MOLÉCULAS DE FUSÃO E FATORES TRANSCRICIONAIS EM MACRÓFAGOS E CÉLULAS MUSCULARES ESQUELÉTICAS DE RATOS: EFEITO DA DESNUTRIÇÃO NEONATAL

JULIANA FÉLIX DE MELO

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Thèse présentée
par
Juliana Félix de Melo

MOLÉCULES DE FUSION ET FACTEURS DE TRANSCRIPTION DANS LES MACROPHAGES ET CELLULES MUSCULAIRES SQUELETTIQUES DE RATS: L'EFFET DE LA DÉNUTRITION NÉONATALE

Pour l'obtention du grade de Docteur de l'Université de Technologie de Compiègne (UTC) et de l'Université Fédérale de Pernambuco (UFPE)

Thèse en co-tutelle dirigée par:

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No dia 27 de fevereiro de 2012, às 10h00, no Auditório do Centro de Ciências Biológicas Universidade Federal de Pernambuco (CCB/UFPE), os Membros Doutores: **a Profª. Drª. Maria Rosângela Cunha Duarte Coêlho – Presidente da Banca (UFPE), a Profª. Drª. Maria do Amparo Andrade (UFPE), a Profª. Drª. Jairza Maria Barreto Medeiros (UFPE), a Profª. Drª. Muriel Vayssade (UTC-FRANÇA) e o Prof. Dr. Cristophe Egles (UTC-FRANÇA),** componentes da Banca Examinadora, em sessão pública, arguiram a doutoranda **JULIANA FÉLIX DE MELO** sobre a sua Tese intitulada **"MOLÉCULAS DE FUSÃO E FATORES TRANSCRICIONAIS EM MACRÓFAGOS E CÉLULAS MUSCULARES ESQUELÉTICAS DE RATOS: EFEITO DA DESNUTRIÇÃO NEONATAL",** a qual foi **orientada pela Profª. Dra. Célia Maria Machado Barbosa de Castro (UFPE) e pela Profª. Drª. Marie Danielle Nagel (UTC-FRANÇA).** Ao final da arguição de cada membro da Banca Examinadora e resposta da doutoranda, as seguintes menções foram publicamente fornecidas.

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"Penso que só há um caminho para a ciência ou para a filosofia: encontrar um problema, ver a sua beleza e apaixonar-se por ele; casar e viver feliz com ele até que a morte vos separe – a não ser que encontrem um outro problema ainda mais fascinante, ou, evidentemente, a não ser que obtenham uma solução. Mas, mesmo que obtenham uma solução, poderão então descobrir, para vosso deleite, a existência de toda uma família de problemas-filhos, encantadores ainda que talvez difíceis, para cujo bem-estar poderão trabalhar, com um sentido, até ao fim dos vossos dias."
(Karl Popper)

"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original."
(Albert Einstein)

RESUMO

Nesta tese avaliamos os efeitos tardios da desnutrição neonatal sobre a expressão/produção de moléculas de fusão e fatores transcricionais em macrófagos alveolares e células musculares esqueléticas. Foram utilizados 36 ratos, machos, Wistar, amamentados por mães que receberam dieta durante a lactação contendo 17% de caseína, grupo nutrido (N) ou 8% de caseína, grupo desnutrido (D). Após o desmame, os animais foram recuperados com dieta Labina ou Teklad Global, até 42 dias (n=12), 60 dias (n=12) e 90 dias de vida (n=12). Metade destes animais (n=18) foi submetida a um procedimento cirúrgico de traqueostomia objetivando a retirada de lavado broncoalveolar e posterior cultura dos macrófagos alveolares por 4 dias. Da outra metade (n=18), foram retirados todos os músculos de ambas as patas dos animais e realizada a cultura das células musculares esqueléticas durante 10 dias. Deste modo, os resultados geraram dois artigos originais. O primeiro intitulado "Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins" permitiu observar que a desnutrição durante a lactação alterou o número de macrófagos em cultura e a produção de proteínas de fusão em ratos jovens e adultos, mas não modificou a expressão das moléculas de adesão caderinas. O segundo intitulado "Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats" demonstrou que a desnutrição neonatal não modificou a expressão de proteínas-chaves do processo miogênico, mas alterou a morfologia e reduziu o número dos miotubos em cultura de animais com 60 dias de vida. Em conclusão, a desnutrição neonatal causou sequelas no organismo jovem e adulto, mesmo após a reposição nutricional. Estas alterações foram evidenciadas no desenvolvimento de macrófagos alveolares em cultura e na miogênese.

Palavras chaves: Desnutrição; "Programming"; Fusão de macrófago; Músculo esquelético; Células satélites; Fusão de mioblasto.

ABSTRACT

In this thesis, we evaluated the late effects of neonatal undernutrition on the expression/production of fusion molecules and transcriptional factors in alveolar macrophages and skeletal muscle cells. Thirty-six male Wistar rats were suckled by mothers fed diets containing 17% casein, control group (C) or 8% casein, undernourished group (UN) during lactation. After weaning, all animals received a normoproteic diet (Labina or Teklad Global), at 42 days (n=12), 60 days (n=12) and 90 days (n=12). Half of these animals (n=18) were submitted to a tracheostomy for the removal of bronchoalveolar lavage and subsequent culture of alveolar macrophages for 4 days. In the other half (n = 18), all muscles of both legs were removed and the skeletal muscle cells cultured for 10 days. This resulted in two original articles. The first of these, entitled "Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins", allowed us to observe that undernutrition during lactation altered the number of macrophages in culture and the production of fusion proteins in young and adult rats, but did not modify the expression of cadherin adhesion molecules. The second article, entitled "Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats", demonstrated that neonatal undernutrition did not modify the expression of key proteins of the myogenic process but altered the morphology and reduced the number of myotubes in culture from 60-day-old rats. In conclusion, neonatal undernutrition caused sequelae in young and adult organisms, even after nutritional recovery. These changes were evidenced in the development of alveolar macrophages in culture and myogenesis.

Keywords: Undernutrition; Programming; Macrophage fusion; Skeletal muscle; Satellite cells; Myoblast fusion.

RÉSUMÉ

Cette thèse en co-tutelle a été effectuée à l'Université de Technologie de Compiègne (UTC - France) et à l'Université Fédérale de Pernambuco (UFPE - Brésil). En France, les activités ont été réalisées dans l'unité de Biomécanique-Bioingénierie (CNRS UMR 6600) de l'UTC, et au Brésil, dans le Laboratoire de Physiologie de la Nutrition Naíde Teodósio (LAFINNT) du Département de Nutrition de l'UFPE, dans le Laboratoire d'Immunopathologie Keizo Asami (LIKA-UFPE) et au sein du Centre de Recherche Aggeu Magalhães (Fiocruz PE).

Une déficience nutritionnelle pendant la période critique du développement des systèmes organiques, comme par exemple les systèmes immunitaire et musculaire squelettique, peut générer des conséquences délétères au niveau structural et fonctionnel de ces systèmes chez l'adulte (OZANNE et HALES, 2002 ; LUCAS, 2005).

Ainsi, il a été mis en évidence qu'une dénutrition protéino-calorique provoquait une réduction de l'immunité à médiation cellulaire, de la fonction phagocytaire, de celle du système du complément, de la production d'anticorps, de la libération de cytokines et de l'expression des molécules d'adhésion cellulaire (CHANDRA, 2002 ; LANDGRAF et al., 2005). Dans cette situation, les mécanismes de défense pulmonaire sont touchés avec une diminution du nombre de macrophages alvéolaires (MA). De plus, plusieurs fonctions des macrophages sont affectées par la dénutrition néonatale (MELO et al., 2008; FERREIRA E SILVA et al., 2009). Pour combattre l'infection, les macrophages peuvent fusionner en réponse à des agents pathogènes et corps étrangers, donnant lieu à des ostéoclastes (os) ou des cellules géantes multinucléées (CGM) (dans divers tissus). Des études montrent que la E-cadhérine contribue à la formation des CGM par l'intermédiaire de la cytokine IL-4 (MORENO et al., 2007; HELMING et GORDON, 2009). En plus de l'IL-4, l'interféron γ (IFN-γ) peut également induire la formation des CGM (HELMING et GORDON, 2008, 2009).

D'autre part, une dénutrition précoce est capable de diminuer la capacité de différenciation des cellules musculaires et de réduire le nombre de fibres présentes dans le muscle (WILSON et al., 1988; BAYOL et al., 2004). Les rats dont les mères ont été dénutries par un régime à

base de caséine 8% présentent une réduction du nombre de fibres musculaires et une diminution de la formation de myotubes secondaires (WILSON et al., 1988). De plus, Bayol et al. (2004) ont montré que la dénutrition pendant la gestation réduisait la cellularité dans le muscle après 3 semaines de restauration nutritionnelle. Cependant, peu d'études ont été consacrées à l'effet d'une dénutrition infligée pendant la période de lactation, sur les propriétés des cellules musculaires de l'adulte.

Certaines molécules d'adhésion cellulaire telles que les cadhérines et les intégrines sont importantes pour la première phase de fusion des cellules musculaires squelettiques (myoblaste x myoblaste), conduisant à la néoformation de myotubes. Horsley et al. (2003) ont démontré que ces myotubes primaires sécrétaient de l'IL-4, qui interagit avec son récepteur (IL-4Rα) présent sur les myoblastes pour promouvoir leur fusion avec les myotubes préexistants (myoblaste x myotube), augmentant ainsi la taille des myotubes. Les facteurs de transcription Pax7 et myogénine sont importants pour le développement musculaire. Pax7 est exprimé dans les cellules satellites quiescentes et en phase de prolifération (FOSCHINI et al., 2004). La myogénine est exprimée dans tous les myoblastes depuis le début de leur différenciation et son expression est maintenue lors de la fusion des cellules, marquant aussi la fin de la prolifération des myoblastes (TE PAS et al., 1999).

Le but de notre étude a été de déterminer quelle influence pouvait, chez des rats jeunes et adultes, avoir l'application d'un régime hypoprotéiné au cours de la période de lactation, sur l'expression/production des molécules de fusion dans les macrophages alvéolaires et sur celle de protéines-clés impliquées dans le développement et la différenciation des cellules musculaires squelettiques.

Nous avons utilisé 36 rats, mâles, Wistar, allaités par des mères ayant été nourries avec un régime contenant 17% de caséine dans le groupe contrôle (C) ou 8% de caséine dans le groupe dénutri (D), pendant la période de l'allaitement. Après le sevrage, les animaux ont été restaurés avec l'alimentation Labina ou de Teklad Global, jusqu'à 42 jours (n=12), 60 jours (n=12) et 90 jours (n=12). La moitié de ces animaux (n=18) a subi une trachéotomie dans le but de pratiquer un lavage bronchoalvéolaire pour ensuite cultiver des macrophages alvéolaires pendant 4 jours. L'autre moitié (n=18), a servi à prélever tous les muscles des deux pattes postérieures de l'animal et à maintenir en culture les cellules musculaires squelettiques pendant 10 jours. Le poids des rats a été enregistré tous les cinq jours pendant

les 21 premiers jours après la naissance, puis une fois par semaine, afin de surveiller le gain de poids pendant la phase de récupération nutritionnelle. Le nombre total de cellules a été évalué indirectement par la libération de l'enzyme lactate déshydrogénase (LDH) après lyse cellulaire provoquée. Nous avons examiné l'expression de Pan-cadhérine (dans les macrophages) et de Pan-cadhérine, β1-intégrine, IL-4Rα, Pax7 et myogénine (dans les cellules musculaires) par Western Blot. Les productions d'IL-4 (dans les macrophages et les cellules musculaires) et IFN-γ (dans les macrophages) ont été mesurées par ELISA.

Les résultats de cette thèse sont rapportés dans (02) articles originaux. Le premier (développé à l'UFPE) intitulé "Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins" indique que la dénutrition pendant la lactation affecte le nombre de macrophages dans les cultures cellulaires et la production de protéines de fusion chez les rats jeunes et adultes, mais ne modifie pas l'expression des cadhérines. Le deuxième (développé à l'UTC), intitulé "Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats" montre que la dénutrition néonatale ne modifie pas l'expression de protéines clés du processus myogénique mais change la morphologie et réduit le nombre de myotubes dans les cultures obtenues à partir des animaux âgés de 60 jours. Des analyses complémentaires portant sur les paramètres de contraction des myotubes mesurés chez les animaux dénutris et leurs témoins ont été réalisées dans le cadre du projet de thèse de Simone Fraga.

En conclusion, la dénutrition néonatale provoque des séquelles chez les organismes jeunes et adultes, même après restauration nutritionnelle. Ces changements observés à partir de cultures cellulaires sont évidents dans le développement des macrophages alvéolaires et dans la myogenèse.

Comme perspectives de ce travail, il peut être envisagé d'évaluer les repercussions de la dénutrition induite dans la période pré-natale sur la fusion des macrophages et des cellules musculaires squelettiques lorsque les animaux sont soumis à une activité physique, mais aussi chez des animaux rendus septiques (durant l'infection on peut noter une perte de muscle généralisée et l'activité physique pourrait moduler ce processus); de réaliser des co-cultures de macrophages alvéolaires et de myotubes nouvellement formés et vérifier l'effet de la dénutrition précoce sur l'index de fusion de ces cellules *in vitro*. En effet, selon Chargé

(2003), la voie de signalisation qui active la fusion des cellules musculaires active aussi celle des macrophages, ce qui suggère la possibilité de fusion illégitime entre macrophages et myotubes.

Mots-clé: Dénutrition; "Programming"; Fusion de macrophage; Muscle squelettique; Cellules satellites; Fusion de myoblastes.

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LISTA DE ABREVIATURAS E SIGLAS

AIN	Instituto Americano de Nutrição
AM	Macrófagos alveolares
BAL	Lavado broncoalveolar
C	Controle
CGM	Células gigantes multinucleares
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
D	Desnutrido
DM	Meio de diferenciação
FAK	Quinase de adesão focal
FAO	Organização das Nações Unidas para Agricultura e Alimentação
FM	Meio de fusão
IFN-ɣ	Interferon-ɣ
IGF	Fator de crescimento semelhante à insulina
IL	Interleucina
IL-4Rα	Receptor IL-4α
LAFINNT	Laboratório de Fisiologia da Nutrição Naíde Teodósio
LBA	Lavado broncoalveolar
LDH	Lactato desidrogenase
LIKA	Laboratório de Imunopatologia Keizo Asami
LPS	Lipopolissacarídeo
MA	Macrófagos alveolares
MGC	Células gigantes multinucleares
MRFs	Fatores regulatórios miogênicos
N	Nutrido
NFATc2	Fator nuclear de ativação de células T
NS	Não significante
PBS	Tampão fosfato-salina
P-caderina	Caderina placentária

LISTA DE ABREVIATURAS E SIGLAS

PCR	Proteína C reativa
PGM	Meio de crescimento primário
SDRA	Síndrome do desconforto respiratório agudo
UFPE	Universidade Federal de Pernambuco
UMR 6600	Unidade de Biomecânica-Bioengenharia
UN	Desnutrido
UTC	Universidade de Tecnologia de Compiègne

SUMÁRIO

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1. APRESENTAÇÃO

Este trabalho de tese em co-tutela foi realizado na Universidade de Tecnologia de Compiègne (UTC - França) e na Universidade Federal de Pernambuco (UFPE - Brasil) no âmbito do projeto: "Papel profilático da atividade física sobre as consequências da desnutrição precoce na função miocitária e nas propriedades neuro-mecânicas do músculo esquelético" (CAPES/COFECUB nº584/07). Na França, as atividades foram realizadas na unidade de Biomecânica-Bioengenharia (UMR 6600) da UTC. No Brasil, no Laboratório de Fisiologia da Nutrição Naíde Teodósio (LAFINNT) do Departamento de Nutrição da UFPE, no Laboratório de Imunopatologia Keizo Asami (LIKA-UFPE) e no Centro de Pesquisas Aggeu Magalhães (Fiocruz PE).

O interesse por esse estudo surgiu quando foram avaliados os dados obtidos da Dissertação de Mestrado "Atividade oxidante-antioxidante de macrófagos alveolares em ratos endotoxêmicos submetidos à desnutrição neonatal" apresentada por mim à banca examinadora do Curso de Pós-graduação em Medicina Tropical da Universidade Federal de Pernambuco (Melo, 2007). Dentre os achados da pesquisa, foi observado que a desnutrição neonatal, mesmo seguida de reposição nutricional, comprometeu a resposta inflamatória pulmonar de ratos adultos, diminuindo o recrutamento de células inflamatórias para o pulmão e a atividade oxidante-antioxidante dos macrófagos alveolares. Assim, surgiu a curiosidade de realizar um estudo envolvendo não só células imunes, mas que pudesse aprofundar os mecanismos envolvidos na programação nutricional também com células não imunes como as células musculares esqueléticas. Parece haver um maior envolvimento da disfunção de células musculares esqueléticas e fusão de macrófagos durante os estados sépticos. Além disso, despertou-se o interesse em entender melhor esses mecanismos também em ratos mais jovens e não somente em ratos adultos.

Os resultados desta tese se encontram apresentados na forma de (02) artigos:

- O Artigo 1: avalia o efeito da desnutrição neonatal sobre o número de macrófagos em cultura e a expressão/produção de proteínas que regulam a fusão macrofágica em ratos jovens e adultos. Tal artigo foi intitulado: "Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the

expression/production of fusion proteins". Enviado para publicação como artigo original ao periódico British Journal of Nutrition.

- O Artigo 2: Neste, foi avaliado o efeito da desnutrição neonatal sobre a morfologia de miotubos em cultura e a expressão de proteínas-chaves que regulam a miogênese em ratos jovens e adultos. Publicado como artigo original no European Journal of Nutrition: MELO, J. F., Aloulou, Nijez, Duval, Jean-Luc, Vigneron, Pascale, Bourgoin, Lee, Leandro, Carol Góis, Castro, Celia M. M. B., Nagel, Marie-Danielle. Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats. European Journal of Nutrition 50: 243-250, 2011.

2. REVISÃO DA LITERATURA

2.1 Processo inflamatório/infeccioso: papel dos macrófagos

Os mecanismos de resistência do hospedeiro podem ser divididos em duas vias principais: a resposta imune específica e a resposta imune inespecífica. As defesas inespecíficas incluem a pele, as membranas mucosas, células fagocíticas, muco, epitélio ciliar, sistema complemento, lisozima, interferon e outros fatores humorais (CHANDRA, 1997). Esses processos inatos, do qual faz parte a reação inflamatória, estão naturalmente presentes, agem como a primeira linha de proteção e retardam o estabelecimento de manifestações infecciosas (CHANDRA, 1997).

A inflamação é definida clinicamente pela associação dos quatro sinais: tumor, rubor, calor e dor. No que diz respeito aos tecidos, esses sinais são consequência de uma vasodilatação, do aumento da permeabilidade vascular e de um afluxo de células fagocitárias (DE CASTRO et al., 1997). No foco de injúria tecidual, fatores quimiotáticos atraem células sanguíneas que, por diapedese, atravessam a barreira endotelial. Dentre estas células, os neutrófilos estão em maior número, seguidos dos monócitos que se transformam em macrófagos nos tecidos.

Os macrófagos são fagócitos que participam ativamente na defesa do hospedeiro frente às infecções. Têm a capacidade de internalizar partículas inertes, células alteradas do indivíduo, micro-organismos e parasitas. Dessa forma, a fagocitose constitui um dos principais fenômenos que ocorrem no processo inflamatório.

Além de apresentarem uma alta capacidade fagocítica, os macrófagos são as células mais frequentes dentre as que residem no pulmão (RUFINO e SILVA, 2006). São derivados da medula óssea, pertencem ao sistema dos fagócitos mononucleares e constituem a segunda maior população celular do sistema imune. Após o contato com o agente agressor, agem liberando citocinas (MÁRTON e KISS, 2000) e outros potentes produtos microbicidas que são responsáveis pela destruição dos micro-organismos fagocitados (AMERSFOORT et al., 2003).

Em geral, os eventos que decorrem da sepse e do choque séptico são causados por endotoxinas produzidas por bacilos Gram negativos. Essas substâncias se localizam na parte

externa da membrana bacteriana e são liberadas a partir da sua replicação e/ou morte (KLEIN et al., 2011). A endotoxemia aguda, caracterizada por altos níveis de endotoxina no sangue, causa reação inflamatória com injúria endotelial, hipotensão, falência múltipla dos órgãos e morte (SUNIL et al., 2002).

Dentre os órgãos envolvidos no choque séptico, o pulmão é o primeiro a ser atingido (D'ACAMPORA et al., 2001). Durante a endotoxemia, as complicações pulmonares, incluindo edema pulmonar e falência respiratória, são a maior causa de morbi-mortalidade em pacientes sépticos (WANG et al., 1994). O lipopolissacarídeo (LPS) ativa macrófagos e com isto, induz a produção e liberação de inúmeras citocinas (MENG e LOWELL, 1997; WEINSTEIN et al., 2000). Estas substâncias induzem a resposta de fase aguda da inflamação e capacitam o sistema imune para uma atividade rápida frente aos processos infecciosos.

2.2 Fusão de macrófagos

Os macrófagos estão presentes em todos os tecidos do corpo e, no combate à infecção, podem fusionar entre si em resposta à patógenos e materiais estranhos dando origem aos osteoclastos (no osso) ou às células gigantes multinucleares (CGM) (em vários tecidos). Também podem fusionar com células somáticas e tumorais (VIGNERY, 2005). Nas alterações teciduais, os osteoclastos e as células gigantes exercem suas funções na osteoporose e nas doenças inflamatórias crônicas, respectivamente. A formação destas últimas é conhecida por aumentar a capacidade defensiva dos macrófagos (MACLAUCHLAN et al., 2009).

Os mecanismos moleculares que permitem a fusão dos macrófagos entre eles ainda são pouco conhecidos (VIGNERY, 2005). Algumas moléculas têm sido implicadas nesse processo o qual pode envolver a ação de glicoproteínas, como as caderinas, que medeiam a fixação da membrana e fusão. Além disso, a fusão entre os macrófagos pode ser induzida por mediadores solúveis como citocinas e fatores de crescimento (HELMING e GORDON, 2008).

As caderinas representam uma família de moléculas de adesão celular transmembranares, dependentes do cálcio, que se ligam através de ligações homofílicas. São importantes não apenas no estabelecimento e manutenção das uniões intercelulares, mas também na determinação da especificidade adesiva das células (CAVALCANTE, 2008). Geralmente, células com poucas moléculas de caderina apresentam menor adesividade. O cálcio é

essencial para a função adesiva das caderinas e na proteção contra a digestão pelas proteases (WHEELOCK e JOHNSON, 2003; CAVALCANTE, 2008).

As caderinas são divididas em subclasses de acordo com sua distribuição tecidual. A nomenclatura deriva-se do tecido no qual estas glicoproteínas foram inicialmente identificadas. A E-caderina foi inicialmente identificada em células epiteliais. A caderina placentária (P-caderina) foi encontrada inicialmente em placenta e no epitélio, embora possa ser expressa em outros tecidos durante o desenvolvimento (CAVALCANTE, 2008). Os anticorpos de amplo espectro anti-pan caderinas são capazes de detectar diversos tipos de caderinas a partir de células em cultura.

Estudos mostram que a E-caderina contribui para a formação das CGM por intermédio da citocina anti-inflamatória IL-4 (MORENO et al., 2007; HELMING e GORDON, 2009). Esta citocina é secretada por várias células imunes (MARTINEZ et al., 2009), incluindo os macrófagos (FERNANDEZ-BOYANAPALLI et al., 2009). Há mais de 10 anos já vem sendo mostrado que a IL-4 induz a formação das CGM *in vitro* (McINNES e RENNICK, 1988; McNALLY e ANDERSON, 1995). Moreno et al. (2007) demonstraram que a IL-4 contribui para um aumento na expressão de E-caderina, e consequentemente, aumento da formação de CGM *in vitro*.

Segundo Helming e Gordon (2009), para se tornarem multinucleares, os macrófagos precisam adquirir um estado de competência para a fusão. Isso acontece através da ação de estímulos endógenos e exógenos. Após se ligar ao receptor IL-4 de cadeia α, a IL-4 induz a competência para a fusão através da ativação do fator de transcrição STAT6 como também pelo contato macrófago-macrófago **(Figuras 1 e 2)** . Somente após isso é que se dá a adesão entre as células mediada pela E-caderina **(Figura 2)**. Além da IL-4, outras citocinas como IL-13 e interferon-γ (IFN-γ) também podem induzir a formação das CGM (HELMING e GORDON, 2008, 2009). Esta última tem um papel na ativação dos macrófagos, porém pouco ainda se sabe sobre os mecanismos envolvidos nesse processo de fusão.

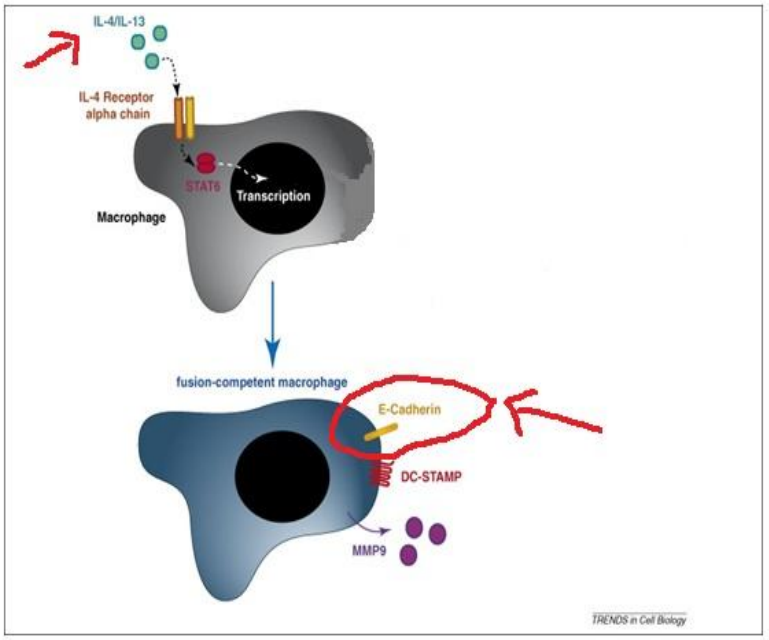

Figura 1. Programação de macrófagos em um estado competente para a fusão. Fonte: Helming e Gordon, 2009

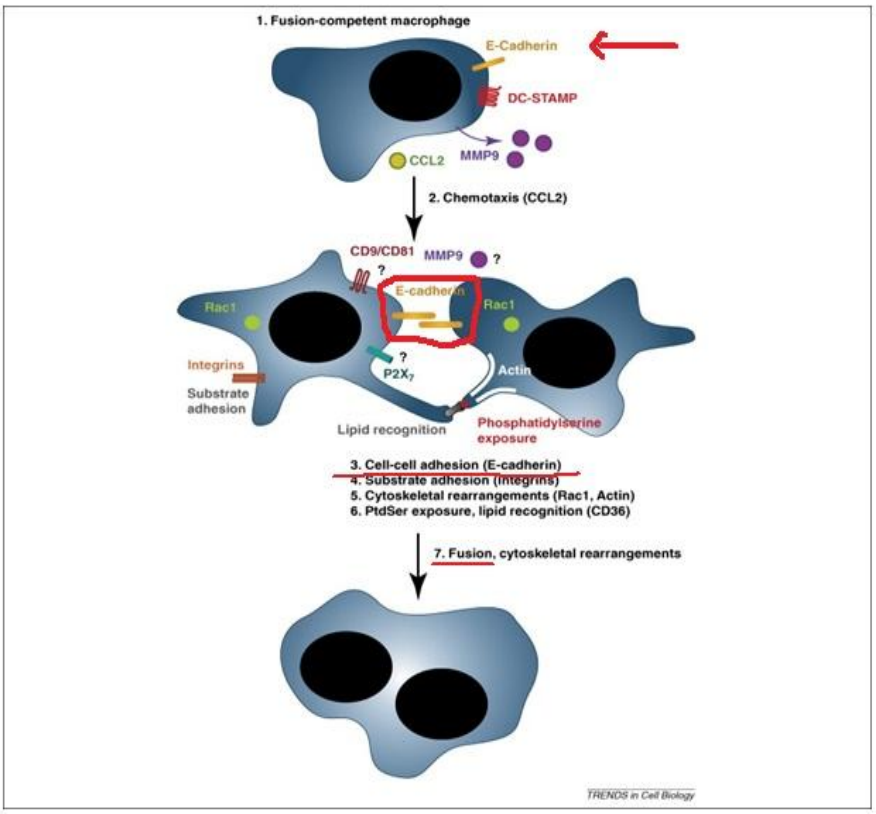

Figura 2. Mecanismo molecular de fusão do macrófago. Fonte: Helming e Gordon, 2009

2.3 Infecção e comprometimento do músculo esquelético

Em condições normais, a massa muscular é mantida pelo balanço entre síntese e degradação protéicas e a perda do músculo pode ocorrer em qualquer situação que perturbe este equilíbrio. Durante a infecção e trauma grave a proteína muscular sofre proteólise, sendo rapidamente mobilizada para atender às necessidades de defesa orgânica na síntese de proteínas de fase aguda como a proteína C reativa (PCR), anticorpos e imunoglobulinas, entre outros (BURET, 2008; FEFERBAUN et al., 2009).

A perda da massa muscular é comumente vista em pacientes sépticos e com febre. Nestes, ocorre uma perda marcante de peso e de proteína corporal, além de uma perda de músculo generalizada (HASSELGREN e FISCHER, 1998). Câncer e AIDS são doenças associadas com perda significante do músculo, e o desenvolvimento da disfunção muscular está associada a uma resposta deficitária ao tratamento (MUSCARITOLI et al., 2006; SUPINSKI e CALLAHAN, 2007).

O prejuízo na capacidade funcional do músculo esquelético é uma das principais causas de morbidade em pacientes com doenças agudas e crônicas tais como pneumonia, infecções generalizadas que causam bacteremia, síndrome do desconforto respiratório agudo (SDRA), uremia, trauma, entre outras (SUPINSKI e CALLAHAN, 2007). Nessas situações, a deficiência nutricional pode contribuir para a perda do músculo e a inflamação sistêmica pode ser o fator chave causador da disfunção do músculo esquelético. Os níveis circulantes de várias citocinas estão aumentados em pacientes com sepse (BELPERIO et al., 2006; CONRAADS, 2006).

Em animais experimentais, a administração de citocinas, como também a indução de produção, têm sido implicadas em causar perda muscular (BUCK e CHOJKIER, 1996; SUPINSKI e CALLAHAN, 2007). De acordo com Li et al. (2003), a incubação de linhagens celulares musculares com citocinas causa uma perda de proteínas celulares. Estudos mostram que a administração de *Streptococcus pneumoniae* (RUFF e SECRIST, 1984) ou endotoxina de *Escherichia coli* (FRICK et al., 2008) é responsável pela perda significante de massa muscular e também pela quebra mais rápida das proteínas musculares em ratos.

2.4 Células musculares esqueléticas e processo de fusão na formação da fibra

O sistema muscular esquelético constitui a maior parte da musculatura do corpo. As células satélites musculares são uma população de células miogênicas, mononucleares e indiferenciadas. Essas células estão presentes no músculo esquelético e estão associadas a todos os tipos de fibras musculares. Quando cultivadas *in vitro*, podem ser caracterizadas através da expressão do gene Pax7 (CHARGÉ e RUDNICKI, 2004). O Pax7 é um fator de transcrição importante para o desenvolvimento do músculo e é expresso em células satélites quiescentes e em proliferação (FOSCHINI et al., 2004). Após proliferarem em resposta a estímulos fisiológicos, as células satélites dão origem à mioblastos que se diferenciam e fusionam.

Os mioblastos, que são as células precursoras das fibras musculares esqueléticas, se fusionam em duas fases. Na primeira fase (mioblasto x mioblasto), os mioblastos diferenciados se fusionam para formação de um miotubo primário com um número limitado de núcleos. Na segunda fase de fusão (mioblasto x miotubo), os mioblastos se fusionam com os miotubos primários originando os miotubos secundários ou maduros (PAVLATH e HORSLEY, 2003) **(Figura 3)**.

Existem moléculas de adesão celular, como caderinas e integrinas, que são importantes para a fusão das células musculares esqueléticas. As caderinas são proteínas transmembranares dependentes do cálcio que conferem a adesão entre células vizinhas (ANGST et al., 2001). As N e M caderinas são cruciais para a fusão dos mioblastos em mamíferos. (MEGE et al., 1992; WRÓBEL et al., 2007).

As integrinas são proteínas de membrana envolvidas em diversos processos biológicos como embriogênese, inflamação e agregação plaquetária (BUTERA WADSWORTH, 2003). São proteínas heterodiméricas, originalmente conhecidas como receptores para moléculas da matriz extracelular. Também medeiam a adesão célula-célula por se ligarem através de ligações heterotípicas a membros da superfamília das imunoglobulinas (CAVALCANTE, 2008). De acordo com Schwander et al. (2003), as integrinas β1, que estão localizadas na superfície dos mioblastos, exercem um papel importante na fusão dessas células.

Estudos vêm mostrando o papel de efetores que fazem parte da resposta imune sobre a proliferação dos mioblastos (CHARGÉ, 2003). Em trabalhos realizados por Horsley et al.

(2003), confirmou-se a atuação da IL-4 sobre proliferação e fusão durante a regeneração muscular. Trata-se da primeira demonstração da expressão de IL-4 por uma célula não imune. O estudo de Horsley et al. (2003) implica a via de sinalização de NFATc2 (fator nuclear de ativação de células T) como responsável pela ativação do gene interleucina 4 (IL-4) e consequente fusão de células musculares (mioblasto x miotubo) através da interação desta citocina, produzida pelo miotubo primário, com o seu receptor (IL-4Rα) presente sobre os mioblastos. Segundo Chargé (2003), a mesma via de sinalização que ativa a fusão de células musculares também atua na fusão de macrófagos, o que sugere a possibilidade de uma fusão ilegítima entre macrófagos e miotubos.

A formação da fibra muscular também depende da presença de um fator de transcrição chamado miogenina. Essa proteína é expressa em todos os mioblastos a partir do início da diferenciação e sua expressão continua durante a fusão celular, marcando também o final da proliferação dos mioblastos (TE PAS et al., 1999). De acordo com Isobe et al. (1998), a expressão da miogenina é necessária em várias etapas da maturação, mesmo após a formação do miotubo.

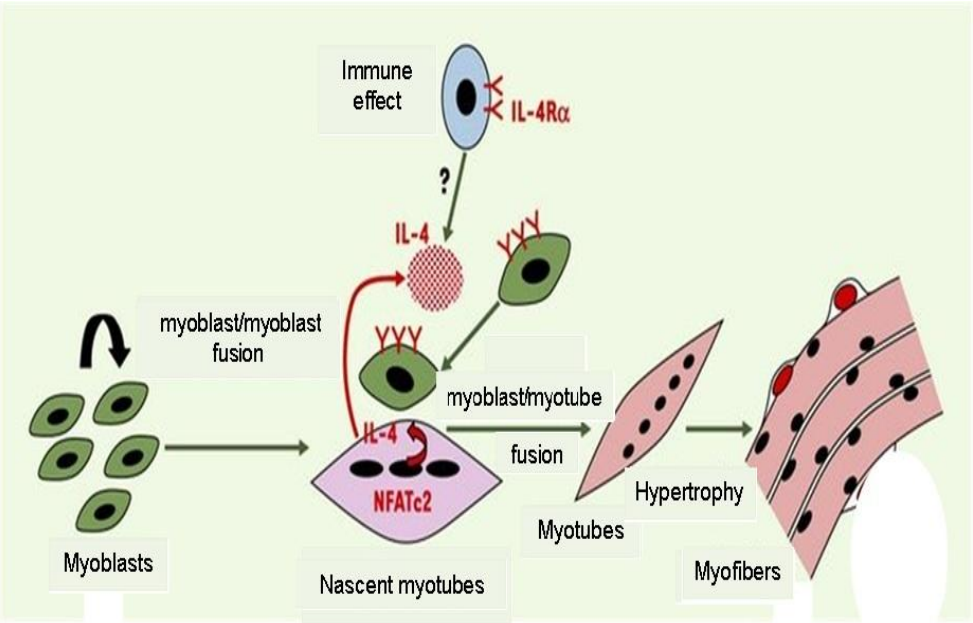

Figura 3. Fusão das células musculares esqueléticas. Fonte: Chargé, 2003

2.5 Desnutrição e sistema imune

A deficiência nutricional durante o período crítico de desenvolvimento dos sistemas de homeostase, entre eles o sistema imune, é capaz de interferir na formação de processos essenciais para a defesa do hospedeiro frente às infecções. Nessa etapa da vida, agressões nutricionais poderão alterar o padrão de eventos celulares, com consequências deletérias tanto na aquisição de padrões fisiológicos maduros do organismo (BORBA et al., 2000) quanto para a ocorrência de eventos metabólicos (OZANNE e HALES, 2002; LUCAS, 2005).

Informações epidemiológicas mostram que a desnutrição protéica é um problema de saúde pública que há tempos acomete grande parcela da população mundial, principalmente nos países em desenvolvimento (GUBERNATIS, 2006; FAO, 2010). Segundo dados da Organização das Nações Unidas para Agricultura e Alimentação (FAO), o número de pessoas desnutridas no mundo chegou a 965 milhões em 2010 (FAO, 2010). No entanto, a evolução do estado nutricional da população brasileira passou por um processo de transição nos últimos anos. Ocorreu um declínio da prevalência de desnutrição infantil e elevação da prevalência de sobrepeso/obesidade em adultos. (BATISTA FILHO e RISSIN, 2003). A desnutrição durante o período crítico de desenvolvimento dos sistemas orgânicos vem sendo apontada como um dos principais fatores não genéticos implicados na etiologia de doenças infecciosas e outras, como por exemplo, diabetes tipo II e obesidade (BARKER, 2003). De acordo com Lucas (2005), o termo programação vem sendo utilizado há alguns anos para explicar os eventos que atuam num período crítico ou susceptível do desenvolvimento e as consequências na estrutura ou função do organismo na vida adulta.

A desnutrição calórico-protéica está associada com a redução da imunidade mediada por células, da função fagocítica, do sistema complemento, da produção de anticorpos e da secreção e liberação de citocinas (CHANDRA, 2002). Nessa situação, os mecanismos de defesa pulmonares são afetados com diminuição no número de macrófagos alveolares (MA) (MELO et al., 2008; Ferreira e Silva et al., 2009). Além disso, várias funções dos macrófagos encontram-se comprometidas (MELO et al., 2008; FERREIRA E SILVA et al., 2009). Estas células, quando ativadas por endotoxinas, liberam produtos microbicidas que são responsáveis pela destruição dos micro-organismos fagocitados (MEYER et al., 1994; AMERSFOORT et al., 2003).

Tem aumentado o interesse dos cientistas pelos eventuais efeitos da privação de alimentos sobre a função imune (ALLENDE et al., 1998). Dados epidemiológicos e clínicos sugerem que deficiências nutricionais alteram a imunocompetência e aumentam o risco de infecções. A desnutrição pode acarretar prejuízos na imunidade celular, mediada por células, e na imunidade humoral, mediada pela produção de anticorpos, além de prejuízo nas funções dos macrófagos e na atividade dos neutrófilos (CHANDRA, 1997).

Estudos realizados pelo nosso grupo de pesquisa (MELO et al., 2008; FERREIRA E SILVA et al., 2009) demonstraram que a desnutrição precoce reduz o número de macrófagos alveolares e compromete a atividade oxidante dessas células em ratos adultos. Várias funções dos macrófagos encontram-se comprometidas na desnutrição (MELO et al., 2008; FERREIRA E SILVA et al., 2009). Além disso, estudos mostram que a deficiência nutricional causa uma redução na produção de anticorpos, na liberação de citocinas e na expressão de moléculas de adesão celular (CHANDRA, 2002; LANDGRAF et al., 2005).

2.6 Desnutrição e suas repercussões no sistema muscular esquelético

Estudos recentes têm contribuído para o esclarecimento de alterações energéticas na célula muscular decorrentes da desnutrição como, por exemplo, depleção enzimática, disfunção mitocondrial, fosforilação oxidativa prejudicada e potencial de membrana celular alterado (KRISTINA et al., 2005).

A desnutrição precoce é capaz de diminuir a capacidade de diferenciação de células musculares e de reduzir o número de fibras presentes no músculo (WILSON et al., 1988; BAYOL et al., 2004). Ratos cujas mães foram desnutridas com dieta à base de caseína 8% apresentaram uma redução no número de fibras musculares e uma diminuição na formação de miotubos secundários (WILSON et al., 1988). Ainda, Bayol et al. (2004) demonstraram que a desnutrição no período gestacional reduziu a celularidade no músculo após 3 semanas de reposição nutricional. Contudo, pouco tem sido pesquisado sobre o efeito da desnutrição somente durante a lactação no desenvolvimento do músculo esquelético.

O processo miogênico é regulado por fatores de transcrição, como: Pax3/Pax7 e fatores regulatórios miogênicos, como: MyoD, miogenina, Myf5 e Myf6 envolvidos diretamente na formação dos músculos (COSSU et al., 1996). Segundo Halevy et al. (2003), a

restrição alimentar diminui a expressão dos marcadores da linhagem miogênica Pax7 e miogenina. De acordo com Landgraf et al. (2005), ocorre uma redução da expressão de moléculas de adesão celular em ratos adultos jovens que foram desnutridos no período gestacional.

Apesar de existirem muitos estudos sobre o efeito da desnutrição sobre os sistemas biológicos, ainda há muito a ser investigado a cerca do seu efeito tardio após reposição nutricional sobre funções de células que atuam em conjunto no processo inflamatório/infeccioso. Diante disso, a realização de trabalhos experimentais utilizando modelos de desnutrição neonatal pode esclarecer os questionamentos a respeito do impacto que este modelo pode causar na fusão de células macrofágicas e miocitárias no organismo jovem e adulto. Isto pode auxiliar na compreensão do processo inflamatório/infeccioso nestes organismos e, desta forma, contribuir na definição de estratégias adequadas para o controle das doenças infecciosas.

3. HIPÓTESES

A desnutrição neonatal, seguida de reposição nutricional, causa na idade jovem e adulta do animal:

- Redução na expressão das moléculas de fusão Pan-caderina de macrófagos alveolares e de células musculares esqueléticas;
- Redução na expressão das moléculas de fusão β1-integrina e IL-4Rα em células musculares esqueléticas;
- Redução na produção de IL-4 por macrófagos alveolares e células musculares esqueléticas;
- Redução na produção de IFN-γ por macrófagos alveolares;
- Redução na expressão dos fatores transcricionais Pax7 e miogenina (marcadores da linhagem miogênica);
- Redução do número de células totais através da diminuição na liberação de lactato desidrogenase (LDH) por macrófagos alveolares e células musculares esqueléticas.

4. OBJETIVOS

4.1 Geral

- Avaliar a expressão/produção de moléculas de fusão e fatores transcricionais em macrófagos alveolares e células musculares esqueléticas de ratos jovens e adultos submetidos à desnutrição neonatal.

4.2 Específicos

Comparar entre grupos de ratos nutridos e desnutridos no período neonatal seguidos de reposição nutricional:

- Expressão de Pan-caderina de macrófagos alveolares e de células musculares esqueléticas em cultura;
- Expressão de β1-integrina de mioblastos em cultura;
- Expressão de IL-4Rα de mioblastos em cultura;
- Níveis de IL-4 em sobrenadantes de cultura de macrófagos alveolares e miotubos;
- Níveis de IFN-γ em sobrenadantes de cultura de macrófagos alveolares;
- Expressão dos marcadores da linhagem miogênica Pax7 e miogenina;
- Quantidade de células através dos níveis de lactato desidrogenase (LDH) em sobrenadantes de cultura de macrófagos alveolares e de células musculares esqueléticas.

5. MATERIAIS E MÉTODOS

5.1 Desenho do estudo

Trata-se de um estudo do tipo experimental. A escolha desse desenho metodológico foi determinada pela grande comparabilidade entre os grupos em termos de variáveis de confundimento e facilidade na formação do grupo controle. A comparabilidade é garantida pela homogeneidade que os grupos apresentam quanto à idade, sexo, raça, tipo de dieta, etc.

5.2 Animais e dietas

Foram utilizados 18 ratos machos, albinos, da linhagem Wistar, provenientes da colônia de criação do Departamento de Nutrição da Universidade Federal de Pernambuco (UFPE) e 18 animais do Centre d'élevage Dépré (Saint-Doulchard, France). Os ratos foram mantidos no biotério sob temperatura de 23 ± 2^0C, ciclo claro-escuro de 12:12 h (com luzes acesas às 6 h), recebendo *ad libitum* as respectivas rações e água até o início dos experimentos.

Ratos com mais de 90 dias de idade foram acasalados pelo sistema poligâmico ou harém, com a combinação de 1 macho/2 ou 3 fêmeas alojados em gaiolas de polipropileno (49x34x16cm). Para obtenção dos animais experimentais, após o nascimento, o número de filhotes por mãe foi padronizado em 6. Isto foi obtido reduzindo-se as proles ou acrescentando-lhes animais da mesma idade, provenientes de outras ninhadas de forma que os filhotes fossem distribuídos aleatoriamente entre as ninhadas do estudo. Após o nascimento dos animais, estes foram divididos em 2 grupos de acordo com a dieta materna durante os primeiros 21 dias pós-natais (período de aleitamento): **Nutrido (N)** (n=9) com dieta normoprotéica à base de caseína a 17% (AIN 93G) **(Tabela 1)**, e **Desnutrido (D)** (n=9**)** com dieta experimental hipoprotéica à base de caseína a 8% (AIN 93G) **(Tabela 1)**. Após o desmame, os filhotes foram separados de suas mães, mantidos em gaiolas coletivas (contendo 3 animais cada), e receberam a dieta do biotério (LABINA – Purina do Brasil S/A ou

TEKLAD GLOBAL – Harlen Teklad) até completarem 42 dias (n=6), 60 dias (n=6) e 90 dias de vida (n=6) **(Figura 4)**.

Tabela 1. Composição das dietas experimentais à base de caseína (17% nutrido e 8% desnutrido)

Ingredientes	**Quantidade para 1 Kg de dieta**	
	8%	**17%**
Caseína	79,3 g	179,3 g
Mistura vitamínica	10 g	10 g
Mistura mineral	35 g	35 g
Celulose	50 g	50 g
Bitartarato de colina	2,5 g	2,5 g
DL-Metionina	3,0 g	3,0 g
Óleo de soja	70 mL	70 mL
Amido de milho	750,2 g	650,2 g

* Composição das dietas segundo a AIN-93G – Reeves et al., 1993

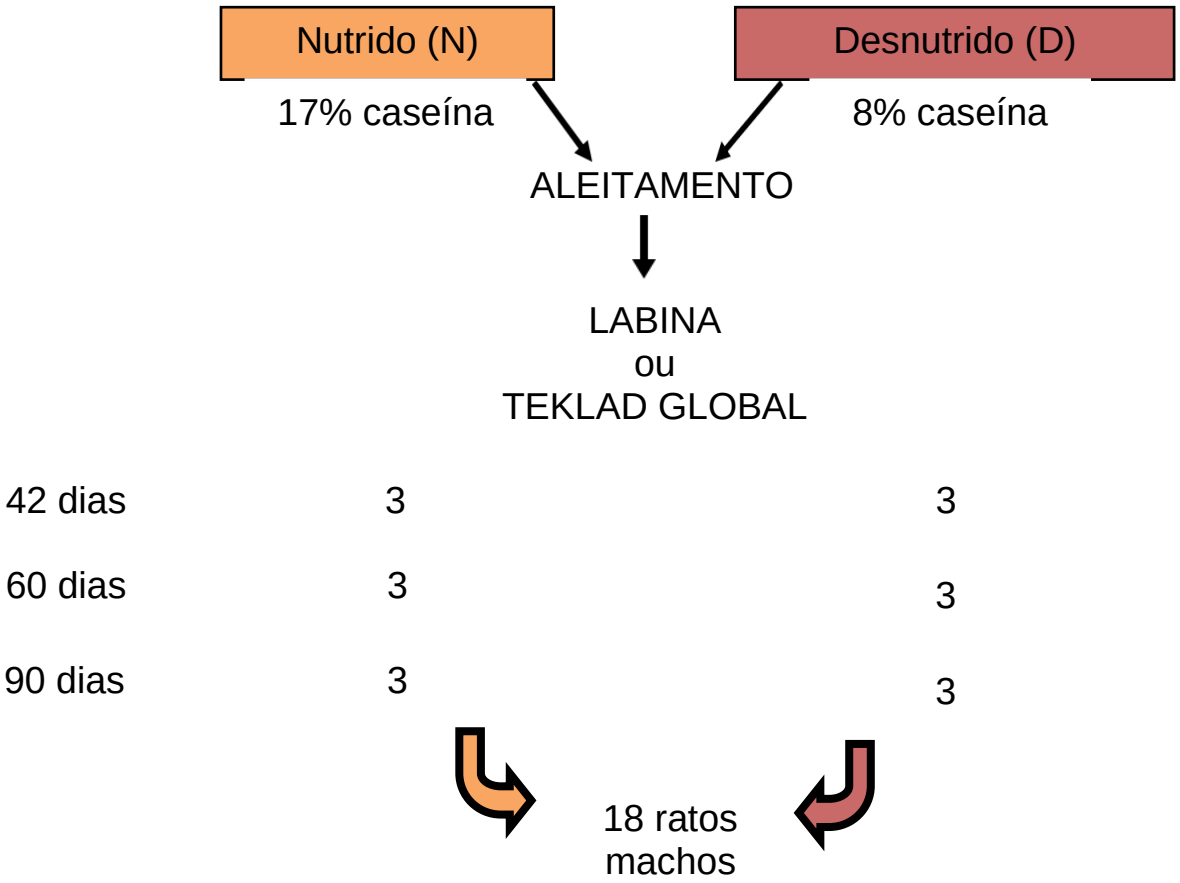

Figura 4- Grupos experimentais

5.3 Operacionalização da pesquisa

5.3.1 Peso Corporal

Durante os primeiros 21 dias após o nascimento, foram registrados a cada cinco dias os pesos corporais de cada animal, a fim de acompanhar o peso durante a manipulação nutricional. A partir do 22° dia de vida até o final do experimento, o peso foi aferido 1 vez por semana, objetivando acompanhar a evolução ponderal durante a fase de reposição nutricional dos animais.

5.3.2 Lavado broncoalveolar (LBA)

O lavado broncoalveolar foi obtido de acordo com a técnica utilizada por De Castro et al. (1995). Os animais foram anestesiados com ketamina (45,2 mg/kg) e xilazina (6,8 mg/kg) via intramuscular. Em seguida, o LBA foi coletado com a injeção de salina a 0,9% através de uma cânula plástica inserida na traquéia **(Figura 5)**. Várias alíquotas de 3 mL foram injetadas e coletadas em tubos cônicos de polipropileno de 50 mL (Falcon, Sigma). Foi recuperado aproximadamente 30 mL de LBA para cada animal.

Figura 5- Coleta do lavado broncoalveolar

5.3.3 Cultura de macrófagos alveolares

O LBA recolhido foi centrifugado a 450 g durante 15min. O precipitado que corresponde às células foi ressuspendido em meio de cultura (RPMI 1640, Gibco-Invitrogen Corporation) contendo soro fetal bovino (3%, Gibco-Invitrogen Corporation) e antibióticos (penicilina 100U/mL e estreptomicina 100µg/mL). As células foram transferidas para placas de cultura de 35 mm de diâmetro (6 poços, Falcon), onde foram dispensados 2 mL da

suspensão em uma proporção de $8x10^5$ células/mL de RPMI 1640 em cada poço. Em placas de cultura de 96 poços (0,33cm^2/poço), foram depositadas $8x10^3$ células/poço em um volume final de 100µL de RPMI 1640 para determinação do número total de células. Após 1h na incubadora a 37°C e 5% CO_2, o sobrenadante com as células não aderentes foi desprezado e foi adicionado 2 mL (placas 6 poços) e 100µL (placas 96 poços) de meio RPMI deixando-se as placas por 4 dias em incubadora **(Figura 6)**. O meio RPMI foi trocado a cada 2 dias durante esse período de cultura, e os macrófagos foram estimulados *in vitro* com LPS (sorotipo de *Escherichia coli*; 055:B5, Sigma) na dose de 10µg/mL em NaCl a 0,9% por 24 horas no 3º dia de cultura.

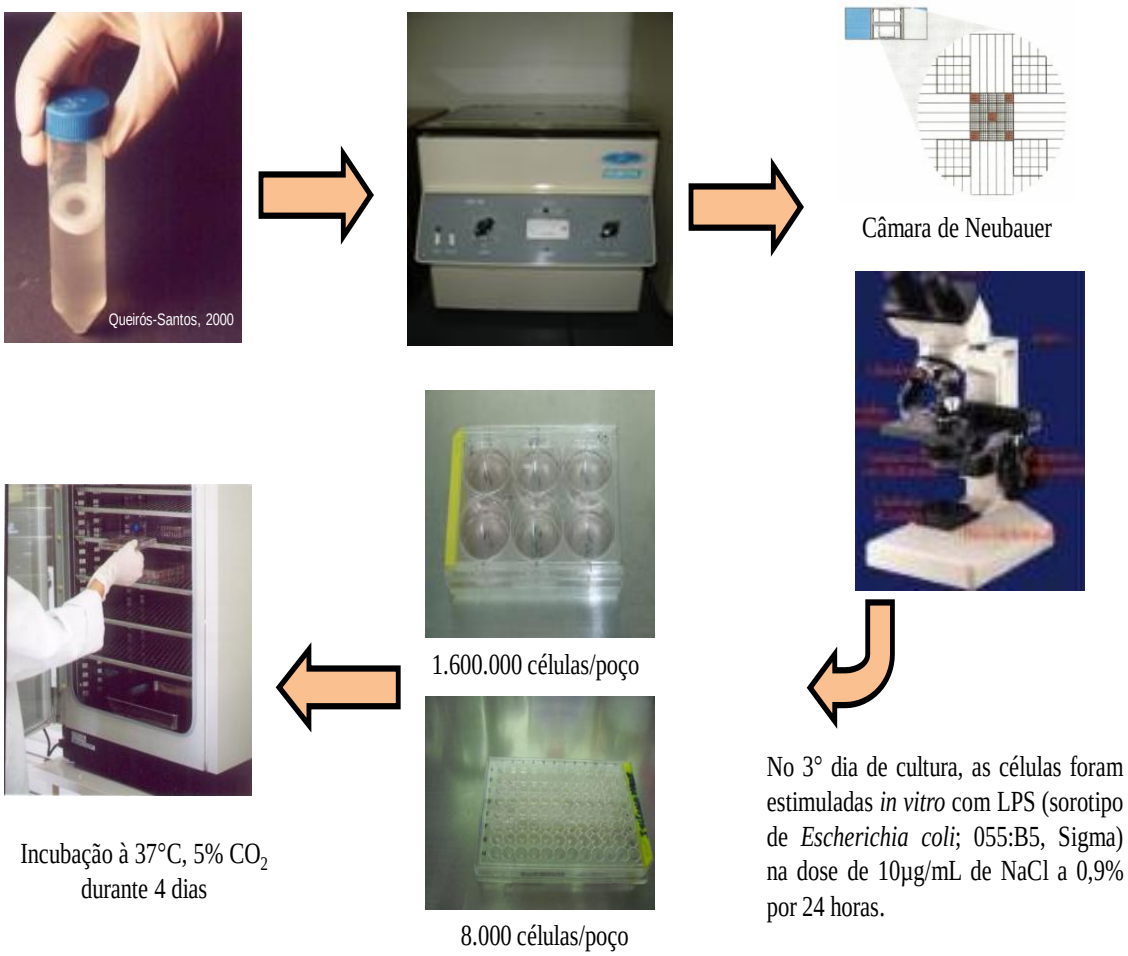

Figura 6- Obtenção e cultura dos macrófagos

5.3.4 Isolamento e cultura de células musculares

Todos os músculos foram retirados de ambas as patas dos animais **(Figura 7)** e isolados em 15 mL de tampão fosfato-salina (PBS) contendo 0,15g de glicose e 1% de solução antibiótica. Os ratos foram anestesiados com ketamina (45,2 mg/kg) e xilazina (6,8 mg/kg) via intramuscular.

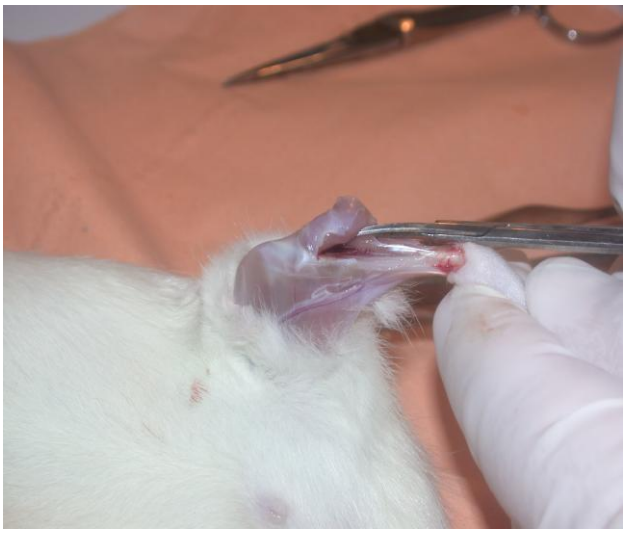

Figura 7- Retirada dos músculos das patas dos ratos para cultura *in vitro*

Um dia antes do experimento, placas de cultura de 80cm^2 (Nunclon®) foram cobertas com 2 mL de Matrigel® (BD Biosciences, Le Pont de Claix, France) diluído 1:500 em meio de cultura DMEM (1g/L glicose) e incubadas à 37°C e 10% CO_2. Os músculos foram cortados em pequenos pedaços e, com o auxílio de tesouras ou bisturis, a gordura e os tendões removidos. Os pedaços de músculo foram triturados e então colocados em 50 mL de meio de crescimento primário (PGM) contendo DMEM (1g/L glicose), soro fetal bovino à 10%, soro de cavalo à 10%, 4mMol/L de L-glutamina e 1% de solução antibiótica e centrifugados a 200 g durante 4min à 4°C. Os sobrenadantes foram coletados e o precipitado ressuspendido em 15mL de DMEM (1g/L glicose) suplementado com 0,03g de colagenase tipo II e 1% de solução antibiótica, e incubados durante 90 min à 37°C sob agitação. Esta operação foi repetida duas vezes utilizando-se 15 mL de DMEM (1g/L glicose) suplementado com 2 mL de tripsina a 0,25% sem EDTA, 2mL de DNAse 1mg/mL de PBS (Sigma) e 1% de solução

antibiótica em adição de 0,04g de colagenase. Os precipitados foram então misturados com os sobrenadantes coletados e filtrados (filtros 50µm, D Deutscher, Issy les Moulineaux, France). As células foram contadas em Câmara de Malassez e transferidas para as placas de cultura na proporção de $1,6x10^6$ células/placa de $80cm^2$ com volume final de 10 mL de PGM. Em placas de cultura de 96 poços ($0,33cm^2$/poço), foram depositadas $8x10^3$ células/poço em um volume final de 100µL de PGM para determinação do número total de células. Após 48h na incubadora a 37°C e 10% CO_2, o PGM foi substituído pelo meio de fusão (FM) contendo DMEM (1g/L glicose), soro de cavalo à 7%, 4mMol/L de L-glutamina e 1% de solução antibiótica. Esse meio foi trocado a cada 2 dias durante um período de cultura de 10 dias **(Figura 8)**. Todos os produtos utilizados para cultura de célula muscular, exceto o Matrigel e a DNAse, foram da marca Gibco (Invitrogen, Cergy-Pontoise, France).

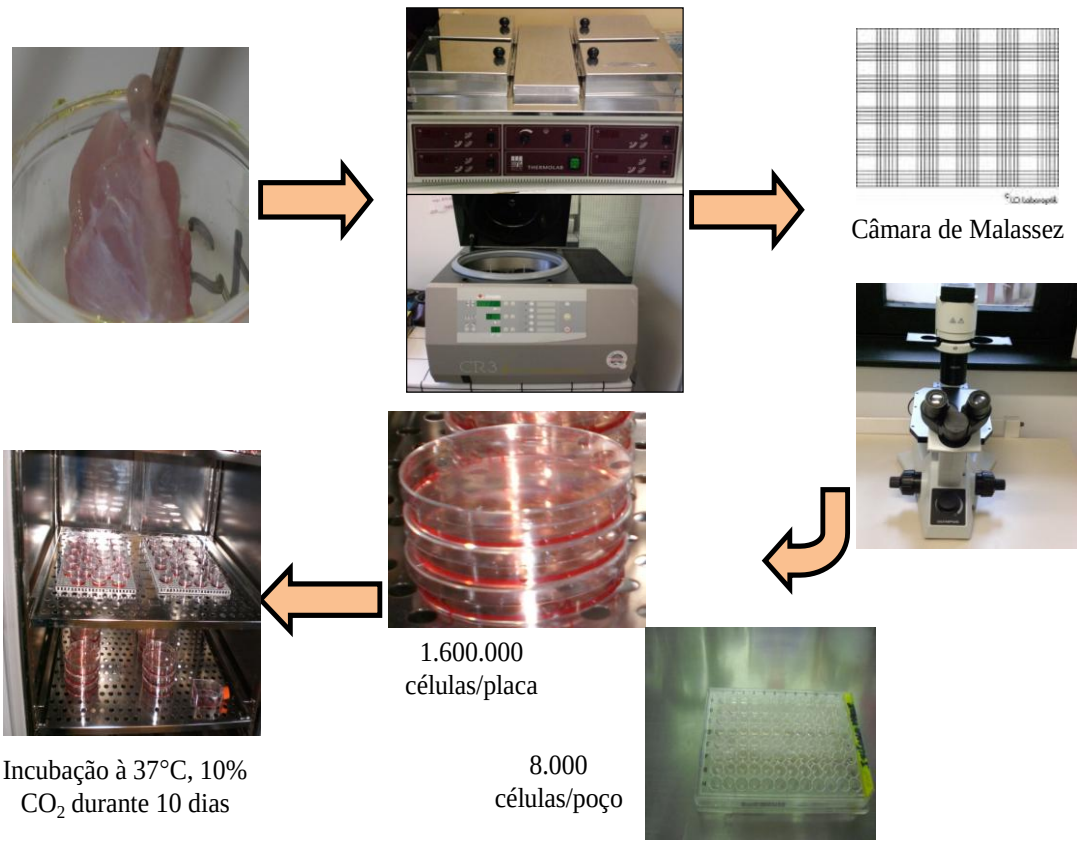

Figura 8- Obtenção e cultura de células musculares esqueléticas

5.3.5 Western Blot

Os precipitados dos macrófagos alveolares e das células musculares foram guardados à -80°C e utilizados para avaliação da expressão de Pan-caderina (em macrófagos) e de Pan-caderina, β1-integrina, IL-4Rα, Pax7 e miogenina (em células musculares) através da técnica de Western Blot. Foram utilizados precipitados de macrófagos estimulados *in vitro* com LPS por 24 horas e precipitados de células musculares cultivadas por 10 dias (0, 4, 7 e 10 dias). Para os macrófagos, a expressão de Pan-caderina foi avaliada no dia 4 de cultura. Para as células musculares, a expressão de Pax7 foi avaliada nos dias 0 e 4 de cultura; Pan-caderina foi avaliada no dia 4 de cultura; β1-integrina nos dias 4 e 7; IL-4Rα no dia 7 e a miogenina no dia 10 de cultura.

As células foram lisadas com tampão de lise (50 mM HEPES, 150 mM NaCl, 10% glicerol, 1% Triton X 100, 1M NaF, 100 mM PMSF, 10mg/ml aprotinina, 10 mM orthovanadato). Alíquotas dos sobrenadantes foram usadas para medir a concentração da proteína total, como descrito por Bradford (1976). Os lisados protéicos das células foram misturados com tampão Laemmli e fervidos durante 5 min. Quantidades iguais de proteínas de cada amostra (40 μg para as células musculares e 160 μg para os macrófagos) foram separadas no SDS PAGE (géis 7,5% ou 12%) e transferidas para membranas de nitrocelulose (BioRad, Marnes-la-Coquette, France). Os filtros de nitrocelulose foram embebidos em solução bloqueadora (PBS contendo 0,1% Tween 20 e 5% de leite desnatado) à temperatura ambiente por 1h e em seguida incubados overnight à 4°C com os anticorpos anti-coelho anti-Pax7 (Santa Cruz Biotechnology, Tebu-Bio, Velizy, France), anti-pan-caderina (Sigma, Saint-Quentin Fallavier, France), ou anti-IL-4Rα (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France), ou o anticorpo anti-β1 integrina de cabra (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France). As membranas foram então incubadas com anticorpo anti-IgG de coelho conjugado à HRP ou anticorpo anti-IgG de cabra ligado à peroxidase (Jackson ImmunoResearch, Interchim, Montluçon, France) durante 3h à temperatura ambiente (para os macrófagos) ou 1h (para as células musculares). As membranas foram incubadas durante 1h à temperatura ambiente com o anticorpo monoclonal anti-miogenina de camundongo (BD Biosciences, Le Pont de Claix, France) seguido por um anticorpo anti-IgG de camundongo conjugado à HRP (Jackson ImmunoResearch, Interchim). A imunoreatividade foi detectada

através de quimioluminescência (ECL e hiperfilme ECL, GE Healthcare Europe, Saclay, France). Os pesos moleculares das bandas imunoreativas foram estabelecidos através de comparação com os padrões corados (BioRad, Marnes-la-Coquette, France; Amersham full-range rainbow molecular weight markers, GE Healthcare, USA). Foram realizados Western blots para as proteínas actina e α-tubulina com o intuito de checar o carregamento do gel. As quantidades de proteínas com relação à actina ou α-tubulina foram determinadas e as intensidades das bandas quantificadas usando-se o software de análise de imagem KODAK 1D (Scientific Image System, Rochester, NY, USA).

5.3.6 ELISA

As produções de IL-4 e IFN-γ foram avaliadas pelo método de ELISA utilizando-se o kit ELISA DuoSet (R&D Systems). Amostras de 100μL foram coletadas a partir do sobrenadante de cultura de macrófagos estimulados por 24 horas com LPS ou a partir do sobrenadante de células musculares cultivadas por 10 dias. As concentrações dessas citocinas foram normalizadas de acordo com a dosagem de LDH.

5.3.7 Número total de células

A quantidade total de células foi avaliada de forma indireta através da liberação da enzima lactato desidrogenase (LDH) em sobrenadantes de células cultivadas em placas de 96 poços (0,33 cm^2/poço). As células macrofágicas estimuladas com LPS por 24 horas e as células musculares cultivadas por 10 dias foram lisadas em meio de cultura sem soro e transferidas para outras placas de cultura de 96 poços. A liberação de LDH foi quantificada com o kit de avaliação de citotoxicidade CytoTox 96 Non-Radioactive (Promega Corporation), seguindo-se as instruções do fabricante. A absorbância (490nm) do vermelho de formazan produzido pela LDH foi medida em um leitor de microplaca (Modelo 680, Bio-Rad) e foi proporcional ao número total de células.

5.4 Análise Estatística

Para comparação de peso corporal, níveis de LDH e níveis das citocinas IL-4 e IFN-γ entre os grupos, foi empregada a Análise de Variância (ANOVA), para os dados paramétricos. Quando a ANOVA revelou a existência de diferença significativa, foi utilizado o Teste de Tukey, a fim de identificar que grupos diferiram entre si. Para os dados não paramétricos, foi utilizado o Teste de Kruskal-Wallis, seguido do Teste de Mann-Whitney. A significância estatística foi considerada admitindo-se um nível crítico de 5% em todos os casos.

6. RESULTADOS

6.1 Artigo 1

Title of paper: Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins

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Running Title of paper: Macrophage fusion in undernourished rats

Keywords: Critical period of development; Programming; Macrophage fusion; Neonatal malnutrition

Abstract

We investigate the effects of a neonatal low-protein diet on the number of macrophages in culture and the expression/production of proteins that regulate macrophage fusion in young and adult rats. Male Wistar rats (n=18) were suckled by mothers fed diets containing 17% protein (controls), or 8% protein (undernourished, UN). All rats were fed a normal protein diet after weaning. Bronchoalveolar lavage was collected from 42-, 60-, and 90-days-old rats. Alveolar macrophages were cultured for 4 days in order to assess the number of cells and the expression of cadherins, key proteins involved in macrophage fusion, by western blotting. IL-4 and IFN-γ levels in culture supernatants were measured by ELISA. Offspring from mothers fed a low-protein diet showed a lower body weight gain. The number of cells in cultured macrophages from UN was evaluated on 42-, 60-, and 90-days-old rats; an increase on the number of cells was observed on 42-, and 60-days-old rats, whereas a decrease was observed on 90-days-old rats. Furthermore, IL-4 production was increased in the supernatants from UN group of 60-days-old rats, but did not affect the expression of cadherins. IFN-γ production increased in the supernatants from UN group of 42-, and 60-days-old rats, and decreased in the later group. This study, thus, demonstrates that dietary restriction during lactation alters the number of alveolar macrophages in culture, and the production of fusion proteins of offspring aged 42, 60, or 90 days, but does not modify the expression of adhesion molecules, which are important for the fusion of those cells.

Introduction

Malnutrition during the critical period of development of systems of homeostasis, including the immune system, may impair the formation of processes essential for host defense against infections. Changes at this stage in response to the environment can cause permanent effects in organs and tissues, a phenomenon known as programming[1]. At this stage of life which includes prenatal and neonatal periods, nutritional assaults may alter the pattern of cellular events, with deleterious consequences both for acquiring mature physiological patterns of the organism[2] and for the occurrence of metabolic events[1-3]. Experimental models in rats have shown that perinatal malnutrition may induce metabolic diseases in adulthood[3,4].

Protein-calorie malnutrition is associated with a reduction in cell-mediated immunity, phagocyte function, complement system, antibody production, cytokine release and expression of cell adhesion molecules[5,6]. In this situation, the pulmonary defense mechanisms are affected with a resulting decrease in the number of alveolar macrophages (AM)[7]. In addition, several functions of macrophages are compromised[7]. These cells, when activated by endotoxins, release microbicide products that are responsible for the destruction of phagocytosed microorganisms[8,9].

Macrophages are present in all body tissues and to fight infection, may fuse with each other in response to pathogens and foreign materials, giving rise either to osteoclasts (bone) or multinucleated giant cells (MGC) (in various tissues). The formation of MGC is known to increase the defensive capacity of macrophages[10]. The molecular mechanisms that allow the fusion of macrophages with one another are still poorly understood[11]. Some molecules have been implicated in this process which may involve the action of glycoproteins such as cadherins, which mediate membrane attachment and fusion. Studies show that IL-4 contributes to an increased expression of E-cadherin and, consequently, an increased formation of MGC *in vitro*[12,13]. After binding to the IL-4 receptor α chain, IL-4 induces competence for fusion through the activation of the transcription factor STAT6 as well as by macrophage-macrophage contact. It is only after this event that adhesion between cells mediated by E-cadherin occurs. In addition to IL-4, other cytokines such as IL-13 and interferon-γ (IFN-γ) can also induce the formation of the MGC[13,14]. The latter has a role in

the activation of macrophages, but little is known about the mechanisms involved in this fusion process.

Previous studies have reported the relationship between neonatal malnutrition and impaired macrophage function in adulthood[7]. However, much remains to be investigated about its late effects, after nutritional recovery on macrophage fusion and on the behavior of these cells in culture. In the present study, we evaluated the effects of neonatal malnutrition on the number of alveolar macrophages in culture and the expression/production of proteins that regulate the macrophage fusion in rats aged 42, 60 and 90 days.

Materials and methods

Ethical Considerations

This research was approved by the Ethics Committee on Animal Experimentation of the Center for Biological Sciences of the Federal University of Pernambuco (protocol number 23076.005512/2008 – 40).

Animals and diet

Pregnant female Wistar rats were obtained from the Biotery of the Department of Nutrition (Federal University of Pernambuco - UFPE, Brazil). The animals were housed under conditions of controlled temperature (22 ± 1°C) in a 12h light-dark cycle. The standard animal laboratory food and water were given *ad libitum*. At the time of delivery, the litter size and pups' birth weight were recorded. During the suckling period, the offspring were kept in groups of six pups, randomly distributed into two nutritional groups according to their mother's diet: a well-nourished group (control, C) fed by mothers receiving a 17% protein (casein) diet, and a low-protein group (undernourished, UN), fed by mothers receiving a 8% protein (casein) diet (Table 1). After weaning (at the age of 22 days), only male offspring were used (nine per group) and kept in the collective cage, receiving animal laboratory food (Labina - Purina do Brasil) until the 90-day old *ad libitum*. Body weights were recorded every five days (Filizola MF-3/1, São Paulo, SP) during lactation and at 42, 60 and 90 days. Body weight gain was calculated as follows: percentage weight gain = [body weight (g) x 100/birthweight (g)] – 100 [11]. The animals were anesthetized with Ketamin (45.2

mg/kg)/Xylazin (6.8 mg/kg) by intramuscular injection at different ages (42, 60 and 90 days, n=6 per age) and sacrificed after the procedures.

Bronchoalveolar lavage fluid (BAL)

The bronchoalveolar lavage was obtained according to techniques developed by De Castro et al.[15]. BAL was collected by injecting 0.9% NaCl through a plastic cannula inserted into the trachea. Several aliquots of 3 mL were injected and collected in 50 mL conical polypropylene tubes (Falcon, Sigma). Approximately 30 mL of BAL was recovered for each animal.

Culture of alveolar macrophages (AM)

The collected BAL was centrifuged at 470 x g for 15 min. The precipitated part, which correspond to the cells, was resuspended in a culture medium (RPMI 1640, Gibco-Invitrogen Corporation, NY, USA) containing heat-inactivated fetal calf serum (3%, Gibco-Invitrogen Corporation, NY, USA), penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL) (Sigma). Cells were placed into $35cm^2$ and 0.33 cm^2 tissue culture wells (Falcon), where 2 mL ($35cm^2$ well) and 100µL ($0.33cm^2$ well) of the suspension was discarded in the proportion of 8×10^5 cells/mL of RPMI 1640 in each $35cm^2$ well and 8×10^3 cells/100µL of RPMI 1640 in each $0.33cm^2$ well. After being incubated for 1h at 37°C and 5% CO_2, the supernatant containing nonadherent cells was discarded, and the remaining monolayers (>95% AM) were incubated for an additional 1h in 2 mL ($35cm^2$ well) and 100µL ($0.33cm^2$ well) of serum-free RPMI medium. After 3 days of culture, the cells were stimulated *in vitro* with lipopolysaccharide (LPS) (sorotype from *Escherichia coli* 055:B5, Sigma) at a dose of 10µg/mL of RPMI 1640, and 24h later the supernatants and cells precipitates were collected for study. The number of cells was measured from $0.33cm^2$ wells and the expression of proteins that regulate macrophage fusion from $35cm^2$ wells.

Number of cells

The number of cells was colorimetrically evaluated by measuring intracellular lactate dehydrogenase (LDH) activity. Cells in culture for 4 days were lysed in serum-free RPMI 1640 and transferred to a 96-well plate. The LDH concentration was quantified with the

CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. The absorbance (490nm) of the red formazan produced by LDH was measured in a microplate reader (Model 680, Bio-Rad). The absorbance was proportional to the total number of cells. LDH activity is given as the number of cells, read off from a standard curve of absorbance plotted against an increasing number of lysed cells.

Expression of Pan-cadherin

Proteins were extracted with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X 100, 1M NaF, 100 mM PMSF, 10mg/ml aprotinin, 10 mM orthovanadate). Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford[16]. Protein lysates from macrophages cultured for 4 days were mixed with Laemmli buffer and boiled for 5 min. Equal amounts of proteins of each sample (160 µg) were subjected to SDS PAGE (7.5% gels) and transferred to nitrocellulose membranes (BioRad, Hercules, CA). The nitrocellulose filters were soaked in blocking solution (PBS containing 0.1% Tween 20 and 5% non-fat dried milk) at room temperature for 1h and subsequently incubated overnight at 4°C with anti-pan-cadherin (Sigma, St. Louis, USA). The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch, Interchim, Montluçon, France) for 3h at room temperature. Immunoreactivity was detected by chemiluminescence (ECL and ECL hyperfilm, GE Healthcare, Buckinghamshire, UK). The molecular weights of the immunoreactive bands were established by comparison with stained standards (Amersham full-range rainbow molecular weight markers, GE Healthcare, USA). Western blots for actin were used to check gel loading. The ratios of proteins to actin were determined and band intensities were quantified using KODAK 1D Image Analysis Software (Scientific Image System, Rochester, NY, USA).

Concentrations of IL-4 and IFN-γ in the supernatants of macrophages in culture

The interleukin-4 (IL-4) and interferon-γ (IFN-γ) released were measured by ELISA in the supernatants from cells cultured for 4 days (DuoSet ELISA Kit, R&D Systems,

Minneapolis, MN, USA). The IL-4 and IFN-γ concentrations were normalized to 10^8 and 10^7 cells, respectively, using the LDH dosage.

Statistical analysis

The data are presented as mean ± S.E.M. For body weight analysis two-way repeated measures analysis of variance ANOVA, followed by Bonferroni's *post hoc* test was used. For LDH, IL-4 and IFN-γ levels in the supernatants of cultured cells and immunoblots quantitation groups were compared using Student's t-test for parametric data and Mann-Whitney test for nonparametric data. Results were considered statistically significant for $p < 0.05$ (NS non-significant, *$p<0.05$). The GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used.

Results

The birth weight of neonates did not differ when groups were compared (C = 6.5 ± 0.24 g and UN = 6 ± 0.27 g). During lactation, offspring from mothers fed a low-protein diet showed a lower body weight than controls at 5, 10, 15 and 21 days. The pups from undernourished mothers continued to exhibit a lower body weight than the control group throughout the experiment: 42 d (C = 187.16 ± 9.16 g; UN = 161.33 ± 1.36 g), 60 d (C = 275 ± 10.83 g; UN = 233.5 ± 5.25 g), and 90 d (C = 360 ± 12.05 g; UN = 300.33 ± 3.92 g) (Figure 1).

After 4 days of culture, LDH measurement showed a lower number of cells in the UN when compared with cells from the C group at 42 d (C group 9.32 ± 0.99 x 10^3, UN group 2.22 ± 0.15 x 10^3) (Figure 2A) and 60 d (C group 3.48 ± 0.17 x 10^3, UN group 0.79 ± 0.18 x 10^3) (Figure 2B). At 90 d, however, there was an increase in the number of cells in the UN group (C group 2.18 ± 0.12 x 10^3, UN group 3.51 ± 0.33 x 10^3) (Figure 2C). A lower number of cells was observed with the age of the C offspring.

The concentration of IL-4 in the supernatants of alveolar macrophages cultured for 4 days, normalized using LDH activity, was increased in the UN group from 60-day-old offspring (C group 54.58 ± 15.70 pg/mL x 10^8, UN group 267.44 ± 46.50 pg/mL x 10^8) (Figure 3). There were no differences between UN and C offspring at 42 d and 90 d.

The cadherin (E) content of cells cultured for 4 days was assayed using a pan-cadherin antibody. The amounts in 42-, 60- and 90-day-old pups appeared to be similar. There was no difference in the expression of cadherin in cells from UN and C pups (Figure 4).

The production of IFN-γ in the supernatants of alveolar macrophages after 4 days of culture, normalized using LDH activity, was increased in the UN group from 42-day-old offspring (C group 21.11 ± 0.02 pg/mL x 10^7, UN group 88.88 ± 0.08 pg/mL x 10^7) (Figure 5A) and 60-day-old offspring (C group 56.46 ± 0.09 pg/mL x 10^7, UN group 251.22 ± 0.50 pg/mL x 10^7) (Figure 5B). Nevertheless, at 90 d, IFN-γ levels decreased in the UN group (C group 90.48 ± 0.13 pg/mL x 10^7, UN group 56.27 ± 0.07 pg/mL x 10^7) (Figure 5C).

Discussion

Nutritional deficits are one of the most studied programming factors acting in the critical period of development[17-19]. Studies have shown that perinatal malnutrition acts in the genesis of metabolic diseases such as obesity, diabetes, and cardiovascular diseases in adulthood[19-22]. In our study, casein (a highly phosphorylated protein) was chosen because it is an important component of breast milk and has been widely used in previous studies as an inducer of perinatal malnutrition.

In this study, we first examined the effects of a low-protein diet on the body weight of the offspring during lactation. We then evaluated the number of alveolar macrophages in culture and the expression/production of proteins known to regulate macrophage fusion, from macrophage cultures of young and adult offspring. The results indicate that offspring from mothers fed a low-protein diet showed a lower body weight during the lactation period from the fifth day of life. These data are consistent with earlier studies using the same standard experimental diet[19,23].

We also verified in this experiment the influence of a low-protein diet on the animals' body weight at 42, 60 and 90 days. Our results show that even with the administration of a balanced diet – Labina (bioterium diet containing 23% mixed protein) after weaning, the body weight of the undernourished animals remained lower than that of the control animals. These results are in agreement with other studies[23-27], in which even after nutritional recovery, the body weight of undernourished animals remained low in adulthood. Passos et al.[28] demonstrated that neonatal maternal protein restriction (8% casein-restricted diet) causes

changes in milk composition and volume of lactating rats, and it may be related to less transfer of nutrients to the offspring and to a long-lasting low body weight.

Protein energy malnutrition leads to the depletion of progenitor hemopoietic populations and changes in cellular development. Hemopoietic tissue requires a high nutrient supply and the proliferation, differentiation and maturation of cells occur in a constant and balanced manner, sensitive to the demands of specific cell lineages and dependent on the stem cell population[29]. Macrophages are cells produced by the differentiation of monocytes in tissues. These latter cells are produced by the bone marrow from haematopoietic stem cell precursors called monoblasts. In the present study, we observed changes in the number of macrophages after 4 days in culture when comparing UN and C offspring. There were fewer macrophages in samples from 42 and 60-day-old UN pups. Nevertheless, in 90-day-old pups it was observed that there was an increase of the macrophage number in the UN group, suggesting that the experimental model used in this study is able to establish a compensatory mechanism, significantly impairing the proliferative capacity of these cells *in vitro*. In samples from C pups, a lower cell number was observed with the age of the offspring. Previous studies[7,25] have shown that neonatal malnutrition, even after nutritional recovery, reduces the number of alveolar macrophages in adult rats. However, in those studies, macrophages were counted from bronchoalveolar lavage fluid and were not maintained in culture. In a previous study[24], using muscle cells from young and adult rats submitted to neonatal malnutrition, a reduction in the number of cells was demonstrated in samples from 60-day-old UN pups after 10 days in culture, but no differences were observed in those from 90-day-old UN pups. Our results thus indicate that more studies are needed to evaluate the behavior of these cells *in vitro*.

In order to test the hypothesis that neonatal malnutrition can impair the formation of multinucleated giant cells (MGC), the capacity of alveolar macrophages from offspring of different ages (42, 60 and 90 days) to produce fusion proteins in culture was assessed. Studies show that cell adhesion molecules such as E-cadherins contribute to the formation of MGC through IL-4[12,13]. This cytokine is secreted by various immune cells[30], including macrophages[31]. It has been known for over 10 years that IL-4 induces the formation of MGC *in vitro*[32,33], and it was recently demonstrated that IL-4 contributes to an increased expression of E-cadherin and, consequently, to an increase in the formation of MGC *in vitro*[12,13]. The

adhesion between cells mediated by E-cadherin occurs only after the fusion competence induced by IL-4 through the activation of the transcription factor STAT6. The antibodies of broad spectrum anti-pan cadherin are able to detect different types of cadherins from cells in culture.

In 42- and 90-day-old animals, the neonatal malnutrition did not affect the production of IL-4 or E-cadherin expression in macrophages, indicating a normal formation of MGC *in vitro*. As for the 60-day-old animals that were malnourished during lactation, we observed an increased production of IL-4, but that did not contribute to an increased expression of E-cadherin. This may suggest that there is no commitment in the formation of MGC of these animals mediated by E-cadherin. In a previous study[34], using cells from the spleen, bone marrow, and peritoneal cavity, it was demonstrated that the levels of IL-4 did not differ in undernourished mice inoculated with LPS when compared to control animals. Other studies show that malnutrition can increase the production of IL-4 in $CD4^+$ and $CD8^+$ cells[35] and in peripheral blood[36] from undernourished children. Regarding E-cadherin, a previous study[24] shows that neonatal malnutrition did not impair the expression of cadherins in skeletal muscle cells of young and adult rats and that the amounts of these molecules appeared to be similar, regardless of the age of the offspring, as observed in the present study.

Lastly, we analysed the production of IFN-γ, which can also induce the formation of the MGC[13,14]. It was observed that a neonatal low-protein diet increased IFN-γ levels in the supernatants of alveolar macrophages from 42- and 60-day-old pups. Nevertheless, at 90 d, it was a reduction in the production of IFN-γ in the UN group. Our results suggest that this increased production of IFN-γ observed in younger animals may have been a compensatory mechanism resulting from the low amount of cells at these ages. IFN-γ is a key cytokine in the development of type 1 immune responses, which are required for the elimination of pathogens[37]. Also, IFN-γ induces differentiation and activation of monocytes/macrophages and enhances their microbicidal effector functions[35,38]. IL-4 is a Th2 cytokine which suppresses cellular immunity[39]. The elevated levels of IL-4 observed in this study in 60-day-old offspring indicate a modulatory mechanism to suppress the high production of IFN-γ in younger animals. As a result, the modulation in the synthesis of IFN-γ started to be observed in older animals (90-day-old) with a low production of this cytokine. Thus, due to a probable existing modulatory mechanism, we cannot suggest that the increase in the production of IFN-

γ is related to an increased formation of MGC *in vitro*. A number of studies show that malnutrition can reduce the production of IFN-γ in $CD4^+$ and $CD8^+$ cells from undernourished children[35] and spleen cells from mice[40]. However, little is known about late effects of early malnutrition on the production of these cytokines by alveolar macrophages.

Conclusion

Thus, based on the results of this study and review of the literature, casein restriction during lactation followed by the restoration of a normal protein diet at weaning has no impact on the expression of cadherins by alveolar macrophages of offspring aged 42, 60 or 90 days. Nevertheless, the number and the production of fusion proteins of macrophages in culture are altered.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures and Legends

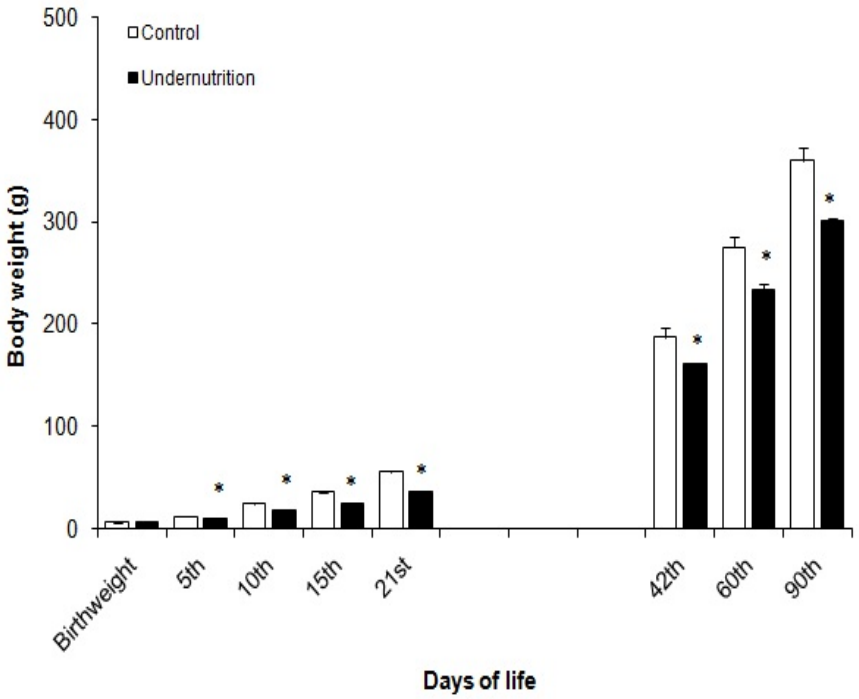

Figure 1 Body weight of pups from mothers fed a low-protein diet (undernutrition, casein 8%) or a control diet (casein 17%) during growth. Analysis was performed every five days during lactation (n=9 in each group), and at 42, 60 and 90 d (n=3 in each group). The values are presented as means + S.E.M. *$p<0.05$ compared with control group using two-way ANOVA and Bonferroni's *post hoc* test.

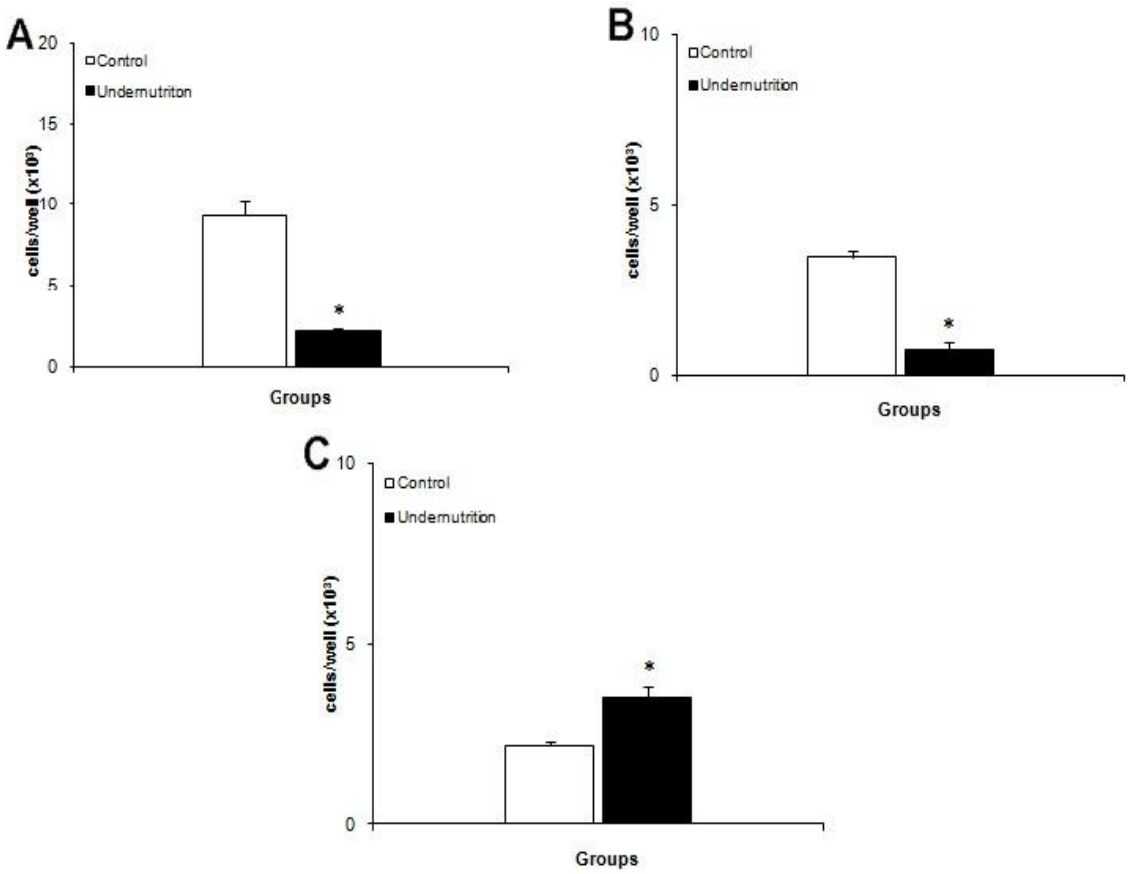

Figure 2 Numbers of macrophages/well (0.33 cm^2) after 4 days in culture (**A**:42-day-old offspring; **B**: 60-day-old offspring; **C**: 90-day-old offspring). Data were obtained from LDH activity of adherent cells. Data are expressed as mean + S.E.M. n=18 in each group. *$p<0.05$ unpaired Student's t-test.

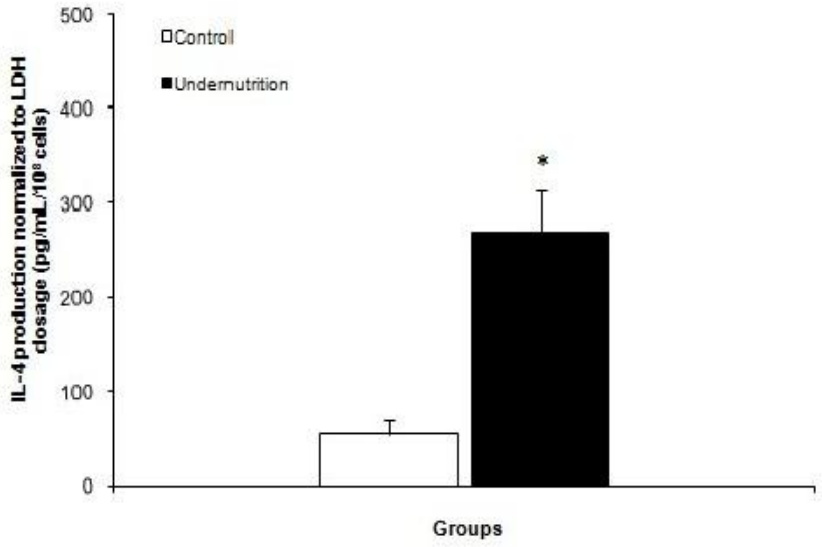

Figure 3 IL-4 production in the supernatants of macrophages from 60 day-old offspring, cultured for 4 days. Results were normalized to 10^8 cells. Data are represent as mean + S.E.M. n=6 in each group. *$p<0.05$ Mann-Whitney test.

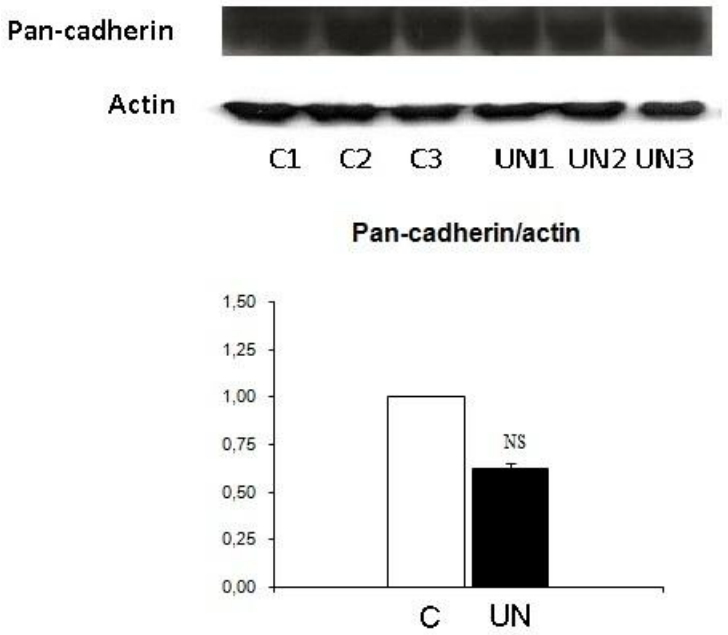

Figure 4 Immunoblots of E (pan) cadherins in muscle cells from 60-day-old rats. Muscle cells from control (C1, C2, C3) and undernourished offspring (UN1, UN2, UN3) were cultured for 4 days. The lysates were separated by 7.5% SDS PAGE. Actin was used as control for gel loading. The amount of cadherin relative to that of actin is shown below. *NS* non-significant.

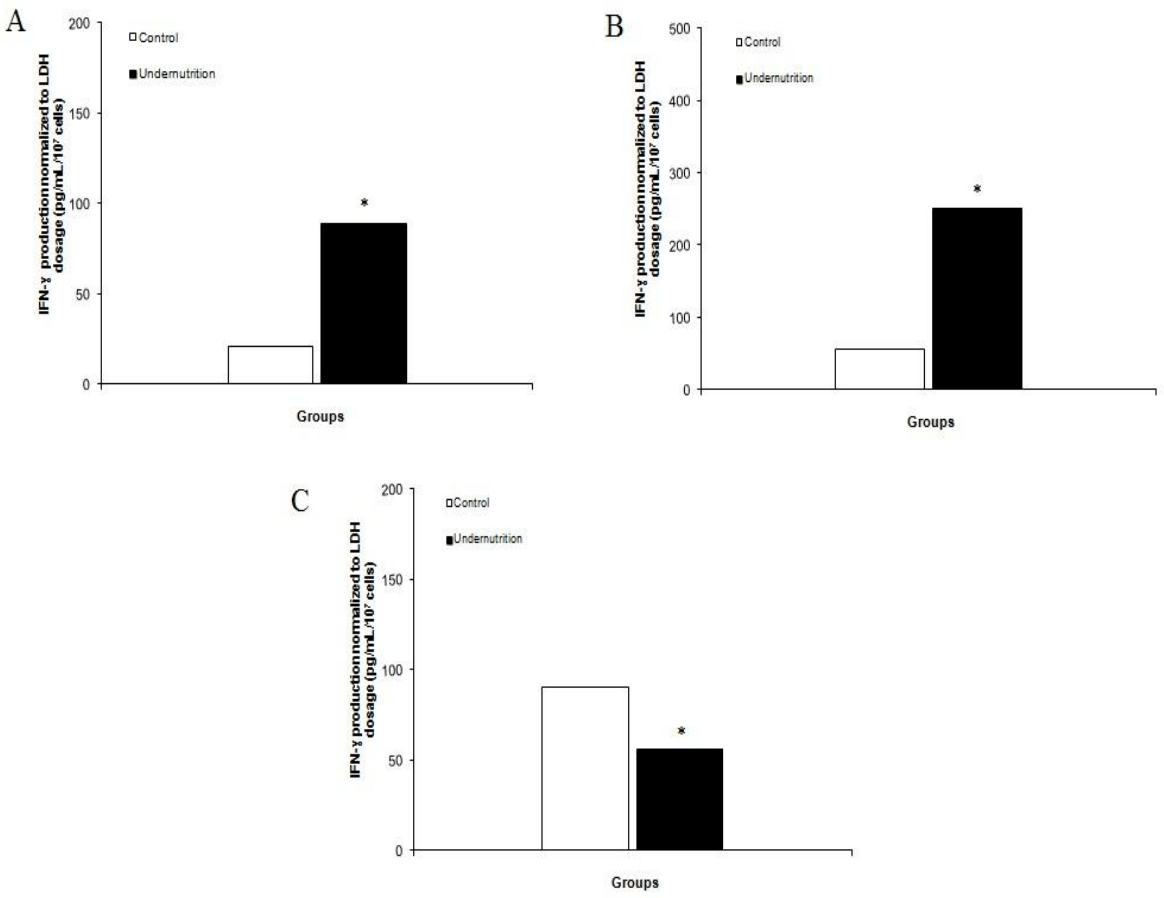

Figure 5 IFN-γ production in the supernatants of macrophages from 42 day-old offspring **(A)**, 60 day-old offspring **(B)** and 90 day-old offspring **(C)** cultured for 4 days. Results were normalized to 10^7 cells. Data are represent as mean + S.E.M. n=6 in each group. *$p<0.05$ Mann-Whitney test.

Table 1. Composition of the diets (control 17% and low-protein 8%)

Ingredients	**Amount for 1 Kg of diet**	
	Low-protein	**Control**
Casein	79,3 g	179,3 g
Vitamin Mix*	10 g	10 g
Mineral Mixture [#]	35 g	35 g
Cellulose	50 g	50 g
Choline bitartrate	2,5 g	2,5 g
DL-Methionine	3,0 g	3,0 g
Soya oil	70 ml	70 ml
Corn Starch	750,2 g	650,2 g

[#] Mineral mixture contained the following (mg/kg of diet): $CaHPO_4$, 17200; KCI, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_2, 120; $FeSO_4 \cdot 7H_2O$, 200; trace elements, 400 ($MnSO_4 \cdot H_2O$, 98; $CuSO_4 \cdot 5H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 80; $CoSO_4 \cdot 7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g [per kg of diet]).

* Vitamin mixture contained the following (mg/kg of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6.

6. RESULTADOS

6.2 Artigo 2

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ORIGINAL CONTRIBUTION

Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats

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Abstract
Aim To investigate the effects of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats.
Methods Male Wistar rats (n = 18) were suckled by mothers fed diets containing 17% protein (controls, C) or 8% protein (undernourished, UN). All rats were fed a normal protein diet after weaning. Muscles were removed from the legs of 42-, 60- and 90-day-old rats. Muscle cells were cultured to assess cell number, morphology and the expression of major proteins involved in myogenesis (Pax7, cadherins, b1 integrin, IL-4Ra and myogenin) by western blotting. IL-4 levels in culture supernatants were measured by ELISA.
Results Offspring from mothers fed a low-protein diet showed a lower body weight gain. Cell number and myotube expansion were reduced in cultured muscle cells from UN, but the expression of myogenic marker proteins was unaltered.
Conclusions Dietary restriction during lactation had no impact on the synthesis of myogenic marker proteins, and myocyte differentiation occurred normally in the muscles of offspring aged 42, 60 or 90 days. Nevertheless, the number and morphology of the myotubes are altered.

Keywords Critical period of development
Programming Satellite cells Neonatal undernutrition
Skeletal muscle Rats

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Introduction

Maternal and neonatal undernutrition not only causes prolonged growth retardation but also modifies the programming of biochemical mechanisms related to endocrine-metabolic control [1]. Fetal programming is the phenomenon whereby changes in the critical period of development in response to the environment may have permanent effects on the organs and tissues [2]. Perinatal undernutrition-induced metabolic diseases in adult life have been demonstrated in experimental models in rats [3, 4]. In humans, epidemiological studies have also revealed an inverse relationship between birthweight and type 2 diabetes mellitus, hypertension, hyperlipidemia, obesity and insulin resistance in adult life [5–7]. It has been speculated that early influences on the growth and development of skeletal muscle fibers may underlie this relationship [8].

Perinatal undernutrition on muscle-fiber development and composition has been described in a variety of mammalian species [9]. Wilson et al. [10] showed a reduction in secondary fibers in both soleus and lumbrical muscles and

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a deficit in the growth rate of the newborn rat, when the maternal diet was restricted to 30% of the control animals. Maternal low-protein exposure (9% casein) during mid- to late pregnancy reduces the numbers of fibers formed within the soleus and gastrocnemius muscles of young rats [9]. On the other hand, Bayol et al. [11] found no effect of maternal undernutrition on numbers of fibers in the semitendinosus muscle at 21 days postpartum but an increased muscle-cell proliferation and a trend toward increased satellite-cell activity.

Satellite cells are responsible for the growth, repair and maintenance of the skeletal muscle [8]. In response to environmental activating signals derived from injury, satellite cells undergo asymmetric cell divisions for the purpose of self-renewal, giving rise to committed myogenic cells, myoblasts, thereby sustaining postnatal muscle repair and growth [8]. Adhesion molecules, growth factors and cytokines released by the neighboring cells can also activate satellite cells [12]. The myogenic process is regulated by transcription factors, such as Pax3/Pax7, and members of the family of the myogenic regulatory factors (MRFs), including MRF4 (Myf6), Myf5, MyoD and myogenin [12].

Myogenin and MyoD may have important functions in later development, since they bind directly to the enhancers and activate transcription of the creatine kinase and myosin light chain [8]. Several gene products are crucial for the fusion of mammalian myoblasts, including cell adhesion molecules (N and M cadherins) and the b1 integrins [13]. It was recently demonstrated that newly formed myotubes also secrete IL-4, which interacts with the IL-4 a receptors (IL-4Ra) present on surrounding myoblasts to promote their fusion with existing myotubes, thereby increasing myotube size [14].

Previous studies have reported the relationship between maternal undernutrition and the expression of genes that control muscle growth in offspring at weaning [11]. Little is known about the long-term effects on the satellite-cell activity in adult rats, and the mechanisms by which maternal nutrition may influence muscle growth. In the present study, we evaluated the effects of neonatal undernutrition on the morphology of myotubes in culture and the expression of proteins that regulate myogenesis in rats aged 42, 60 and 90 days.

Materials and methods

Animals and diet

Pregnant female Wistar rats were purchased from Centre d'élevage Dépré (Saint-Doulchard, France). Animals were handled in accordance with the guidelines and regulations of the Ethics and Safety Committee of the Compiègne University of Technology and housed under conditions of controlled temperature (22 ± 1 C), 12-h light–dark cycle. The standard animal laboratory food and water were given ad libitum. At the time of delivery, the litter size and pups' birth weight were recorded. During the suckling period, the offspring were kept in groups of six pups, randomly distributed into two nutritional groups according to their mothers' diet: a well-nourished group (control, C) fed by mothers receiving a 17% protein (casein) diet and a low-protein group (undernourished, UN), fed by mothers receiving a 8% protein (casein) diet (Table 1). After weaning (at the age of 22 days), only male offspring were used (nine per group) and kept in the collective cage, receiving standard animal laboratory food (Teklad Global 18% protein rodent diet; Harlen Teklad) until the 60-day-old ad libitum. Subsequently, offspring were fed by a 14% protein diet (Teklad Global 14% protein rodent diet; Harlen Teklad) until the 90th day of life. Body weights were recorded every 5 days (Mettler Toledo XS4001S, 0.1 g readability, Inc, Columbus, OH) during lactation and at 42, 60 and 90 days. Body weight gain was calculated as follows: percentage weight gain = [body weight (g) x 100/birthweight (g)] – 100 [11].

Offspring were anesthetized with Ketamin (45.2 mg/kg)/Xylazin (6.8 mg/kg) by intramuscular injection at different ages (42, 60 and 90 days, n = 6 per age). The muscles from both legs were removed and immersed in ice-cold 15 mL phosphate-buffered saline (PBS) containing 0.15 g glucose

Table 1 Composition of the diets (control 17% and low protein 8%)

Ingredients	Amount for 1 kg of diet	
	Low protein	Control
Casein	79.3 g	179.3 g
Vitamin mix[a]	10 g	10 g
Mineral mixture[b]	35 g	35 g
Cellulose	50 g	50 g
Choline bitartrate	2.5 g	2,5 g
DL-methionine	3.0 g	3,0 g
Soya oil	70 mL	70 mL
Corn starch	750.2 G	650.2 g

[a] Vitamin mixture contained the following (mg/kg of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2,720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6

[b] Minerals mixture contained the following (mg/kg of diet): $CaHPO_4$, 17,200; KCI, 4,000; NaCl, 4,000; MgO, 420; $MgSO_4$, 2,000; Fe_2O_2, 120; $FeSO_4$ $7H_2O$, 200; trace elements, 400 ($MnSO_4$ H_2O, 98; $CuSO_4$ $5H_2O$, 20; $ZnSO_4$ $7H_2O$, 80; $CoSO_4$ $7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g [per kg of diet])

and 1% antibiotic solution (Gibco, InVitrogen Cergy Pontoise, France). The animals were killed after the procedures.

Primary culture of rat skeletal muscle cells

All products for cell culture except Matrigel and DNAse were from Gibco (InVitrogen, Cergy-Pontoise, France). Primary culture of rat skeletal muscle cells was performed as previously described [15]. Briefly, the soleus, gastrocnemius and quadriceps muscles were dissected, cut into small pieces and digested with 2% type II collagenase, 0.25% trypsin and 0.1% DNAse (Sigma) for isolation of myoblasts. Cells were placed into 80-cm^2 Pétri dishes (Nunclon) coated with 2 mL Matrigel (BD Biosciences, Le Pont de Claix, France) diluted 1:500 in DMEM. Cells (1.6 x 10^6 cells/80-cm^2 dishes) were cultured in DMEM containing 10% fetal bovine serum, 10% horse serum,
4mMol/L L-glutamine and 1% antibiotic solution. After 48 h of culture, a differentiation medium (DM) with 7% horse serum, 4mMol/L L-glutamine and 1% antibiotic solution was used and changed every 2 days for a total of 10 days.

Cell number and morphology of the myotubes in culture

Cell number was colorimetrically evaluated by measuring intracellular lactate dehydrogenase (LDH) activity. Cells in culture for 10 days were lysed in serum-free DMEM and transferred to a 96-well plate. The LDH concentration was quantified with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. The absorbance (490 nm) of the red formazan produced by LDH was measured in a microplate reader (Model 680, Bio-Rad). The absorbance was proportional to the total number of cells. LDH activity is given as the number of cells, read off from a standard curve of absorbance plotted against an increasing number of lysed cells.

The morphology of myotubes in culture for 10 days was observed using a contrast microscope (Olympus CKX41, Japan) coupled to a digital camera (Canon A620). Qualitative analysis was performed from 5 fields by dish at 100x magnification.

Expression of myogenic marker proteins

Proteins were extracted with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Tritonx 100, 1 M NaF, 100 mM PMSF, 10 mg/mL aprotinin, 10 mM orthovanadate). Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford [16]. Protein lysates from muscle cells cultured for 0, 4, 7 and 10 days were mixed with Laemmli buffer and boiled for 5 min. Equal amounts of proteins of each sample (40 µg) were subjected to SDS–PAGE (7.5% or 12% gels) and transferred to nitrocellulose membranes (BioRad, Marnes-la-Coquette, France). The nitrocellulose filters were soaked in blocking solution (PBS containing 0.1% Tween 20 and 5% non-fat dried milk) at room temperature for 1 h and subsequently incubated overnight at 4 C with rabbit anti-Pax7 (Santa Cruz Biotechnology, Tebu-Bio, Velizy, France), anti-pan-cadherin (Sigma, Saint-Quentin Fallavier, France), anti-IL-4Ra (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France) antibodies or a goat anti-b1 integrin antibody (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France). The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G or a peroxidase-linked donkey anti-goat immunoglobulin G (Jackson ImmunoResearch, Interchim, Montluçon, France) for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with mouse monoclonal anti-myogenin antibody (BD Biosciences, Le Pont de Claix, France), followed by a goat anti-mouse IgG coupled to horseradish peroxidase (Jackson ImmunoResearch, Interchim, France). Immunoreactivity was detected by chemiluminescence (ECL and ECL hyperfilm, GE Healthcare Europe, Saclay, France). The molecular weights of the immunoreactive bands were established by comparison with stained standards (BioRad, Marnes-la-Coquette, France). Western blots for actin and a-tubulin were used to check gel loading. The ratios of proteins to actin or a-tubulin were determined, and band intensities were quantified using KODAK 1D Image Analysis Software (Scientific Image System, Rochester, NY, USA).

Concentration of IL-4 in the supernatants of muscle cells in culture

Interleukin-4 (IL-4) released was measured by ELISA in the supernatants from cells cultured for 10 days (DuoSet ELISA Kit, R&D Systems, Minneapolis, MN, USA). The IL-4 concentration was normalized to 10^5 cells using the LDH dosage.

Statistical analysis

The data are presented as mean ± SEM. For body weight analysis, two-way repeated measures analysis of variance ANOVA, followed by Bonferroni's post hoc test, was used. For LDH and IL-4 levels in the supernatants of cultured cells and immunoblots quantitation groups were compared using Student's t-test. Results were considered statistically significant for $p < 0.05$ (NS non-significant, $*p < 0.05$). The GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used.

Results

The birth weight of neonates did not differ when groups were compared (C = 5.9 ± 0.4 and UN = 5.8 ± 0.3). During lactation, offspring from mothers fed a low-protein diet showed a lower body weight than controls at 10, 15 and 21 days. The pups from undernourished mothers continued to exhibit a lower body weight than the control group throughout the experiment: 42 days (C = 208.6 ± 4.3; UN = 183.3 ± 4.3), 60 days (C = 308.7 ± 6.6; UN = 246.1 ± 5.6) and 90 days (C = 378.2 ± 7.7; UN = 313.1 ± 4.3; Fig. 1).

Between days 4 and 7 in differentiation medium (DM), both myoblasts from controls and undernourished rats began to form myotubes into the standardized mode. Examination of cultures under the phase contrast microscope suggested that on the 10th day of culture, myotubes from control rats are large and numerous, while the number and the size of myotubes were low in undernourished rats (Fig. 2a). LDH measurement showed a lower cell number in the UN group when compared with cells from the C group (C group 21.8 ± 0.19 x 10^3, UN group 18.9 ± 0.2 x 10^3; Fig. 2b).

Myotubes from UN 90-day-old offspring were less ramified than those from their controls (Fig. 3).

The expression of Pax7 protein in lysates of cells taken from 42-, 60- and 90-day-old pups and cultured for 0 and 4 days was studied by western blotting. The expression remained roughly constant in cells from offspring aged between 42 and 90 days at all culture times (Fig. 4). There was no difference in the Pax7 content of cells from UN and C pups. Similarly, the cadherin (N and M) content of cells cultured for 4 days was assayed using a pan-cadherin antibody. The amounts in 42-, 60- and 90-day-old pups appeared to be similar. There was no difference in the expression of cadherin in cells from UN and C pups (Fig. 4).

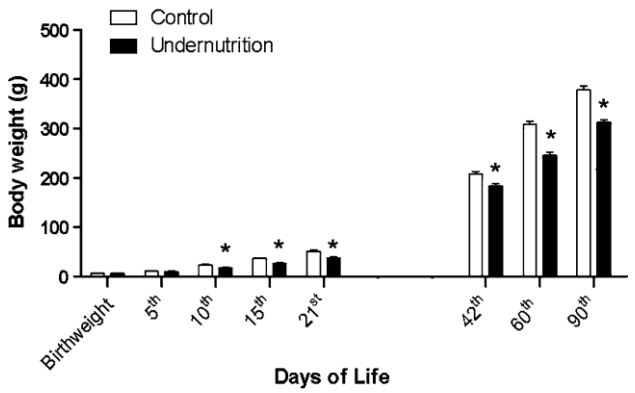

Fig. 1 Body weight of pups from mothers fed a low-protein diet (undernutrition, casein 8%) or a control diet (casein:17%) during growth. Analysis was performed every 5 days during lactation (n = 9 in each group) and at 42, 60 and 90 days (n = 3 in each group). The values are presented as means + SEM. $p < 0.05$ compared with control group using two-way ANOVA and Bonferroni's post hoc test

The expression of the b1 integrin subunit in cells cultured for 4 or 7 days did not vary with the age of the pups providing the muscle samples. The content of the b1 integrin subunit in muscle cultures of UN and C offspring was similar. Cells from 42-, 60- and 90-day-old pups cultured for 7 days showed similar contents of IL-4Ra, but IL-4Ra was not detected in cells from 90-day-old offspring. The expression of IL-4Ra protein in cells from UN and C offspring cultured for 7 days was the same.

Muscle cells cultured for 10 days all showed the same content of myogenin protein, regardless of the age of the offspring providing the muscle. There was also no difference in the expression of myogenin in cells cultured for 10 days from 60-day-old UN and C offspring.

The concentration of IL-4 in the supernatants of muscle cells from 60-day-old offspring cultured for 10 days, normalized using LDH activity, showed no significant differences between UN and C offspring.

Discussion

Perinatal undernutrition has been one of the most thoroughly studied programming factors acting in the genesis of metabolic disease in adult life [17]. The mechanisms for the induction of programming appear to include the following: certain clones of cells may be altered by environmental adversity during development; nutrient environment may permanently alter gene expression; and early nutrition may permanently reduce cell numbers in the organs and tissues [18]. In our study, casein (a highly phosphorylated protein) was chosen because it is an important component of breast milk. Casein absorption induces an increase in the concentrations of essential (threonine, valine, methionine, isoleucine, leucine, phenylalanine and lysine) and non-essential amino acids (histidine and arginine) in the neonatal plasma [19]. Moreover, it has been shown that dietary casein intake is the key factor in the stimulation of ribosome aggregation in liver that reflects the protein biological value [19]. Thus, casein has been widely used in previous studies as an inducer of perinatal undernutrition.

In the present study, we first examined the effects of a low-protein diet during lactation on the body weight of the offspring. We then studied the morphology of myotubes and the expression of some key proteins known to regulate myogenesis, from muscle-cell cultures of young and adult offspring. The results indicate that offspring from mothers fed a low-protein diet showed a lower body weight at adult age (60 and 90 days) than their controls. Our results are in

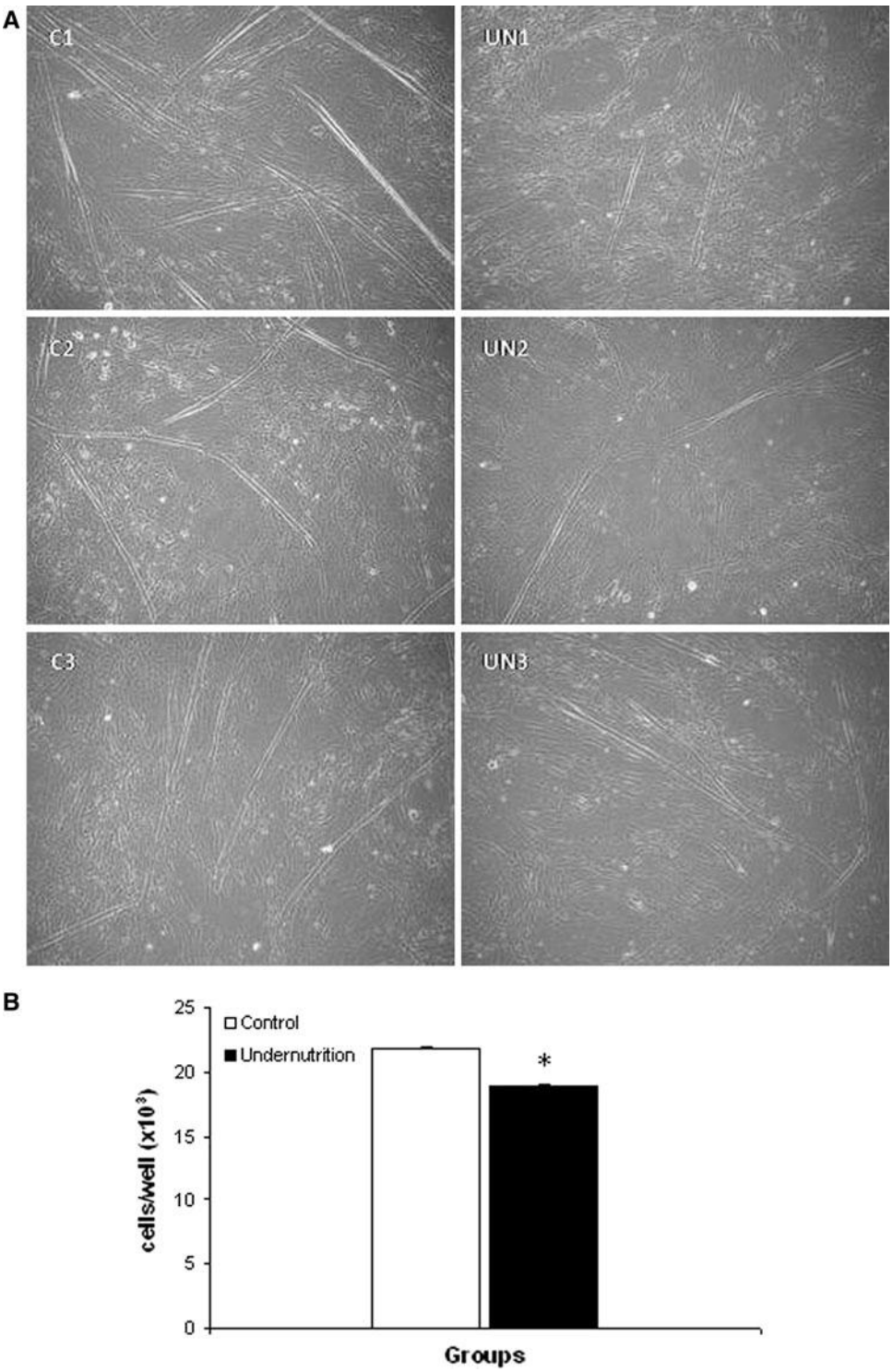

Fig. 2 Morphological observation of muscle cells in culture (a) and numbers of muscle cells/well (0.33 cm^2) (b) after 10 days in culture (60-day-old offspring). Mothers were submitted to a control diet (C1, C2, C3) or a low-protein diet (UN1, UN2, UN3) during lactation. a Cells were cultured in a differentiation medium (DM) and observed at 10 days. Phase contrast objective 10x. Bar 100 µm. b Data were obtained from LDH activity of adherent cells. Data are expressed as mean + SEM n = 18 in each group. *$p < 0.05$ unpaired Student's t-test. C control group, UN undernourished group

agreement with earlier studies using the same experimental model [11, 20, 21]. Nevertheless, 90-day-old rats were not evaluated in those studies. Neonatal maternal protein restriction (8% casein-restricted diet) causes changes in milk composition and volume of the lactating rats, and it may be related to less transfer of nutrients to the offspring and to a long-lasting low body weight [22]. In addition, it has been demonstrated that reduced body weight induced by maternal undernutrition is related to the low number of muscle fibers in offspring [8, 18]. Thus, it can be suggested that changes in the numbers and/or types of fibers present in skeletal muscle may contribute to the risk of developing type 2 diabetes, and/or obesity in later life (developmental origin of adult disease) [17]. However, little is known

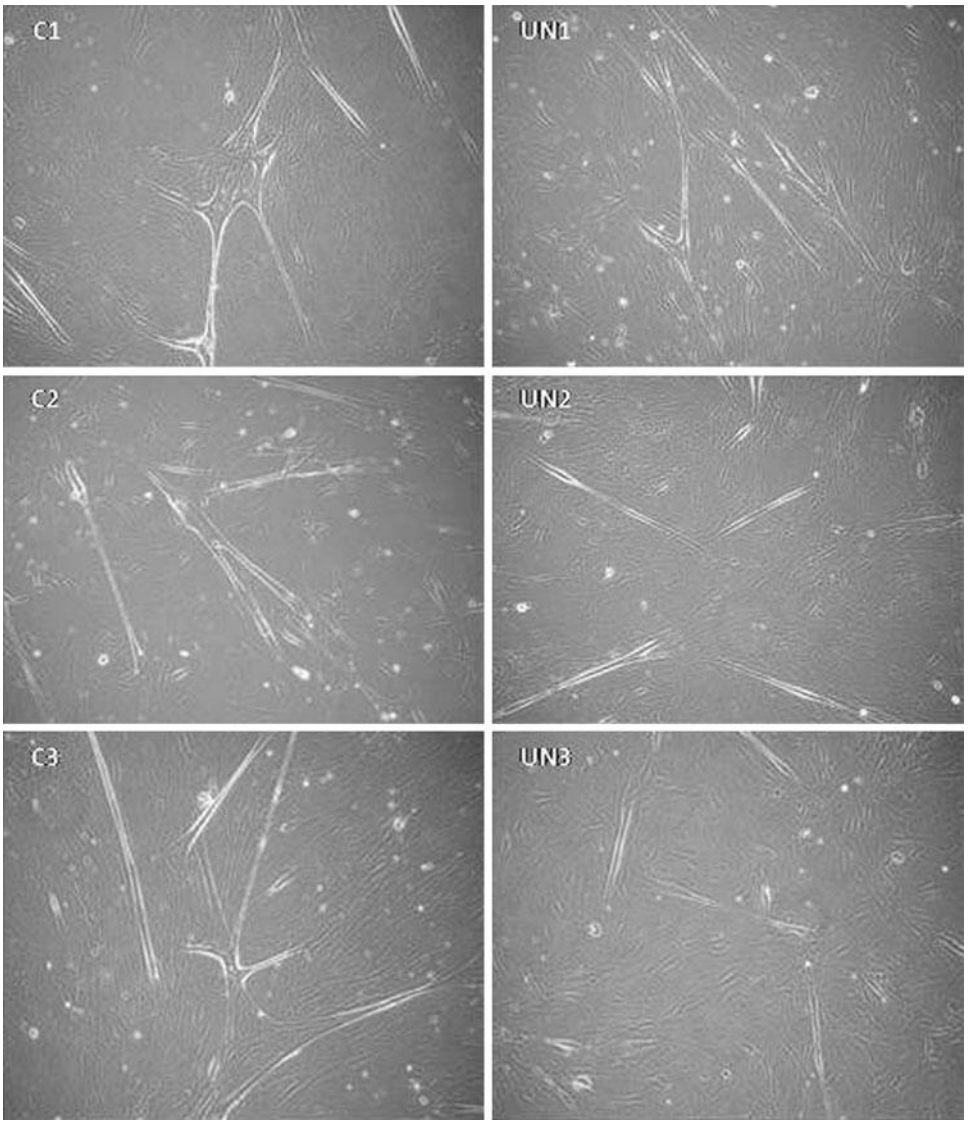

Fig. 3 Morphological analysis of muscle cells in culture (90-day-old offspring). Mothers' offspring were submitted to a control diet (C1, C2, C3) or a low-protein diet (UN1, UN2, UN3) during lactation. Cells were cultured in a differentiation medium (DM) and analyzed on the 10th day. Phase contrast objective 10x. Bar 100 μm

about the molecular mechanisms by which maternal nutrition influences muscle growth.

In order to test the hypothesis that neonatal undernutrition can impair myogenesis, the capacity of muscles from offspring of different ages (42, 60 and 90 days) to produce myogenic markers of satellite cells, myoblasts and myotubes in culture was assessed. Our data demonstrate that muscle cells from 60-day-old UN offspring cultured for 10 days grew more slowly than did muscle cells from C offspring. There were fewer myotubes in samples from 60-day-old UN offspring, and the myotubes from 90-day-old offspring expanded more slowly than those from their C counterparts. It has been demonstrated that muscle-fiber formation and postnatal growth are regulated by insulin-like growth factor (IGF)-1 [23]. Systemic IGF-1 levels are low in both mothers and fetuses following maternal undernutrition, suggesting that impaired myogenesis could be the result of diminished levels of IGF-1 [24]. After birth, IGF-1 has been shown to promote muscle-fiber hypertrophy by a combination of protein synthesis and activation of satellite cells [25]. We therefore performed key protein-expression analyses of satellite cells, myoblasts and myotubes in the offspring.

Pax7 transcription factor and cadherins (especially N and M cadherins) are involved in initial cell to cell recognition during myogenesis and play an important role as regulators of muscle-cell specification and tissue formation during development [12, 13]. Pax7 is expressed in quiescent satellite cells, serving as a marker for their localization beneath the basal lamina [12]. M-cadherin accumulates with b-catenin at the areas of contact between fusing myoblasts and b-catenin colocalizes with actin in pre-fusing myoblasts [13]. Satellite cells contain b-catenin that remains present in them during activation and proliferation.

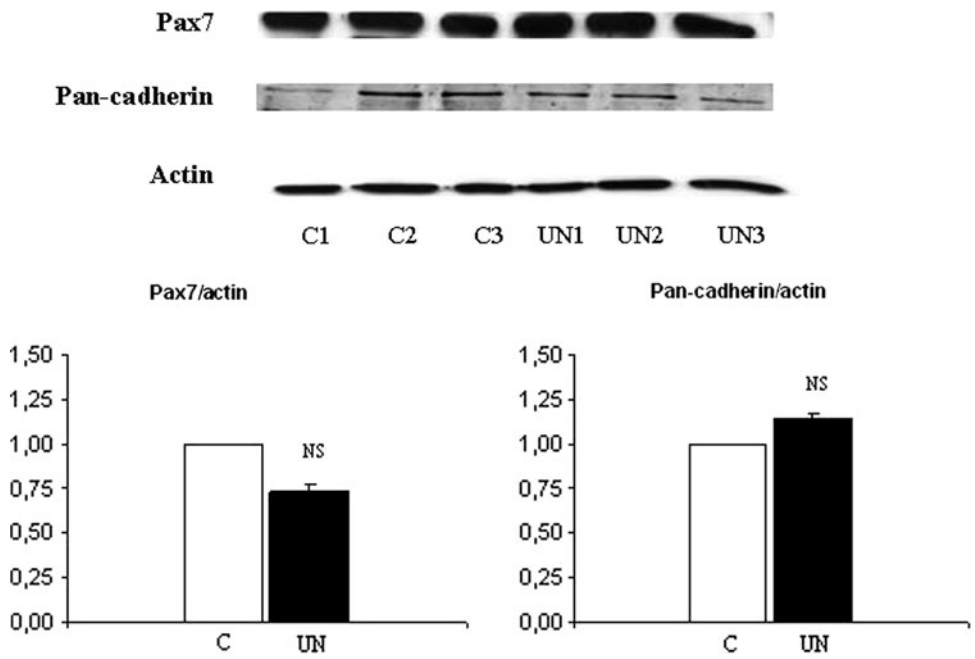

Fig. 4 Immunoblots of Pax7 and M–N (pan) cadherins in muscle cells—from 60-day-old rats. Muscle cells from control (C1, C2, C3) and undernourished offspring (UN1, UN2, UN3) were cultured for 4 days. The lysates were separated by 7.5% SDS PAGE. Actin was used as control for gel loading. The amount of each factor relative to that of actin is shown below. NS non-significant

In the present study, we found no difference in the expression of Pax7 and cadherin proteins in cells from UN and C offspring, regardless of the age of the donor offspring or the time in culture. The expression of those proteins seems to remain roughly the same in offspring aged between 42 and 90 days. Most satellite cells expressing Pax7 up-regulate MyoD and enter into a proliferative state [12, 26]. N and M cadherins play a role in the initiation of myoblast fusion to form multinuclear myotubes [27]. Our results show that the initial processes of proliferation and differentiation in myogenesis were not affected by neonatal undernutrition.

Skeletal muscle cells are known to produce a wide variety of integrins. The b1 subunit is produced throughout myogenesis, and different variants are present in myoblasts and myotubes [28]. Pure populations of b1-null myoblasts and satellite cells isolated from b1-null chimeric embryos and chimeric newborn mice differentiate in vitro and fuse to form multinucleated myotubes [29]. Other studies have demonstrated that b1-deficient myoblasts adhere to each other, but that their plasma membrane breakdown is defective [30]. The amounts of the b1 integrin protein did not seem to change with the age of the donor pups, and we found no differences in its expression between cells from UN and C pups. Many cell adhesion proteins, including the integrins and cadherins, have been shown to be important for myoblast fusion, but how exactly they regulate cell fusion remains largely unknown. However, the focal adhesion kinase (FAK) involved in integrin signaling appears to be a mediator by which integrins regulate myoblast fusion [31]. IL-4 is not required for fusion between mononucleated myoblasts, but it is required for myotube maturation. It may favor myogenic cell migration and the integrin-modulated interaction of these myoblasts with newly formed myotubes [14].

The newly formed myotubes recruit myoblasts for fusion by secreting IL-4, leading to muscle growth. The transcription factor NFATc2 controls myoblast fusion after the formation of a myotube and is necessary for further cell growth [14]. IL-4 has been identified as a molecular signal that binds to the IL-4Ra subunit on myoblasts to promote fusion and growth [14]. The IL-4Ra subunit of the IL-4R is present on both myoblasts and myotubes and is required for muscle growth. IL-4 controls cell fusion by binding to the IL-4R of myoblasts rather than myotubes, and signaling via the IL-4R in myoblasts is required for cell fusion with nascent myotubes [14]. We believe that the IL-4Ra protein is absent from the muscles of pups aged over 60 days, and this was true for both UN and C rats. Our results suggest that this regulation no longer exists in adult rats (60-day-old). Cells from 60-day-old UN and C rats in culture for 10 days secrete the same amount of IL-4 into the medium. This suggests that myotube function is not altered in UN rats, in spite of the difference observed in their expansion.

Lastly, we analyzed the production of the myogenic regulatory factor myogenin, which is abundant in the muscles of newborn pups. It resides in the nucleus and may function as a sequence-specific DNA-binding factor that interacts directly with muscle-specific genes during myogenesis [12]. The content of myogenin protein in 10-day muscle-cell cultures was not influenced either by the age of the donor rats or whether they were UN or C rats.

Conclusion

In conclusion, casein restriction during lactation followed by the restoration of a normal protein diet at weaning has no impact on the synthesis of myogenic marker proteins, and myocyte differentiation occurred normally in the muscles of offspring aged 42, 60 and 90 days. Nevertheless, the number and morphology of myotubes are affected.

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Conflict of interest The authors declare that they have no conflict of interest.

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7. CONCLUSÕES

Os resultados desse estudo permitem concluir que:

- A desnutrição neonatal, mesmo seguida de reposição nutricional, modifica a quantidade de macrófagos alveolares em cultura de ratos jovens e adultos, o que pode interferir no desenvolvimento dessas células em cultura. Além disso, altera a produção de proteínas de fusão (IL-4 e IFNγ), sem modificar a expressão de caderinas na superfície dos macrófagos. Apesar dessas alterações observadas, devido a um provável mecanismo compensatório existente nos animais desnutridos, pode-se sugerir que a formação das células gigantes multinucleares mediada por caderinas e IFNγ parece não ser afetada na vida adulta do animal pela restrição protéica na lactação.

- A desnutrição neonatal, mesmo seguida de reposição nutricional, reduz a quantidade de miotubos em cultura de ratos com 60 dias de vida e altera a morfologia dessas células em ratos adultos com 90 dias de vida, os quais apresentam miotubos com menos ramificações. Dessa forma, pode-se sugerir que esse modelo de desnutrição compromete o desenvolvimento da miogênese. No entanto, a expressão de proteínas-chaves do processo miogênico permanece inalterada.

8. RECOMENDAÇÕES/PERSPECTIVAS

Perspectivas elaboradas como objetivo para dar continuidade ao estudo:

- Avaliar as repercussões tardias da desnutrição numa fase mais precoce do desenvolvimento, o período gestacional, sobre a fusão de macrófagos e células musculares esqueléticas.

- Estudar o efeito da desnutrição neonatal sobre a contração de miotubos em cultura e a ação da insulina na formação dessas células *in vitro* (tese de doutorado da aluna Simone do Nascimento Fraga).

- Utilizar o aleitamento cruzado para estudo das repercussões tardias da desnutrição neonatal.

- Avaliar a influência da atividade física sobre a expressão de proteínas de fusão macrofágica e miocitária em ratos submetidos à desnutrição precoce.

- Realizar co-cultura de macrófagos alveolares com miotubos recém formados e verificar o efeito da desnutrição precoce no índice de fusão dessas células *in vitro*.

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ANEXOS

Anexo A. Aprovação do Comitê de Ética em Experimentação Animal da UFPE

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

Ofício nº 214/09

Recife, 06 de novembro de 2009.

Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Profa.: Célia Maria Machado Barbosa de Castro**
Departamento: Medicina Tropical- CCS / UFPE
Processo nº 23076.005512/2008-40

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado*:* ***"Efeito da desnutrição neonatal sobre a fase de maturação macrofágica e miocitária em ratos jovens e adultos."***

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

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

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

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

Observação: Origem dos animais: Biotério do Departamento de Nutrição/UFPE ; Animais: Ratos, linhagem: Wistar; Sexo: Machos; Idade: 42; 60 e 90 dias ; Nº de Animais previsto no projeto: 18 animais . Mudança de título referente ao processo no 23076.005512/2008-40.

CCB: Integrar para desenvolver

Anexo B. Documentação do Artigo 1

De: **edoffice@nutsoc.org.uk**
Enviada: sexta-feira, 3 de fevereiro de 2012 00:34:05
Para: julemelo@hotmail.com

Dear Mrs. MELO,

On 2nd Feb 2012, we received your manuscript entitled "Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins" by Juliana Melo, Thacianna Costa, Tamara Lima, Maria Chaves, Muriel Vayssade, Marie-Danielle Nagel, and Célia Castro.

The manuscript has been assigned the Paper number: BJN-2012-018000.

If we have any queries regarding your submission we will contact you within the next few days.

Sincerely,

Claire Goodstein
Publications Office
British Journal of Nutrition
The Nutrition Society, 10 Cambridge Court, 210 Shepherds Bush Road, London W6 7NJ, UK
Tel: +44 (0)20 7371 6225
Fax: +44 (0)20 7602 1756
E-mail: edoffice@nutsoc.org.uk

Anexo C. Documentação do Artigo 2

Date: 30-08-2010

To: "Juliana Melo" julemelo@hotmail.com

From: "Gerhard Rechkemmer" ejn@bfel.de

Subject: EJN: Your manuscript entitled Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats

Ref.: Ms. No. EJN-D-10-00050R1
Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats
European Journal of Nutrition

Dear Mrs Melo,

I am pleased to tell you that your work has now been accepted for publication in European Journal of Nutrition.

It was accepted on 30-08-2010.

Thank you for submitting your work to this journal.

With kind regards

Gerhard Rechkemmer
Editor-in-Chief
European Journal of Nutrition

Resumos da tese publicados em congressos:

- Effect of a neonatal low-protein diet on the cell number and morphology of myotubes in adult rats In: XV Meeting of the Brazilian Society for Cell Biology, 2010, São Paulo-SP.

- Expressão de Pax7 e miogenina por células satélites musculares e miotubos de ratos jovens e adultos submetidos à desnutrição neonatal In: V Congresso Brasileiro de Células-Tronco e Terapia Celular, 2010, Gramado-RS.

- Cell number quantification and IL-4 production of macrophages in young and adult rats submitted to neonatal malnutrition In: XXVI Reunião Anual da Federação de Sociedades de Biologia Experimental –FESBE, 2011, Rio de Janeiro-RJ.

- IL-4Rα/IL-4 in myoblasts and myotubes of young and adult rats submitted to neonatal malnutrition In: VI Congresso Brasileiro de Células-Tronco e Terapia Celular, 2011, Salvador-BA.

SBBC

MEETING OF THE BRAZILIAN SOCIETY FOR CELL BIOLOGY
July 24-27th 2010
Bourbon Convention Ibirapuera
São Paulo, SP Brazil

We certify that

MELO, J.F.', ALOULOU, N., DUVAL, J.L., VIGNERON, P., BORGOIN, L., LEANDRO, C.G., DE CASTRO, C.M.M.B., NAGEL, M.D.

presented the Poster entitled

Effect of a neonatal low-protein diet on the cell number and morphology of myotubes in adult rats

at the XV Meeting of the Brazilian Society for Cell Biology held in São Paulo, Brazil on July 24-27th, 2010.

President
Organizing Committee
SBBC 2010

Organizing Committee

Vilma Regina Martins
Estela Bevilacqua
Luiz Fernando Lima Reis
Patrícia Gama
Marinilce F Santos
Chao Yun Irene Yan
Marimélia Porcionatto
Flávia A Gomes
Silvana Allodi

Agência de Fomento: CNPq, FAPERJ, INCa, MS

E-122 THE INTERFACE OF ENDOPLASMIC RETICULUM STRESS AND ABERRANT GLYCOSYLATION: EVIDENCE FOR N-GLYCAN ADDICTION IN MELANOMA CELLS

Luciana Maria Dorneles de Oliveira, Renata de Freitas Saito, Andréia Hanada Otake, Roger Chammas
1. FMUSP, Faculdade de Medicina da Universidade de São Paulo, Av Dr Arnaldo, 455 sala 4122

Glycan structures are often altered in tumor cells, a phenomenon known as aberrant glycosylation. Among these alterations, there are several clinical markers for tumor progression. Here, we have exploited a syngeneic model of melanomagenesis comprised of a "normal" melanocyte cell line (Melan-a) and two melanoma cell lines (TM1 and TM5) derived from impediment of melan-a cell adhesion(Oba-Shinjo et al., 2006). Upon transformation, melanoma cells were characterized for (1)survival in a prooxidant state, like human melanoma cells; (2)an increase in the global content of L-PHA reactive oligosaccharides, an N-glycan-dependent pattern of aberrant glycosylation. Both features are related with endoplasmic reticulum(ER) homeostasis. As melanoma cells survive in a prooxidant state, which in turn leads to ER stress, we wondered if alterations in N-glycan biosynthesis would play a role in the cellular adaptation response. To elucidate this phenomenon in melanoma, we studied the induction of ER stress by tunycamycin, an N-glycosylation inhibitor, in human (LB373) and murine (melan-a,TM1 and TM5) melanoma cells. Tumorigenic murine cell lines were more sensitive to tunicamycin-induced cell death, via the unfolded protein response(UPR), than melan-a. Murine melanoma cells accumulated both GRP78 and GADD153 (>2-fold increase) upon exposure to tunicamycin, as compared to accumulation of the same UPR effectors in melan-a cells. Consistently with tunicamycin activities, a significant decrease in the amounts of L-PHA reactive oligosaccharides was also observed. This exquisite sensitivity of the tumorigenic lines to tunicamycin suggests that tumor cells are addicted to N-linked glycosylation.

E-123 CYTOTOXICITY OF A DERIVATIVE OF PODOPHYLLOTOXIN IN HT-29 AND HEPG2 CELLS

Alethéia Lacerda da Silveira, Caio César Barbosa Bomfim, Glaucia Verissimo Faheina-Martins, Ana Maria Laurindo Silva, Aderson de Farias Dias, Demetrius Antonio Machado de Araujo
1. UFPB, Universidade Federal da Paraíba, Campus I João Pessoa PB

The cytotoxicity assays have been used as a starting point in the discovery of a new antitumor agent, whose metabolic profile can be measured using cultured hepatocytes. Therefore, the present study aimed to determined the cytotoxicity of a novel podophyllotoxin derivative on human tumor cell lines HT-29 (colon cancer) and HepG2 (hepatoma), evaluating whether this effect is associated with metabolism. Cells were seeded at a concentration of 3x104 cells/well and then treated with the derivative A5 (5 - 250 µM). In hepatocytes, the substance was added in the absence or presence of dexamethasone (CYP3A inducer), ketoconazole (CYP3A inhibitor) or borneol (inhibitor of glucuronidation). After 24 and 72 hours of treatment, the MTT assay was used to assess cell viability. Compound A5 was more toxic in HT-29 cells, with IC50 values corresponding to 18.35 µM (24h) and 14.14 µM (72h). In HepG2, the substance was little cytotoxic even after 72 hours of treatment (IC50 = 226.6 µM). This low cytotoxicity in hepatocytes may be a reflection of the process of detoxification. Co-incubation with ketoconazole (IC50=133 µM) or borneol (IC50=170 µM) increased the cytotoxic effect of A5 derivative in hepatocytes. Pretreatment with dexamethasone decreases the cytotoxicity of the drug (IC50>250 µM). These results indicate that CYP3A isoform and glucuronidation reactions are involved in the degradation of the derivative A5. The derivative A5 was more cytotoxicity in HT-29 than in HepG2 cells. This hepatocyte protection is possibly due the pathways of detoxification involving the CYP3A isoform and reactions of glucuronidation.
Agência de Fomento: CAPES, CNPq

E-124 MODULATION OF EGFR/ERBB RECEPTORS AND ITS LIGANDS BY DERMCIDIN IN BREAST CANCERS

Dayson Friaça Moreira, José Ernesto Belizário
1. USP, USP, Av Prof Lineu Prestes ICB I

We identified Dermcidin (DCD) as a gene overexpressed in human breast cancer with high and low HER2 amplification. To further investigate their cooperative role in breast tumorogenesis, we transfected MCF-7 breast cancer cell line, a low HER2 amplification, with pcDNA-DCD plasmid and selected stable cell clones. Next, we evaluated the expression of the EGFR/ERBBs receptors, its growth factor ligands and their downstream signaling pathways by real time RT-PCR and immunofluorescence analysis. MCF-7-DCD cell clones expressed over 3-fold more mRNA and proteins for ErbB1/EGFR and ErbB2/HER2 than the MCF cells. In addition, mRNA expression for AREG, EGF, HB-EGF, NGR-3 and NGR-4 increased up to 10-folds ($p<0.05$). Overexpression of DCD in the MCF-7 cells increased the activation of the receptor tyrosine kinase (RTK) signaling pathways as confirmed by increasing in the phosphorylation status of transmembrane protein tyrosine residues, pAKT (ser308 and thr473), pSTAT3, pERK and pMAKP38 as well as the levels of cyclin B and c-MYC. Our studies suggest DCD promotes tumor growth and resistance in breast cancer cells by modulating EGFR/ERBBs expression and signaling.
Agência de Fomento: FAPESP

F- DEVELOPMENTAL BIOLOGY

F-01 EXPRESSION OF DAPPER1 AND DAPPER2 DURING CHICKEN LIMB DEVELOPMENT

Thais Rirsch, Denner Jefferson Peterlini, Angelica Vasconcelos Pedrosa, Lucimara Aparecida Sensiate, Lucia Elvira Alvares
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The *Dapper* (*Dpr*) gene family has been associated to several processes of vertebrates' development, including morphogenetic movements during gastrulation, mesoderm and neural tissue specification and morphogenesis of encephalon, eye and heart. Because of the importance of this gene family, we used whole-mount *in situ* hybridization to characterize the expression pattern of the two *Dpr* paralogues present in the chicken genome (*Dpr1* and *2*) during limb development. To allow comparisons, the expression domains of markers for skeletal muscle (*MyoD*) and cartilage (*Sox9*) development were also established. At HH24, *Dpr1* transcripts are observed in the proximal and medial mesenchyme of fore and hindlimbs while *Dpr2* mRNAs are clearly associated to the developing cartilage elements. At HH25, *Dpr1* is expressed throughout the limbs but a stronger signal is observed around the cartilage elements of zeugopod and autopod. In comparison, at the same stage *Dpr2* has a shaper expression pattern with transcripts being found almost exclusively in the cartilage elements of the autopod. At HH28, *Dpr1* and *Dpr2* expression is restricted to the autopod with higher levels of expression above the carpus/tarsus. Interestingly, at this stage *Dpr2* is strongly expressed in the digits 1 and 5 and also in the tips of developing digits. Finally, at stage HH34, both *Dpr* genes are expressed in the joints that are being formed in the digits. Our data strongly suggest a role for the *Dpr* genes during chicken limb development.
Agência de Fomento: FAPESP; PIBIC/CNPq

F-02 EFFECT OF A NEONATAL LOW-PROTEIN DIET ON THE CELL NUMBER AND MORPHOLOGY OF MYOTUBES IN ADULT RATS

Juliana Félix de Melo[1,3], Nijez Aloulou[3], Jean-Luc Duval[3], Pascale Vigneron[3], Lee Bourgoin[3], Carol Góis Leandro[2], Célia M.M.B de Castro[1], Marie-Danielle Nagel[3]
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The aim of this study was to investigate the effects of a neonatal low-protein diet on the cell number and morphology of

myotubes in adult rats. Male Wistar rats (n=6) were suckled by mothers fed diets containing 17% protein (controls, C) or 8% protein (undernourished, UN). All rats were fed a normal protein diet after weaning. Body weights were recorded every five days during lactation. Muscles were removed from the legs of 60 day-old rats and cells cultured (1.6 x 106 cells/80cm2 dishes) for 10 days in DMEM. Cell number was colorimetrically evaluated by measuring intracellular lactate dehydrogenase (LDH) activity. The morphology of myotubes in culture was observed using a contrast microscope coupled to a digital camera. Offspring from mothers fed a low-protein diet showed a lower body weight gain at 10th (C = 23.1 ± 1.6; UN = 17.8 ± 1.4), 15th (C = 36.3 ± 1.1; UN = 25.9 ± 1.7), and 21st day of life (C = 50.7 ± 2.8; UN = 36.7 ± 2.5). Pups from undernourished mothers remain with their lower body weight in comparison with control group at 60 d (C = 308.7 ± 6.6; UN = 246.1 ± 5.6). $p<0.05$ unpaired Student's t-test. Examination of cultures under the phase contrast microscope suggested that, at 10th d of culture, myotubes from control rats are large and numerous, while the number and the size of myotubes was reduced in undernourished rats. LDH measurement showed a lower cell number in UN group when compared with cells from C group (C group 21.8 ± 0.19 x 103, UN group 18.9 ± 0.2 x 103) $p<0.05$ unpaired Student's t-test. The dietary restriction during lactation alters the number and morphology of myotubes in culture of adult rats.

Agência de Fomento: CAPES-COFECUB (grant 584/07)

F-03 MATRIPTASE INITIATES NETHERTON SYNDROME THROUGH EPIDERMAL PRO-KALLIKREIN ACTIVATION

Katiúchia Uzzun Sales[1], Andrius Masedunskas[1], Alexandra L Bey[1], Amber Rasmussen[1], Roberto Weigert[1], Karin List[2,1], Roman Szabo[1], Paul A Overbeek[3], Thomas H Bugge[1]
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Deficiency in the serine protease inhibitor LEKTI is the etiological origin of Netherton syndrome. The principal morbidities of the disease are stratum corneum detachment and chronic inflammation. We show that the membrane protease, matriptase, initiates Netherton syndrome by premature activation of a pro-kallikrein-related cascade. Auto-activation of pro-inflammatory and stratum corneum detachment-associated pro-kallikrein-related peptidases was either low or undetectable, but they were efficiently activated by matriptase. Ablation of matriptase dampened inflammation, eliminated aberrant protease activity, prevented stratum corneum detachment, and improved epidermal barrier function in a LEKTI-deficient mouse model of Netherton syndrome. The study uncovers a pathogenic matriptase-pro-kallikrein pathway that could be operative in several human skin and inflammatory diseases. Agência de Fomento: NIDCR Intramural Research Program

F-04 EFFECTS OF NEONATAL IRON TREATMENT ON BEHAVIOR AND OXIDATIVE STRESS ON ZEBRAFISH (DANIO RERIO) LARVAE

Lídia Farias Martins[2,3], Martina Blank[2,3], Laura Nery[2,3], Angélica Tamborski[2,3], Mônica Vianna[2,3]
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Iron is necessary for normal neural function. Iron deficiency during critical periods of development is associated with disruptions of behavioral performance. However, excessive iron in the brain plays an important role in neurodegenerative disorders. The present study aims to evaluate the effects of neonatal iron overloading on zebrafish behavior and oxidative stress. Embryos were obtained from natural mating of wild-type adults in our fish facility. At 72 hpf embryos were divided into a iron treated group (20mM) and a conditioned water group for 48h. At 7 dpf larvae were individually assigned for exploratory behavior. Data was analyzed using ANYmaze (Stoelting). Larvae treated presented lower average velocity and consequently less distance travelled in comparison to control larvae. The optomotor response, in which groups of zebrafish larvae move in the same direction as a moving pattern of stripes, showed that control larvae responds better to this stimulus than larvae treated with iron. Our preliminary results using the HPLC assay for detection of malondialdeide shows that larvae treated with iron presented higher lipidic peroxidation (167,9 nmol/g) versus control larvae (81,1 nmol/g). Despite iron did not affect hatching and survival, the treatment had evident effects on larvae behavior, locomotion and oxidative stress suggesting a correlation with data of previous studies obtained in rodents.

Agência de Fomento: CNPq, PUCRS, CAPES

F-05 NUCLEAR DIVISION AND MOVEMENTS DURING BLASTODERM FORMATION OF THE BROWN SPIDER (LOXOSCELES INTERMEDIA)

Mônica Akemi Okada, Juliano Morimoto Borges, Cláudia Feijó Ortolani-Machado, Flavia Sant'Anna Rios
1. UFPR, Universidade Federal do Paraná, Centro Politécnico, Cx. Postal 19031, 81531-990, Curitiba - PR

Classic studies of arthropod development are based in the knowledge from insects. However, embryos of spiders have played an important role in recent studies about arthropod evolution. In this study, eggs of L. intermedia were processed by three ways in order to examine the early embryogenesis: (1) live embryos were kept in liquid paraffin; (@) fixed 4% Paraformaldehyde in 0.1M Phosphate Buffer; and (3) fixed with Carnoy's fixative (100% ethanol:chloroform:glacial acetic acid, 6:3:1). Embryos fixed with Paraformaldehyde were embedded in historesin. Serial sections (5 im) were stained with Hematoxylin-Eosin (HE) and toluidine blue. Images of both live embryos and histological sections were acquired with a digital camera on a light microscope. After fication with Carnoy's fixative, the chorion was spontaneously ruptured and remove. Histochemistry ang Immunohistochemistry was performed to detect nucleus and cytoskeleton. The nuclei divide in the central region, being contained within a common cytoplasm. During this stage, the yolk granules get a radial disposition. Nuclei gradually migrate from the center to the periphery, probably using the paths of cytoplasm among granules, and forming the syncytial blastoderm. In the egg cortex, discrete portions of cytoplasm become to limit each nucleus. The cell limits are difined, forming the cellular blastoderm. This process involves a delicate interplay between microtubules and microfilaments. The period between oviposition and blastoderm celularization take about fou days, being considered a slow process when campared with others arthropods.

F-06 EFFECTS OF EARLY EXPOSURE TO LITHIUM ON ZEBRAFISH DEVELOPMENT AND LARVAE BEHAVIOR

Laura Roesler Nery[1,2], Lídia Farias Martins[1,2], Martina Blank[1,2], Angélica Rech Tamborski[1,2], Mônica Ryff Moreira Vianna[1,2]
1. PUCRS, Pontificia Universidade Católica do Rio Grande do Sul, Av. Ipiranga 6681 - Porto Alegre/RS 2. INCT-TM, National Institute for Translational Medicine, 90035-003 Porto Alegre, RS, Brazil

Lithium comprises the most widely used mood stabilizer, despite reports that it may affect normal neurodevelopment, causing transient and permanent neurological effects. In this study we evaluated morphogenetic and behavioral effects of lithium during zebrafish early development. Embryos were obtained from natural mating of wild-type adults in our facility. Embryos were individually treated from 2-72hpf with conditioned water or LiCl at 0.05, 0.5 and 5mM. No significant effect was observed on mortality rates, but a significant delay on hatching occurred at 5mM treatment. Animals treated with 5mM LiCl eventually displayed eye and body deformities. At 10dpf larvae with no evident abnormality were behaviorally tested and data was analyzed using ANYmaze (Stoelting). Control larvae spent more time in the central portion of the area than treated larvae. In accordance, larvae treated with lithium at 5mM spent significantly more time in the external area and also made more body turns than other groups. Larvae treated with 0.5mM LiCl travelled longer distances and with higher speed. Our results can be integrated to previous data demonstrating opposing

CERTIFICADO

Conferimos o presente certificado a

MELO,I.F***; ALOULOU, N*; DUVAL, J.L*; VIGNERON, P*; BOURGOIN, L*; LEANDRO, C.G**; DE CASTRO, C.M.M.B***; NAGEL, M.D*

pela sua participação, na qualidade de

Autor do trabalho científico:
EXPRESSÃO DE PAX7 E MIOGENINA POR CÉLULAS SATÉLITES MUSCULARES E MIOTUBOS DE RATOS JOVENS E ADULTOS SUBMETIDOS À DESNUTRIÇÃO NEONATAL

Gramado / RS, 02 de outubro de 2010.

Renato Abdala Karam Kalil
Presidente
V Cong. Bras. de Células-tronco e Terapia Celular

Nance Beyer Nardi
Coordenadora da Comissão Científica
V Cong. Bras. de Células-tronco e Terapia Celular

Paulo Roberto Slud Brofman
Presidente
ABTECel - Assoc. Bras. de Terapia Celular

PROMOÇÃO E REALIZAÇÃO

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APOIO

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formation was 100%; in group 1, 34,45% in 2; 46,02% in 3; 26,63% in 4; 75% in 5; 14,8% in 6; 83,3% in 7 and 60,1% in 8. The medial area did not differ in any group. We can conclude that the DS inhibits the neointimal formation in both animal types by avoiding SMC proliferation. The MNC transfusion (alone or with DS) only inhibited the neointimal formation in C57BL06 mice; in ApoE-/- mice, neither MNC nor MNC+DS treatment could avoid neointimal formation. These results suggested that in C57BL06 mice all treatments proved efficient, but when we used an animal that developed atherosclerosis, the treatment with MNC could contribute to the inflammatory response promoted by atherosclerosis and increase neointimal formation what can be caused by the fact that MNC contains not only endothelial progenitor cells but also inflammatory cells.

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EXPRESSÃO DE PAX7 E MIOGENINA POR CÉLULAS SATÉLITES MUSCULARES E MIOTUBOS DE RATOS JOVENS E ADULTOS SUBMETIDOS À DESNUTRIÇÃO NEONATAL

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MELO,J.F***; ALOULOU, N*; DUVAL, J.L*; VIGNERON, P*; BOURGOIN, L*; LEANDRO, C.G**; DE CASTRO, C.M.M.B***; NAGEL, M.D*

Introdução. O processo miogênico é regulado por fatores de transcrição, como: Pax3/Pax7 e fatores regulatórios miogênicos, como: MyoD, miogenina, Myf5 e Myf6 envolvidos diretamente na formação dos músculos. Apesar de existirem estudos sobre o efeito da desnutrição precoce na capacidade de diferenciação de células musculares, pouco tem sido pesquisado a cerca do seu efeito tardio após um período de reposição nutricional. Assim, o objetivo deste estudo foi investigar se a desnutrição neonatal interfere na expressão de marcadores da linhagem miogênica Pax7 e miogenina, em ratos jovens e adultos, após reposição nutricional. Materiais e Métodos. Ratos machos Wistar (n=18) foram amamentados por mães cuja dieta durante a lactação continha 17% de proteína à base de caseína (AIN-93G) no grupo nutrido (N) e 8% de proteína (AIN-93G) no grupo desnutrido (D). Após o desmame, todos os animais foram recuperados com dieta à base de caseína a 17% até a vida adulta. Os pesos corporais dos animais foram registrados a cada cinco dias durante a lactação. Os músculos foram retirados de ambas as patas dos ratos com 42, 60 e 90 dias de vida e as células cultivadas (1.6 x 106 células/placa 80cm2) por 10 dias em DMEM. As expressões das proteínas Pax7 e miogenina foram avaliadas através da técnica de Western Blot nos dias 0 e 4 de cultura para Pax7 e no dia 10 para miogenina. Resultados. Os pesos corporais dos animais desnutridos (D) foram menores quando comparados aos dos animais nutridos (N) a partir do 10° dia (N = 23.1 ± 1.6; D = 17.8 ± 1.4) até o 21° dia pós-natal (N = 50.7 ± 2.8; D = 36.7 ± 2.5), permanecendo valores menores nas idades de 42 (N = 208.6 ± 4.3; D = 183.3 ± 4.3), 60 (N = 308.7 ± 6.6; D = 246.1 ± 5.6) e 90 dias (N = 378.2 ± 7.7; D = 313.1 ± 4.3), $p<0.05$, teste t de Student. Não houve diferença nas expressões de Pax7 e miogenina em células satélites musculares e miotubos entre ratos nutridos e desnutridos nas idades de 42, 60 e 90 dias. Conclusões. Esses resultados sugerem que a desnutrição neonatal não altera a expressão dos marcadores miogênicos Pax7 e miogenina por células satélites musculares e miotubos em ratos jovens e adultos, em caso de haver um período de reposição nutricional. Apoio Financeiro: CAPES-COFECUB (584/07)

175.

GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) TREATMENT CAUSES IMMUNOMODULATION IN MICE WITH CHRONIC CHAGASIC CARDIOMYOPATHY

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Juliana Fraga Vasconcelos1,2; Bruno Solano de Freitas Souza1,3; Letícia Maria da Silva Garcia3, Adriano Alcântara3,4, Carine Machado Azevedo1,3, Ricardo Ribeiro-dos-Santos1,3; Milena Botelho Pereira Soares1,3

INTRODUCTION: Approximately 30% of chagasic patients develop a chronic disease typically characterized by myocarditis associated with prominent fibrotic scarring and organ dysfunction. In a previous work we have demonstrated that administration of granulocyte colony-stimulating factor (G-CSF) in mice with heart lesions caused by chronic Trypanosoma cruzi infection improved the heart structure and function. In this study we investigated the effects of G-CSF treatment the immune-inflammatory response in mice with chronic chagasic cardiomyopathy (CCC).

FeSBE 2011

24 a 27 de agosto de 2011
Rio de Janeiro – RJ

CERTIFICADO

Certificamos que o resumo 18.004 – CELL NUMBER QUANTIFICATION AND IL-4 PRODUCTION OF MACROPHAGES IN YOUNG AND ADULT RATS SUBMITTED TO NEONATAL MALNUTRITION, autoria de GALVAO, A. M.; MELO, J. F. D.; COSTA, T. B. D.; CHAVES, M. E. C.; NAGEL, M.; CASTRO, C. M. M. B. D., foi apresentado na XXVI Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, realizado de 24 a 27 de agosto de 2011 no Rio de Janeiro, RJ.

Comissão Organizadora

Para verificar a autenticidade deste certificado, acesse www.fesbe.org.br/certificados

Mascarin, L. Z. ; Silva, E. S. ; Tonussi, C. R.
Universidade federal de santa catarina, UFSC

17.060
TRAMADOL AND MAPROTILINE WERE EFFECTIVE TO REDUCE HYPERALGESIA AND ALLODYNIA OF NEUROPATHIC PAIN IN ANIMAL MODELS.
Monteiro, C. E. S. [1]; Zapata-sudo, G. [1]; Barreiro, E. J. [1]; Sudo, R. T. [1]
[1] Programa de Desenvolvimento de Fármacos(Ciências Biomédicas), UFRJ[2] Faculdade de Farmácia (Centro de Ciências da Saúde), UFRJ

17.061
ANTI-HYPERNOCICEPTIVE EFFECT OF CITRONELLAL IS DEPENDENT OF NO--CGMP-ATP-SENSITIVE-K+ CHANNEL ACTIVATION.
Santana, M. T. [1]; Guimarães, A. G. [1]; Camargo, E. A. [1]; Santana, D. G. [1]; Oliveira, M. G. B. [1]; Santana, M. F. [1]; Souza, D. P. [1]; Almeida, J. R. G. S. [2]; Lima, J. T. [2]; Quintans--júnior. L. J. [1]
[1] Departamento de Fisiologia. Universidade Federal de Sergipe, UFS[2] Colegiado de C. Farmacêuticas, U. F. Vale do São Francisco, UNIVASF

17.062
ADENOSINE AND INOSINE ANTI-INFLAMMATORY EFFECTS IN PLEURISY-INDUCED INFLAMMATION: EVIDENCE FOR THE PARTICIPATION OF ADENOSINE RECEPTORS AND ADENOSINE METABOLISM.
Lapa, F. R. [1]; Cabrini, D. A. [1]; Santos, A. R. S. [2]
[1] Depto de Farmacologia, UFPR[2] Depto. de ciências Fisiológicas, UFSC

17.063
ANTI-INFLAMMATORY ACTIVITY OF OMEGA-3: AN IN VIVO AND IN VITRO EVALUATION
Chakraborty, S. A. [1]; Sampaio, T. M. A. [1]; Correia, A. O. [1]; Nobre, M. E. P. [1,2,4]; Felipe, C. F. B. [1]; Lucetti, D. L. [1]; Lopes, A. D. A. [3]; Leal, L. K. A. M. [3]; Viana, G. S. D. B. [1,3]; Arida, R. M. [4]
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17.064
NEURAL MOBILIZATION IN NEUROPATHIC PAIN CONTROL: EVALUATION OF SATELLITE CELLS AND NEURAL GROWTH FACTOR IN DORSAL ROOT GANGLION.
Silva, J. T. D. [1]; Santos, F. M. D. [1]; Giardini, A. C. [1]; Silva, A. A. D. [2]; Chacur, M. [1]
[1] Depto de Anatomia, ICB III, USP, ICB III - USP[2] Depto de Fisiologia e Biofísica, ICB I, USP, ICB I - USP

17.065
GENETIC BACKGROUND DETERMINES MOUSE STRAIN DIFFERENCES IN INFLAMMATORY ANGIOGENESIS
Marques, S. M. ; Campos, P. P. ; Castro, P. R. ; Cardoso, C. C. ; Ferreira, M. A. N. D. ; Andrade, S. P.
Fisiologia/Instituto de Ciências Biológicas, UFMG

17.066
EFFECT OF DEXAMETHASONE ON OROFACIAL NEUROPATHIC THERMAL NOCICEPTION
Coelho, S. C. ; Chichorro, J. G. ; Zampronio, A. R.
Farmacologia / Universidade Federal do Paraná, UFPR

17.067
EFFECT OF APPLICATION OF ALTERNATING ELETRIC CURRENTS OF MEDIUM FREQUENCY (NEMEC), EVALUATED BY SCAPULAR POSITION, ON THE CERVICOBRACHIALGY – CASE STUDY
Lemes, E. V. ; Beirith, A.
Department of Physiotherapy and Natural Sciences, FURB

18. Imunologia Celular e Molecular

18.001
DOUBLE COLLECTION: AN IMPROVEMENT ON TUBERCULOSIS DIAGNOSIS
Laux, L. C. ; Michelon, C. T. ; Rosso, F. ; Schmid, K. B. ; Ribeiro, A. W. ; Verza, M. ; Dalla Costa, E. R. ; Rossetti, M. L. R.
Centro de Desenvolvimento Científico e Tecnológico, FEPPS

18.002
BLOCKADE OF THE CXCL-ELR+ CHEMOKINE / RECEPTOR CXCR2 AXIS DURING NEUTROPHILIC PHASE ACCELERATES WOUND CLOSURE IN MICE
Castro, T. B. R. [1,2]; Canesso, M. C. C. [1,2]; Almeida, B. G. [1,2]; Cisalpino, D. [1,4]; Colotta, F. [5]; Bertini, R. [5]; Proudfoot, A. [6]; Andrade, S. P. [2]; Teixeira, M. M. [1,3]; Barcelos, L. S. [1,2]
[6] Merck Serono Geneva Research Centre, UNIGE[4] Microbiology, ICB-UFMG[5] Dompé Research and Development, Dompé S.p.A[1] Immunopharmacology group, ICB-UFMG[2] Department of Physiology and Biophysics, ICB-UFMG[3] Biochemistry and Immunology , ICB-UFMG

18.003
THE ROLE OF EXTRACELLULAR ATP ON PROMASTIGOTES OF LEISHMANIA AMAZONENSIS
Uribe; G. D. C. S. ; Marques-da-silva; C. ; Coutinho-silva; R.
Instituto de Biofísica Carlos Chagas Filho/ UFRJ, IBCCF

18.004
CELL NUMBER QUANTIFICATION AND IL-4 PRODUCTION OF MACROPHAGES IN YOUNG AND ADULT RATS SUBMITTED TO NEONATAL MALNUTRITION
Galvao, A. M. [1]; Melo, J. F. D. [1,2]; Costa, T. B. D. [1]; Chaves, M. E. C. [1]; Nagel, M. [2]; Castro, C. M. M. B. D. [1,2]
[1] Laboratório de Imunopatologia Keizo Asami, LIKA[2] Université de Technologie de Compiègne, BMBI

18.005
EFFECT OF PALMITATE IN THE CELLULAR DYE REDUCTION OF 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) AND PRODUCTION OF REACTIVE OXYGEN SPECIES IN LEUKOCYTES FROM TYPE 2 DIABETIC PATIENTS
Volpe, C. M. O. [1]; Fagundes-netto, F. S. [1]; Veloso, C. A. [2]; Fernandes, J. S. [1]; Chaves, M. M. [2]; Nogueira-machado, J. A. [1]
[1] Instituto de Ensino e Pesquisa da Santa Casa de BH, IEP - Santa Casa BH[2] Departamento de Bioquímica e Imunologia UFMG, ICB - UFMG

18.006
BRAIN MICROCIRCULATORY EFFECTS OF LOVASTATIN IN PLASMODIUM BERGHEI ANKA-INDUCED EXPERIMENTAL CEREBRAL MALARIA.
Estato, V. [1]; Reis, P. A. [2]; Tibiriçá,[1]; Castro--faria-neto, [2]
[1] Laboratório de Investigação Cardiovascular, FIOCRUZ[2] Laboratório de Imuno--farmacologia, FIOCRUZ

18.007
MODULATION OF EXPERIMENTAL GLOMERULONEPHRITIS BY INVARIANT NATURAL KILLER T CELLS AGONISTS.
Khaled, N. A. [2,3]; Monteiro, A. P. F. S. [2,3]; Reis, V. O. [2,3]; Silva, J. C. [2,3]; Trindade, G. [2,3]; Savage, P. B. [4]; Keller, A. C. [2,3]
[3] Departamento de M.I.P./ Universidade Federal de São Paulo, UNIFESP[2] Laboratório de Imunopatologia Experimental, LIPE/ UNIFESP[4] Department of Chemistry and Biochemistry, Brigham Young Univ, BYU

18.008
INFLAMMATORY BIOMARKERS IN WORKERS EXPOSED TO SILICA
Mendonça, V. A. [1]; Souto, M. F. O. [2]; Carneiro, A. P. S. [2]; Borges, V. O. [3]; Mendonça, A. L. [2]; Amorim, M. R. [1]; Teixeira, M. M. [3]; Teixeira, A. L. [3]
[1] Laboratório de Imunologia/Departamento de Fisioterapia/FCBS, UFVJM[2] Centro de Referencia Estadual em Saúde do Trabalhador , CEREST[3] Laboratório de Imunofarmacologia – ICB , UFMG

18.009
PRODUCTION OF IFN- , IL-12 AND IL-10 BY ALVEOLAR MACROPHAGES AND LYMPHOCYTES: STUDY OF IN VITRO CELLULAR INFECTION BY METHICILLIN--SENSITIVE AND METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS IN RATS WITH NEONATAL MALNUTRITION
Costa, T. B. [1]; Morais, N. G. [1]; Almeida, T. M. D. [2]; Ribas, K. H. S. [2]; Araújo, F. R. G. [2]; Castro, C. M. M. B. [1]
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18.010
REDUCTION OF THE NUMBER AND REACTIVITY OF MAST CELLS INDUCED BY GLUCOCORTICOIDS DEPENDS OF ADVANCED GLYCATION END PRODUCTS
Santoro, T. ; Torres, R. C. ; Cordeiro, R. S. B. ; Silva, P. M. R. ; Martins, M. A. ; Carvalho, V. F.
Laboratório de Inflamação/ Instituto Oswaldo Cruz, IOC/FIOCRUZ

18.011
TRYPANOSOMA CRUZI INVADES HOST CELLS THROUGH THE ACTIVATION OF ENDOTHELIN AND KININ RECEPTORS: A CONVERGING PATHWAY LEADING TO CHAGASIC VASCULOPATHY
Andrade, D. D. S. [1]; Serra, R. R. [1]; Svensjö, E. [1]; Morandi, V. [2]; Soeiro, ;. M. D. N. C. [3]; Tanowitz, H. B. [4]; Scharfstein, J. [1]
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18.012
B7-CD28/CTLA-4 PATHWAY IN ALTERED IN ALZHEIMER AND FRONTOTEMPORAL DEMENTIA PATIENTS
Lima,; Torres, K. C. L. ; Santos, R. R. D. ; Fiamoncini, C. ; Ferreira, R. O. S. ; Moraris, E. N. ; Silva, M. A. R.
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18.013
PERIPHERAL BIOMARKERS RELEASED BY HUMAN FIBROBLASTS AFTER STIMULA-


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Juliana Félix de Melo;Renata Almeida Raele;Simone do Nascimento Fraga;Nijez Aloulou;Jean-Luc Duval;Pascale Vigneron;Lee Bourgoin;Carol Góis Leandro;Célia Maria Machado Barbosa De Castro;Marie-Danielle Nagel
apresentaram, no VI Congresso Brasileiro de Células-Tronco e Terapia Celular realizado no período de 28 de setembro a 01 de outubro de 2011, em Salvador - Bahia, trabalho intitulado "IL-4Rα/IL-4 IN MYOBLASTS AND MYOTUBES OF YOUNG AND ADULT RATS SUBMITTED TO NEONATAL MALNUTRITION", sob a forma de Poster.
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Salvador, 01 de outubro de 2011
Ricardo Ribeiro dos Santos
Presidente do VI Congresso Brasileiro de Células-Tronco e Terapia Celular
Rosalia Mendez-Otero
Presidente da ABTCel
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IL-4Rα/IL-4 IN MYOBLASTS AND MYOTUBES OF YOUNG AND ADULT RATS SUBMITTED TO NEONATAL MALNUTRITION

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Introduction and Objectives. The myogenic process can be triggered by the fusion of muscle cells through the interaction of the cytokine IL-4 produced by newly formed myotubes with IL-4Rα receptor present on the myoblasts. Although there are studies about the effect of early malnutrition on the ability of differentiation of muscle cells, little has been researched about the late effect after a period of nutritional supplementation. The objective of this study was to investigate whether neonatal malnutrition interferes with the receptor IL-4Rα expression and IL-4 production in young and adult rats after nutritional supplementation. Methods and Results. Male Wistar rats (n=18) were handled in accordance with the guidelines and regulations of the Ethics and Safety Committee of the Compiègne University of Technology. The animals were suckled by mothers fed diets containing 17% protein (controls, C) or 8% protein (undernourished, UN). All rats were fed a normal protein diet after weaning. Body weights were recorded every 5 days during lactation. Muscles were removed from both legs of 42-, 60- and 90-day-old rats and the cells (1.6 x 106 cells/ 80cm2 dishes) were cultured in DMEM for a total of 10 days. The expression of IL-4Rα protein in cells cultured for 7 days was studied by western blotting. IL-4 released was measured by ELISA in the supernatants from cells cultured for 10 days. During lactation, offspring from mothers fed a low-protein diet showed a lower body weight than controls at 10, 15 and 21 days. The pups from undernourished mothers continued to exhibit a lower body weight than the control group throughout the experiment: 42 days (C = 208.6 ± 4.3; UN = 183.3 ± 4.3), 60 days (C = 308.7 ± 6.6; UN = 246.1 ± 5.6) and 90 days (C = 378.2 ± 7.7; UN = 313.1 ± 4.3), $p<0.05$ unpaired Student's t-test. There was no difference in the IL-4Rα content of myoblasts from 42-, 60- and 90-day-old UN and C pups. The concentration of IL-4 in the supernatants of myotubes showed no significant differences between UN and C offspring. Conclusion. These results suggest that neonatal malnutrition do not modifie the expression of IL-4Rα receptor in myoblasts neither IL-4 production in myotubes of young and adult rats, when there is a period of nutritional supplementation