Universidade Federal de Pernambuco
Centro de Ciências da Saúde
Programa de Pós-Graduação em Nutrição

EFEITO DA DESNUTRIÇÃO NEONATAL SOBRE A FUNÇÃO E O METABOLISMO MIOCITÁRIO DE RATOS ADULTOS JOVEM

SIMONE DO NASCIMENTO FRAGA

Prof[a] Célia Maria Machado Barbosa de Castro

Prof[a] Marie Danielle Nagel

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

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Tese aprovada em 15 de janeiro de 2013 pela banca examinadora:

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Recife
2013

Ao meu pai *(in memoriam)*

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"Compreendi que viver é ser livre... Que ter amigos é necessário... Que lutar é manter-se vivo... Que pra ser feliz basta querer... Aprendi que o tempo cura... Que mágoa passa... Que decepção não mata... Que hoje é reflexo de ontem... Compreendi que podemos chorar sem derramar lágrimas... Que os verdadeiros amigos permanecem... Que dor fortalece... Que vencer engrandece... Aprendi que sonhar não é fantasiar... Que pra sorrir tem que fazer alguém sorrir...Que a beleza não está no que vemos, e sim no que sentimos... Que o valor está na força da conquista... Compreendi que as palavras tem força... Que fazer é melhor que falar... Que o olhar não mente... Que viver é aprender com os erros... Aprendi que tudo depende da vontade... Que o melhor é ser nós mesmos...

"...E umas das coisas que aprendi é que se deve viver apesar de. Apesar de, se deve comer. Apesar de, se deve amar. Apesar de, se deve morrer. Inclusive muitas vezes é o próprio apesar de que nos empurra para frente. Foi o apesar de que me deu uma angústia que insatisfeita foi criadora de minha própria vida."

Clarice Lispector

RESUMO

Para estudar o efeito da desnutrição neonatal sobre o tecido muscular esquelético, foi avaliada a sensibilidade dos miotubos em cultura à insulina, bem como os parâmetros biomecânicos das células musculares cultivadas. Para isso, 12 ratos machos Wistar foram divididos em dois grupos, nutrido (N), n = 6, e desnutrido (D), n = 6, segundo a dieta oferecida à mãe durante o período de lactação (21 dias) de sua prole. Para o acompanhamento da evolução ponderal, os animais foram pesados a cada 5 dias durante o período de aleitamento, e uma vez por semana no período após a lactação. Após o desmame, os animais receberam dieta padrão de biotério até o dia da coleta do músculo esquelético (60-70 dias). Através da otimização de protocolos anteriores, um novo protocolo para a cultura de células musculares primárias foi estabelecido com o objetivo de simplificar os procedimentos, reduzir o tempo para a confecção da cultura e torná-lo menos dispendioso. Assim, as células musculares das patas posteriores dos animais foram cultivados na presença de insulina (Ni, Di) ou na ausência desta (grupos controles Nc, Dc) e, ao 10^{o} de cultura, foram observados a taxa de proliferação celular e o percentual de incorporação de núcleos pelos miotubos entre os animais N e D. Além disso, os parâmetros biomecânicos dos miotubos foram caracterizados através da frequência de contração, do percentual de amplitude, bem como pelo período de contração. Ao 21^{o} dia, o peso dos animais D mostrou-se inferior quando comparado ao peso dos animais N (N = 67.8 ± 3.0; D = 40.7 ± 1.9). Esta condição permaneceu até o dia da coleta dos músculos, entre 60-70 dias (N = 394 ± 28.4; D = 342.7 ± 20.7). A insulina não interferiu na taxa de proliferação dos mioblastos (Nc = 2.61 ± 0.56; Ni = 2.49 ± 0.51; Dc = 3.12 ± 0.92; Di = 2.77 ± 0.81), mas reduziu o percentual de incorporação de núcleo pelos miotubos dos animais D (Dc = 11.5 ± 8.9; Di = 7.0 ± 5.6). Miotubos de animais N e D, tratados com insulina, apresentaram maior número de contrações por minuto (Nc = 17.6 ± 1.8, Dc = 16.4 ± 3.6; Ni = 29.2 ± 3.8; Di = 28.2 ± 4.2). O percentual de amplitude não se mostrou diferente entre N e D ($p=0.0687$). A presença da insulina aumentou o período de contração nos animais N (Ni = 1.2 ± 0.9; Dc = 0.8 ± 0.3), enquanto que no grupo D provocou redução (Di = 0.8 ± 0.1; Dc = 0.9 ± 0.2). Houve redução no período de contração no grupo Di, quando comparado ao grupo Ni ($p<0.05$). Assim, este estudo permitiu complementar os conhecimentos sobre o efeito da desnutrição neonatal sobre a ação da insulina no tecido muscular, além de possibilitar o surgimento de novas perspectivas sobre o estudo do músculo esquelético em cultura.

Palavras-chaves: desnutrição, programação metabólica, músculo esquelético, miotubos, cultura de células.

ABSTRACT

In order to study the effect of neonatal undernutrition on skeletal muscle tissue, the sensitivity of culture myotubes to insulin, as well as the biomechanical parameters of muscle cells cultivated were assessed. For this purpose, twelve male Wistar rats were divided into two groups according to the diet provided to the mother during lactation period (21 days) of their offspring: 6 nourished (N) and 6 undernourished (U). For monitoring the weight gain, the animals were weighed every 5 days during the suckling period and once a week in the period after lactation. After weaning, the animals were fed with standard animal diet until the day of the collection of skeletal muscle (60-70 days). Through improvement in previous protocols, a new protocol for the culture of primary muscle cells was established with the aim to simplify procedures, reduce the time for the making of culture and make it less expensive. So, the muscle cells of the hind legs of the animals were cultured in the presence of insulin (Ni, Ui) or in its absence (control groups Nc, Uc). At culture day 10, we have observed the cell proliferation rate and the percentage nuclei incorporation by myotubes between animals N and U. Furthermore, the biomechanical parameters of myotubes were characterized by the frequency of contraction, the percentage of amplitude and the period of contraction. At day 21, the weight of animals U proved inferior when compared to the weight of animals N (N = 67.8 ± 3.0; U = 40.7 ± 1.9). This condition remained until the day of muscle collection, between 60-70 days (N = 394 ± 28.4; U = 342.7 ± 20.7). Insulin did not affect the rate of myoblasts proliferation (Nc = 2.61 ± 0.56, Ni = 2.49 ± 0.51; Uc = 3.12 ± 0.92, Ui = 2.77 ± 0.81), but decreased the percentage of nuclei incorporation of the myotubes animal U (Uc = 11.5 ± 8.9; Ui = 7.0 ± 5.6). Myotubes of animals N and U, treated with insulin, presented a higher number of contractions per minute (Nc = 17.6 ± 1.8, Uc = 16.4 ± 3.6; Ni = 29.2 ± 3.8, Ui = 28.2 ± 4.2). The amplitude percentage has not presented difference between N and U ($p = 0.0687$). The presence of insulin increased the contraction period in N animals (Ni = 1.2 ± 0.9; Uc = 0.8 ± 0.3) while group U showed a reduction (Ui = 0.8 ± 0.1; Uc = 0.9 ± 0.2). There was a reduction in the contraction period in Ui group, when compared to Ni ($p < 0.05$). Thus, this study provided additional knowledge about effect of neonatal undernutrition on insulin action in muscle tissue, besides allowing the emergence of new perspectives on study of skeletal muscle in culture.

Key Words: undernutrition, metabolic programming, skeletal muscle, myotubes, cell culture.

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LISTA DE ABREVIATURAS E SIGLAS

%A	Percentual de amplitude
AIN-93G	Instituto Americano de Nutrição - 93 para crescimento
CEEA	Comitê de Ética em Experimentação Animal
D	Desnutrido
DMEM	Dulbecco's Modified Eagle Medium
DNA	Ácido Desoxirribonucléico
F	Frequência de contração
FAO	Organização das Nações Unidas para a Agricultura e Alimentação
FM	Meio de fusão para células musculares esqueléticas
GLUT-1	Transportador de Glicose - 1
GLUT-4	Transportador de Glicose - 4
HGF	Fator de crescimento do hepatócito
IGF-I	Fator de crescimento insulínico - I
IL-4	Interleucina - 4
ISSN	Padrão Internacional do Número de Série
L6	Linhagem de célula muscular esquelética de rato
MF	Meio de Fusão
MRF	Fator de Regulação Miogênica
MRF 4	Fator de Regulação Miogênica 4
mRNA	RNA mensageiro
Myf 5	Proteína que regula a diferenciação muscular
Myo D	Proteína para diferenciação muscular
N	Nutrido
P	Período de contração
PAF	Paraformaldeído
PBS	Tampão Salina Fosfato
PGM	Meio de crescimento para células musculares esqueléticas

SUMÁRIO

1 APRESENTAÇÃO

A desnutrição neonatal é um dos principais problemas de saúde pública que ainda persiste nos países em desenvolvimento, nos quais a privação econômica leva ao retardo do crescimento infantil associado a alterações metabólicas severas (TORUN; CHEW, 1994). Em ratos, a deficiência nutricional materna no período crítico de crescimento e desenvolvimento da prole é capaz de interferir na função dos sistemas orgânicos em decorrência de alterações no padrão de eventos celulares, que levam a consequências deletérias na maturação dos sistemas orgânicos (BORBA *et al.*, 2000) e no metabolismo. De acordo com Guedes, Melo e Teodósio (2004), as consequências da desnutrição neonatal, à longo prazo, estão correlacionadas de forma proporcional à intensidade e à duração das alterações nutricionais, e podem afetar tanto a estrutura quanto o metabolismo de órgãos e sistemas, fenômeno conhecido como programação metabólica (SRINIVASAN; PATEL, 2008; WEELS, 2003; GODFREY; BARKER, 2001).

A desnutrição calórico-proteica está associada ao déficit funcional da imunidade mediada por células e da imunidade humoral (CHANDRA, 2002), ao aumento do tempo do ciclo celular das linhagens da medula óssea (GÓMEZ *et al.*, 1996) e à modificações na taxa de apoptose (RIVADENEIRA *et al*., 2001; ORTIZ *et al*., 2009). O tecido muscular esquelético, em especial, se mostra sensível à desnutrição por apresentar-se susceptível às alterações na diferenciação das fibras musculares (IHEMELANDU, 1985; NASCIMENTO *et al.*, 1990; ALVES; DÂMASO; DAL PAI, 2008; OLIVEIRA *et al.* 1999), cuja formação é regulada pela ativação, proliferação e diferenciação de várias linhagens de células miogênicas, além de depender da expressão e da atividade de fatores transicionais (DAL PAI-SILVA; CARVALHO, 2007; MELO et al., 2011).

O controle do processo de fusão celular tem grande importância no desenvolvimento de bioterapias futuras utilizando as células miogênicas precursoras (células satélites). Mas antes disso, a fusão de células miogênicas precursoras é um evento chave no desenvolvimento e na reparação do músculo esquelético (FALZONI *et al*., 2000; TAYLOR, 2000; BAILEY *et al*., 2001; MELO et al., 2011). Fatores de crescimento como a inervação, a IL-4 e sinais hormonais tal como a insulina, se estabelecem como condições imprescindíveis para o desenvolvimento dos miotubos (JACQUEMIN et al., 2005) e, consequentemente, do desempenho da função muscular.

O músculo, por ser alvo da ação da insulina (WOLFE, 2000; LEVINE *et al*., 1949), tem suas funções mecânicas diretamente relacionadas à atuação deste hormônio polipeptídio, que vão desde o transporte de aminoácidos, de íons, de nucleotídeos, do potencial elétrico até a síntese e degradação de proteínas e do metabolismo do RNA (GOLDFINE, 1981; BRUNETTI *et al*., 1989). A regulação do tamanho do miotubo também é importante para o bom funcionamento do músculo esquelético, e neste caso, a insulina poderá ser um fator determinante para o seu desenvolvimento.

Vários fatores de crescimento foram implicados no processo de ativação de células miogênicas, mas a fusão destas células é um processo complexo que requer uma série de eventos celulares e moleculares até então pouco conhecidos (CHARGÉ, 2003). O mecanismo molecular de aumento na assimilação de glicose estimulado por insulina, por exemplo, pode estar relacionado a um aumento na expressão e na atividade de proteínas chaves conhecidas por regular o metabolismo da glicose no músculo esquelético (ZIERATH, 2002).

O tamanho da fibra muscular foi a primeira medida a ser avaliada sobre a repercussão da desnutrição no músculo (HALBAN, 1983: apud WALLS, 1960), e em outros trabalhos, a desnutrição foi capaz de reduzir o diâmetro das fibras musculares e o seu tamanho (MONTGOMERY, 1962), bem como de alterar a frequência dos tipos de fibras (MARCONDES, 1976). Em decorrência destes achados, outros estudos envolvendo as adaptações das fibras esqueléticas musculares também têm sido alvo das alterações metabólicas proporcionadas pela desnutrição (HENRIKSSON, 1990; MAXWELL *et al*., 1992).

No intuito de responder estas questões, a comunidade científica vem investindo em pesquisas envolvendo desnutrição e seus efeitos metabólicos. Apesar da desnutrição provocar depleções e disfunções enzimáticas, mitocondriais (NORMAN; LOCHS; PIRLICH, 2005) e aumento da sensibilidade à insulina no músculo solear (GRACE *et al*., 1990), pouco se sabe ainda, o quanto ela é capaz de afetar a atividade biomecânica muscular. Em trabalho publicado por Melo *et al.* (2011), o modelo experimental de desnutrição utilizado em ratos Wistar (dieta a base de 8% de caseína durante a lactação, AIN-93G) mostrou uma diferença na formação de miotubos em animais com idade entre 60 e 70 dias de vida no que se refere à morfologia e à atividade mecânica destes miotubos em cultura.

Baseado no exposto, nota-se uma importante disfunção muscular em decorrência da desnutrição e a perda de massa muscular contribui inegavelmente para o prejuízo desta função. Assim, tais estudos nos motivou a estudar o perfil biomecânico de miotubos de ratos que foram submetidos à desnutrição neonatal, e que passaram por um período de reposição nutricional, visto que grande parte dos estudos é realizada em animais desnutridos, mas que não foram recuperados totalmente ou parcialmente da desnutrição, o que deixa lacunas no esclarecimento sobre os eventos celulares após recuperação total ou parcial do quadro de desnutrição promovido durante o período neonatal.

Além disso, a dificuldade de se encontrar na literatura um protocolo de coleta, isolamento e cultura de células musculares primárias que fosse de fácil reprodução, somado à variabilidade nos protocolos publicados em artigos de circulação internacional, nos levou a buscar um método mais simples e rápido que fosse capaz de manter a viabilidade celular, além de promover a formação de miotubos em cultura.

Diante do exposto, o presente estudo teve como objetivos avaliar o efeito da desnutrição neonatal sobre o sistema muscular de ratos adultos a partir de culturas de células musculares primárias; analisar o efeito da insulina sobre a proliferação celular e a formação de miotubos em cultura de ratos desnutridos; avaliar os parâmetros biomecânicos *(frequência, amplitude e período de contração)* de miotubos em cultura e estudar a capacidade de incorporação de núcleos pelos miotubos de ratos desnutridos na presença de insulina, além de otimizar o protocolo para coleta e cultura de células musculares utilizado por Melo *et al.* (2011), a fim de proporcionar aos pesquisadores uma possibilidade simplificada de realizar mais estudos que envolvam o músculo esquelético.

Para tais fins, foram utilizadas, no período de aleitamento, dieta materna à base de 17% de caseína para o grupo controle, denominado Nutrido (N), bem como à base de 8% de caseína para o grupo experimental, denominado desnutrido (D). Entre 60 e 70 dias de vida, os ratos N (n=6) e D (n=6) foram eutanasiados para coleta e cultura das células musculares primárias. As células foram estimuladas em cultura com insulina, com o intuito de possibilitar a formação do combustível metabólico (glicogênio) nos miotubos já formados. Filmes foram realizados para avaliação do perfil biomecânico dos miotubos, os quais também foram imunocitoquimicamente marcados para a observação do percentual de núcleos incorporados e, assim, do potencial de formação dos miotubos pelos ratos D.

Ao longo deste processo, e em consonância com estudo piloto realizado, o protocolo de coleta e cultura de células musculares foi otimizado para que estudos com células musculares primárias em cultura se tornem mais frequentes, em razão da simplificação do protocolo de coleta e isolamento destas células.

Assim, o estudo das eventuais relações entre a desnutrição neonatal, seguida de reposição nutricional e as células miocitárias, poderá cooperar no esclarecimento sobre as consequências que este modelo de desnutrição acarreta nestas células quando adultas, em especial nas células satélites, precursoras inicial dos miotubos que estas estão relacionadas com o reparo muscular diante da injúria.

Esta pesquisa resultou na redação de dois artigos. O primeiro deles, intitulado IMPROVED PROTOCOL FOR THE COLLECTION AND CULTURE OF WISTAR RAT PRIMARY SKELETAL MUSCLE CELLS, foi submetido ao *The Journal of Physiological Sciences*, ISSN 1880-6546, cuja norma de submissão se encontra no Anexo D. Neste artigo, foi descrito o método otimizado de coleta e cultura de células musculares primárias de ratos Wistar.

O segundo artigo intitulado EFFECT OF INSULIN ON THE FORMATION AND FUNCTION OF MYOTUBE CULTURES FROM YOUNG ADULTS RATS SUBJECTED TO NEONATAL UNDERNUTRITION, demonstra o efeito que a insulina exerce em cultura de miotubos sobre os parâmetros morfológicos e biomecânicos frente à desnutrição neonatal. Este artigo, por sua vez, foi submetido ao *British Journal of Nutrition*, ISSN 0007-1145, cuja norma de submissão se encontra no Anexo E.

2 REVISÃO DA LITERATURA

2.1 Desnutrição

As diferentes formas de má nutrição, resultantes do déficit ou do excesso de nutrientes essenciais e de alto valor biológico, são consideradas um grave problema de saúde pública. Caracterizada pela redução de quantidades ideais de macro e de micronutrientes, e apesar do atual processo de transição nutricional, a desnutrição continua sendo um grave problema de saúde pública mundial, uma vez que ela pode desequilibrar grande parte das funções fisiológicas normais do organismo em decorrência da privação de nutrientes essenciais para a manutenção da homeostase (GODFREY; BARKER, 2001; BERTRAM; HANSON, 2001; ARMITAGE et al. 2004, 2005; BURDGE et al., 2007).

De acordo com a Organização das Nações Unidas para Agricultura e Alimentação (FAO), cerca de 925 milhões de pessoas no mundo foram acometidas pela desnutrição energético-proteica em 2010, cuja maioria é proveniente de países de economia emergente (FAO, 2010).

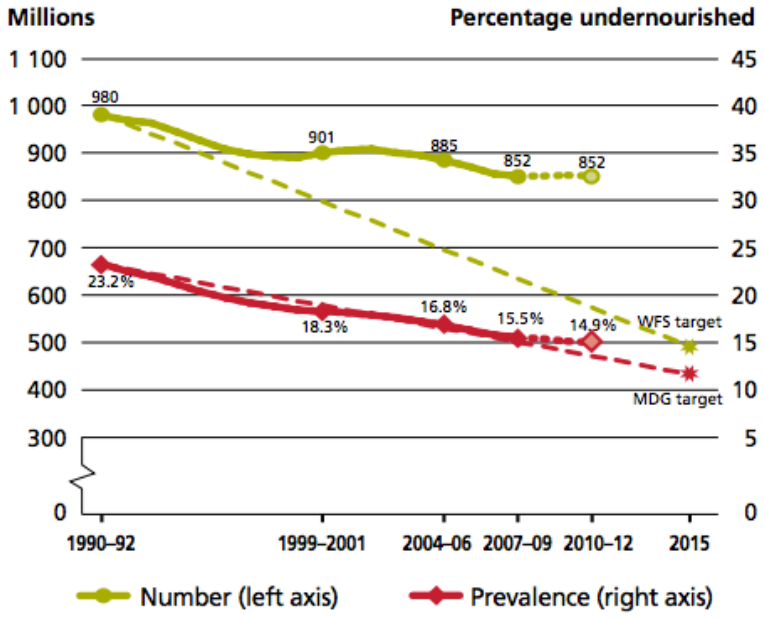


Figura 1. Desnutrição no mundo em desenvolvimento. Dados de estimativas provisórias para 2010-12. Fonte: FAO, 2012

A transição nutricional, na qual se observa um aumento da incidência do binômio sobrepeso/obesidade, é uma tendência epidemiológica mundial que se contrapõe à diminuição do número de pessoas acometidas pela desnutrição (SOUZA, 2010). Mas apesar dos índices mundiais decrescentes, com cerca de 852 milhões de indivíduos desnutridos (14,9% da população) em 2012 (FAO, 2012), como mostra a **Figura 1**, a desnutrição não se encontra erradicada na sociedade brasileira e demais países em desenvolvimento, sendo ainda considerada como um fenômeno resultante de processos sociais de exploração, na qual o indivíduo se submete a uma pauta dietética de baixo valor biológico (SCHAIBLE; KAUFMANN, 2007).

Existem dois tipos básicos de desnutrição. A primeira e mais importante é a desnutrição energético-proteica, que se caracteriza pela baixa ingestão de alimentos ricos em carboidratos e proteínas. Este é o tipo mais letal, sendo referida quando o problema da fome mundial é discutido. O segundo tipo se refere à deficiência de micronutrientes, como vitaminas e minerais (WORLD HUNGER, 2011). A deficiência de vitamina A, por exemplo, pode resultar em cegueira noturna ou adaptação ao escuro prejudicada, em anemia, no aumento da vulnerabilidade às infecções respiratórias, no crescimento deficiente e desenvolvimento ósseo retardado, podendo ocasionar até a morte (UCHENDU, 2011). A **Figura 2** esquematiza os principais efeitos da desnutrição sobre diversas estruturas e sistemas orgânicos.

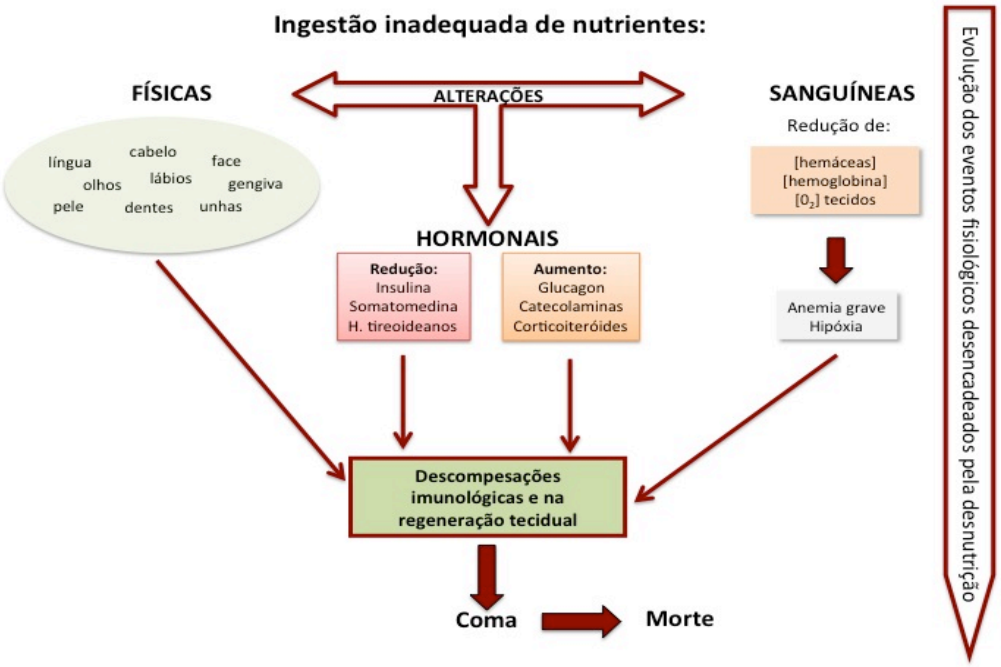


Figura 2. Evolução da desnutrição: consequência sobre diversos sistemas e estruturas do corpo. Fonte: PRODIET Nutrição Clínica, 2009 (modificado)

A desnutrição precoce pós-natal, comumente denominada desnutrição neonatal, é um subtipo particular de desnutrição que ainda persiste nos países em desenvolvimento, nos quais as privações econômicas e sociais levam ao retardo do crescimento infantil associado às alterações metabólicas severas nestas crianças (TORUN; CHEW, 1994). Em ratos, a deficiência nutricional materna no período crítico de crescimento e desenvolvimento da prole é capaz de interferir na função dos sistemas orgânicos em decorrência de alterações no padrão de eventos celulares, que levam a consequências deletérias tanto na aquisição de padrões fisiológicos maduros do organismo (BORBA *et al.*, 2000) quanto na ocorrência de reações metabólicas necessárias à manutenção da homeostase fisiológica.

Estudo de Morley e Lucas publicado em 1993 relata a consequência da desnutrição em diversos sistemas. Segundo eles, a desnutrição é capaz de causar várias deficiências que se refletem ao longo da vida, tais como a redução de força muscular e a fadiga precoce, deterioração da função muscular respiratória, cardíaca, sexual e imunológica, além de predispor ao estado de depressão, irritabilidade, ansiedade, incapacidade de manter a concentração e redução do desejo sexual. Em crianças, a desnutrição prejudica ainda o crescimento e provoca atraso na maturação sexual.

Várias pesquisas, ao longo dos anos, têm investido em estudos que buscam relacionar os processos de desenvolvimento que ocorrem durante estágios iniciais de vida com o estado de saúde na vida adulta do indivíduo. Estes estudos foram intensificados desde a época de Hipócrates, passando por Forsdahl em 1977 e Barker e Osmond em 1986. Este último, que estuda temas relacionados ao crescimento e desenvolvimento, foi um pioneiro na realização de pesquisas que evidenciassem a associação do baixo peso ao nascer com a ocorrência de doenças relacionadas à síndrome metabólica no adulto como a hipertensão, a diabetes tipo II e doenças cardiovasculares. Anteriormente, tais fatos eram considerados apenas uma hipótese, conhecida como *Hipótese de Barker*. Hoje, entretanto, esta hipótese mantem-se confirmada depois de vários estudos que mostraram que a subnutrição fetal ou no primeiro ano de vida (neonatal), principalmente quando seguida de plena recuperação nutricional ou até excesso de peso/ obesidade, que representa um fator de risco para o desenvolvimento de doença cardiovascular coronariana (BARKER, 1995).

Outros estudos mostram ainda que a desnutrição neonatal é capaz de alterar outros sistemas fisiológicos, tais como o sistema imune, com o enfraquecimento da imunidade mediada por células e da imunidade humoral (CHANDRA, 2002), o aumento do tempo do

ciclo celular das linhagens da medula óssea (GÓMEZ *et al.*, 1996) e modificações na taxa de apoptose (RIVADENEIRA *et al*., 2001; ORTIZ *et al*., 2009).

O tecido muscular esquelético, em especial, se apresenta sensível à desnutrição proteica por ser um reservatório de proteínas no organismo, se tornando alvo de depleção quando há déficit proteico na dieta, o que leva a alterações nas fases de crescimento, desenvolvimento e diferenciação das fibras musculares (IHEMELANDU, 1985; NASCIMENTO *et al.*, 1990; OLIVEIRA *et al.* 1999, ALVES; DÂMASO; DAL PAI, 2008). Assim, a hipótese de Barker foi reforçada, o que significa que as ações de fatores ambientais, como a desnutrição, às quais o organismo é submetido durante período fetal e pós-natal imediato - os estágios iniciais de vida - possivelmente podem programar as funções orgânicas na vida adulta como efeito tardio da desnutrição.

2.2 Miogênese e atuação dos fatores de crescimento do músculo esquelético

O músculo esquelético é a unidade do sistema muscular derivada de células precursoras mesodérmicas provenientes dos somitos que se liga diretamente aos ossos, cartilagens, fáscias e pele (JUNQUEIRA; CARNEIRO, 2008). Durante o desenvolvimento embrionário, os precussores celulares do mesoderma para linhagem miogênica são regulados por sinais químicos liberados pelos tecidos adjacentes. Estes sinais estão envolvidos com a regulação do processo de formação miogênica, e envolvem a ativação, a proliferação e a diferenciação de várias linhagens de células miogênicas dependentes da expressão e da atividade de fatores transcricionais, conhecidos como fatores de regulação miogênica (DAL PAI-SILVA; CARVALHO, 2007). Os fatores transcricionais envolvidos nesta missão são o M*yo*D e o M*yf*5, que são ligados à família *myogenic regulation factor* (MRF) (HASTY *et al*., 1993; NABESHIMA *et al*., 1993).

Após a diferenciação decorrente da expressão dos fatores M*yo*D e M*yf*5, as células miogênicas são então chamadas de mioblastos ou células musculares jovens. Após a expressão do fator *MRF*-4, pertencente à mesma família MRF, as células são reconhecidas como miócitos, que são células adultas diferenciadas que exercem plenamente as funções atribuídas às células musculares (**Figura 3**). Estas células contêm genes específicos que

expressam a cadeia pesada de miosina e a creatina quinase, que caracterizam os miócitos (LOWEY *et al*., 1969).

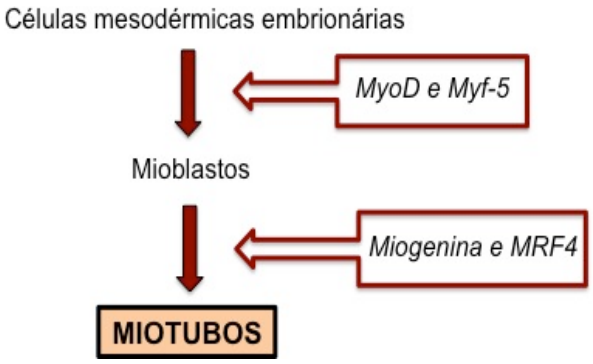


Figura 3. Atuação dos fatores de crescimento nas fases de desenvolvimento do tecido muscular

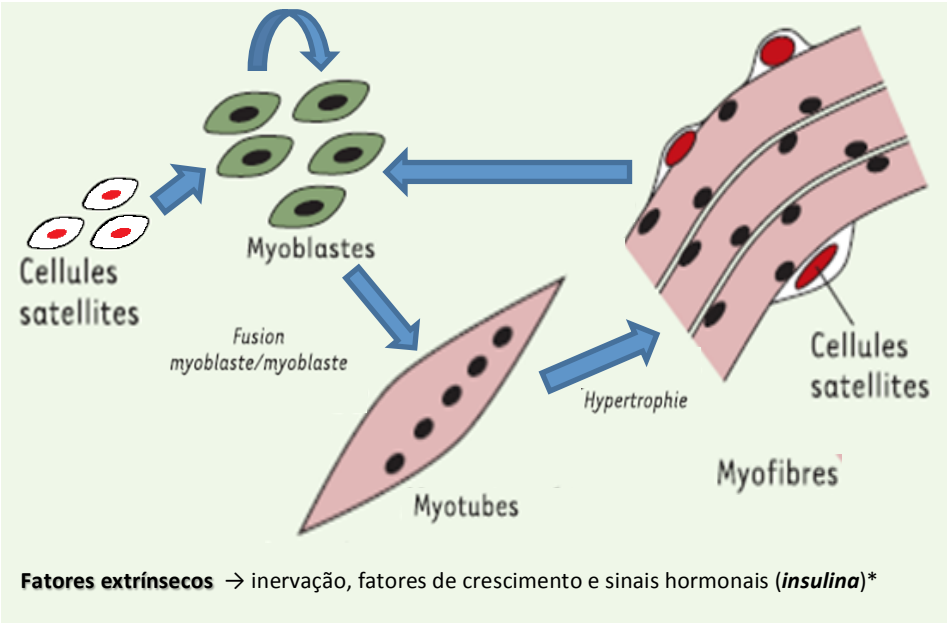


Figura 4. Etapas de formação dos miotubos. Fonte: Chargé, 2003 (modificado)

Durante esta fase, os miócitos permanecem engajados num processo de diferenciação, que culmina com a fusão com os outros miócitos e a formação de um sincício mononucleado chamado miotubo (**Figura 4**), que atinge a maturidade celular mostrando-se contrátil, ao ser então conhecido como a unidade morfofuncional do músculo esquelético (LAFUSTE, 2004).

Ao mesmo tempo, durante o desenvolvimento muscular, uma população distinta de mioblastos permanecem indiferenciadas, e fica associada à superfície das fibras musculares em desenvolvimento, servindo como células fonte musculares quiescentes, as células satélites (CHARGE; RUDNICKI, 2004). Estas células são células tronco unipotentes que estão prontas para se diferenciar unicamente em miócitos a partir do momento que são estimuladas para tal atividade (McARDLE, 1998).

As propriedades funcionais do músculo esquelético dependem da manutenção de uma rede completa de miofibras, motoneurônios, vasos sanguíneos e da matriz extracelular do tecido conjuntivo. Esse conjunto de estruturas, bem como a organização entre elas, é o que determina a função muscular. Entretanto, para que o músculo seja funcional, durante seu processo de formação e diferenciação, é necessário a atuação de alguns fatores de crescimento, também conhecidos como somatomedinas ou IGF (ROSENDAL *et al.*, 2002; PRESTES *et al.*, 2006).

O fator de crescimento insulínico-I (IGF-I) e seus componentes se trata de um hormônio polipeptídeo pertinente ao anabolismo celular e exerce importante efeito sobre o crescimento somático e tecidual de muitas espécies. No músculo, o IGF-I exerce efeito central na hipertrofia e na hiperplasia das fibras musculares, bem como em tecidos associados a sua função, como os tendões (LEVINE *et al*., 1949; PRESTES *et al*., 2006). Ainda no músculo, o IGF-I é responsável por regular uma variedade de funções, incluindo o transporte de aminoácidos, de íons e de nucleotídeos, sobre o potencial elétrico, a síntese e a degradação de proteínas, além de regular o metabolismo do RNA (GOLDFINE, 1981; BRUNETTI *et al*., 1989), eventos celulares essenciais para o metabolismo das células musculares.

A regulação do tamanho do miotubo também é importante para o bom funcionamento do músculo esquelético e, para isso, outros fatores de crescimento estão envolvidos nesse processo. Portanto, além do IGF-I, o fator de crescimento do hepatócito (HGF) está implicado no processo de ativação de células miogênicas. A fusão destas células é um processo mais complexo que requer uma série de eventos celulares orquestrados, como a migração e o alinhamento de células, seu reconhecimento, sua aderência e por fim a fusão da membrana. Mas atualmente, os mecanismos moleculares que controlam esses processos de fusão miogênica são pouco conhecidos (CHARGÉ, 2003).

Por outro lado, o mecanismo molecular de aumento na assimilação de glicose estimulado por insulina, por exemplo, pode estar relacionado a um aumento na expressão e na atividade de proteínas chaves conhecidas por regular o metabolismo da glicose no músculo esquelético (ZIERATH, 2002).

No período pós-prandial, a glicose é distribuída em vários tecidos do corpo, nos quais pode ser utilizada como fonte energética imediata ou estocada como glicogênio (PREISS; WALSH, 1981). Para que a glicose seja capturada pelas células musculares, há um processo de transporte desempenhado por proteínas transportadoras conhecidas como GLUT-1 e GLUT-4, cujas atividades são influenciadas pela insulina. Este evento vem sendo estudado há uma tempo por pesquisas que envolvem cultura de células musculares esqueléticas de humanos e de animais, como o estudo de Klip e Pâquet (1990), que utilizou cultura de células musculares L6 submetida ao tratamento agudo (1 hora) com insulina, e cuja resposta foi uma elevação no recrutamento dos transportadores de glicose para a membrana plasmática das células musculares. Entretanto, este estudo também realizou um teste com a exposição prolongada (24 horas) das células à insulina num meio com a concentração de glicose reduzida, tendo como resposta uma redução da expressão do GLUT-1 mRNA e GLUT-1 proteína. Assim, é provável que seja atribuída à insulina um possível efeito modulador sobre a expressão dos transportadores de glicose no músculo.

2.3 Engenharia tecidual e desnutrição

Estima-se que no músculo esquelético de um rato adulto normal, 1 a 2% dos núcleos das miofibras são substituídos a cada semana (SHMALBRUCH; LEWIS, 2000). O músculo esquelético dos mamíferos tem uma rápida e alta capacidade regenerativa, principalmente em resposta a um dano severo, como no caso de uma necrose miocitária. A fase precoce desta regeneração está associada à ativação de células mononucleadas, como macrófagos e células miogênicas. Neste caso, os macrófagos infiltram nos miócitos necrosados para fagocitar os restos celulares, mas também para participar diretamente no processo de regeneração muscular ativando as células miogênicas, embora pouco se conheça a respeito do relacionamento entre macrófagos e miócitos.

A proliferação celular é um evento necessário à regeneração muscular, e a diferenciação e a fusão miogênica ocorrem tanto com as fibras musculares danificadas, quanto com os miócitos já existentes, formando novos miotubos no processo de regeneração do músculo.

A engenharia tecidual, técnica que consiste na regeneração de órgãos por meio do recrutamento de células do próprio tecido que migram naturalmente para o local injuriado ou por meio de implante *in situ*, tem sido atualmente objeto de grande perspectiva para a resolução de injúrias teciduais com poucas alternativas terapêuticas (GRIFFITH; NAUGHTON, 2002; ZHANG *et al*., 2012). Através das técnicas inerentes à engenharia tecidual, é possível reconstruir os tecidos, além de devolvê-los sua função.

Neste contexto, o músculo esquelético encontra-se numa posição privilegiada devido ao fato dele conter células satélites que são capazes de repor as fibras musculares perdidas no momento da injúria, fundindo-se às demais existentes no músculo e reconstituíndo, então, os miotubos (CHARGE; RUDNICKI, 2004). Esse fenômeno ocorre naturalmente por mecanismos fisiológicos estimulados pelos diversos fatores de crescimento, tais como os presentes na **Figura 3**, mas é possível ter uma melhor eficiência na reconstrução muscular se tais células forem inseridas em *in situ* na lesão muscular.

Tais afirmações são reforçadas pelas publicações mais recentes, que mostram um fenômeno semelhante observado através da aplicação de Plasma Rico em Plaquetas (PRP) em lesões ósseas, ligamentares e musculares (BARBOSA *et al*., 2008; SILVA; CARMONA; REZENDE, 2012; VENDRUSCOLO; CARVALHO; MORAES *et al*., 2012), cujo mecanismo de ação se baseia na estímulo da regeneração de tais tecidos através da alta quantidade de fatores de crescimento que contém este material. Entretanto, embora seja esperançosa a utilização de fatores de crescimento na regeneração da injúria muscular, bem como a utilização de células do próprio músculo, não se sabe se o organismo que passou por desnutrição, em algum fase da vida, apresentaria os mesmos resultados.

Sabe-se que a desnutrição é capaz de alterar eventos metabólicos, mas pouco se conhece sobre as possíveis alterações nas vias metabólicas em um organismo desnutrido, em especial do seu músculo. Por isso, os estudos referentes à engenharia tecidual em animais desnutridos no período neonatal que receberam reposição nutricional ao longo da vida serão

pertinentes na descoberta de técnicas que auxiliem na reconstrução do tecido muscular deste grupo de organismos.

Assim, estudos que envolvam cultura de células musculares de animais desnutridos no período neonatal, mas que passaram por reposição nutricional ao longo da vida, podem ser úteis no desenvolvimento de técnicas de replicação de miócitos e formação de miotubos em cultura, fazendo com que este novo campo terapêutico possa ser tão eficaz nos organismos que passaram por privação alimentar, como são nos organismos nutricionalmente normais.

2.4 Nutrição e Programação Metabólica

O desenvolvimento normal de organismos multinucleares se baseia em uma série de eventos sincronizados dirigidos por instruções genéticas adquiridas desde a concepção. Durante o período neonatal (período crítico de crescimento e desenvolvimento), o organismo apresenta a habilidade de desenvolver respostas diferenciadas às situações ambientais estranhas ao desenvolvimento normal. Neste contexto, cabem as adaptações em níveis moleculares, celulares e bioquímicos, caracterizadas como resultados de resposta a um estímulo (stress) nutricional que altera permanentemente a fisiologia e o metabolismo de um organismo, e que continuam a ser expressos mesmo na ausência dos estímulos iniciais (LUCAS, 1991). A este processo, foi dado o nome de *programação metabólica*, situação fisiológica que resulta em vários problemas de saúde frequentes nas sociedades do mundo atual.

Estudos epidemiológicos realizados desde 1995 (BARKER, 1995) indicam que a programação metabólica pode ocorrer em humanos. Neste trabalho, foi reconhecido que o tamanho desproporcional dos recém-nascidos eram resultados da má nutrição materna, e que este fato estava correlacionado ao risco aumentado de situações adversas à saúde da prole, tais como diabetes tipo II, hipertensão arterial e demais doenças cardiovasculares na vida adulta.

A programação metabólica resultante da desnutrição também tem sido evidenciada em estudos com roedores realizados anteriormente aos estudos em humanos. McCance (1962) demonstrou que a quantidade de alimentos ingerida durante o período neonatal acarreta

diversas consequências sobre o crescimento e o desenvolvimento. A má nutrição caracterizada pelos baixos teores proteico e/ ou calórico é capaz de alterar a estrutura e consequentemente a função de vários órgãos na prole. Estes achados foram confirmados com os estudos de Latorraca *et al*. (1998) e Bennis-Taleb *et al.* (1999), que mostraram que a dieta hipoprotéica em ratas grávidas é capaz de reduzir a vascularização das ilhotas pancreáticas e, consequentemente, a capacidade proliferativa de células β, que são produtoras de insulina pelo pâncreas. Além disso, outro achado demonstrado por este estudo foi a redução da sensibilidade insulínica na presença de glicose no músculo.

A capacidade metabólica do fígado, do músculo e do tecido adiposo da prole também é comprometida pela restrição proteica materna durante o período de gestação e lactação (OZANNE; HALES, 1999). Quando a mãe é acometida por diabetes gestacional, é comum observar um nível elevado de insulina na prole durante o período crítico de desenvolvimento (perinatal), fato que geralmente leva a uma permanente má organização do núcleo hipotalâmico ventromedial, seguida pela intolerância à glicose durante a vida adulta (HARDER *et al*., 1998). Estes dados nos auxiliam a interpretar o fato dos bebês nascidos de mães com diabetes gestacional apresentarem uma tendência ao sobrepeso pelo menos durante a infância. Todos estes estudos em modelo animal indicam que, definitivamente, a manipulação dietética em breves períodos da vida tem consequências variadas na saúde de um indivíduo adulto.

2.5 Músculo e epigenética

Perturbações nutricionais no ambiente uterino são capazes de interferir na formação do embrião, além de repercutir sob vários parâmetros fisiológicos na vida adulta, com modificação ou retardo no crescimento, qualitativa e quantitativamente. Tais situações podem predispor o indivíduo a problemas como a síndrome metabólica ou doenças correlatas na vida adulta, tais como intolerância à glicose, resistência à insulina, doenças cardiovasculares, hipertensão e obesidade (GLUCKMAN *et al*., 2005; LANGLEY-EVANS, 2006; SYMONDS *et al*., 2007). Entretanto, quando as perturbações nutricionais acometem o indivíduo no início da vida, especialmente no período neonatal, elas também são capazes de influenciar a

fisiologia ao longo da vida adulta.

A epigenética (estudo epigenômico), um campo emergente da pesquisa biológica, é um fenômeno reconhecido pelas metilações e acetilações nas histonas da molécula do ácido desoxirribonucléico (DNA), e está em pauta nas discussões sobre como o organismo é capaz de se programar em resposta a alterações ambientais e nutricionais (LIOTTO *et al*., 2009). Este fenômeno tem dado um novo sentido à estrutura da cromatina, e desempenha um papel importante no controle da atividade de transcrição e expressão dos genes.

Ao contrário das mutações genéticas ou polimorfismos, a epigenética não resulta em alterações na sequência de DNA. Além das metilações e acetilações que são de ocorrência comum, sutis modificações nas bases nitrogenadas também podem ser responsáveis pela alteração na expressão dos genes (BAYLIN, 2005; ZWETSLOOT; LAYE; BOOTH, 2009).

Modificações de histonas podem ser tanto transitórias, responder rapidamente a alterações dentro da célula ou do meio ambiente circundante, ou mantida de forma estável ao longo da vida, quando obtidas no início da vida fetal (CLAYTON; HAZZALIN; MAHADEVAN, 2006). Tais modificações são capazes de induzir, restringir ou silenciar o desenvolvimento de estruturas somáticas permanentes ou sistema fisiológico (GUILLOTEAU *et al*., 2009).

Assim, na vida pós-natal, os músculos esqueléticos adultos mantêm uma população heterogênea de células progenitoras (células musculares satélites), que parecem estar sob o controle de uma rede transcricional que opera durante a somitogênese (SNIDER; TAPSCOTT, 2003; PARKER; SEALE; RUDNICKI, 2003). Entretanto, tais células mostram diferenças anatômicas e histológicas nas interações com as células vizinhas, além de outras distinções morfológicas e funcionais que sugerem a existência de redes reguladoras embrionárias específicas *versus* progenitores do músculo esquelético adulto (TAJBAKHSH, 2009).

Estes estudos reforçam a hipótese de que, mesmo que a miogênese aconteça no período embrionário-fetal, é possível que os estímulos ambientais, tais como as alterações nutricionais, sejam capazes de promover alterações do desempenho fisiológico dos tecidos, inclusive do músculo esquelético.

Apesar da existência de muitos estudos que relacionam o efeito da desnutrição sobre os sistemas biológicos, ainda se faz necessário investigar diversas variáveis que estão relacionadas ao desempenho muscular dos organismos que sofreram desnutrição. Por isso, estudos experimentais que utilizem a desnutrição neonatal através de aleitamento, seguida de reposição nutricional, podem esclarecer quais são os caminhos metabólicos e epigenômicos do músculo esquelético para, então, serem estabelecidas estratégias biológicas capazes de superar as possíveis disfunções musculares neste grupo.

3 MÉTODOS

3.1 Desenho Experimental

Estudo do tipo experimental, com desenho metodológico planejado para a comparação das variáveis entre os grupos controle (nutrido-N) e teste (desnutrido-D), graças à homogeneidade entre eles no que se refere à idade, ao sexo, à raça e à dieta experimental.

3.2 Considerações éticas

Este trabalho foi previamente apreciado e aprovado pelo biotério da Universidade de Tecnologia de Compiègne-França, sob protocolo de número 60-20 (Anexo A).

3.3 Grupos e Dietas

Ratos albinos, da linhagem Wistar, provenientes do Centre d'élevage Dépré (Saint-Doulchard, France), foram utilizados com cerca de 90 dias de idade, em sistema de acasalamento poligâmico (combinação de 1 macho com 2 ou 3 fêmeas), para a obtenção dos animais experimentais. O acasalamento foi realizado em gaiolas de polipropileno (49 x 34 x 16cm) e 1 dia após o nascimento, os filhotes foram numericamente padronizados em n=6 filhotes/mãe. Para isso, houve tanto redução quanto acréscimo de filhotes de outra ninhada por mãe, para que este número fosse padronizado em 6.

Após o nascimento, foram utilizados 12 filhotes machos de várias ninhadas, selecionados aleatoriamente, e divididos em 2 grupos (n=6) de acordo com a dieta materna durante os primeiros 21 dias pós-natais (período de aleitamento ou de lactação). Durante a lactação (**Figura 5**), 6 ratos foram amamentados por mães que receberam dieta experimental à base de caseína à 17% (AIN-93G) (Rhoster® - Brasil) – grupo nutrido (N), e os outros 6

foram amamentados por mães que receberam dieta hipoproteica, à base de 8% de caseína (AIN-93G) (Rhoster® - Brasil) – grupo desnutrido (D) (**Tabela 1**). Após este período, os grupos de animais N e D receberam dieta padrão de biotério com 18% de proteína (Teklad Global) até o dia dos procedimentos experimentais.

Figura 5. Filhotes em lactação

Tabela 1. Composição da dieta experimental oferecida à ratas lactantes (período de lactação - 21 dias após o nascimento da prole). Fonte: Melo et al. (2012) (modificado)

Ingredientes	**Quantidade/ kg de dieta**	
	Dieta controle (17% caseína)	**Dieta hipoproteica (8% caseína)**
Caseína	179.3 g	79.3 g
Mix vitamínico*	10 g	10 g
Mix de minerais**	35 g	35 g
Celulose	50 g	50 g
Bitartarato de colina	2.5 g	2.5 g
DL-metionina	3 g	3 g
Óleo de soja	70 mL	70 mL
Amido de milho	650.2 g	750.2 g

* Conteúdo do mix vitamínico (mg/kg de dieta): retinol, 12; colecalciferol, 0.125; tiamina, 40; riboflavina, 30; ácido pantotênico, 140; piridoxina, 20; inositol, 300; cianocobalamina, 0.1; menadiona, 80; ácido nicotínico, 200; colina, 2720; ácido fólico, 10; ácido paraaminobenzóico, 100; biotina, 0.6.

**Conteúdo do mix de minerais (mg/kg de dieta): $CaHPO_4$, 17200; KCI, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_2, 120; $FeSO_4 \cdot 7H_2O$, 200; elementos traço, 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; amido suficiente para o prepare de 40 g [por kg de dieta]).

Ao longo do experimento, os ratos foram mantidos no biotério sob temperatura de 23°C ± 2°C e ciclo claro-escuro 12:12h. Dietas (experimental e controle) e água foram oferecidas *ad libitum* até o início dos procedimentos experimentais (**Figura 6**).

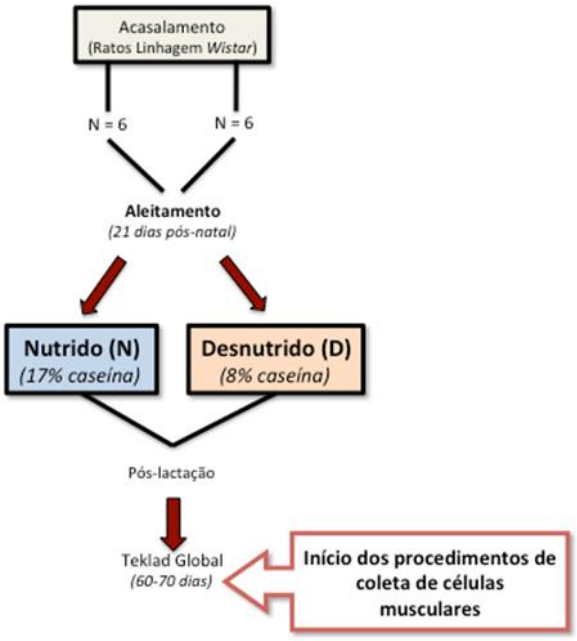


Figura 6. Grupos experimentais segundo a dieta: nutrido (N) e desnutrido (D)

3.4 Curva de Crescimento

Desde o nascimento, e durante todo o período de lactação, os animais foram pesados a cada 5 dias (Hettler Toledo XS4001S). Em seguida, o peso foi aferido uma vez por semana até o 60^{o} dia de vida. Uma curva de evolução ponderal foi construída para comparar o peso adquirido entre os dois grupos (N e D) e, assim, comprovar a eficácia da dieta experimental em desnutrir os animais (**Figura 7 A-B**).

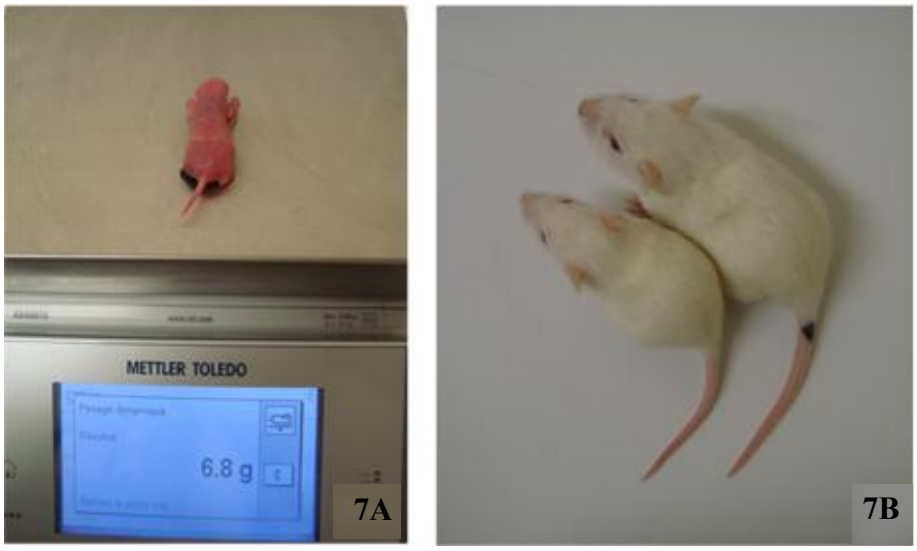
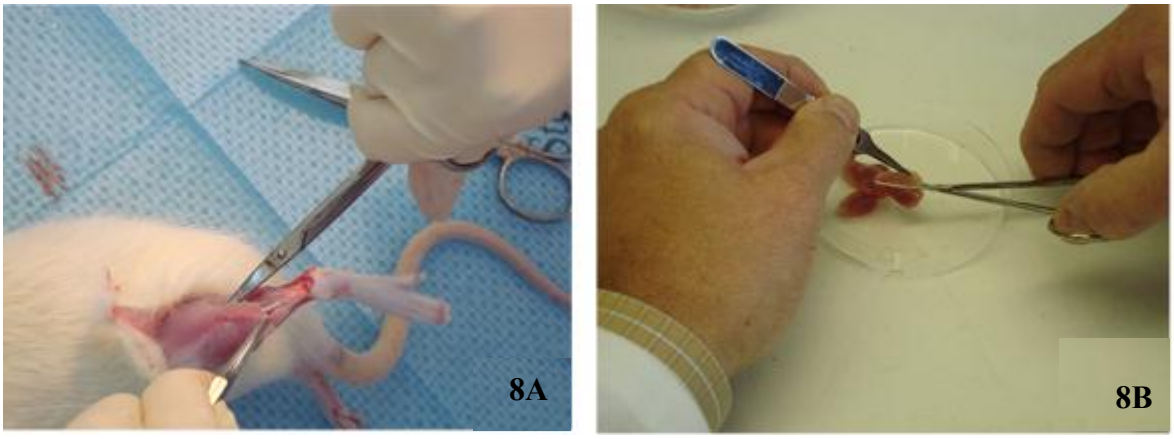


Figura 7 A-B. (7A) Rato no primeiro dia de lactação; **(7B)** Ratos N (calda marcada) e D no último dia de lactação (21° dia de vida)

3.5 Coleta e isolamento de células musculares primárias

Entre 60 e 70 dias de vida, os ratos foram previamente anestesiados e, em seguida, procedeu-se a eutanásia com xilazina e ketamina (1:1, 100µL/100g), para a coleta dos músculos solear, gastrocnêmio e quadríceps das duas patas posteriores (**Figura 8A**). Inicialmente, os músculos coletados foram submersos na solução A, contendo 15mL de PBS, 0,15g de glicose e 80µL Penicilina/Estreptomicina, à 4°C (caixa térmica contendo gelo), durante todo o procedimento de coleta (**Tabela 2**). Em seguida, foram retirados dos músculos toda gordura e tendões, para que eles fossem colocados num béquer de 30mL, contendo 15mL da mesma solução (A) para, logo em seguida, serem fragmentados com o auxílio de uma tesoura (**Figura 8B**). A solução foi, então, completada com PBS para o volume de 50mL em tubo Falcon®.



Figuras 8 A-B. (8A) Coleta dos músculos das patas posteriores; **(8B)** Remoção da gordura e dos tendões, com fragmentação do músculo

Esta solução foi centrifugada (Jouan CR3i) à 1000rpm/4min, à 4°C, e o seu sobrenadante foi recuperado e centrifugado, nas mesmas condições, durante 10min. O *pellet* de células recuperado através da centrifugação do sobrenadante foi ressuspendido em 1mL de meio de crescimento (DMEM 1g/L glicose, 10% de soro fetal bovino, 10% de soro de cavalo, 2% L-glutamina 4mM e 1% Penicilina/Estreptomicina) e acondicionado à 37°C na incubadora, em atmosfera de 10% de CO_2. As células recuperadas receberam 15mL da solução C, contendo DMEM 1g/L glicose e 1% Penicilina/Streptomicina (solução B) e 0,03g colagenase II, e incubadas durante 90 minutos em banho-maria, à 37°C, com agitações em vórtex a cada 10 minutos. Durante os últimos 10 minutos, as células foram ressuspendidas manualmente com a utilização de uma pipeta automática. Esta suspensão de células foi, então, centrifugada durante 20min, à 1000 rpm e à 4°C. Nos casos em que não houve separação entre células e sobrenadante, a suspensão foi diluída com 35mL de PBS estéril (50mL final), e centrifugada novamente à 4°C, à 2000 rpm, durante 20 min.

Uma vez recuperado o sobrenadante das células, este foi separado para uma posterior centrifugação. Adicionou-se 10mL de solução D (15mL da solução B; 0,04g colagenase tipo II; 2mL Tripsina; 2mL DNAse tipo I à 1g/L de PBS), e o *pellet* desta suspensão foi ressuspendido com uma pipeta automática, incubado em banho-maria à 37°C, por 20min e, em seguida, centrifugado à 1000rpm, à 4°C, por 20 min. O sobrenadante foi recuperado e reservado em tubo Falcon. Em seguida, 15mL da solução D foi adicionado ao *pellet* e esta solução foi levada novamente ao banho-maria, à 37°C, durante 20min. Homogeneizações manuais foram realizadas a cada 5 minutos.

A partir desta etapa, com o objetivo de substituir os meios enzimáticos, 15mL de meio de crescimento foi adicionado, homogeneizado e centrifugado à 1000rpm por 20min. O sobrenadante foi recuperado num tubo Falcon, e o *pellet* de células recebeu 30mL de meio de crescimento. A partir desta etapa, a suspensão de mioblastos estava pronta para ser filtrada numa membrana de poros de 50µm. Todos os sobrenadantes recuperados foram filtrados no mesmo filtro, a fim de recuperar o número máximo de mioblastos. Foi obtido, ao menos, 30mL do filtrado para as culturas e contagem de células.

As células musculares primárias foram finalmente contadas em câmara de Malassez, e semeadas em placas de cultura de $10cm^2$, tratadas para culturas celulares (**Figura 9 A-B**).

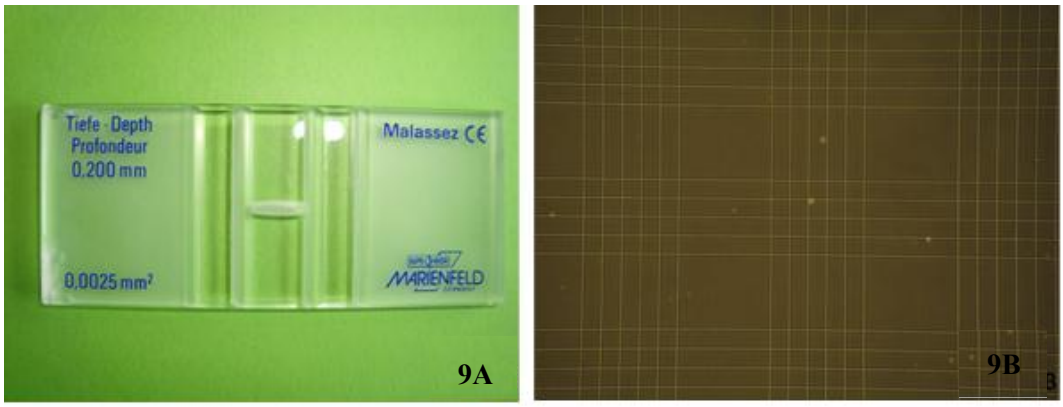


Figura 9 A-B. Câmara de Malassez

A imagem a seguir (**Figura 10**), resume as etapas de coleta, isolamento e cultura das células musculares primárias.

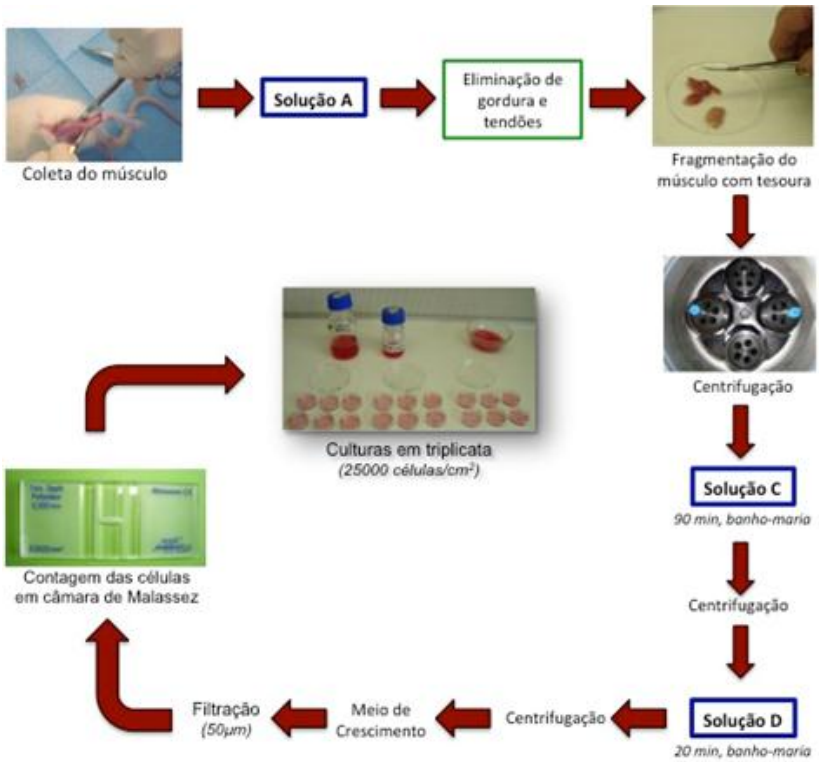


Figura 10. Sequência da coleta, isolamento e cultura de células musculares primárias

Tabela 2. Soluções utilizadas na coleta e cultura de células musculares primárias

Solução A	15mL de PBS 0,15g de glicose 80µL de Penicilina/Estreptomicina
Solução B (reagentes para as soluções C e D)	DMEM 1g/L glicose 1% Penicilina/Estreptomicina
Solução C	DMEM 1g/L glicose 1% Penicilina/Estreptomicina 0,03g de Colagenase II
Solução D	15mL da Solução B 0,04g de Colagenase II 2mL de Tripsina 2mL de DNAse I
Meio de crescimento	DMEM 1g/L glicose 10% SFB 10% SC 2% L-glutamina 4mM 1% Penicilina/Streptomicina
Meio de fusão	140mL DMEM 1g/L glicose 10mL Soro de Cavalo 2% Glutamina 4mM 1% Penicilina/Estreptomicina

3.6 Cultura de células musculares primárias

Cerca de 6h antes do semeio das células, as placas de cultura de 10cm^2 foram preparadas, adicionando-se 750µL de matrigel (1:500) em DMEM 1g/L glicose (MELO *et al*., 2011) (**Figura 11**).

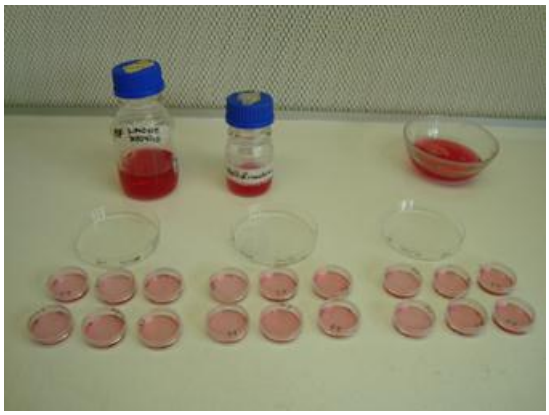


Figura 11. Placas de cultura semeadas em triplicata

Em seguida, as células foram semeadas em triplicata numa densidade de 25.000 células/cm^2 (**Figura 11**), incubadas à 37°C, 10% CO_2, durante 10 dias (**Figura 12 A-B**). Para cada animal de cada grupo, foram semeadas três placas controle, e três placas às quais foram adicionadas insulina. O meio de fusão (DMEM 1g/L de glicose, 5% de soro de cavalo, 2% de L-glutamina 4mM e 1% de penicilina/estreptomicina) foi adicionado a partir do segundo dia, no qual as células foram mantidas até o décimo dia de cultura. Este meio foi utilizado para induzir a fusão dos mioblastos e assim, formar miotubos.

Figura 12 A-B. Incubadora de células (37^{o}C, 10% CO_2)

3.7 Estimulação dos miotubos com insulina

A insulina foi adicionada no segundo dia de cultura, juntamente com o meio de fusão, numa concentração de 10μU/mL (CASSAR-MALEK *et* al., 1999) e, a cada troca de meio, a mesma foi reposta na mesma concentração (**Figura 13**).

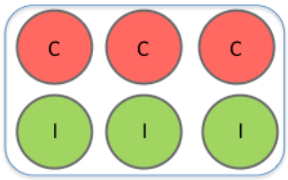


Figura 13. Para cada rato, as células foram semeadas em triplicata. C = controle; I = insulina

3.8 Imunocitoquímica

A imunocitoquímica foi realizada com o objetivo de avaliar o potencial de formação dos miotubos, baseando-se no protocolo utilizado por Jacquemin *et al*. (2005).

Ao décimo dia de cultura, as células foram então fixadas em paraformaldeído (PAF) a 4% e, para a marcação imunocitoquímica, foi utilizado o anticorpo policlonal Anti-Myosin Rabbit pAb (Ref. 476126, lote D00075180), segundo o protocolo proposto pela Calbiochem® (**Figura 14 A-B**).

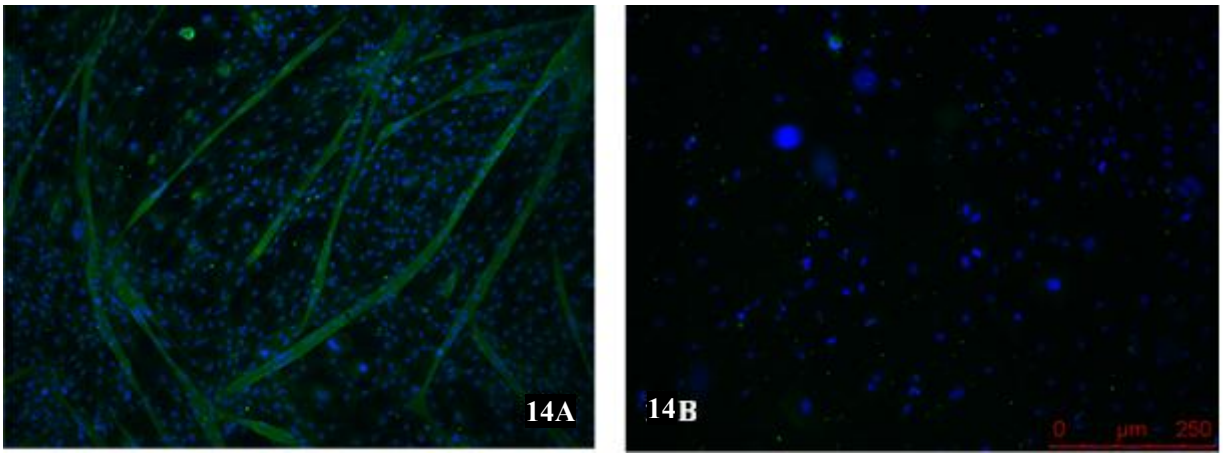


Figura 14 A-B. (14A) Miotubos imunomarcados com anti-myosin Rabbit pAb; **(14B)** Núcleos contra-corados com DAPI

De cada amostra dos Grupos N e D, foram obtidas 3 fotos, nas quais foram contados os núcleos incorporados e não incorporados pelos miotubos. Esta contagem foi realizada em triplicata para ambos os grupos (3 amostras controle e 3 amostras com insulina, por rato), e o resultado foi obtido através da média entre as 3 contagens. A relação encontrada entre a quantidade de núcleos incorporados *versus* núcleos não incorporados revelou a capacidade de formação de miotubos entre os grupos estudados.

3.9 Análise biomecânica das células musculares em cultura

Em cada placa de cultura, 3 filmes de no mínimo 30 segundos foram realizados através de uma câmara digital (Canon A620) acoplada a um microscópio de contraste de fase (Olympus CKX41, Japan) (**Figura 15**). Estes filmes foram referentes tanto às 3 amostras controle, quanto às 3 amostras com insulina de cada rato.

Figura 15. Realização dos filmes em microscópio de contraste de fase

O resultado foi baseado na média entre as três análises. Para tal, os seguintes parâmetros foram analisados (**Quadro 1**):

1. Frequência de contração (número de contrações por minuto);
2. Amplitude de contração (relação entre as medidas da contração e do relaxamento medidas através do percentual de encurtamento do miotubo);

3. Período de contração ou duração da contração analisada (relação entre o número de imagens entre a contração e o relaxamento pelo número de imagens por segundo, obtida pela câmara fotográfica).

Quadro 1. Fórmulas utilizadas para o cálculo dos parâmetros biomecânicos

Parâmetros biomecânicos	Representação	Fórmulas
Frequência de Contração	F	$F = \frac{n° \, contrações}{30} \times 60$
Amplitude de Contração	A	$\%A = \frac{\Delta \, distância}{distância \, do \, relaxamento} \times 100$
Período de Contração	P	$P = \frac{n° \, imagens \, entre \, contração \, e \, relaxamento}{n° \, imagens \, por \, segundo}$

Para a análise dos parâmetros de contração 2 e 3, foi necessário fazer a extração de duas fotografias por cada filme. Estas fotografias representaram a contração máxima do miotubo (encurtamento), bem como o relaxamento máximo (alongamento) do mesmo. Para extrair as imagens dos filmes, foi utilizado o programa XnView (versão 1.97, Libformat 5.54), enquanto que, para a análise das imagens, o programa ImageJ (versão 1.37v, USA) se mostrou eficaz na medida do centro de massa dos pontos escolhidos para análise de cada imagem.

Esses três parâmetros foram capazes de revelar uma ideia biomecânica temporária dos miotubos em cultura.

3.10 Padronização do protocolo de coleta e cultura de células

Diante da necessidade de otimizar os procedimentos de coleta de células musculares, um estudo piloto foi previamente realizado com ratos Wistar do grupo controle, na mesma faixa etária dos ratos que foram utilizados em nosso experimento, com o objetivo de simplificar os procedimentos de coleta e isolamento destas células. Através do estudo piloto, no qual foram utilizados 6 ratos machos, foi possível padronizar os procedimentos de coleta e

de cultura de células musculares primárias aqui utilizados e, assim, torná-los mais simples para serem reproduzidos pela comunidade científica.

É importante ressaltar que todas as etapas foram otimizadas no que se refere ao seu tempo de duração para a obtenção do número máximo de células viáveis para a cultura.

3.11 Análise Estatística

Os grupos experimentais propostos apresentaram uma mesma distribuição nos parâmetros avaliados e, portanto, para todas as análises, foi utilizado o teste estatístico não paramétrico de *Mann-Whitney* para comparar as diferenças entre os parâmetros analisados nos grupos (N e D), em ambas as condições (com e sem insulina). Considerou-se uma significância de 95%, e $\alpha<0.05$ para todos os casos.

4 RESULTADOS

O resultado deste estudo está representado sob forma de dois artigos científicos, conforme regulamentação do Colegiado de Pós-Graduação em Nutrição, do Centro de Ciências da Saúde, da Universidade Federal de Pernambuco.

Artigo 1: **IMPROVED PROTOCOL FOR THE COLLECTION AND CULTURE OF WISTAR RAT PRIMARY SKELETAL MUSCLE CELLS**

Submetido ao *The Journal of Physiological Sciences* (Qualis B2)

Artigo 2: **EFFECT OF INSULIN ON THE FORMATION AND FUNCTION OF MYOTUBE CULTURES FROM YOUNG ADULTS RATS SUBMITTED TO NEONATAL UNDERNUTRITION**

Submetido ao *British Journal of Nutrition* (Qualis A2)

ARTIGO 1

IMPROVED PROTOCOL FOR THE COLLECTION AND CULTURE OF WISTAR RAT PRIMARY SKELETAL MUSCLE CELLS

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Simone do Nascimento Fraga participated in the preparation of research project, of the experimental strategy, data collection, tabulation and discussion of results and drafting of paper. **Jean-Luc Duval** and **Pascale Vigneron** participated of the experimental strategy, data collection, discussion of results and drafting of paper. **Francis Canon** and **Juliana Félix de Melo** participated in the drafting of article. **Célia Maria Machado Barbosa De Castro** and **Marie-Danielle Nagel** participated in the preparation of research project, tabulation and discussion of results and drafting of paper.

Abstract

Cell culture is a useful technique for *in vitro* studies due to the ability to evaluate cellular responses to various stimuli; however, the available culture protocols for muscle cells are difficult to reproduce. Here, we propose a simple and reproducible protocol for primary muscle cell culture, which was developed based on the observation of morphofunctional myotube formation, the rate of cell proliferation and the potential formation of myotubes (myotube morphology and percentage of nuclei incorporated using immunocytochemistry). Although evaluations of the rate of cell replication showed no significant difference ($p>0.05$), there was intense cell fusion for the formation of myotubes from the 6^{th} day of culture. Immunocytochemical labelling was effective for identifying myotube formation on the 10^{th} day. Our results indicate that this new protocol could be useful for the culture of primary muscle cells.

Keywords: muscle cells, myoblasts, cell culture, myotubes and tissue engineering.

Introduction

Cell culture has been widely used as a tool to reproduce tissues in the laboratory for both basic and applied research [1], with applications in cell biology procedures, pharmacology for the large-scale production of biological materials and in the engineering cells and tissues. Tissue engineering consists of the regeneration of living tissues and organs through cell recruitment of the patient's own tissue and has great potential for the resolution of various tissue injuries that have few therapeutic alternatives thus far [2, 3].

The field of cell culture has increased in recent years due to the potential of cells to grow and proliferate *in vitro*, providing a favourable milieu for procedures that would otherwise require experimental models animal [4]. Although there are some variations in the morphophysiological responses of cultured cells, the cells are usually able to accurately represent the tissue *in vivo*.

The study of muscle cells in culture is a powerful research technique for assessing the differentiation of muscle growth at the cellular level. However, the collection and culture of this cell line presents barriers to the development of muscle tissue engineering, in part due to the difficulty of muscle cell isolation, culture and differentiation [5] and the difficulty to reproduce the protocols available in the literature.

Knowing that the muscle can regenerate after injury, research on the characterisation and function of muscle cells has been performed with the goal of understanding the behaviour of these cells. Based on this, myoblasts were initially studied with regard to the treatment of muscular dystrophy [6], but other applications have been developed over the years, for example, in cardiology through a study [7] testing a cold injury dog model, with little known about the regenerative potential of cells at that time. As the results of the study were encouraging, the researchers engaged in preclinical studies aiming to demonstrate the benefits achieved in the previous study.

Research to simplify and standardise protocols for the collection, isolation and culture of cells is an important step in muscle tissue engineering. Primary muscle cell culture, which contains satellite cells (unipotent adult stem cells), can function as an alternative for easy manipulation and acceptance in autologous transplantation; specifically, to preserve the genetic and immunological characteristics [8, 9]. However, there are differences in the protocols available for the use of this cell line.

Based on the difficulty of handling this cell line, we developed this protocol for the collection and primary culture of muscle cells, simplified and adapted, with the aim of helping the scientific community with a simple and reproducible method for the culture of muscle cells. This method is a potential research tool in the development of new technologies for the therapeutic treatment of muscle injury of various etiologies. Accordingly, we discuss the advantages and disadvantages of the application of the muscle cell culture method proposed in this study.

Methods

This study was conducted in accordance with the guidelines and regulations of the committee of ethics and safety of the University of Technology of Compiègne-France under protocol n° 60-24. We used 6 male

Wistar rats, aged between 60 and 70 days, which were housed in an air-conditioned bioterium (22±2°C), with a 12 h light-dark cycle and food and water *ad libitum*.

The solutions used for collection and culture of the primary muscle cells are detailed in Table 1. To preserve the properties of the reagents used in each solution, they should be stored at 4°C until use.

Collecting and isolating primary muscle cells of Wistar rats

The rats were first weighed and anaesthetised with xylazine and ketamine (1:1), intraperitoneally in the at the dosage 100 mL/100 g bodyweight. The *soleus* and *quadriceps* muscles of the hind legs were collected in a sterile container containing 30 mL of Solution A (see Table 1) and maintained at 4°C during the collection procedure.

The cell processing was performed in a Class II laminar flow hood to maintain the sterility of the cell cultures. The steps of the protocol the primary muscle cells are described below.

1. The muscles were washed in sterile phosphate-buffered saline (PBS). All the tendinous tissue and fat were removed with the aid of scissors. The muscles were again washed in PBS before being subjected to size reduction by manual cutting performed using surgical instruments (a scissors or a scalpel).
2. The muscles were triturated in the presence of PBS, and these smaller fragments, together with PBS, were transferred to a conical tube. PBS was added to a final volume of 50 mL, and the sample was centrifuged at 150 x g, for 10 minutes at 4°C.

   During the centrifugation, Solutions C and D, which were refrigerated at 4°C, were prepared.
3. The supernatant was discarded, and 15 mL of Solution C was added. This suspension was incubated in a water bath at 37°C for 90 minutes, with moderate vortex agitation every 15 minutes. The last 10 minutes of the incubation are important for the separation of the cells, and manual homogenisation using an automatic pipette helps to disperse the still intact portion of the tissue.
4. After 90 minutes, the muscle fragments mixed in Solution C became gelatinous. PBS was added to a 50 mL final volume, and the sample was centrifuged at 599 x g for 20 minutes at 4°C. The supernatant was discarded.

In cases in which the quantification of the primary muscle cell recovery is required, it is advisable to retain the supernatants after centrifugation. The supernatants should be diluted again in PBS and centrifuged at 599 x g for 20 minutes at 4°C to identify a cell pellet.

5. A 10 mL aliquot of Solution D was added to the pellet of Step 4 and incubated in a water bath at 37°C for 30 minutes, with medium-intensity shaking every 15 minutes.
6. PBS was added to a final volume of 50 mL, and this suspension was centrifuged at 599 x g at 4°C for 20 minutes. The pellet was suspended in 15 mL Prime Growth Medium (PGM), homogenised with a electronic pipette, and centrifuged at 150 x g, 4°C for 20 minutes.

   The PGM used in this step only serves to dilute and wash the cells in suspension, inactivating all the enzymes used to this point. The cells were then suspended in 30 mL PGM.
7. The cell suspension was filtered through nylon of 50 μm pore diameter, placed in a glass funnel. The cells obtained after filtration were counted in a Malassez chamber.

Primary muscle cell culture of Wistar rats

On the same day of cell collection, 750 μL of Matrigel® solution (BD Biosciences, Le POnt de Claix, France) in DMEM 1 g/L glucose (1:500) was added to 10 cm^2 culture dishes (Nunclon®, USA). The plates were stored in an incubator under an atmosphere of 10% CO_2, for about 6 hours, before the cells were added; 25.000 cells/cm^2 were plated in each dish. This number was determined by a pilot study with the L6 cell line (LGC, CRL-1458™ , England).

For each rat, the cells were seeded in triplicate using 2 mL PGM for dish culture; the cells were maintained in an incubator at 10% CO_2. On the second day, the culture medium was partially replaced with Fusion Medium (FM) - 1 mL; every two days, only half of the total medium in each culture dish was replaced, with order to provide growth factors necessary, such as horse serum and others, for growth and differentiation of muscle cells into myotubes were included. The cells were maintained in culture for 10 days, time necessary to form myotubes.

Cellular differentiation

The cultures were observed daily, and images were captured for each culture dish every two days to observe cell fusion and the formation of myotubes using a phase-contrast microscope (Olympus CKX41, Japan) coupled to a digital camera (Canon A620, Japan).

Recovery and counting of cells on the 10^{th} day of culture

The cells were recovered using a standard trypsinisation technique. By day 10, two culture dishes for each animal were used to count the rate of cellular proliferation. For this, the culture medium was removed, and 1 mL of 0.25% trypsin-EDTA (Invitrogen®, USA) was added to each dish; the samples were then incubated for 3 minutes under the same atmospheric conditions as the culture.

The enzymatic activity was stopped by adding 0.5 mL bovine foetal serum (Invitrogen®, USA), and the cells were centrifuged at 216 x g at 4°C for 4 minutes.

After centrifugation, the supernatant was discarded, and the cells were suspended in 1 mL PGM. The cells were counted using a Malassez chamber.

Immunocytochemistry

In parallel to the progress of the muscle cell culture, on the 10^{th} day, 6 dishes, one for each animal, were evaluated using an immunocytochemical technique, with the aim of observing the myotube nuclei [10]. The myotubes were fixed in 4% paraformaldehyde, and the protocol for the use of polyclonal anti-myosin (Calbiochem®) was followed according to the manufacturer's data sheet. The nuclei were counter-stained with DAPI.

This technique allowed a better visualisation of the myotubes.

Statistical analysis

The nonparametric Mann-Whitney test was performed to evaluate the rate of cell replication. The significance level was $p < 0.05$. GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used.

Results

Rate of cell replication

The cells were evaluated for their replication rate on the 10th day of culture. In 18 cell cultures, we observed a cell growth rate of 2.5 ± 0.55 (Fig. 1). However, although the number of cells recovered at the end of culture was twice the initial number ($6.2 \times 10^5 \pm 1.3$), the Mann-Whitney statistical test showed that this result was not significant ($p > 0.05$).

Culture and cell differentiation

On the 4th day of culture, confluence was observed in all the culture dishes, and the first myotubes resulting from the fusion of primary muscle cells could be observed on the 6th day (Fig. 2).

In addition to the visualisation of the myotubes, their first contractions were also observed on the 6th day of culture. On the 10th day, the myotubes were numerous and contractile; in addition, it was possible to observe numerous branches.

Immunocytochemistry

Through immunocytochemical labelling, we observed the myotube morphology and the incorporation of nuclei (Fig. 3).

Discussion

This study used the primary muscle cells of Wistar rats to test the efficiency of a modified method for the culture of this cell line. The method tested in this study resulted in viable cells from the time of collection until the end of culture, and the formation of myotubes and their contraction were achieved. The optimisation of the length of time for the collection and processing of the cells was important to the success of this method.

The choice of II collagenase is important in the cultivation of primary muscle cells for performing the dissociation of the cells, in addition to the preservation of the insulin receptor, which, in this case, is essential for the cells to be able to absorb the glucose provided by DMEM and, thus, perform their biomechanical function, i.e., the contraction of the myotubes [11, 12]. Due to this, we have chosen continue using this enzyme.

Previous protocols used for the cultivation of these cells, including the protocol of a study published in 2005 [13], entailed a longer incubation time with II collagenase (3 hours), whereas only 2 hours were used in the present study. The reduction in the contact time of any cell type with the enzyme used for the dissociation of tissue is important in maintaining cellular viability because the enzymes used in cell culture are able to disrupt cytoskeletons, thereby, decreasing the capacity for proliferation and differentiation in culture [14]. Thus, the decrease in the dissociation treatment time was an important difference in the present protocol, particularly in view of the replication and myotube differentiation rate observed after 10 days of culture.

In cell culture, ethylenediaminetetracetic acid (EDTA) acts as a chelating agent that binds calcium and prevents the association of proteins, called cadherins, between cells. These proteins, in turn, are transmembrane proteins that play an important role in cellular adhesion and provide a link between the cells of the same tissue. Therefore, we chose to use trypsin without EDTA to ensure free calcium in the culture medium for the myotube biomechanical function: contraction. Trypsin-EDTA was used at the 10th day of culture only to assist in the removal of the cells from their dishes.

The reduction of the initial concentration of horse serum (10%) in the PGM to 5% in the FM was another modification of the protocols available in the literature. Even with this reduction in the horse serum concentration, it was possible to observe the differentiation of the muscle cells into myotubes. This result confirms the findings of previous research [5, 15], which reported that a low horse serum concentration (2%) in the fusion medium achieved good cell differentiation. These studies, however, included other growth and differentiation factors, such as linoleic acid, ascorbic acid, biotin, vitamin E and extracellular matrix, which increase the cost and complexity of the culture, even with using a horse serum concentration that was lower than that reported here.

The formation of a myotube, the morphological and functional unit of muscle, is the result of several fusions that are stimulated with FM in cell culture. The contraction of these structures is the phenotypic expression of differentiated cells arranged into myotubes. The developmental process of excitation-contraction myotubes has been demonstrated in foetal rat cells using Rhodamine (RYR) and Dihydropyridine (DHPR) receptors [16]; according to this study, the cluster of such receptors was observed after 30 days in myotubes cultivated according to a conventional method proposed in that work. Although we did not analyse such receptors, it was observed in our study that the contraction process of the myotubes was already initiated on the 6th day of culture, indicating that the adjustments made in our protocol facilitated both structural maturation and also the functional maturation of the myotubes in an *in vitro* system.

On the 10th day, immunocytochemistry using polyclonal anti-myosin was performed to assess the contractile proteins actin and myosin. Another study [10] used this technique to observe the capability of the myotubes formed in culture by counting the respective incorporated nuclei, obtaining a percentage compared to the number of nuclei not incorporated into these structures. In our study, this technique was effective to observe the exact delineation of the myotubes formed, thus confirming the proposed method of cultivation.

The use of extracellular matrix is critical to assist in the organisation of the tissue to be formed, and this study used Matrigel® (BD, USA) as a scaffold to perform this function. Matrigel® is able to promote the transport of nutrients, growth factors and metabolites and to develop the important role of attachment and cell differentiation [17]; the dilution used for this matrix (1:500 in DMEM 1 g/L glucose) was consistent with that was used in other study [18]. This high dilution helped in the homogenisation of the cells seeded on the scaffold. In addition, the low concentration of Matrigel® made the method less costly. It was also observed that the addition of Matrigel® to the culture plates on the same day of seeding did not interfere with cellular proliferation and myotube formation because the culture period required for myotube development (6^{th} day) is in agreement with a previous report [18] in which Matrigel® was used since the day before cells seeding and myotubes were observed between days 4 and 7.

The cellular replication rate on the 10^{th} day of culture, even though not significant, was relevant to our study, showing that the cells that proliferated during culture fused to form myotubes, thus confirming the success of this method. The resulting fusion of the cells was well measured by labelling and immunocytochemistry through which it was possible to observe each nucleus inside of the myotubes formed, with the nuclei originating from the replicated myoblasts.

The primary muscle cell culture system presented here is a powerful tool for the development of myotubes *in vitro* system. The protocol proposed here proved to be reproducible, and it was possible to obtain results consistent with previous studies because both the morphology and functionality of myotubes were confirmed by this technique.

Accordingly, we suggest that this study will serve as the basis for the development of muscle tissue engineering due to both the method simplification and the quality of the material obtained in culture. Thus, we propose that this method will contribute to the development of strategies that make possible the treatment of muscular dystrophy, tumours, and other diseases that promote the loss of skeletal muscle tissue.

Acknowledgments

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Conflict of interest

All the authors contributed to the collection and interpretation of the data and to the drafting of the manuscript. The authors declare that they have no conflict of interest.

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Figure Captions

Fig. 1. Cell number seeded (day 0) and recovered at final culture (10^{th} day), n=18. The results are the mean values, with the standard error of the mean represented by vertical bars (significance level 5%) using the nonparametric Mann-Whitney test.

Fig. 2. Representative picture of the development of primary muscle cell cultures of a random rat. (**a)** 2^{nd} day of culture; (**b**) 4^{th} day of culture; (**c)** 6^{th} day of culture; (**d)** 8^{th} day of culture; **(e)** 10^{th} day of culture.

Fig. 3. (a) Myotubes of a random rat labelled with a polyclonal antibody against myosin. The nuclei were stained with DAPI. **(b)** Negative control. (x 100).

Fig. 1

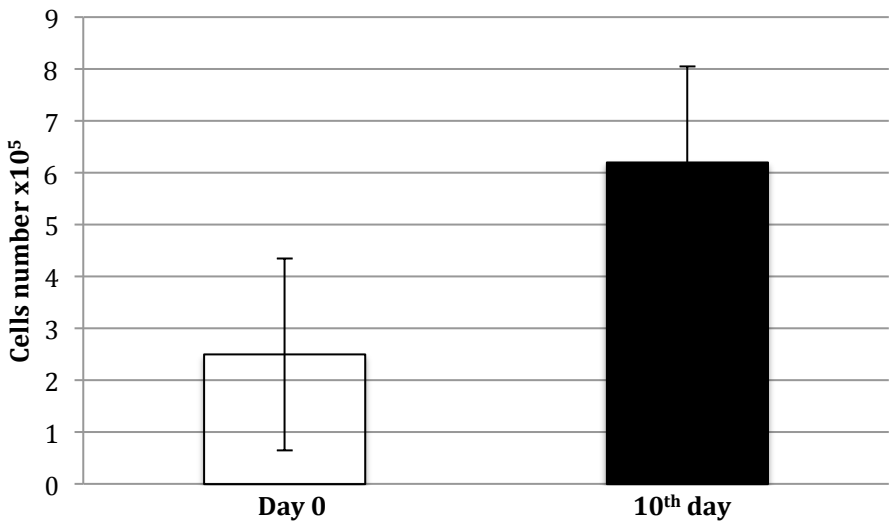

Fig. 1

Fig. 2
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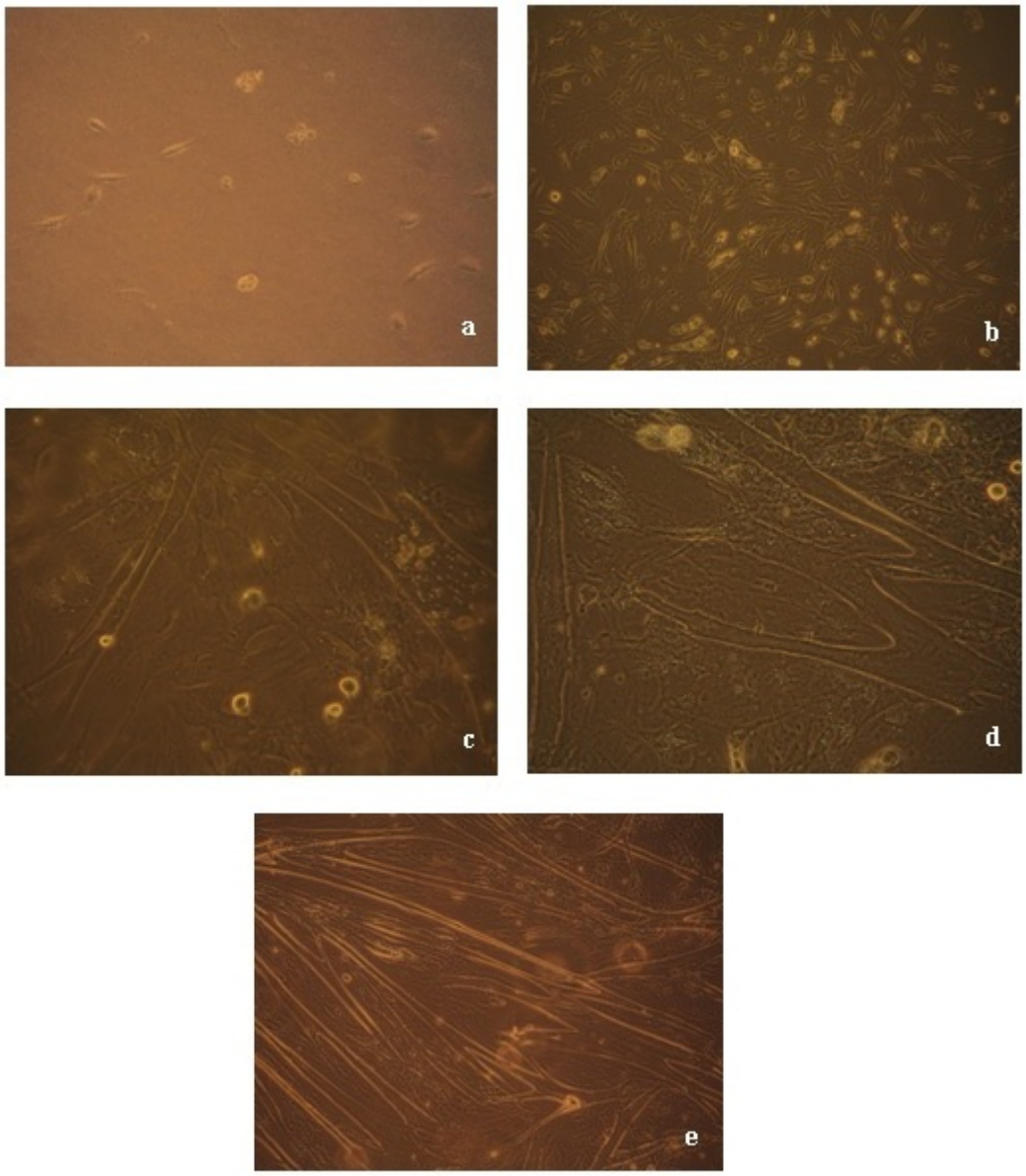

Fig.2

Fig. 3
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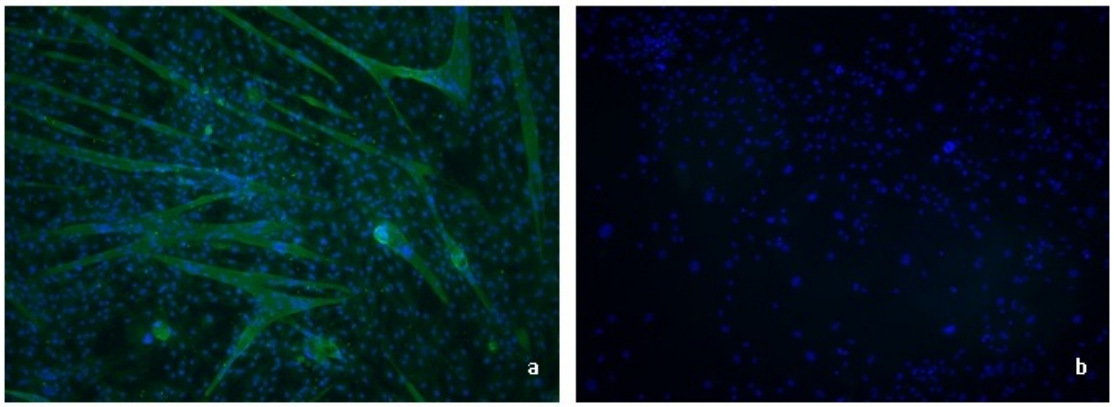

Fig. 3

Table

Table 1. Solutions used in the primary muscle cell collection and culture

SOLUTION	COMPOSITION	APPROXIMATE VOLUME USED PER RAT
SOLUTION A	30 mL PBS 0.3 g Glucose 160 μL Penicillin/Streptomycin	30 mL
SOLUTION B (Reagent C and D solution)	45 mL DMEM 1 g/L glucose 450 μL Penicillin/Streptomycin	15 mL
SOLUTION C	15 mL solution B 0.03 g II Collagenase	15 mL
SOLUTION D	15 mL solution B 0.04 g II Collagenase 2 mL Trypsin without EDTA 2 mL I DNase (stock solution:10 mg I DNase in 10 mL of PBS)	10 mL
PGM (Prime Growth Medium)	120 mL DMEM 1 g/L glucose 15 mL Foetal Bovine Serum 15 mL Horse Serum 2% Glutamine (final concentration 4 mM) 1% Penicillin/Streptomycin	45 mL
FM (Fusion Medium)	140 mL DMEM 1 g/L glucose 10 mL Horse Serum 2% Glutamine (final concentration 4 mM) 1% Penicillin/Streptomycin	Variable

ARTIGO 2

EFFECT OF INSULIN ON THE FORMATION AND FUNCTION OF MYOTUBE CULTURES FROM YOUNG ADULTS RATS SUBJECTED TO NEONATAL UNDERNUTRITION

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Running Title of paper: Undernutrition: insulin action on myotubes

Key words: neonatal undernutrition, skeletal muscle, myotube, insulin, rats

Abstract

Maternal nutritional deficiency during the lactation period can influence the formation and maturation of several physiological systems in adulthood of offspring. Skeletal muscle tissue, as a protein reserve in the body, is particularly sensitive to protein undernutrition, and its formation and differentiation is regulated by several transcription factors, such as insulin. In the present study, we determined whether the presence of insulin in primary muscle cell cultures of rats that were undernourished in the neonatal period alters the cytological and biomechanical profiles of the myotubes formed in culture. Lactanting Wistar rats were fed a casein-based diet from 1-21 days of lactation (state how undernutrition was achieved). When the offspring were 60-70 days of age, primary muscle cells of the leg (*soleus*, *gastrocnemius* and *quadriceps*) were harvested and cultured in the presence or absence of insulin and biomechicanical and cytological parameters measured. The experimental diet promoted a decrease in weight gain until adulthood in the undernourished rat group and changed the insulin sensitivity of the muscle cells in the primary cultures. The myotubes from undernourished offspring were narrower and less branched. Insulin reduced the incorporation capacity of nuclei to form myotubes, increased the number of contractions per minute and reduced the period of contraction in undernourished rats. These data highlight the possible metabolic and biomechanical changes are unchained by nutritional deficiencies in the neonatal period.

Introduction

Maternal nutritional deficiency during the crucial period of offspring growth and development can interfere with the formation of several organ systems due to changes in the pattern of cellular events, with deleterious consequences both for the acquisition of the physiological patterns of a mature organism[1] and for the occurrence of necessary metabolic reactions to maintain physiological homeostasis. According to a previous study[2,3], the long-term consequences on the body will depend on the intensity and duration of the nutritional changes.

During the critical growth period, known as the plasticity developmental period[4], the nutritional, hormonal and metabolic environments offered by the mother can permanently programme the structure and physiology of her offspring, a phenomenon known as metabolic programming[5,6]. Through this phenomenon, a certain genotype is able to express diverse morphological and physiological states in response to different environmental conditions during development[7], as shown by a study[8] in which the authors affirm that mechanisms induced by this programming alter genetic expression. A permanent reduction of the number of cells in the organs and tissues occurs in consequence of adverse environmental effects, such as a protein deficiency during this critical period.

Protein-calorie undernutrition is associated with the functional impairment of various systems and tissues, such as weakening of the cell and humoral immunity[9], a prolonged cell cycle of bone marrow lineages[10] and changes in the rate of apoptosis[11,12]. The skeletal muscle tissue, as a protein reserve in the body, is particularly sensitive to protein undernutrition; thus, protein deficiencies that are usually dietary lead to changes in the phase of growth, development and muscle fibre differentiation[13-16].

The formation of muscle fibres is regulated by several transcription factors, such as MyoD, myogenin, Myf5 and Myf6[17], while the differentiation of these fibres depends on the innervation and performance of hormonal factors, such as insulin and IGF-1[18-20]. These hormonal factors are involved in the activation and proliferation of various myogenic cells and act as important intracellular regulators, such as PI3-K/Akt, resulting in a cascade of activation of targets required for protein synthesis and, consequently, for myotube formation.

Some studies have contributed to the elucidation of energy changes in the muscle cell resulting from undernutrition, since it undernutrition is capable of causing enzymatic deplation, mitochondrial disorders and changes in the transmembrane potential and oxidative phosphorylation[21] and increase sensitivity to insulin in the *soleus* muscle of undernourished

rats[22]. Futhermore, the protein focal adhesion kinase (FAK) regulates insulin action in skeletal muscle[23], but did not correlate those findings with any undernourished animals.

The fusion of myogenic precursor cells is a key event in the development and repair of skeletal muscle[24-26], and the control of this process is important for the development of future biotherapies using myogenic precursor cells (satellite cells). However, few studies investigate the effects of neonatal undernutrition on cell fusion and biomechanical profiles of muscle cells in culture.

Thus, the objective of this study was to evaluate the effect of neonatal undernutrition on cell fusion and myotube formation in culture and to examine the contraction capacity of myotubes in response to insulin. For that purpose, both morphological and biomechanical aspects were addressed.

Materials and Methods

Animals and diets

This study was conducted according to the guidelines and regulations laid down in the committee of ethics and safety of the University of Technology of Compiègne (n° 66-20). We used 12 male Wistar rats, under the conditions of a controlled temperature (22 ± 1°C), 12 h light-dark cycle and water and food available *ad libitum*. During lactation (21 days), mothers received an experimental diet based on 17% casein (Rhoster®-Brasil) – Nourished group (N), while another group of mothers was subjected to a low-protein diet based on 8% casein (Rhoster®-Brasil) – Undernourished group (U), according to the recommendations of the American Institute of Nutrition 93G[27]. Were standardized 6 animals per mother, and offspring were birth weights similar. After this period, at weaning, both the N and U animal groups received a standard diet with 18% protein (Teklad Global 18% protein rodent diet; Harlen Teklad-France) until the day of the experimental procedures, to provide these animals essential elements for their development. The animals were weighed every 5 days during lactation (0-21 days), and then the weight was measured every week until the day 60 of life.

Collection and isolation of primary muscle cells

Between the 60 and 70 days of age, the animals were anesthetised and euthanised with ketamine and xylazine (1:1, 100 μL/100 g) for the collection of *soleus*, *gastrocnemius* and *quadriceps* muscles in both of the hind legs. Fat and tendons were removed, and the muscles were cut into small pieces. Then, the muscles cells were separated into two enzyme solutions (15 mL DMEM 4.5 g/L of glucose, 1% antibiotic solution, 0.2% type II Collagenase and 15 mL DMEM 4.5 g/L of glucose, 1% antibiotic solution, 0.3% type II Collagenase, 10.5% Trypsin and 10.5% DNase) for 90 and 30 minutes, respectively. The cells were filtered in a 50 μm sieve, counted in a Malassez hemocytometer, and then seeded in Pétri dishes, as previously described[3].

Primary muscle cell cultures

All products for cell culture except Matrigel were from Gibco (InVitrogen®, Cergy-Pontoise, France). Approximately 6 h before the experiment, 10 cm^2 Pétri dishes (Nunclon®) were coated with 750 μL Matrigel® (BD Biosciences, Le Pont de Claix, France) diluted 1:500 in DMEM 4.5 g/L of glucose and kept in a 100% humidified incubator at 37°C with 10% CO_2. Later, the cells were seeded ($2.5x10^5$ cells/10 cm^2 dish) in 2 mL Prime Growth Medium (PGM) containing DMEM 4.5 g/L of glucose, 10% foetal bovine serum, 10% horse serum, 4 mmol/L L-glutamine and 1% antibiotic solution, and they were grown in a 100% humidified incubator at 37°C in an atmosphere of 90% air/10% CO_2 for 48 h. For both the N and the U animal groups, the dishes were seeded in triplicate, where three dishes were seeded without insulin (control, Nc and Uc) and three dishes with insulin (insulin, Ni and Ui). The PGM was then replaced by Differentiation Medium (DM) containing DMEM 4.5 g/L of glucose, 5% horse serum, 2% L-glutamine and 1% antibiotic solution) and changed every 2 days. Insulin (10 μU/mL) was added from the 2^{nd} day of culture[28]. The cells were cultured for 10 days.

Cell proliferation and immunocytochemistry

To evaluate the rate of cell proliferation after 10 days of culture, cells of all dishes of cultures, control group and the insulin group of N and U animals, were trypsinised and counted in a Malassez hemocytometer.

Immunocytochemistry was assessed to evaluate the potential formation of myotubes, based on the protocol used with human myoblasts[29].

On the 10th day of culture, cells were fixed in 4% paraformaldehyde (PAF), and rabbit polyclonal anti-myosin antibody (Calbiochem®- Germany) was used according to the protocol according to the manufacturers instructions.

From each sample of the N and U groups, 3 images were captured randomly and were used to count the incorporated and unincorporated nuclei in myotubes[29]. This count was performed in triplicate for each animal in all groups (3 control samples: Nc and Uc and 3 insulin samples: Ni and Ui of each animal), and the result was obtained using the average of the three counts. The association between the number of incorporated and unincorporated nuclei revealed the ability to form myotubes.

Biomechanical analysis of myotubes in culture

In every culture dish, three 30-second films were captured on the 10th day of culture using a digital camera with a frequency of 30 frames per second (Canon A620-France) coupled with a phase contrast microscope (Olympus CKX41-Japan). Images of the contraction and relaxation of myotube films were captured through the programme XnView (version 1.97, Libformat 5.54-France) and analysed with the programme ImageJ (version 1.37v, USA). Three parameters were analysed based on the measures of distance between the relaxation and contraction points (*xy* coordinates) of the chosen points in the studied images: (1) *contraction number* (per minute); (2) *shortening due to contraction* (%) and (3) *period of contraction*[30-32]. These parameters were calculated according to the formulas given in table 1.

Statistical analysis

For all analyses, we used the nonparametric statistical Mann-Whitney test to compare the differences among groups. The data are presented as means ± SD, and the results were considered statistically significant for $p<0.05$. GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used.

Results

Growth Curve

During of lactation period, the weight of the Nourished (N) and Undernourished (U) animals differed from the 6th day of age (N = 17.9 ± 1.7 and U = 14.4 ± 1.0) (Figure 1). At the 21st day, the weight difference (approximately 27 g) between the N and U groups was significant (N = 67.8 ± 3.0 and U = 40.7 ± 1.9).

In the post-lactation period, in which offspring were fed a normal protein diet with nutritional supplementation, there was also a reduction in weight gain in the U group compared to the N group (N = 394 ± 28.4 and U = 342.7 ± 20.7) (Figure 2). At the sixth week after the end of lactation, the weight difference between the groups was approximately 50 g.

Cell proliferation

In all conditions, no difference was found in the rate of cell proliferation in myoblast cultures (Nc = 2.61 ± 0.56; Ni = 2.49 ± 0.51; Uc = 3.12 ± 0.92 and Ui = 2.77 ± 0.81). However, Figure 3 shows that cell proliferation tended to increase in animals that suffered undernutrition, regardless of the presence of insulin.

Immunocytochemistry

Immunocytochemistry was used to evaluate the potential formation of myotubes between the N and U groups (Figure 4). Myotubes were well developed and branched in Nc and Ni cultures (Figure 4 A and 4 B), and they were poorly developed in Uc and Ui cultures (Figure 4 C and 4 D). Based on the percentage of nuclei incorporated into myotubes (Figure 5), there was no difference between the Nc and Ni cultures (14.9 ± 3.3 and 16.9 ± 9.5, respectively). The presence of insulin did not change the percentage of nuclei incorporated in Ni cultures ($p>0.05$). A lower number of incorporated nuclei ware measured in Ui cultures (7.0 ± 5.6) compared to that in Uc culture (11.5 ± 8.9) (Figure 5).

Biomechanical analysis

Three parameters were evaluated for the biomechanical analysis of myotubes in culture: (1) the contraction number, (2) the shortening due to contraction and (3) the period of contraction.

The contraction number was presented in Figure 6. Comparing groups Nc and Uc, no difference was found (Nc = 17.6 ± 1.8, Uc = 16.4 ± 3.6). However, comparing control and insulin-treated cultures, it was observed that approximately 11 contractions more per minute were found in the Ui and Ni cultures than in the Nc and Uc cultures, respectively (Ni = 29.2 ± 3.8 and Ui = 28.2 ± 4.2).

The shortening percentages due to contraction (Figure 7) were not significantly different in Uc vs Nc cultures, though values tended to be lower in Uc cultures. Insulin did not change the results in Ni vs Nc cultures. Even though Figure 7 shows an increase in this variable in Ui group, there was no statistical difference when compared to the Uc group ($p = 0.0687$).

The results presented in Figure 8 shows the period of contraction (duration of a contraction). Were not different in Uc vs Nc cultures. The insulin presence in group N cells increased the contraction period (Ni = 1.2 ± 0.9 vs Nc = 0.8 ± 0.3), while in the U group, a decrease was observed (Ui = 0.8 ± 0.1 vs Uc = 0.9 ± 0.2), with $p>0.05$. The analyses revealed that there were reduced contraction periods in the Ui group compared to the Ni group ($p<0.05$). The Nu and Uc groups were similar.

Discussion

In this work, we studied the effect of maternal diet with a low protein concentration during the lactation period on the weight gain of the offspring. Previous studies have shown that nutritional deficiency during the critical growth period has an effect throughout adulthood, and the consequences are evident in the weight reduction until the appearance of metabolic diseases[33]. In our study, we demonstrated that a maternal diet with 8% casein, even though it was able to provide essential amino acids[34] during the lactation period, effectively reduced the weight of offspring, confirming studies previously conducted with the same model of undernutrition[3].

The skeletal muscle tissue plays a central role in the energy metabolism of the body[35] and is formed and developed during pregnancy, when the number of muscle fibres has already

been established. Therefore, maternal nutrition during this period has several effects that may recur throughout the adult life of the offspring, like explain the epigenetics[36]. The immediate postnatal period also represents a stage in which nutritional manipulation can still alter the maturation of the muscle fibres formed earlier[37]. During muscle development, embryonic precursor cells undergo differentiation into myotubes (fusion of myoblasts) and maturation in muscle fibres[38-40].

Our study also evaluated cell proliferation and the ability to form myotubes in cell cultures of rats submitted to undernutrition that were stimulated or not with insulin. However, the results only reflected the effects of undernutrition on the body, which include a tendency towards an increasing rate of cell proliferation, such as showed in Figure 5, but without a significant difference, and reductions of the ability to incorporate nuclei and to form myotubes.

In the immediate postnatal period, several physiological and metabolic mechanisms have not yet reached full maturity and continue in the process of maturation. Studies[41,42] have shown that during this postnatal period, the pancreatic islets and neurons of rodents continue to develop, confirming the hypothesis of a critical window period for development. Therefore, nutritional changes at this time are able to programme certain metabolic functions throughout life. Based on reported results, the cell proliferation and formation of myotubes during this period may be influenced by a maternal protein deficiency during lactation. In a study conducted in 2004[37], our model of undernutrition was able to reduce the number of nuclei in the muscle fibres of offspring whose mothers received protein-restricted diets, indicating a reduction in muscle cell proliferation. In our study, we did not observe a difference in cell proliferation among the groups, suggesting that undernutrition does not alter the mitogenic effect of insulin sensitivity in muscle cells. However, despite an increasing tendency towards cell proliferation in undernourished animals, our data show that the presence of insulin still reduced the incorporation of nuclei during myotube formation, resulting in the development of narrower and less branched myotubes. Although insulin stimulates myogenesis, its effect can be influenced by undernutrition during lactation. Our study shows that in group N cultures, insulin did not change the incorporation of nuclei into myotubes, although the insulin shown a myogenic ability[29]. Therefore, the effect of insulin is still susceptible to the nutritional conditions imposed during the critical period of development, were nutritional changes can affect several molecular and physiological mechanisms in throughout the life[1-6].

Muscle dysfunction is a common occurrence in malnutrition, and loss of muscle mass contributes significantly to the damage of this condition. A study[43] that investigated animals that passed through a prolonged period of malnutrition observed that the animals had deleterious changes in muscle structure, whereas the dry weight and wet muscle, thickness, composition and fibre size were not modified. The results reported in this study indicate that prolonged malnutrition can cause deleterious changes in the structure of this tissue that may impair its ability to generate force. In our study, we observed a similar result in cultured primary muscle cells.

Biomechanical parameters were measured at the 10th day of culture; we observed the contraction number, shortening percentage due to contraction and the period of myotube contraction. The contraction numbers observed among the groups showed that myotubes of Ni and Ui animals were increased in the presence of insulin, significantly in both groups when compared to their respective controls (Nc and Uc). Such results indicate that the myotubes from U animals had the capability to respond to insulin under these experimental conditions. To account for the total metabolism of a man, it is estimated that the muscle is capable of storing 20% more glucose than the liver (glycogen); because of this, insulin is a major player in muscles' ability to absorb glucose and to generate glycogen. When phosphorylated, the insulin receptor IRS-1 increases the affinity of glucose with the GLUT-4 transporter in muscle cells, thus increasing the main metabolic fuel supply to muscles when starting their contractions. A limitation in protein intake by the mother during pregnancy and/or lactation in rats alters the expression of genes involved in lipid homeostasis[44], in glucocorticoid receptor expression and in the enzymes that can inactivate its expression[45]. Based on these roles, we might hypothesise that the model of undernutrition used in this study could have changed the contraction numbers of the myotubes treated with insulin in both the Ni and the Ui groups. However, the contraction numbers remained similar between the Nc/Uc and the Ni/Ui groups. Comparing the groups that received insulin and the control group, there was a greater number of contractions in the groups with insulin (Ni and Ui), regardless of the diet during lactation.

Striated muscles may develop into various sizes according to the function each performs; therefore, the amplitude of contraction is proportional to its size. A long muscle has a higher amplitude, so its ability to shorten is greater than that of a muscle-type penniform with smaller fibres but a larger potency[46]. A recent study[3] using a maternal low protein diet during lactation showed that myotubes in culture from the offspring were less branched than their respective controls, similar to our results. In our study, the shortening percentages due to contraction of all the groups presented were similar, despite a reduction shown on the graph in

the shortening between the Uc and Nc groups. However, the myotubes in the Ui groups compared with those in the Uc groups have a slight tendency to increase the shortening percentage in the presence of insulin, which attests to the ability of myotubes from U animals to respond to insulin under our experimental conditions. Our results agree with a study conducted in 2011[3]; the decrease of the ramifications in myotube cultures from undernourished rats can contribute to a longer length and consequently can increase the contraction amplitude (shortening percentage due to contraction) among them.

To complete the analysis of biomechanical parameters, was evaluated the period of contraction in the myotube cultures, which was measured by the time that a myotube remained contracted. Muscle contraction is a result of the interaction between the proteins actin and myosin that requires the availability of Ca^{2+} and glucose as glycogen for performing this function, both provided by the culture medium. The period of contraction will be obviously proportional to the amount of glycogen stored in the myotube, which in turn depends on how much glucose was absorbed. If neonatal undernutrition can alter the metabolic and physiological functions in adulthood, it is likely that it is also responsible for the behaviour of glucose uptake by muscles, which involves glucose absorption through the translocation of GLUT-4 and the expression of insulin receptors in these cells. In our study, the period of contraction in the Ui group decreased compared to the Uc group. In this case, we believe that the amount of glycogen in the Ui group was insufficiently available to maintain the myotube contraction for a longer period of time. Epigenetic studies could explain this fact[37], as it is based on gene expression secondary to environmental influence. Thus, a change may have occurred in the expression of the genes that regulate the expression of insulin receptors in myotubes from undernourished groups.

Conclusions

In conclusion, protein restriction during lactation followed by nutritional supplementation at weaning altered the morphological and biomechanical profiles in cultures of adult rat myotubes. Although myocyte differentiation occurred normally, undernutrition was able to reduce the fusion capacity of primary muscle cells in myotubes, suggesting that the muscle tissue exhibited a reduced sensitivity to insulin. However, in the case of biomechanical parameters, undernutrition altered insulin sensitivity, which was demonstrated by a decrease in the period of contraction that resulted from metabolic changes unchained by nutritional hostility in the neonatal period.

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Conflict of interest

All the authors contributed to the collection and interpretation of the data, as well as to the drafting of the manuscript. The authors have no conflicts of interest to declare.

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Figure Captions

Figure 1. Body weight of pups from mothers fed a low-protein and normal-protein diet (Nourished, casein 17% or Undernourished, casein 8%) during the lactation period. Analysis was performed every 5 days (n = 6 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. (*p<0.05) compared with the N group using the nonparametric Mann-Whitney test.

Figure 2. Body weight of pups from mothers fed a low-protein and normal-protein diet (Nourished, casein 17% or Undernourished, casein 8%) during the post-lactation period. Analysis was performed once a week (n = 6 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. (*p<0.05) compared with the N group using nonparametric Mann-Whitney test.

Figure 3. Rate of cell proliferation from the 10th day of culture (n = 18 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. We used the nonparametric Mann-Whitney test, with p<0.05.

Figure 4. Myotubes of Nourished (N) and Undernourished (U) animals, with insulin (i) and without insulin (c), labelled with rabbit anti-myosin PAb (Calbiochem®). Nuclei were stained with DAPI. **(A)** Nc, **(B)** Ni, **(C)** Uc and **(D)** Ui. (x 100).

Figure 5. Percentages of incorporated nuclei in myotubes (n = 9 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. We used the nonparametric Mann-Whitney test, with * p<0.05 between Ui and Uc groups.

Figure 6. Contractions (number/min) of myotubes in culture between N and U animals, stimulated or not with insulin (n = 18 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. (*p<0.05) compared with the Nc and Uc groups using the nonparametric Mann-Whitney test.

Figure 7. Shortening percentage due to the contraction of myotubes in the cultures of the N and U rats, stimulated or not with insulin. Analysis was performed on the 10th day of culture (n = 18 in each group). The results are mean values, with the standard error of the mean represented by vertical bars. Nonparametric Mann-Whitney test, with p=0.0687 between Nc and Uc.

Figure 8. Periods of contraction in myotube cultures of Nourished (N) and Undernourished (U) rats, stimulated with insulin (i) or without it (c) (n = 20). The results are mean values, with standard error of the mean represented by vertical bars. (*p<0.05) compared with Ni using the nonparametric Mann-Whitney test.

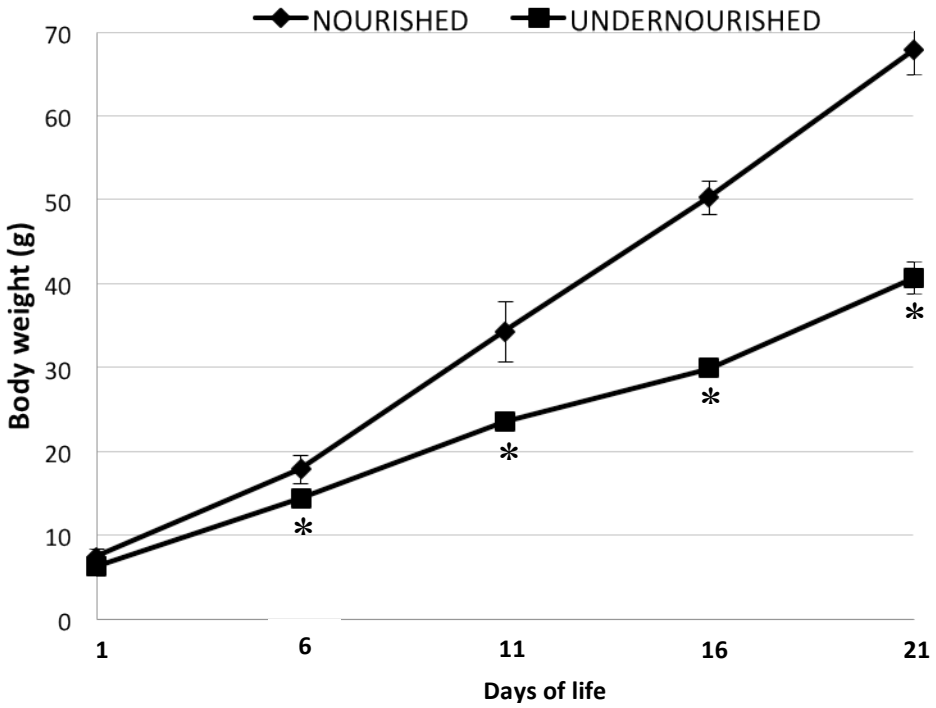
NOURISHED
UNDERNOURISHED
Body weight (g)
70
60
50
40
30
20
10
0
1
6
11
16
21
Days of life
*
*
*
*

Figure 1

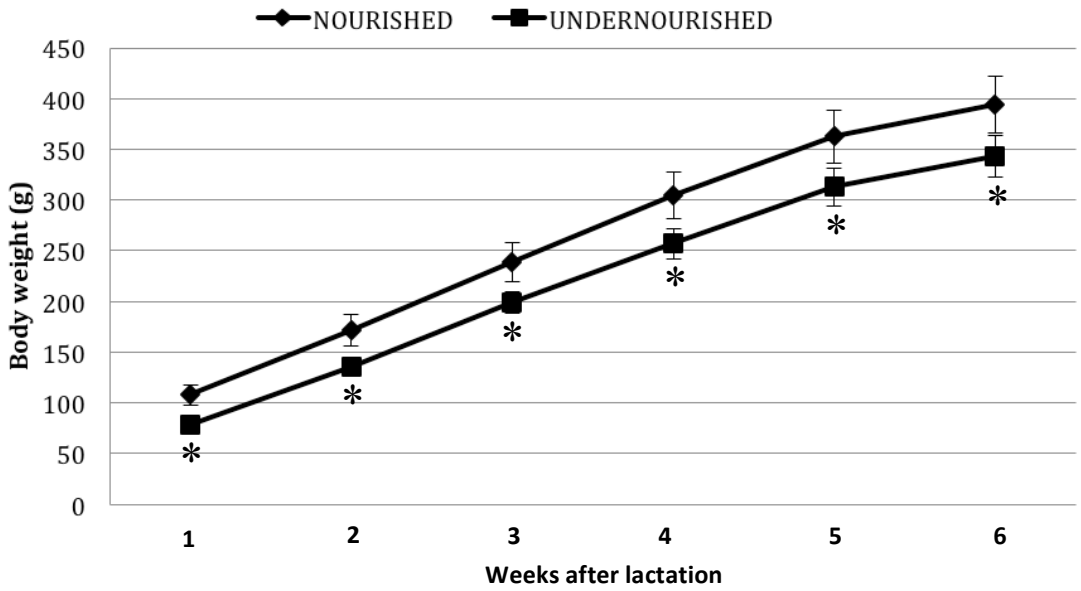
NOURISHED
UNDERNOURISHED
Body weight (g)
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
Weeks after lactation
*
*
*
*
*
*

Figure 2

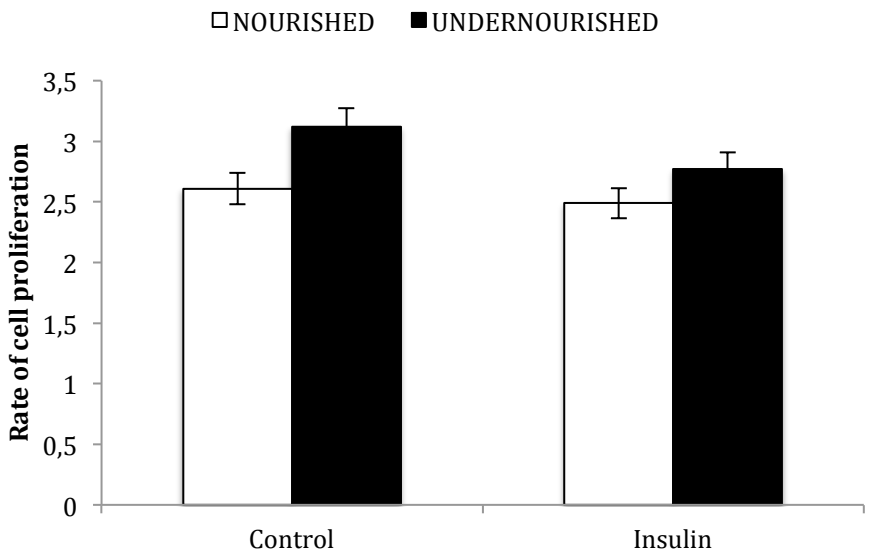
NOURISHED
UNDERNOURISHED
Rate of cell proliferation
3,5
3
2,5
2
1,5
1
0,5
0
Control
Insulin

Figure 3

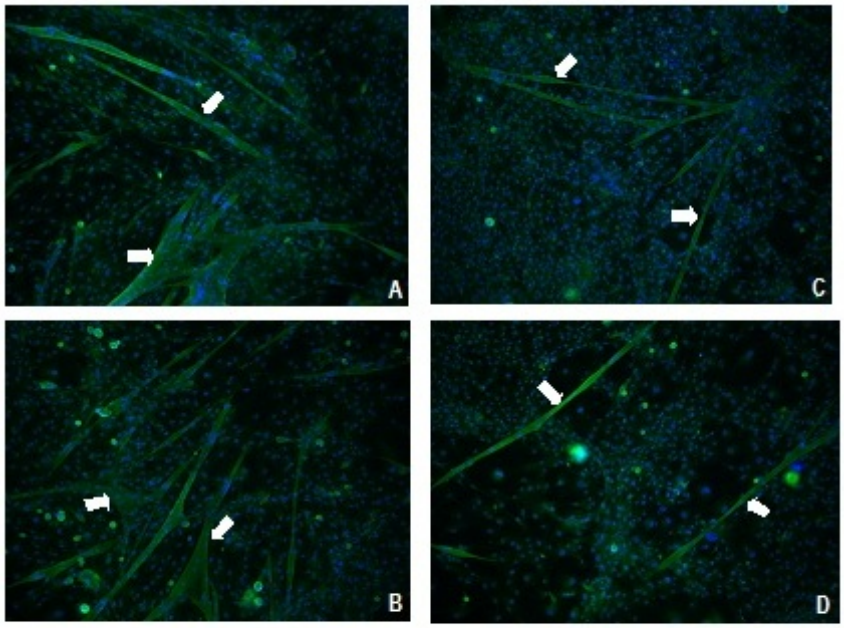

Figure 4

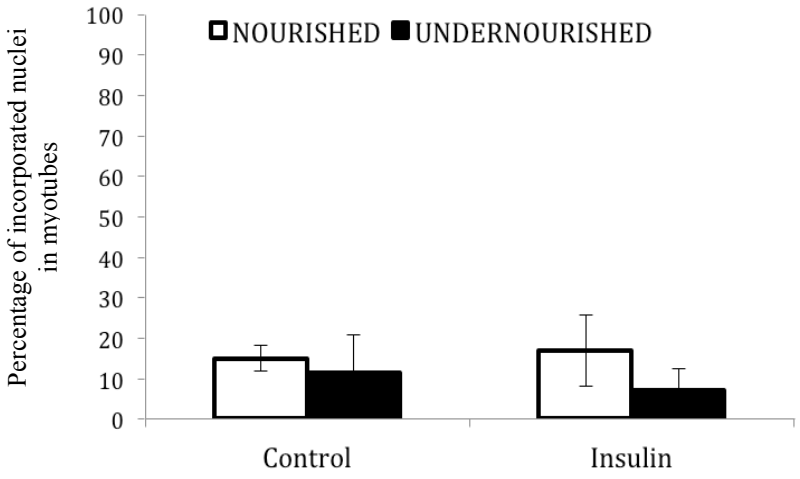
NOURISHED
UNDERNOURISHED
Percentage of incorporated nuclei in myotubes
100
90
80
70
60
50
40
30
20
10
0
Control
Insulin

Figure 5

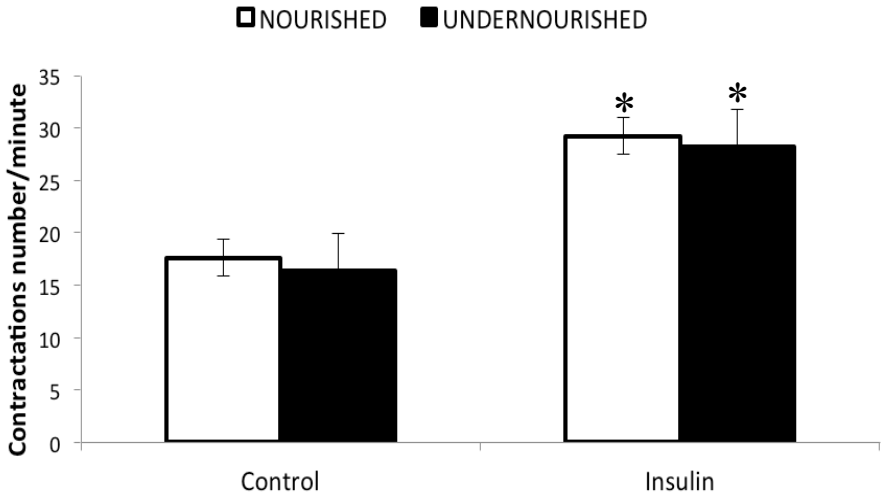
NOURISHED
UNDERNOURISHED
Contractations number/minute
35
30
25
20
15
10
5
0
*
*
Control
Insulin

Figure 6

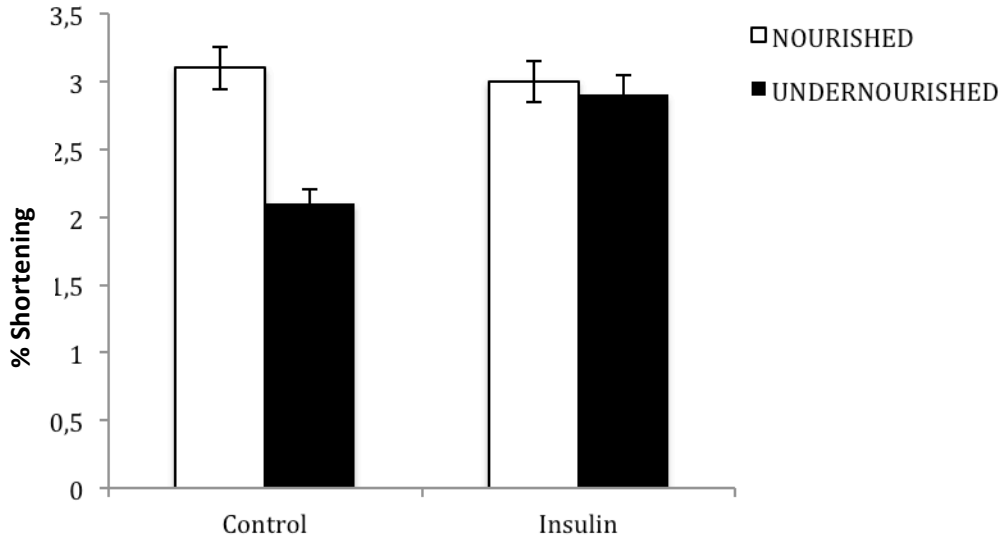
3,5
3
2,5
2
1,5
1
0,5
0
% Shortening
Control
Insulin
NOURISHED
UNDERNOURISHED

Figure 7

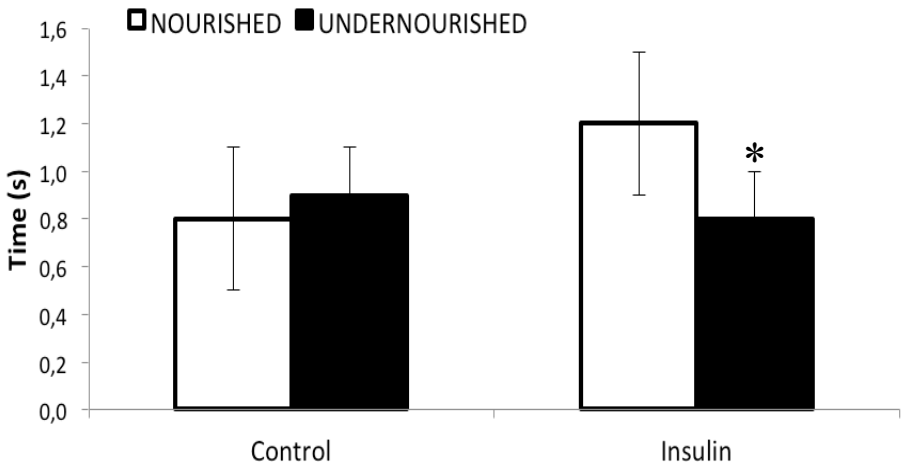
NOURISHED
UNDERNOURISHED
Time (s)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
Control
Insulin

Figure 8

Table 1. Formulas used to calculate the biomechanical parameters of myotubes in culture

Parameter analysed	**Formulas**
Contraction number	$CN = \frac{contraction\ number}{30}\ x\ 60$
Shortening due to contraction	$\%\ SC = \frac{\Delta\ distances}{relaxation\ distances}\ x\ 100$
Period of contraction	$P = \frac{number\ of\ images\ between\ contraction\ and\ relaxation}{number\ of\ images\ per\ second}$

5 CONSIDERAÇÕES FINAIS

As últimas linhas dessa tese se designam a uma tentativa de reunir os achados desta pesquisa ao longo do seu desenvolvimento. A princípio, tínhamos o objetivo de encontrar um método que fosse capaz de esclarecer as diferenças morfológicas e funcionais de miotubos em cultura entre os animais N e D, na presença ou não da insulina. Entretanto, ao longo de seu desenvolvimento, foi percebida a necessidade de otimizar e de padronizar o protocolo de coleta e de cultura das células musculares primárias de ratos Wistar utilizado em estudos anteriores (CARRASCO *et al.*, 2003; MELO et al., 2011), em decorrência da diversidade e das dificuldades entre os protocolos disponíveis na literatura.

A otimização de tal protocolo se mostrou eficiente no desenvolvimento das células musculares cultivadas assim como os demais protocolos disponíveis na literatura, uma vez que possibilitou o desenvolvimento morfológico e funcional destas células através da formação e da contração dos miotubos em cultura. Entretanto, algumas vantagens observadas foram destacadas após a sua otimização, tais como a simplificação dos procedimentos, o tempo reduzido desde o cultivo até a formação dos miotubos, bem como o menor custo.

A otimização do protocolo de cultura de células musculares também foi capaz de viabilizar o desenvolvimento de miotubos em cultura e, portanto, se apresentou como uma ferramenta importante para a engenharia de tecidos. Além disso, através da metodologia otimizada nesta pesquisa, foi possível desenvolver análises dos miotubos, em apenas 10 dias, que permitiu identificar e caracterizar algumas alterações musculares desencadeadas pelo modelo experimental de desnutrição proposto, principal propósito deste estudo.

Em pesquisa anterior realizada por Melo *et al*. (2011), foi observado que a desnutrição neonatal, mesmo seguida de reposição nutricional, é capaz de alterar tanto a morfologia quanto a fisiologia dos miotubos em cultura de ratos Wistar. Em nosso estudo, o perfil morfofuncional destes miotubos, traçado através do método desenvolvido para avaliar os seus parâmetros biomecânicos (contração e relaxamento) no mesmo modelo de desnutrição, possibilitou a caracterização das alterações morfofuncionais encontradas no trabalho supracitado e, assim, comprová-las.

O método otimizado nesta tese foi, então, capaz de confirmar os resultados do estudo anterior por meio das medidas de *frequência de contração dos miotubos*, através da qual foi avaliado o número de contrações por minuto; da *amplitude de contração*, que se referiu ao potencial de encurtamento dos miotubos; e do *tempo de contração (período de contração)*, medida que se relacionou à força muscular. Além disso, e como complementação deste trabalho, o *percentual de incorporação de núcleos*, técnica adotada por Jacquemin *et al*. (2005), denotou a capacidade que cada um dos grupos experimentais apresentava em formá-los e, portanto, se seriam capazes de apresentar maior ou menor força muscular durante o processo de contração.

Através de tais procedimentos, foi possível caracterizar o perfil biomecânico das células musculares em cultura, e assim confirmar nossa hipótese inicial de que a desnutrição neonatal é capaz de alterar a biomecânica do músculo de ratos.

Alguns estudos realizados anteriormente, tais como os de Oliveira *et al*. (1999); Alves, Dâmaso e Dal Pai (2008), além do estudo de Melo *et al*. (2011), mostram que a desnutrição neonatal produz um déficit no ganho energético. Assim, nós esperávamos que a perda energética promovida pela desnutrição também fosse refletida nos parâmetros relacionados à contração e ao relaxamento dos miotubos. Por isso, esta pesquisa procurou verificar a ação que a insulina exerce no músculo de ratos submetidos à desnutrição, uma vez que ela é fundamental para a captação da glicose pelas células musculares, para que esta seja estocada em forma de glicogênio, a reserva de energia muscular.

Através dos dados coletados sobre a ação da insulina nas células musculares em cultura, é possível afirmar que a desnutrição neonatal aumenta a sensibilidade dos miotubos à tal hormônio peptídeo, que culmina na modificação de alguns de seus parâmetros biomecânicos. Este dado comprovou outra hipótese, a de que o organismo sofre alterações de suas características epigenômicas quando exposto a estímulos nutricionais, tal como a manipulação nutricional no período neonatal. Com isso, é possível inferir que, embora a miogênese aconteça nos períodos embrionário e fetal, o período neonatal ainda se comporta como uma "janela de plasticidade celular" da fase crítica de crescimento e desenvolvimento para o tecido muscular.

Apesar da insulina atuar como fator estimulador da miogênese, é possível considerar que a desnutrição compromete esta função, visto que o estímulo à replicação das células

musculares em cultura não resultou no aumento da fusão de tais células. Neste caso, a função miogênica da insulina se resumiu apenas ao aumento da replicação celular, provavelmente como uma forma de garantir o número de células necessárias para formar os miotubos, ao passo que a fusão destas mesmas células, fenômeno inerente à miogênese, ficou comprometida neste modelo experimental, e acarretou na modificação do perfil biomecânico dos miotubos em cultura destes animais.

Neste sentido, este estudo corrobora com a tese de que os organismos neonatos também são sensíveis às variações nutricionais maternas, cujas consequências repercutem ao longo da vida. Entretanto, a maior parte dos estudos que envolvem desnutrição e o tecido muscular está relacionada ao período pré-natal, provavelmente devido ao fato da miogênese acontecer nesta fase da vida. Portanto, a linha de pesquisa que envolve os estudos epigenéticos pode ser capaz de justificar os resultados aqui apresentados, além de mostrar que o período em que ocorreu a manipulação nutricional (período neonatal) pode promover interferências significativas na biomecânica muscular, ao contrário do que alguns pesquisadores acreditavam.

Assim, é importante o desenvolvimento de estudos dispostos a elucidar quais seriam as modificações epigenéticas que justificariam as alterações observadas nos miotubos de animais desnutridos no período neonatal, em decorrência da alteração à sensibilidade insulínica. Talvez estudos que envolvam a dinâmica de assimilação da glicose, através da translocação do GLUT-4 e a consequente produção de glicogênio pelos miotubos, bem como o padrão de acetilação e metilação de histonas e de bases nitrogenadas de genes das células musculares, associados à análise de possíveis polimorfimos dos receptores de insulina nessas células, constituam novas perspectivas de estudo no campo da repercussão da desnutrição neonatal na vida adulta.

Além disso, tal elucidação epigenética do tecido muscular esquelético durante o desenvolvimento neonatal e na vida adulta, poderá ser uma estratégia experimental para gerar células-tronco musculares, seja por reprogramação de células-tronco embrionárias, ou por induzir pluripotência nas células musculares esqueléticas adultas, bem como para elucidar a capacidade muscular em se contrair e se manter em contração, conforme pesquisa realizada nesta tese.

Portanto, é importante continuar esta pesquisa para que os questionamentos que surgiram ao longo desse tempo possam ser respondidos, e que dessa maneira, eles possam esclarecer de que forma as alterações nas células musculares de animais que sofreram desnutrição podem ser revertidas, para que estas células possam ter o mesmo desempenho das que não passaram por esse tipo de agressão nutricional.

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ANEXOS

ANEXO A – Autorização para utilização do biotério e realização da pesquisa com animais de experimentação da UTC

PREFET DE L'OISE

MINISTÈRE DE L'ALIMENTATION DE L'AGRICULTURE ET DE LA PÊCHE

Le Directeur départemental

de la protection des populations

CERTIFICAT D'AUTORISATION D'EXPÉRIMENTER SUR ANIMAUX VIVANTS - Extension

Code Rural articles L. 214-3 et L 215-7, R 214-87 à R.214-122 et R. 215-10 relatifs aux expériences pratiquées sur les animaux vertébrés
Arrêté du 19 avril 1988 fixant les conditions d'attribution de l'autorisation de pratiquer des expériences sur les animaux

Numéro de l'autorisation : **60-24**

Identité du demandeur: DUVAL Jean Luc

Adresse de(s) l'établissement(s) :Universite de Technologie de Compiègne BP 20529 60205 Compiègne cedex

Est autorisé (e) à réaliser des expériences sur animaux vertébrés vivants dans les conditions suivantes :

DOMAINES D'ACTIVITÉ

- Recherche fondamentale ;
- Essais d'activité d'efficacité ou de toxicité des médicaments et d'autres substances biologiques et chimiques;
-

TYPE DE PROTOCOLES EXPÉRIMENTAUX MIS EN ŒUVRE ET ESPÈCES ANIMALES UTILISÉES

- Administration de substances sur animaux vigiles : souris ; rats
- Injection intra-veineuse de cellules tumorales : souris
- Injection sous-cutanée de biomatériaux : rats
- Injection intra-dermique de biomatériaux : rats
- Prélèvement de substances sur animaux vigiles : souris, rats
- Euthanasie des animaux en vue d'examens et de prélèvements : souris, rats

Autorisation valable jusqu'au 11 avril 2012
Renouvelable sur **demande écrite**

Fait à Beauvais le 23 décembre 2010

P/Le Directeur départemental de la protection des populations

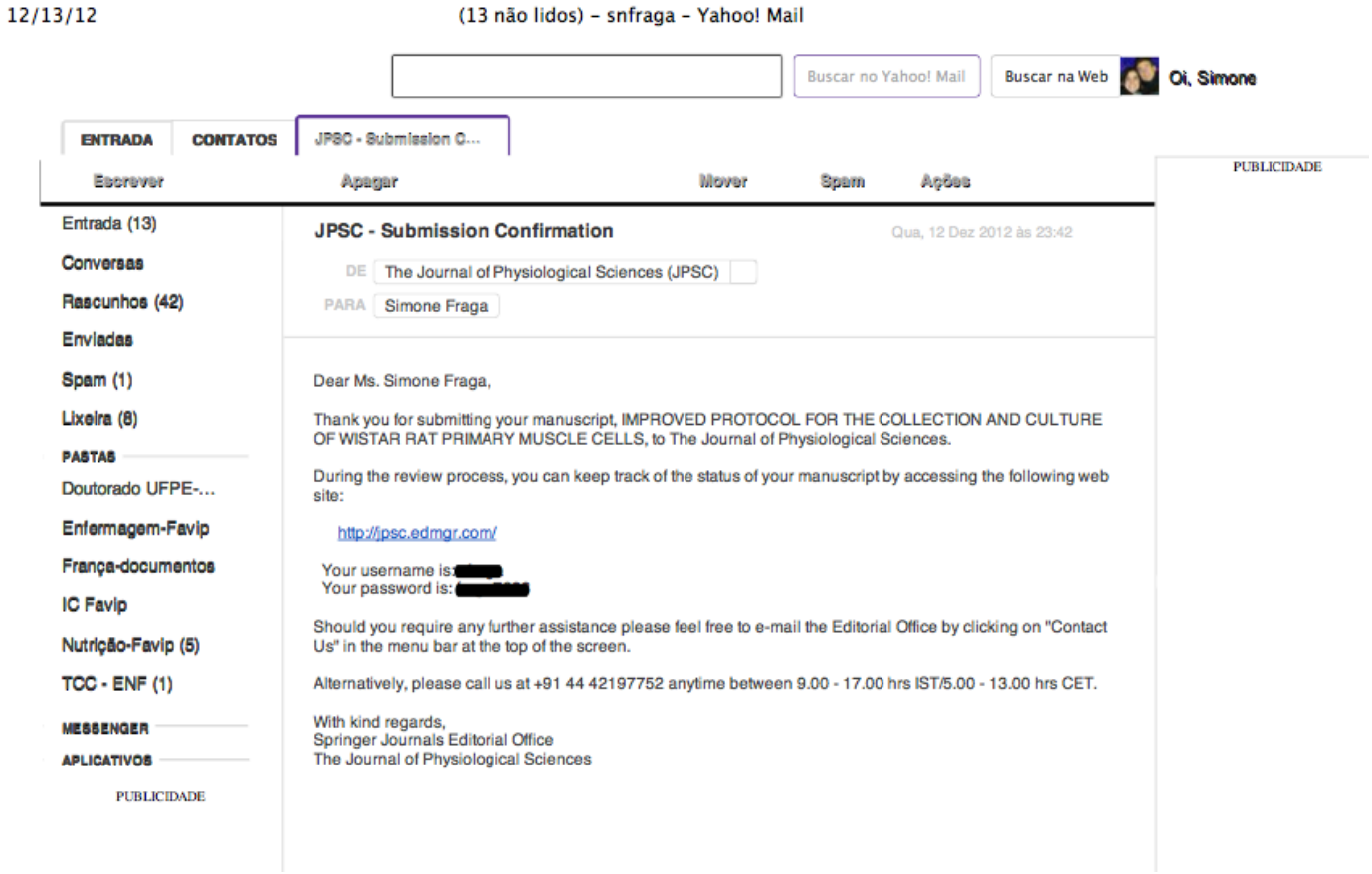
Dr Jacques FAVRE

PREFECTURE DE L'OISE SERVICES VETERINAIRES

Voir Informations importantes au verso

Direction départementale de la protection des populations de l'Oise BP 708304 60007 Beauvais cedex
Tél. :03 44 06 32 95 – E-Mail : ddsv60@agriculture.gouv.fr

ANEXO B – Carta de submissão do artigo 1 ao *The Journal of Physiological Sciences*



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Qua, 12 Dez 2012 às 23:42

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PARA Simone Fraga

Dear Ms. Simone Fraga,

Thank you for submitting your manuscript, IMPROVED PROTOCOL FOR THE COLLECTION AND CULTURE OF WISTAR RAT PRIMARY MUSCLE CELLS, to The Journal of Physiological Sciences.

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With kind regards,
Springer Journals Editorial Office
The Journal of Physiological Sciences

ANEXO C – Carta de submissão do artigo 2 ao *British Journal of Nutrition*

Assunto:	BJN-2012-019287 BJN submission received
De:	edoffice@nutsoc.org.uk (edoffice@nutsoc.org.uk)
Para:	snfraga@yahoo.com.br;
Data:	Quarta-feira, 12 de Dezembro de 2012 13:00

Dear Ms. Fraga,

On 12th Dec 2012, we received your manuscript entitled "EFFECT OF INSULIN ON THE FORMATION AND FUNCTION OF MYOTUBE CULTURES FROM ADULT RATS SUBMITTED TO NEONATAL UNDERNUTRITION" by Simone Fraga, Jean-Luc Duval, Pascale Vigneron, Francis Canon, JULIANA MELO, Marie-Danielle Nagel, and Célia De Castro.

The manuscript has been assigned the Paper number: BJN-2012-019287.

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Sincerely,

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ANEXO D - Normas para publicação no *The Journal of Physiological Sciences*

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The Journal of Physiological Sciences

Editor-in-Chief: Yasuo Sakuma

ISSN: 1880-6546 (print version)
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Journal no. 12576

Instructions for Authors

Instructions for Authors

GENERAL INFORMATION

The Journal of Physiological Sciences publishes peer-reviewed original papers, reviews, short communications, technical notes, and letters to the editor, based on the principles and theories of modern physiology.

Original papers should describe novel ideas based on new results that provide new perspectives in the scientific community. Reviews should be overview articles related to other authors' original research, usually solicited by the editor. Short communications should be short articles, peer reviewed as quickly as possible, and with no subheadings in the sections: materials and methods, results, and discussion. Technical notes should be reports on new methods or techniques. Short communications and technical notes should be no longer than 6 printed pages. Letters to the editor should be short discussions on recently published articles in this journal, published together with response(s) from the original article's author(s). Its text should be no longer than 750 words. All manuscripts should be written in English.

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1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

• Reference list

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anion channels in acidosis-induced cell death in human epithelial cells. Pflügers Arch 454: 223–233

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Boini KM, Graf D, Kuhl D, Häussinger D, Lang F (2008) SGK1 dependence of insulin induced hypokalemia. Pflügers Arch. doi:10.1007/s00424-008-0559-5

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

- Book

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- Book chapter

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

- Online document

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ANEXO E - Normas para publicação no *British Journal of Nutrition*

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British Journal of Nutrition

(Revised January 2012)

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Reviews and Meta-Analyses (PRISMA) Statement, a guideline to help authors report a systematic review and meta-analysis http:/prisma-statement.org (see *British Medical Journal* (2009) **339**, b2535). A systematic review or meta-analysis of randomised trials and other evaluation studies should follow the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http:/prisma-statement.org).

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If the paper is one of a series of papers that have a common main title followed by a subtitle specific to the individual paper, numbering should not be used to indicate the sequence of papers. The format should be 'common title: specific subtitle', with a short common title, e.g. Partitioning of limiting protein and energy in the growing pig: testing quantitative rules against experimental data.

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Authors are asked to supply three or four key words or phrases (each containing up to three words) on the title page of the typescript.

(b) *Abstract*: each paper must open with an abstract of **not more than 250 words**. The abstract should be a single paragraph of continuous text outlining the aims of the work, the experimental approach taken, the principal results and the conclusions and their relevance to nutritional science.

(c) *Introduction*: it is not necessary to introduce a paper with a full account of the relevant literature, but the introduction should indicate briefly the nature of the question asked and the reasons for asking it. It should be **no longer than two pages**.

(d) *Experimental methods*: methods should appear after the introduction.

The notice of contributors is drawn to the guidelines in the World Medical Association (2000) Declaration of Helsinki: ethical principles for medical research involving human subjects, with notes of clarification of 2002 and 2004 (http://www.wma.net/en/30publications/10policies/b3/), the *Guidelines on the Practice of Ethics Committees Involved in Medical Research Involving Human Subjects* (3rd ed., 1996; London: The Royal College of Physicians) and the Guidelines for the ethical conduct of medical research involving children, revised in 2000 by the Royal College of Paediatrics and Child Health: Ethics Advisory Committee (*Arch Dis Child* (2000) **82**, 177–182). A paper describing any experimental work on human subjects must include the following statement in the materials/methods section: "This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the [insert name of the ethics committee; a specific ethics number may be inserted if you wish]. Written [or Verbal] informed consent was obtained from all subjects/patients. [Where verbal consent was obtained this must be followed by a statement such as: Verbal consent was witnessed and formally recorded]."

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(h) *References*: these should be given in the text using the Vancouver system. They should be numbered consecutively in the order in which they first appear in the text using superscript Arabic numerals in parentheses, e.g. 'The conceptual difficulty of this approach has recently been highlighted[1,2–4]'. If a reference is cited more than once the same number should be used each time. References cited only in tables and figure legends and not in the text should be numbered in sequence from the last number used in the text and in the order of mention of the individual tables and figures in the text. At the end of the paper, on a page(s) separate from the text, references should be listed in numerical order. When an article has more than three authors only the names of the first three authors should be given followed by '*et al.*' The issue number should be omitted if there is continuous pagination

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1. Setchell KD, Faughnan MS, Avades T *et al.* (2003) Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women. *Am J Clin Nutr* **77**, 411–419.
2. Barker DJ, Winter PD, Osmond C *et al.* (1989) Weight in infancy and death from ischaemic heart disease. *Lancet* **ii**, 577–580.
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4. Bradbury J, Thomason JM, Jepson NJA *et al.* (2003) A nutrition education intervention to increase the fruit and vegetable intake of denture wearers. *Proc Nutr Soc* **62**, 86A.
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8. Hall WL, Vafeiadou K, Hallund J *et al.* (2005) Soy isoflavone enriched foods and inflammatory biomarkers of cardiovascular risk in postmenopausal women: interactions with genotype and equol production. *Am J Clin Nutr* (In the Press).
9. Skurk T, Herder C, Kraft I *et al.* (2004) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology* (Epublication ahead of print version).
10. Skurk T, Herder C, Kraft I *et al.* (2005) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology* **146**, 1006–1011; Epublication 2 December 2004.
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13. Bruinsma J (editor) (2003) *World Agriculture towards 2015/2030: An FAO Perspective*. London: Earthscan Publications.
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16. International Agency for Research on Cancer (2004) *Cruciferous Vegetables, Isothiocyanates and Indoles. IARC Handbooks of Cancer Prevention* no. 9 [H Vainio and F Bianchini, editors]. Lyon, France: IARC Press.
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18. World Health Organization (2003) *Diet, Nutrition and the Prevention of Chronic Diseases*. *Joint WHO/FAO Expert Consultation*. *WHO Technical Report Series* no. 916. Geneva: WHO.
19. Keiding L (1997) *Astma, Allergi og Anden Overfølsomhed i Danmark – Og Udviklingen 1987–199I* (*Asthma, Allergy and Other Hypersensitivities in Denmark, 1987–1991*). Copenhagen, Denmark: Dansk Institut for Klinisk Epidemiologi.

References to material available on websites should include the full Internet address, and the date of the version cited. Thus:

20. Department of Health (1997) Committee on Toxicity of Chemicals in Food Consumer Products and the Environment. Statement on vitamin B_6 (pyridoxine) toxicity. http://www.open.gov.uk/doh/hef/B6.htm
21. Kramer MS & Kakuma R (2002) *The Optimal Duration of Exclusive Breastfeeding: A Systematic Review*. Rome: WHO; available at http://www.who.int/nut/documents/optimal_duration_of_exc_bfeeding_review_eng.pd
22. Hooper L, Thompson RL, Harrison RA *et al.* (2004) Omega 3 fatty acids for prevention and treatment of cardiovascular disease. *Cochrane Database of Systematic Reviews*, issue 4, CD003177. http://www.mrw.interscience.wiley.com/cochrane/clsysrev/articles/CD003177/frame.html
23. Nationmaster (2005) HIV AIDS – Adult prevalence rate. http://www.nationmaster.com/graph-T/hea_hiv_aid_adu_pre_rat (accessed June 2005).

(j) *Supplementary data:* Additional data (e.g. data files, large tables) relevant to the paper can be submitted for publication online only, where they are made available via a link from the abstract and the paper. The paper should stand alone without these data. Supplementary data should be supplied as a PDF for the review process and must be cited in a relevant place in the text of the paper.

Mathematical modelling of nutritional processes. Papers in which mathematical modelling of nutritional processes forms the principal element will be considered for publication provided: (a) they are based on sound biological and mathematical principles; (b) they advance nutritional concepts or identify new avenues likely to lead to such advances; (c) assumptions used in their construction are fully described and supported by appropriate argument; (d) they are described in such a way that the nutritional purpose is clearly apparent; (e) the contribution of the model to the design of future experimentation is clearly defined.

Units. Results should be presented in metric units according to the International System of Units (see Quantities, Units and Symbols in Physical Chemistry, 3rd ed. (2007) Cambridge: RSC Publishing), and Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences (1972) London: The Royal Society – as reproduced in *Proceedings of the Nutrition Society* (1972) **31**, 239–247). SI units should be used throughout the paper. The author will be asked to convert any values that are given in any other form. The only exception is where there is a unique way of expressing a particular variable that is in widespread use. Energy values must be given in Joules (MJ or kJ) using the conversion factor 1 kcal = 4·184 kJ. If required by the author, the value in kcal can be given afterwards in parentheses. Temperature is given in degrees Celsius (ºC). Vitamins should be given as mg or µg, not as IU.

For substances of known molecular mass (Da) or relative molecular mass, e.g. glucose, urea, Ca, Na, Fe, K, P, values should be expressed as mol/l; for substances of indeterminate molecular mass (Da) or relative molecular mass, e.g. phospholipids, proteins, and for trace elements, e.g. Cu, Zn, then g/l should be used.

Time. The 24 h clock should be used, e.g. 15.00 hours.

Units are: year, month, week, d, h, min, s, kg, g, mg, µg, litre, ml, µl, fl. To avoid misunderstandings, the word litre should be used in full, except in terms like g/l. Radioactivity should be given in becquerels (Bq or GBq) not in Ci. 1 MBq = 27·03 µCi (1Bq = 1 disintegration/s).

Statistical treatment of results. Data from individual replicates should not be given for large experiments, but may be given for small studies. The methods of statistical analysis used should be described, and references to statistical analysis packages included in the text, thus: Statistical Analysis Systems statistical software package version 6.11 (SAS Institute, Cary, NC, USA). Information such as analysis of variance tables should be given in the paper only if they are relevant to the discussion. A statement of the number of replicates, their average value and some appropriate measure of variability is usually sufficient.

Comparisons between means can be made by using either confidence intervals (CI) or significance tests. The most appropriate of such measures is usually the standard error of a difference between means (SED), or the standard errors of the means (SE or SEM) when these vary between means. The standard deviation (SD) is more useful only when there is specific interest in the variability of individual values. The degrees of freedom (df) associated with SED, SEM or SD should also be stated. The number of decimal places quoted should be sufficient but not excessive. Note that pH is an exponential number, as are the log(10) values often quoted for microbial numbers. Statistics should be carried out on the scalar rather than the exponential values.

If comparisons between means are made using CI, the format for presentation is, e.g. 'difference between means 0·73 (95 % CI 0·314, 1·36) g'. If significance tests are used, a statement that the difference between the means for two groups of values is (or is not) statistically significant should include the level of significance attained, preferably as an explicit *P* value (e.g. *P*=0·016 or *P*=0·32) rather than as a range (e.g. *P*<0·05 or *P*>0·05}. It should be stated whether the significance levels quoted are one-sided or two-sided. Where a multiple comparison procedure is used, a description or explicit reference should be given. Where appropriate, a superscript notation may be used in tables to denote levels of significance; similar superscripts should denote lack of a significant difference.

Where the method of analysis is unusual, or if the experimental design is at all complex, further details (e.g. experimental plan, raw data, confirmation of assumptions, analysis of variance tables, etc.) should be included.

Figures. Figures should not be incorporated into the article file and should be supplied as separate electronic files. Figure legends should be grouped in a section at the end of the text. Each figure should be clearly marked with its number and separate panels within figures should be clearly marked (a), (b), (c) etc. so that they are easily identifiable when the article and figure files are merged for review.

In curves presenting experimental results the determined points should be clearly shown, the symbols used being, in order of preference, ○, ●, Δ, ▲, □, ■, ×, +. Curves and symbols should not extend beyond the experimental points. Scale-marks on the axes should be on the inner side of each axis and should extend beyond the last experimental point. Ensure that lines and symbols used in graphs and shading used in histograms are large enough to be easily identified when the figure is reduced to fit the printed page.

Figures and diagrams can be prepared using most applications but please do not use the following: cdx, chm, jnb or PDF. All figures should be numbered and legends should be provided. Each figure, with its legend, should be comprehensible without reference to the text and should include definitions of abbreviations. Latin names for unusual species should be included unless they have already been specified in the text. Each figure will be positioned near the point in the text at which it is first introduced unless instructed otherwise.

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Plates. The *British Journal of Nutrition* will now also consider the inclusion of illustrations and photomicrographs. The size of photomicrographs may have to be altered in printing; in order to avoid mistakes the magnification should be shown by scale on the photograph itself. The scale with the appropriate unit together with any lettering should be drawn by the author, preferably using appropriate software.

Tables. Tables should carry headings describing their content and should be comprehensible without reference to the text. Tables should not be subdivided by ruled lines. The dimensions of the values, e.g. mg/kg, should be given at the top of each column. Separate columns should be used for measures of variance (SD, SE etc.), the ± sign should not be used. The number of decimal places used should be standardized; for whole numbers 1·0, 2·0 etc. should be used. Shortened forms of the words weight (wt) height (ht) and experiment (Expt) may be used to save space in tables, but only Expt (when referring to a specified experiment, e.g. Expt 1) is acceptable in the heading.

Footnotes are given in the following order: (1) abbreviations, (2) superscript letters, (3) symbols. Abbreviations are given in the format: RS, resistant starch. Abbreviations appear in the footnote in the order that they appear in the table (reading from left to right across the table, then down each column). Abbreviations in tables must be defined in footnotes. Symbols for footnotes should be used in the sequence: *†‡§‖¶, then ** etc. (omit * or †, or both, from the sequence if they are used to indicate levels of significance).

For indicating statistical significance, superscript letters or symbols may be used. Superscript letters are useful where comparisons are within a row or column and the level of significance is uniform, e.g. '^{a,b,c}Mean values within a column with unlike superscript letters were significantly different (P<0·05)'. Symbols are useful for indicating significant differences between rows or columns, especially where different levels of significance are found, e.g. 'Mean values were significantly different from those of the control group: *P<0·05, **P<0·01, ***P<0·001'. The symbols used for P values in the tables must be consistent.

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Please refer to a recent copy of the journal for examples of tables.

Chemical formulas. These should be written as far as possible on a single horizontal line. With inorganic substances, formulas may be used from first mention. With salts, it must be stated whether or not the anhydrous material is used, e.g. anhydrous $CuSO_4$, or which of the different crystalline forms is meant, e.g. $CuSO_4.5H_2O$, $CuSO_4.H_2O$.

Descriptions of solutions, compositions and concentrations. Solutions of common acids, bases and salts should be defined in terms of molarity (M), e.g. 0·1 M-NaH_2PO_4. Compositions expressed as mass per unit mass (w/w) should have values expressed as ng, μg, mg or g per kg; similarly for concentrations expressed as mass per unit volume (w/v), the denominator being the litre. If concentrations or compositions are expressed as a percentage, the basis for the composition should be specified (e.g. % (w/w) or % (w/v) etc.). The common measurements used in nutritional studies, e.g. digestibility, biological value and net protein utilization, should be expressed as decimals rather than as percentages, so that amounts of available nutrients can be obtained from analytical results by direct multiplication. See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences*. London: The Royal Society, 1972 (para. 8).

Cell lines. The Journal expects authors to deposit cell lines (including microbial strains) used in any study to be published in publicly accessible culture collections, for example, the European Collection of Cell Cultures (ECACC) or the American Type Culture Collection (ATCC) and to refer to the collection and line or strain numbers in the text (e.g. ATCC 53103). Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory line or strain designations and donor sources as well as original culture collection identification numbers.

Gene nomenclature and symbols. The use of symbols and nomenclature recommended by the HUGO Gene Nomenclature Committee (http://www.genenames.org/) is encouraged. Information on human genes is also available from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene), on mouse genes from the Mouse Genome Database (http://www.informatics.jax.org/) and on rat genes from the Rat Genome Database (http://rgd.mcw.edu/).

Nomenclature of vitamins. Most of the names for vitamins and related compounds that are accepted by the Editors are those recommended by the IUNS Committee on Nomenclature. See *Nutrition Abstracts and Reviews* (1978) **48**A, 831–835.

Acceptable name	*Other names**
Vitamin A	
Retinol	Vitamin A_1
Retinaldehyde, retinal	Retinene
Retinoic acid (all-*trans* or 13-*cis*)	Vitamin A_1 acid
3-Dehydroretinol	Vitamin A_2
Vitamin D	
Ergocalciferol, ercalciol	Vitamin D_2 calciferol
Cholecalciferol, calciol	Vitamin D_3
Vitamin E	
α-, β- and γ-tocopherols plus tocotrienols	
Vitamin K	
Phylloquinone	Vitamin K_1
Menaquinone-n (MK-n)†	Vitamin K_2
Menadione	Vitamin K_3,

	menaquinone, menaphthone
Vitamin B_1	
Thiamin	Aneurin(e), thiamine
Vitamin B_2	
Riboflavin	Vitamin G, riboflavine, lactoflavin
Niacin	
Nicotinamide	Vitamin PP
Nicotinic acid	
Folic Acid	
Pteroyl(mono)glutamic acid	Folacin, vitamin B_c or M
Vitamin B_6	
Pyridoxine	Pyridoxol
Pyridoxal	
Pyridoxamine	
Vitamin B_{12}	
Cyanocobalamin	
Hydroxocobalamin	Vitamin B_{12a} or B_{12b}
Aquocobalamin	
Methylcobalamin	
Adenosylcobalamin	
Inositol	
Myo-inositol	*Meso*-inositol
Choline	
Pantothenic acid	
Biotin	Vitamin H
Vitamin C	
Ascorbic acid	
Dehydroascorbic acid	

*Including some names that are still in use elsewhere, but are not used by the *British Journal of Nutrition*.

†Details of the nomenclature for these and other naturally-occurring quinones should follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature (see *European Journal of Biochemistry* (1975) **53**, 15–18)

Generic descriptors. The terms **vitamin A**, **vitamin C** and **vitamin D** may still be used where appropriate, for example in phrases such as 'vitamin A deficiency', 'vitamin D activity'.

Vitamin E. The term **vitamin E** should be used as the descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol. The term **tocopherols** should be used as the generic descriptor for all methyl tocols. Thus, the term **tocopherol** is not synonymous with the term **vitamin E**.

Vitamin K. The term **vitamin K** should be used as the generic descriptor for 2-methyl-1,4-naphthoquinone (menaphthone) and all derivatives exhibiting qualitatively the biological activity of phylloquinone (phytylmenaquinone).

Niacin. The term **niacin** should be used as the generic descriptor for pyridine 3-carboxylic acid and derivatives exhibiting qualitatively the biological activity of nicotinamide.

Vitamin B_6. The term **vitamin B_6** should be used as the generic descriptor for all 2-methylpyridine derivatives exhibiting qualitatively the biological activity of pyridoxine.

Folate. Due to the wide range of C-substituted, unsubstituted, oxidized, reduced and mono- or polyglutamyl side-chain derivatives of pteroylmonoglutamic acid that exist in nature, it is not possible to provide a complete list. Authors are encouraged to use either the generic name or the correct scientific name(s) of the derivative(s), as appropriate for each circumstance.

Vitamin B_{12}. The term **vitamin B_{12}** should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. The term **corrinoids** should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term **corrinoid** is not synonymous with the term **vitamin B_{12}**.

Vitamin C. The terms **ascorbic acid** and **dehydroascorbic acid** will normally be taken as referring to the naturally-occurring L-forms. If the subject matter includes other optical isomers, authors are encouraged to include the L- or D- prefixes, as appropriate. The same is true for all those vitamins which can exist in both natural and alternative isomeric forms.

Amounts of vitamins and summation. Weight units are acceptable for the amounts of vitamins in foods and diets. For concentrations in biological tissues, SI units should be used; however, the authors may, if they wish, also include other units, such as weights or international units, in parentheses.

See *Metric Units, Conversion Factors and Nomenclature in Nutritional and Food Sciences* (1972) paras 8 and 14–20. London: The Royal Society.

Nomenclature of fatty acids and lipids. In the description of results obtained for the analysis of fatty acids by conventional GLC, the shorthand designation proposed by Farquhar JW, Insull W, Rosen P, Stoffel W & Ahrens EH (*Nutrition Reviews* (1959), **17**, Suppl.) for individual fatty acids should be used in the text, tables and figures. Thus, 18 : 1 should be used to represent a fatty acid with eighteen carbon atoms and one double bond; if the position and configuration of the double bond is unknown. The shorthand designation should also be used in the abstract. If the positions and configurations of the double bonds are known, and these are

important to the discussion, then a fatty acid such as linoleic acid may be referred to as *cis*-9,*cis*-12-18 : 2 (positions of double bonds related to the carboxyl carbon atom 1). However, to illustrate the metabolic relationship between different unsaturated fatty acid families, it is sometimes more helpful to number the double bonds in relation to the terminal methyl carbon atom, *n*. The preferred nomenclature is then: 18 : 3*n*-3 and 18 : 3*n*-6 for α-linolenic and γ-linolenic acids respectively; 18 : 2*n*-6 and 20 : 4*n*-6 for linoleic and arachidonic acids respectively and 18 : 1*n*-9 for oleic acid. Positional isomers such as α- and γ-linolenic acid should always be clearly distinguished. It is assumed that the double bonds are methylene-interrupted and are of the *cis*-configuration (see Holman RT in *Progress in the Chemistry of Fats and Other Lipids* (1966) vol. 9, part 1, p. 3. Oxford: Pergamon Press). Groups of fatty acids that have a common chain length but vary in their double bond content or double bond position should be referred to, for example, as C_{20} fatty acids or C_{20} PUFA. The modern nomenclature for glycerol esters should be used, i.e. triacylglycerol, diacylglycerol, monoacylglycerol *not* triglyceride, diglyceride, monoglyceride. The form of fatty acids used in diets should be clearly stated, i.e. whether ethyl esters, natural or refined fats or oils. The composition of the fatty acids in the dietary fat and tissue fats should be stated clearly, expressed as mol/100 mol or g/100 g total fatty acids.

Nomenclature of micro-organisms. The correct name of the organism, conforming with international rules of nomenclature, should be used: if desired, synonyms may be added in parentheses when the name is first mentioned. Names of bacteria should conform to the current Bacteriological Code and the opinions issued by the International Committee on Systematic Bacteriology. Names of algae and fungi must conform to the current International Code of Botanical Nomenclature. Names of protozoa should conform to the current International Code of Zoological Nomenclature.

Nomenclature of plants. For plant species where a common name is used that may not be universally intelligible, the Latin name in italics should follow the first mention of the common name. The cultivar should be given where appropriate.

Other nomenclature, symbols and abbreviations. Authors should consult recent issues of the *British Journal of Nutrition* for guidance. The IUPAC rules on chemical nomenclature should be followed, and the recommendations of the Nomenclature Committee of IUBMB and the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature and Nomenclature Commission of IUBMB in *Biochemical Nomenclature and Related Documents* (1992), 2nd ed., London: Portland Press (http://www.chem.qmul.ac.uk/iupac/bibliog/white.html). The symbols and abbreviations, other than units, are essentially those listed in *British Standard* 5775 (1979–1982), *Specifications for Quantities, Units and Symbols*, parts 0–13. Day should be abbreviated to d, for example 7 d, except for 'each day', '7th day' and 'day 1'.

Elements and simple chemicals (e.g. Fe and CO_2) can be referred to by their chemical symbol (with the exception of arsenic and iodine, which should be written in full) or formula from the first mention in the text; the title, text and table headings, and figure legends can be taken as exceptions,. Well-known abbreviations for chemical substances may be used without explanation, thus: RNA for ribonucleic acid and DNA for deoxyribonucleic acid. Other substances that are mentioned frequently (five or more times) may also be abbreviated, the abbreviation being placed in parentheses at the first mention, thus: lipoprotein lipase (LPL), after that, LPL, and an alphabetical list of abbreviations used should be included. Only accepted abbreviations may be used in the title and text headings. If an author's initials are mentioned in the text, they should be distinguished from other abbreviations by the use of stops, e.g. 'one of us (P. J. H.)…'. For UK counties the official names given in the *Concise Oxford Dictionary* (1995) should be used and for states of the USA two-letter abbreviations should be used, e.g. MA (not Mass.) and IL (not Ill.). Terms such as 'bioavailability' or 'available' may be used providing that the use of the term is adequately defined.

Spectrophotometric terms and symbols are those proposed in *IUPAC Manual of Symbols and Terminology for Physicochemical Quantities and Units* (1979) London: Butterworths. The attention of authors is particularly drawn to the following symbols: m (milli, 10^3), μ (micro, 10^6), n (nano, 10^9) and p (pico, 10^{12}). Note also that ml (millilitre) should be used instead of cc, μm (micrometre) instead of μ (micron) and μg (microgram) instead of γ.

Numbers. Numerals should be used with units, for example, 10 g, 7 d, 4 years (except when beginning a sentence, thus: 'Four years ago...'); otherwise, words (except when 100 or more), thus: one man, ten ewes, ninety-nine flasks, three times (but with decimal, 2·5 times), 100 patients, 120 cows, 136 samples.

Abbreviations. The following abbreviations are accepted without definition by the *British Journal of Nutrition:*

ADP (GDP)	adenosine (guanosine) 5'-disphosphate
AIDS	acquired immune deficiency syndrome
AMP (GMP)	adenosine (guanosine) 5'-monophosphate
ANCOVA	analysis of covariance
ANOVA	analysis of variance
apo	apolipoprotein
ATP (GTP)	adenosine (guanosine) 5'-triphosphate
AUC	area under the curve
BMI	body mass index
BMR	basal metabolic rate
bp	base pair
BSE	bovine spongiform encephalopathy
CHD	coronary heart disease
CI	confidence interval
CJD	Creutzfeldt-Jacob disease
CoA and acyl-CoA	co-enzyme A and its acyl derivatives
CV	coefficient of variation

CVD	cardiovascular disease
Df	degrees of freedom
DHA	docosahexaenoic acid
DM	dry matter
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
Expt	experiment (for specified experiment, e.g. Expt 1)
FAD	flavin-adenine dinucleotide
FAO	Food and Agriculture Organization (except when used as an author)
FFQ	food-frequency questionnaire
FMN	flavin mononucleotide
GC	gas chromatography
GLC	gas–liquid chromatography
GLUT	glucose transporter
GM	genetically modified
Hb	haemoglobin
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
Ig	immunoglobulin
IHD	ischaemic heart disease
IL	interleukin
IR	infra red
kb	kilobases
K_m	Michaelis constant
LDL	low-density lipoprotein
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MS	mass spectrometry
MUFA	monounsaturated fatty acids
NAD+, NADH	oxidized and reduced nicotinamide-adenine dinucleotide
NADP+, NADPH	oxidized and reduced nicotinamide-adenine dinucleotide phosphate
NEFA	non-esterified fatty acids
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NS	not significant
NSP	non-starch polysaccharide
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
RDA	recommended dietary allowance
RER	respiratory exchange ratio
RIA	radioimmunoassay
RMR	resting metabolic rate
RNA, mRNA etc.	ribonucleic acid, messenger RNA etc.
rpm	revolutions per minute
RT	reverse transcriptase
SCFA	short-chain fatty acids
SDS	sodium dodecyl sulphate
SED	standard error of the difference between means
SFA	saturated fatty acids
SNP	single nucleotide polymorphism
TAG	triacylglycerol
TCA	trichloroacetic acid
TLC	thin-layer chromatography
TNF	tumour necrosis factor
UN	United Nations (except when used as an author)
UNICEF	United Nations International Children's Emergency Fund

UV	ultra violet
VLDL	very-low-density lipoprotein
V_{O2}	O_2 consumption
V_{O2max}	maximum O_2 consumption
WHO	World Health Organization (except when used as an author)

Use of three-letter versions of amino acids in tables: Leu, His, etc.
CTP, UTP, GTP, ITP, as we already use ATP, AMP etc.

Disallowed words and phrases. The following are disallowed by the *British Journal of Nutrition*:
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