weight of visceral fat ($P < 0.01$), 15% in the weight of the liver ($P < 0.01$) and 15% in the weight of the soleus muscle ($P < 0.01$) when compared the GC. No differences were found in the length of the soleus muscle or the relative weights of the soleus muscle, visceral fat or liver between the groups (Table 2).

Muscle fiber morphology and morphometry

The muscle fibers of the soleus muscle were seen to be arranged in fascicles surrounded by connective tissue of normal appearance (perimysium), with each fiber also surrounded by connective tissue (endomysium). The soleus muscle of the RG animals presented approximately 19% less collagen when compared to the CG ($P < 0.05$, Fig 1). No differences were found between the groups regarding the number of muscle fibers and nuclei (Table III). Nevertheless, there was a noteworthy presence of central nuclei in both groups. While the area of muscle fibers was found to be 5% smaller in the RG when compared to CG ($P < .001$), there was no difference in the total cross-sectional area of the soleus muscle between the GC and RG (Table 3) groups. Histoenzymological analysis showed the fibers could be characterized into: type I, IIa and IIb fibers (Brooke and Kaiser 1970) (Figs. 2A and 2B). In the RG there was a 20% reduction in the cross-sectional area of type I fibers ($P < 0.001$) and of approximately 5% in type IIa fibers ($P < 0.05$) compared to the CG. By contrast, there was an increase in the cross-sectional area of the type IIb fibers of approximately 5% ($P < 0.01$) in the RG compared to the CG (Fig. 2C). There was no significant difference between the groups in relation to the quantification of the different types of muscle fibers (Fig. 2D).

Ultrastructural analysis of the muscle fibers

In the GC, the morphology of the soleus muscle showed sarcoplasm with the presence of well-defined myofibrils and sarcomeres with organized A-, I-bands and Z-line (Fig. 3A). By contrast, the RG showed a large amount of fibers with disorganized myofibrils and Z line (Fig. 3B). In both experimental groups, clusters of mitochondria were found in various regions of the soleus muscle in all types of analyzed fibers (Figs. 3C and 3D).

Morphological and morphometric analysis of the NMJs

A high degree of polymorphism was seen among the analyzed NMJs. Among the most common shapes are the round, oval and elliptical NMJs (Figs. 4A and 4B). The morphometric analysis showed that the RG presented reductions of 10% in the area ($P < 0.001$; Fig. 4C) and 17% in the small diameter of the NMJs ($P < 0.001$; Fig. 4E) compared to the CG. However, there was a 7% increase in the large diameter of these structures in the RG compared to the CG ($P < 0.05$, Figure 4D).

DISCUSSION

The results show that maternal protein restriction reduced the weight of the animals in senescence, even when a standard diet was offered after the lactation period. This is one of the parameters used to prove the efficacy of the diet applied in experiments using protein restriction (Bayol et al. 2004; Toscano et al. 2008; Parade-Simon et al. 2011; Aragon Da Silva et al. 2013). The animals in the RG showed a reduction of approximately 19% in body weight compared to the GC, showing that protein deficiency in the initial developmental phase is determinant for the growth of the offspring. There was also a reduction in the absolute weight of the soleus muscle, the visceral fat and the liver in the restricted animals compared to controls. In their study, Cabeço et al. (2012) found that at 30 days old the absolute weight of the soleus muscle and the extensor digitorum longus was lower in animals fed a low protein diet during pregnancy. Alves et al. (2008) also reported that restriction may cause a deficit in the concentration of tissue proteins. Thus, it is believed that the reductions in the weight of the soleus muscle and liver observed in this study may be associated with this lower amount of protein available for tissue development, since these organs are mainly composed of protein.

The fetal period is important for the development of muscle fibers (Nissen et al. 2003). Following birth, there may be growth (hypertrophy) (Dal Pai Silva and Carvalho 2007) or reduction (atrophy or hypertrophy) (Powers et al. 2007) in the area of these fibers. In the RG animals, no difference was found in the number of fibers when compared to the CG. Moreover, there was no difference between experimental groups in relation to the total cross-sectional area of the soleus muscle, although a tendency towards decrease was observed a decrease in RG. This tendency towards reduction in RG can be explained by muscle fibers atrophy, associated with a decrease in the percentage of intramuscular collagen. One of the first studies to evaluate the effects of protein restriction during pregnancy and lactation on muscle, created by Haltia et al. (1978), showed a significant reduction in the cross-sectional area of muscle fibers in the EDL muscle of restricted animals. Parada-Simão et al. (2011), using low-protein diets during pregnancy, also observed a reduction in the amount of collagen in the brachial biceps muscle. The mechanisms by which protein restriction during pregnancy affects the morphology of striated skeletal muscle remain to be elucidated, but an alteration in the pathway involved in protein synthesis may be related to the hypertrophy of muscle fibers (Zhu et al. 2004; Cabeço et al. 2012). It is known that, with aging, satellite cells can be activated and incorporated into a muscle fiber to form new fibers. In the intermediary stage of this process, these newly formed muscle fibers present centralized nuclei, which subsequently migrate to the periphery (Carosio et al. 2011). The appearance of centrally located nuclei in both experimental groups aged 365 days may be due to this muscle remodeling process.

In mammals, the skeletal musculature is composed of different fiber types with different morphological and functional characteristics (Pette and Staron 2000). In this study, no differences were found between the restricted and the control groups in terms of the proportion of different types of muscle fibers. At 112 days of age, rats submitted to protein restriction (6%) during the gestation period and that underwent nutritional recovery also showed no differences in the

proportion of muscle fibers in the soleus muscle (Cabeço et al. 2012). However, the different types of fibers presented a modified cross-sectional area after protein restriction during pregnancy and lactation. There is no consensus in the literature regarding the type of fiber altered by this intervention. In adult rats, Cabeço et al. (2012) showed that after protein restriction during pregnancy there only occurred a decrease in the area of type I fibers, while Nascimento et al. (1990) observed a more marked reduction in type II fibers. More specifically, Oliveira et al. (1999) found a decrease in the area of type IIa and IIb fibers and no change in type I fibers in rats at 21 days of age that had undergone maternal protein restriction during pregnancy. In the present study, there was a significant reduction in cross-sectional area of type I and IIa muscle fibers, and an increase in type IIb fibers in the RG compared with the CG. The consequences of changes in the level of the different types of muscle fibers after protein restriction in pre-and perinatal period have yet to be elucidated.

The ultrastructural analysis of the muscle fibers of the soleus muscle showed the CG presented normal morphology, with well-defined myofibrils and sarcomeres with presence of organized A-, I-bands and Z-line. However, the myofibrils in the RG were disorganized and the Z line was fragmented in a significant amount of the muscle fibers. A previous study in adult rats supplied with a low protein content feed during two weeks showed the presence of changes in the sarcomere and the Z-line fragmented or with an irregular appearance (Oliveira et al. 1999). It is suggested that ultrastructural damage induced by the low protein-level diet may be due to lipid membrane peroxidation and excessive entry of Ca^{2+} in the cytosol, with subsequent activation of endogenous protease (Oumi et al. 2000). The increased Ca^{2+} concentration may also have been aggravated by aging, making the structural damage more evident (Teixeira et al. 2012). Furthermore, clusters of mitochondria were observed in both experimental groups, which may directly affect the contraction and functionality of the muscle due to a potential mitochondrial dysfunction (Jones et al. 2009; Waters and Baumgartner 2011).

This is the first report to describe the effect of maternal protein restriction on the morphology of the NMJs in aged rats. In the RG, we observed a reduction in the area and small diameter of the NMJs, and an increase in large diameter compared to the CG. The NMJs may affect the morphology of the skeletal muscles, thus determining their functional pattern (Jang et al. 2012). Polymorphism was observed among the analyzed NMJs, with the occurrence of round, oval and elliptical shapes. The variation in the shapes of the NMJs is related to the different types of fibers present in the skeletal muscle (Ogata and Yamasaki 1985). The morphometric analysis revealed that RG had a reduction of 10% in area and 17% in the small diameter of the NMJs compared to the CG. The observed decrease in area of the NMJs is probably related to the reduction in the fiber area, since these structures are interconnected and act together. However, there was a 7% increase in the large diameter of these structures in the RG compared to the CG. It is postulated that the increase in the large diameter of the NMJs, may be related to the age of the animals. As previously described by Chai et al. (2011), the NMJs of aged mice (29 months old) become diffuse and have a larger diameter when compared to young rats.

Inadequate diet of the mother and offspring during the gestational and neonatal periods may also lead to protein malnutrition, even in more favorable socioeconomic conditions. In conclusion, at 365 days of age it was still possible to observe that protein deficiency during pregnancy and lactation affected the morphology of the muscle fibers of the soleus muscle and the adjacent NMJs in the offspring, despite the nutritional recovery offered during adulthood. The effects of this type of dietary restriction on skeletal striated muscles appear to be durable and non-reversible, affecting the long-term functioning of the muscle fibers and neuromuscular junctions.

ACKNOWLEDGMENT

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES- Brazil) for the financial support. Also we thank UNIOESTE - Cascavel e UNESP – Botucatu, especially to technician Gelson Rodrigues of anatomy department for the great help in all steps of research.

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TABLES TITLES

FIGURE LEGENDS

Figure 1. Light microscopy images of the collagen present in the soleus muscle of 365-day-old rats. A: CG without polarized light, B: RG without polarized light, C: CG with polarized light and D: RG with polarized light. Cross section. Picrosirius red. E: Percentage of collagen in soleus muscle. Values expressed as mean ± standard deviation. * Represent statistical significance $p < 0.05$. Student's *t* test.

Figure 2. Light microscopy images showing the different fiber types (I, IIa and IIb) of the soleus muscle of 365-day-old rats. A: CG; B: RG. Small-diameter fibers (I); medium-diameter fibers (IIa) and large-diameter fibers (IIb). Cross section. NADH-TR. C: Cross-sectional area of the fibers and D: Fiber count. Values expressed as mean±standard deviation. Statistical significance set at *$p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. Student's *t* test.

Figure 3. Electron microscopy images of muscle fibers from the soleus muscle of 365-day-old rats. Longitudinal section. A: CG, organized Z-line (thin arrows), A-band and I-band; B: RG, disorganized Z-line (thin arrow), loosely arranged myofibrils (thick arrow); C: CG and D: RG. Presence of a cluster of mitochondria (star).

Figure 4. Light microscopy images of NMJs from 365-day-old rats. A: CG; B: RG. Longitudinal section. Nonspecific esterase. C: NMJs areas; D: Large-diameter of the NMJs and E: Small-diameter of the NMJs. Values are expressed as mean±standard deviation. Statistical significance set at * $p < 0.05$ and *** $p < 0.001$. Student's *t* test.

TABLES

Table. 1

Ingredients*	**Normal-protein diet (17% protein)**	**Low-protein diet (6% protein)**
Casein (84% protein**)	202.00	71.50
Corn Starch	397.00	480.00
Dextrin	130.50	159.00
Saccharose	100.00	121.00
Soybean oil	70.00	70.00
Fiber (microcelulose)	50.00	50.00
Mineral mix***	35.00	35.00
Vitamin mix ***	10.00	10.00
L – cysteine	3.00	1.00
Choline Chlorine	2.50	2.50

* Diet for gestation stage in rodents - AIN-93G
** Values corrected according to protein content of the casein
*** According to AIN-93G

Table. 2

Parameters	CG	RG
Weight body (g)	563.00 ± 26.60	456.90 ± 21.54***
Visceral fat weight (g)	25.37 ± 5.26	16.04 ± 4.54**
Visceral fat weight/body weight (g)	4.48 ± 0.75	3.49 ± 0.91
Liver weight (g)	15.67 ± 1.01	13.36 ± 1.134**
Liver weight/ body weight (g)	2.783 ± 0.080	2.925 ± 0.205
Soleus muscle weight (g)	0.538 ± 0.040	0.458 ± 0.029**
Soleus muscle weight/body weight (g)	0.095 ± 0.006	0.100 ± 0.005
Soleus muscle length (mm)	35.11 ± 3.52	34.98 ± 2.13

Values expressed as mean±standard deviation. Statistical significance set at **p<0.01 and ***p<0.001. *Mann Whitney* test and Student's *t* test.

Table. 3

Parameters	CG	RG
Cross sectional area (μm^2)	5764 ± 605,5	4734 ± 983.8
Fibers muscle number	125.4 ± 11.95	125.0 ± 17.41
Nuclei peripheral number	278.8 ± 24.46	292.4 ± 24.17
Nuclei central number	1.2 ± 2,16	1.0 ± 1.22
Fibers muscle area (μm^2)	2491 ± 952.5	2365 ± 986**

Values expressed as mean±standard deviation. Statistical significance set at **p<0.01. *Mann Whitney* test and Student's *t* test.

FIGURES

Figure 1

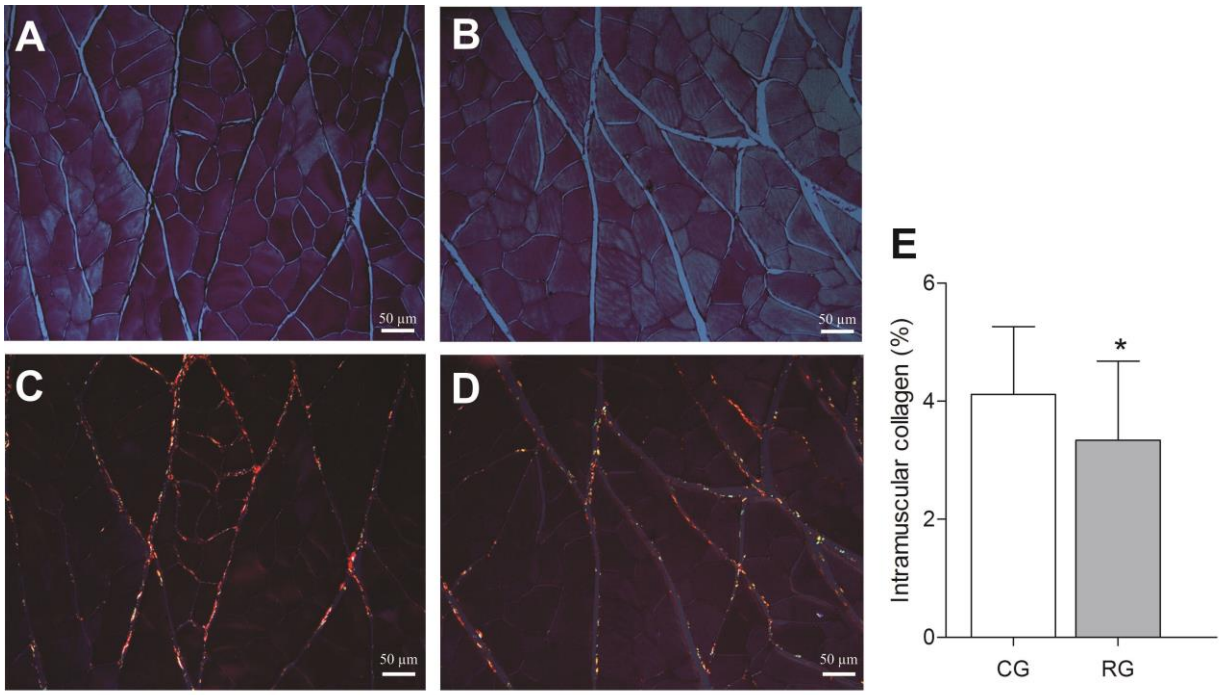


Figure 2

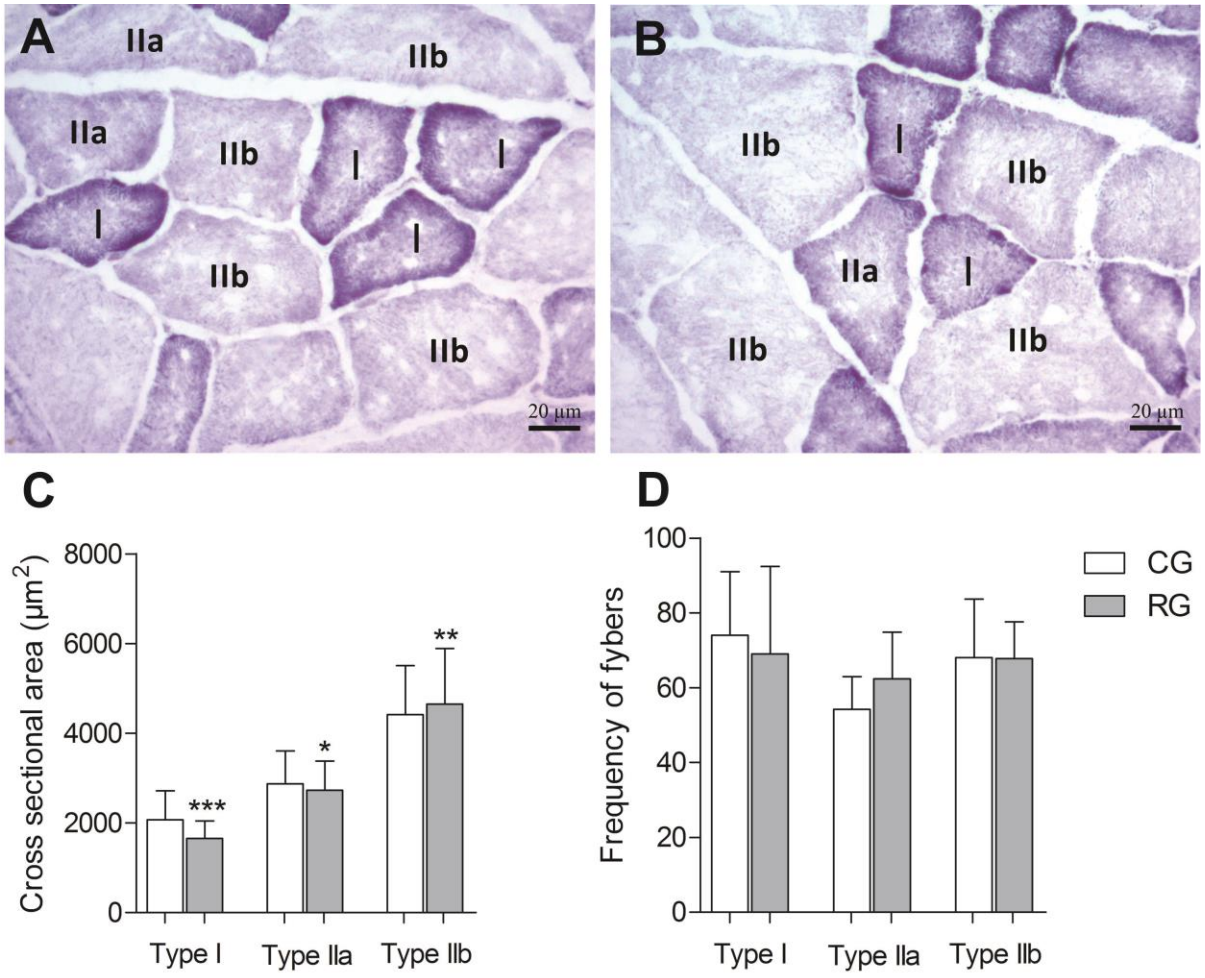

A
IIa
IIb
IIa
IIb
I
I
I
I
IIb
IIb
20 µm
B
IIb
I
IIb
IIa
I
IIb
IIb
20 µm
C
Cross sectional area (µm2)
8000
6000
4000
2000
0
***
*
**
Type I
Type IIa
Type IIb
D
Frequency of fybers
100
80
60
40
20
0
CG
RG
Type I
Type IIa
Type IIb

Figure 3

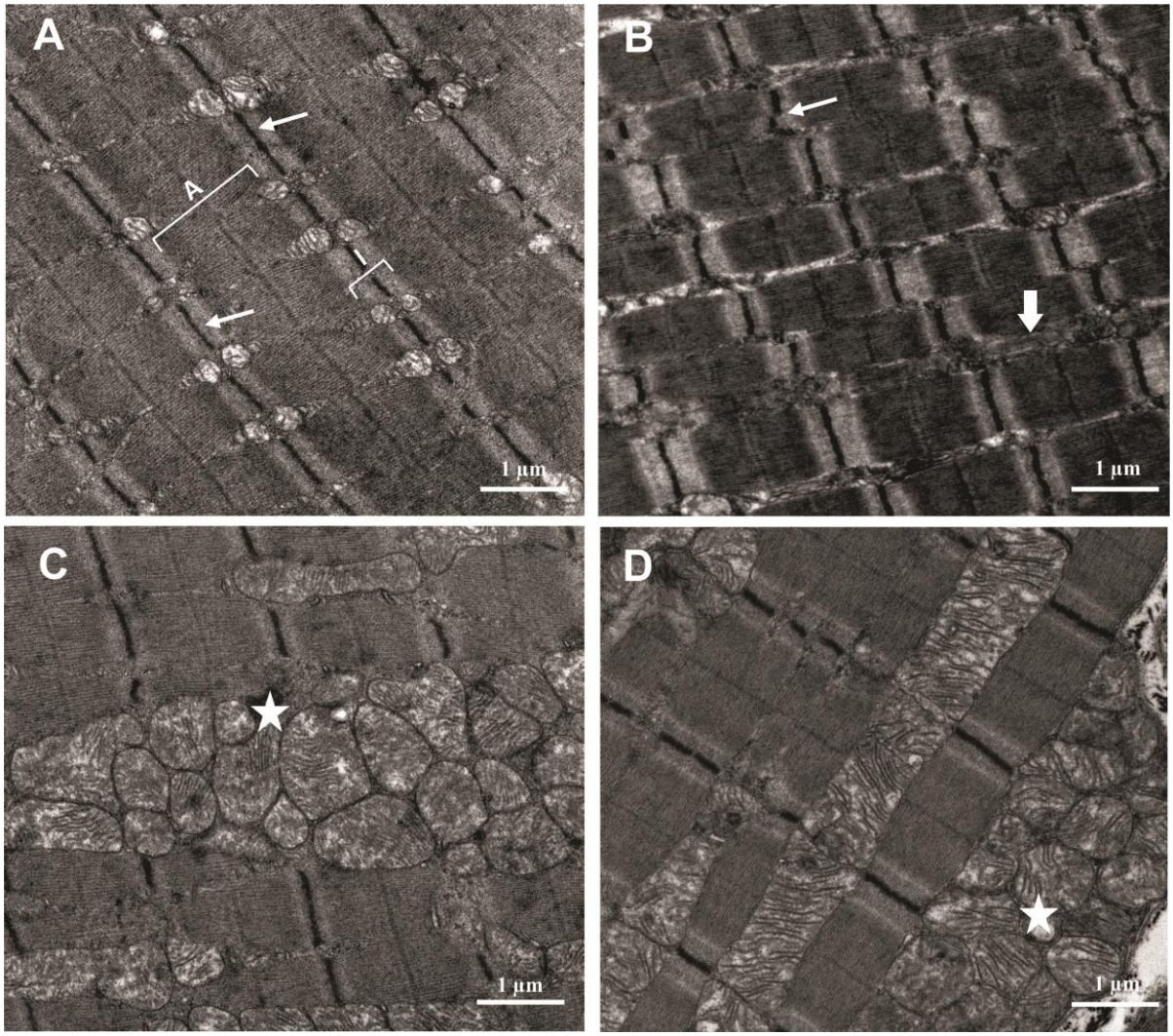
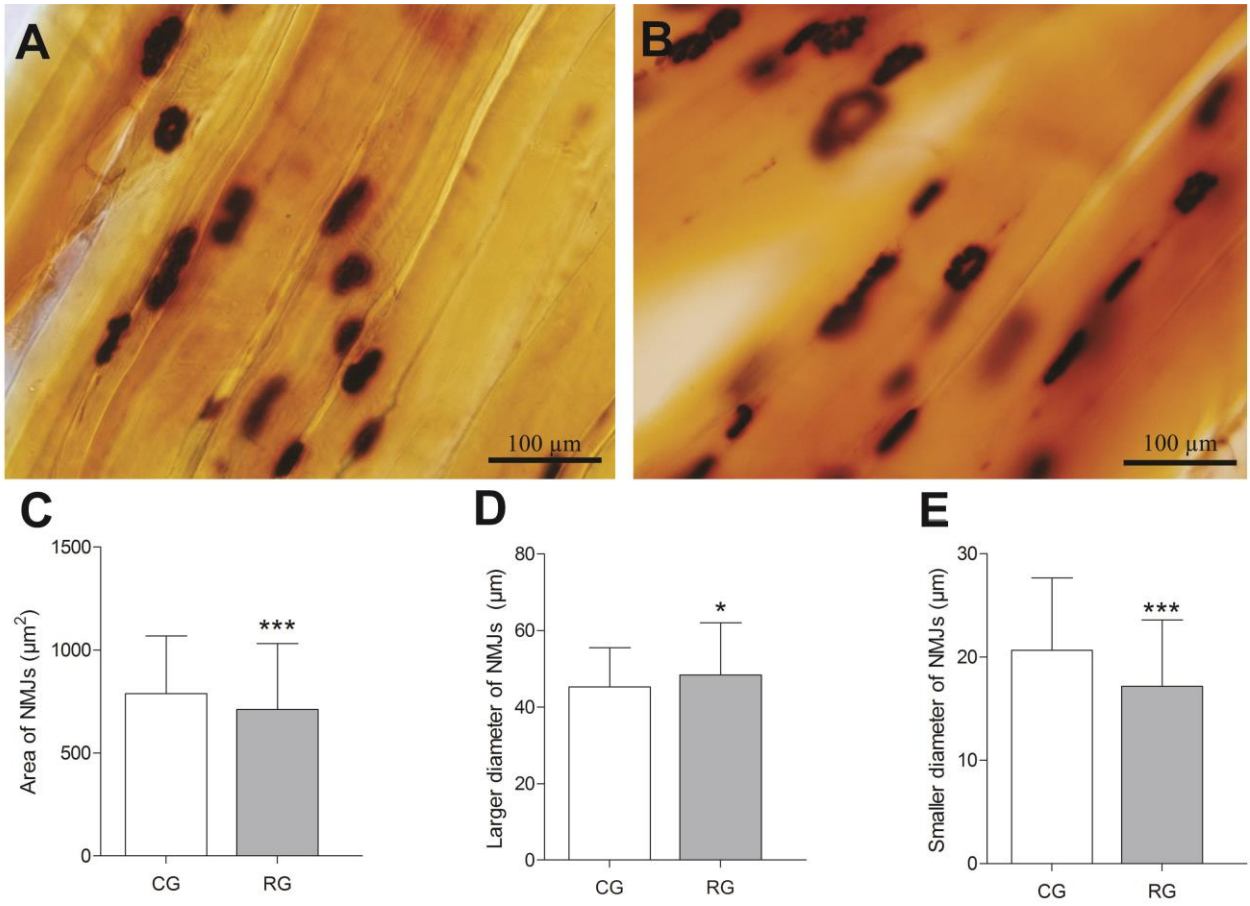


Figure 4


A
100 µm
B
100 µm
C
Area of NMJs (µm²)
1500
1000
500
0
***
CG
RG
D
Larger diameter of NMJs (µm)
80
60
40
20
0
*
CG
RG
E
Smaller diameter of NMJs (µm)
30
20
10
0
***
CG
RG

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CERTIFICADO

Certificamos que o Protocolo nº **264-CEEA**, sobre "Programação fetal por restrição proteica maternal em ratos machos", sob a responsabilidade de **Patrícia Fernanda Felipe Pinheiro**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **03/12/2010.**

Botucatu, 03 de dezembro de 2010.

Profª Drª PATRÍCIA FERNANDA FELIPE PINHEIRO
Presidente - CEEA

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ANEXO B:

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AUTHOR INFORMATION PACK

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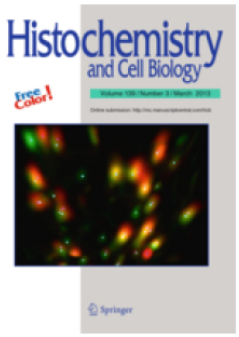

Histochemistry and Cell Biology

Editors-in-Chief: D. **Drenckhahn**; J. **Roth**

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- Negotiation research spans many disciplines (Thompson 1990).
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- This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086
- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the
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- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
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- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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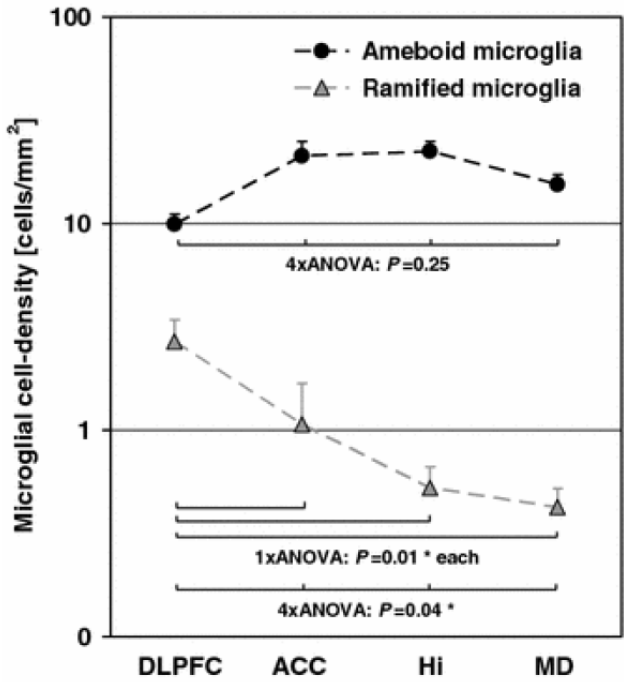

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

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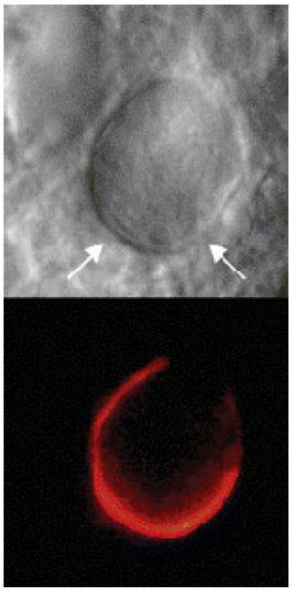
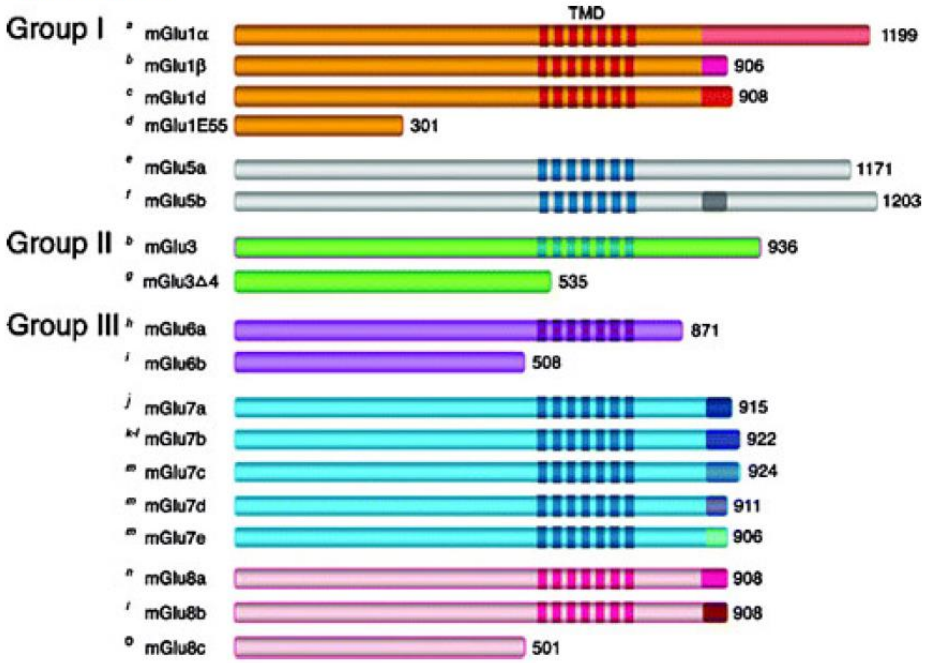


Combination Art



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