Kelli Nogueira Ferraz Pereira Althoff

Efeitos precoces da restrição protéica neonatal sobre a morfofisiologia da mastigação em ratos

Recife/2012

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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.

Orientador: Prof. Dr. Raul Manhães de Castro

Co-orientador: Profa. Dra. Ana Elisa Toscano

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Tese aprovada em 19 de dezembro de 2012.

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Prof Dr Rubem Carlos Araújo Guedes

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Prof Dr Cristiano Mendes da Silva

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Profa Dra Ana Elisa Toscano Meneses da Silva Castro

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Profa Dra Wylla Tatiana Ferreira e Silva

_____

Profa Dra Raquel da Silva Aragão

Recife

2012

Ficha catalográfica elaborada pela
Bibliotecária: Mônica Uchôa, CRB4-1010

A467e Althoff, Kelli Nogueira Ferraz Pereira.
Efeitos precoces da restrição protéica neonatal sobre a morfofisiologia da mastigação em ratos / Kelli Nogueira Ferraz Pereira Althoff.– Recife: O autor, 2012.
106 f.: il.; tab.; quadr.; 30 cm.

Orientador: Raul Manhães de Castro.
Tese (Doutorado) – Universidade Federal de Pernambuco, CCS. Programa de Pós-Graduação em Nutrição, 2012.
Inclui referências, apêndices e anexos.

1. Desnutrição. 2. Núcleos do trigêmeo. 3. Masseter. 4. Mastigação. I. Castro, Raul Manhães de (Orientador). II. Título.

612.3 CDD (23.ed.) UFPE (CCS2013-137)

Dedico esta tese a todos da minha família.

Sem ela não conseguiria chegar a nenhum lugar.

AGRADECIMENTOS

*À **Tiago**, que foi essencial nesta fase da minha vida, sempre me dando força e apoio, suportando alguns momentos dificéis.*

*À minha mãe **Zeza**, uma mulher forte e de muita garra, que, apesar de todos os problemas e dificuldades, sempre está sorrindo, é uma lição de vida para mim. E, ao meu pai **Tadeu**, que, me apoiou e ajudou de todas as formas possíveis.*

*À minha irmã **Keilla** que sempre esteve ao meu lado, me ajudando no que fosse possível, minha melhor amiga.*

*Ao **prof. Raul**, que acreditou em mim desde o início e que me fez crescer muito, não só como pesquisadora, mas como pessoa. Com as nossas conversas, sempre procurei absover todos os seus conselhos e ensinamentos.*

*À **Ana Elisa**, que desde o começo me ajudou muito, me estimulando para que eu realizasse os experimentos da melhor forma possivel.*

*À **Raquel**, amiga eterna. Sei que sempre posso contar com ela.*

*Aos estagiários, **Diego, Camila** e **Tomas**.*

*Ao antigo **grupo de pesquisa NNI**. É muito bom fazer parte de um grupo tão forte e dedicado, que procura crescer e trabalhar junto.*

“Há tempo de nascer, e tempo de morrer; tempo de plantar, e tempo de arrancar o que se plantou;
Tempo de matar, e tempo de curar; tempo de derrubar, e tempo de edificar;
Tempo de chorar, e tempo de rir; tempo de prantear, e tempo de dançar;
Tempo de espalhar pedras, e tempo de ajuntar pedras; tempo de abraçar, e tempo de afastar-se de abraçar;
Tempo de buscar, e tempo de perder; tempo de guardar, e tempo de lançar fora;
Tempo de rasgar, e tempo de coser; tempo de estar calado, e tempo de falar;
Tempo de amar, e tempo de odiar; tempo de guerra, e tempo de paz.
Que proveito tem o trabalhador naquilo em que trabalha?
Tenho visto o trabalho que Deus deu aos filhos dos homens, para com ele os exercitar.
Tudo fez formoso em seu tempo; também pôs o mundo no coração do homem, sem que este
possa descobrir a obra que Deus fez desde o princípio até ao fim.
Já tenho entendido que não há coisa melhor para eles do que alegrar-se e fazer bem na sua
vida;
E também que todo o homem coma e beba, e goze do bem de todo o seu trabalho; isto é um dom de Deus.
Eu sei que tudo quanto Deus faz durará eternamente; nada se lhe deve acrescentar, e nada se lhe deve tirar; e isto faz Deus para que haja temor diante dele.
O que é, já foi; e o que há de ser, também já foi; e Deus pede conta do que passou.
Vi mais debaixo do sol que no lugar do juízo havia impiedade, e no lugar da justiça havia iniqüidade.
Eu disse no meu coração: Deus julgará o justo e o ímpio; porque há um tempo para todo o propósito e para toda a obra.
Disse eu no meu coração, quanto a condição dos filhos dos homens, que Deus os provaria, para que assim pudessem ver que são em si mesmos como os animais.
Porque o que sucede aos filhos dos homens, isso mesmo também sucede aos animais, e lhes sucede a mesma coisa; como morre um, assim morre o outro; e todos têm o mesmo fôlego, e a vantagem dos homens sobre os animais não é nenhuma, porque todos são vaidade.
Todos vão para um lugar; todos foram feitos do pó, e todos voltarão ao pó.
Quem sabe que o fôlego do homem vai para cima, e que o fôlego dos animais vai para baixo da terra?
Assim que tenho visto que não há coisa melhor do que alegrar-se o homem nas suas obras, porque essa é a sua porção; pois quem o fará voltar para ver o que será depois dele?”

Eclesiaste 3

RESUMO

Analisar os efeitos precoces da desnutrição protéica neonatal sobre a morfofisiologia da mastigação em ratos. Ratos machos da linhagem Wistar foram divididos em grupos experimentais conforme a manipulação nutricional imposta às mães durante o período de lactação. O grupo nutrido, consistiu de oito filhotes machos cujas mães foram alimentadas com caseína 17%, ao passo que o grupo desnutrido foi composto por mães que foram alimentadas com dieta caseína 8%. O peso corporal dos animais foi mensurado durante o período de lactação, para estabelecer o ganho de peso corporal. Dos 14 aos 21 dias de idade, foi realizada a avaliação das propriedades de membrana intrínseca de neurônios localizados na parte dorsal do núcleo sensorial principal do trigêmeo (NVsnpr) por meio de patch-clamp. Aos 17, 19 e 21 dias de idade, os animais foram filmados para posterior análise dos parâmetros da mastigação. E, aos 25 dias de idade, o feixe superficial do músculo masseter foi dissecado para avaliação da composição dos tipos de fibras musculares, por meio da técnica de ATPase miofibrilar, bem como da área e perímetro das fibras musculares. A restrição de proteína precoce foi associada com a redução no peso corporal. Os animais desnutridos apresentaram uma menor capacidade de gerar disparos ritmicos e uma redução na frequência de disparos. Contudo, demonstraram um potencial de membrana para geração de disparos mais despolarizado. Ademais, os filhotes desnutridos apresentaram um menor número de sequências e ciclos mastigatórios. Os ratos desnutridos apresentaram uma maior proporção de fibras do tipo IIa e uma menor quantidade de fibras do tipo IIb que os animais controle; e, uma menor área e perímetro tanto das fibras do tipo IIa quanto do tipo IIb quando comparado ao grupo nutrido. A desnutricão protéica neonatal atrasa o desenvolvimento morfológico e funcional da mastigação. Contudo, parece que estes animais são capazes de se adaptarem, de forma que conseguem manter os movimentos mandibulares durante a sequência mastigatória.

Palavras-chaves: Desnutrição. Núcleo sensorial principal do trigêmeo. Masseter. Mastigação.

ABSTRACT

To analyze the early effects of neonatal protein malnutrition on mastication morphology and physiology in rats. Rats were divided into experimental groups according to nutritional manipulation imposed on mothers during the lactation period. The nourished group consisted of eight male pups whose mothers were fed casein 17%, while the malnourished group was composed of mothers that were fed casein diet with 8%. Body weight of animals was measured at 3, 8, 14, 15, 16, 17, 18, 19, 20 and 21 days of age during the lactation period, to establish the gain in body weight. From 14 to 21 days old, was evaluated from intrinsic membrane properties of neurons located in the dorsal part of the trigeminal main sensory nucleus (NVsnpr) by patch-clamp. At 17, 19 and 21 days of age, the animals were videotaped for later review the parameters of mastication. And, at 25 days of age, the superficial masseter muscle was dissected to assess the composition of muscle fiber types, using the technique of myofibrillar ATPase as well as the area and perimeter of the muscle fibers. Early protein restriction was associated with a reduction in body weight. The malnourished animals showed a reduced ability to generate rhythmic bursts and a reduction in the frequency of burst. However, the membrane showed a potential for generating of burst more depolarized. Moreover, malnourished pups had a lower number of sequences and chewing cycles. The undernourished rats showed a greater proportion of type IIa fibers and a smaller amount of fiber type IIb than the control animals, and a smaller area and perimeter of both type IIa fibers as type IIb compared to the nourished group. The neonatal protein malnutrition delays the morphological and functional development of chewing. However, it seems that these animals are able to adapt, so they can keep the jaw movements during the masticatory sequence.

Keywords: Malnutrition. Trigeminal main sensory nucleus. Masseter. Mastication.

SUMÁRIO

1. Introdução

A maturação das estruturas chaves para a mastigação ocorre concomitantemente com os estágios críticos do desenvolvimento do sistema nervoso central. Em humanos, estes períodos correspondem ao terceiro trimestre de gestação e se estendem até o final dos primeiros 2 a 4 anos de vida pós-natal. Ao passo que, nos ratos, se estende desde a gestação até o final das três primeiras semanas de vida pós-natal. Assim, alterações ambientais incidentes nos estágios críticos do desenvolvimento têm efeitos permanentes na estrutura e função dos órgãos. Estudos epidemiológicos e em animais têm demonstrado consequências deletérias na vida adulta como resultado de agressões ambientais durante os períodos fetal, neonatal ou da infância. Portanto, uma nutrição adequada durante os períodos de gestação e lactação é essencial para o crescimento e desenvolvimento normal dos diferentes órgãos e sistemas do corpo. Dessa forma, a desnutrição precoce pode induzir consequências permanentes ou duradouras nas diferentes estruturas e funções do organismo. Estudos relatam que a desnutrição protéica resulta em retardo do crescimento dos filhotes e no desenvolvimento craniofacial. A desnutrição pós-natal em ratos pode influenciar o crescimento cerebral, o comportamento alimentar, a ontogênese dos reflexos, as propriedades mecânicas do músculo esquelético, e a atividade locomotora.

O estado nutricional também está associado com vários problemas de saúde oral. Estudos experimentais sugerem que a desnutrição pós-natal parece resultar em atraso na maturação craniofacial, causando atrofia muscular, mudanças no tamanho da mandíbula e suas propriedades biomecânicas. Tambem são observadas mudanças nos dentes, incluindo atraso na erupção, no tamanho e na morfologia dentária, e maior susceptibilidade à cárie.

Portanto, podemos observar que há grandes evidências de que a desnutrição pré e/ou pós-natal pode levar a danos nas estruturas que atuam nos processos de mastigação. Com isso,

sugere-se que as alterações morfológicas relatadas levam, possivelmente, ao comprometimento funcional, podendo, dessa forma, acarretar mudanças no padrão de ingestão alimentar, com déficit no consumo de nutrientes, ou, até mesmo, aumento da probabilidade de desenvolver doenças digestivas e reduzir a absorção intestinal. Tais mudanças podem levar à seleção inadequada dos alimentos.

Diante desses eixos que fundamentam o interesse científico, o objetivo geral da presente tese foi analisar os efeitos precoces da desnutrição neonatal sobre a morfofisiologia da mastigação em ratos. À princípio, a pergunta de condução da pesquisa foi: Pode filhotes de mães submetidas à dieta hipoprotéica durante o período de lactação apresentarem alterações morfológicas e fisiológicas relacionadas à mastigação? Nossos resultados, como poderá ser verificado nesse trabalho, nos deram respostas e nos trouxeram outras questões. Esse conjunto de evidências e hipóteses provenientes do labor experimental permitiu a elaboração de três artigos científicos, um de revisão da literatura e dois originais. O primeiro, intitulado "Effect of early malnutrition on masticatory function: review of the literature" foi submetido à revista *Archives of Oral Biology* (ANEXO A). Ao passo que, os artigos originais intitulados, respectivamente, "Neonatal low-protein diet reduces the rhythm of chewing in rats?"; e, "A low-protein diet during lactation changes the phenotype of the fibers and alters the morphology of the masseter muscle in rats", foram submetidos à revista indexada *Nutrition Research* (ANEXO B e C).

Para testar as hipóteses que deram origem ao primeiro artigo original, a doutoranda realizou um estágio de doutorado na Université de Montréal/Canadá, por um período de 1 ano, sob a orientação da Profa Dra Arlette Kolta. Na mencionada instituição, a pesquisadora aplicou uma técnica refinada de eletrofisiologia, conhecida como patch-clamp, com o objetivo de avaliar os efeitos precoces da desnutriçao neonatal sobre o ritmo da mastigação. Em

seguida, a fim de complementar os resultados observados no Canadá, uma outra análise mais simples foi realizada para verificar os efeitos da desnutrição neonatal sobre as fases constituintes da sequência mastigatória. Além disso, foi realizada uma outra avaliação, que deu origem ao segundo artigo original. Esta última consistiu da aplicação da técnica de ATPase miofibrilar. O método empregado permitiu a análise dos tipos de fibras e da morfologia do feixe superficial do músculo masseter, que possui ação decisiva na redução das partículas de alimento durante a ingestão alimentar.

- **Objetivo Geral**

Analisar os efeitos precoces da restrição neonatal sobre a morfofisiologia da mastigacão em ratos.

- **Objetivos Específicos**

- Analisar os efeitos da desnutrição neonatal sobre as propriedades intrínsecas e padrões de disparo de neurônios da parte dorsal do núcleo sensorial principal do trigêmeo;

- Avaliar os efeitos da restrição nutricional, durante o período de lactação, sobre parâmetros da função mastigatória;

- Investigar os efeitos da restrição nutricional precoce sobre a distribuição dos tipos de fibras e da morfologia do feixe superficial do músculo masseter.

- **Hipóteses**

Filhotes de mães submetidas à restrição protéica durante o período de lactação apresentam um ritmo de mastigação mais lenta, necessitando de mais ciclos mastigatórios e um tempo maior para triturar o alimento durante a ingestão alimentar.

Filhotes de mães submetidas à desnutrição protéica durante o período de lactação modificam seu fenótipo de fibras e altera a morfologia do músculo masseter.

2. Revisão da literatura

A revisão da literatura está apresentada na forma de um artigo de revisão da literatura (Apêndice A).

3.Métodos

3.1. Animais e manipulação nutricional

Ratos machos Wistar (Rattus norvegicus) foram obtidos do Departamento de Nutrição, Universidade Federal de Pernambuco, Brasil, e da Université de Montréal, Canadá. Os ratos foram mantidos à temperatura ambiente de 23±1°C e em um ciclo claro-escuro (6:00-18:00 hs). Ratas fêmeas virgens foram acasaladas com machos (2x1). O dia em que os espermatozóides estavam presentes no esfregaço vaginal foi designado como o dia da concepção. No primeiro dia depois do nascimento (24 hs após o parto), as ninhadas foram ajustadas para oito filhotes, e durante o período de lactação, suas mães foram alimentadas com uma dieta de caseína 8% ou caseína 17%. Do 1° dia de nascimento até o dia do desmame (por volta de 22 dias de idade), conforme a dieta oferecida às mães, os filhotes foram divididos em dois grupos experimentais, nutrido (N, n=40), cujas mães foram alimentadas com uma dieta normoprotéica, caseína 17% (AIN-93G: REEVES, NIELSEN, FAHEY, 1993); e, desnutrido (D, n=40), cujas mães foram alimentadas com uma dieta hipoprotéica, caseína 8% (Tabela 1). Após o desmame, os filhotes foram colocados em gaiolas coletivas e receberam a comida padrão do biotério (Labina) ad libitum.

Tabela 1: Composição das dietas (caseína 17% e caseína 8%).

Ingredientes	Quantidade para 1 Kg de Caseína	
	8%	17%
Caseína	79,3g	179,3g
Mix Vitamínico	10g	10g
Mix Mineral	35 g	35 g
Celulose	50 g	50 g
Bitartarato de colina	2,5 g	2,5 g
α-metionina	3,0 g	3,0 g
Óleo	70 mL	70 mL
Amido	750,2 g	650,2 g

3.2. Peso corporal dos filhotes

O peso corporal dos filhotes foi aferido diariamente durante os experimentos com uma balança de precisão (Marte Scale, AS-1000). A taxa de crescimento foi calculada pelo número de gramas de peso corporal por dia (BAYOL et al., 2004).

3.3. Análise eletrofisiológica

A análise eletrofisiológica dos neurônios do núcleo sensorial principal do trigêmo foi realizado no departamento de fisiologia e estomatologia da Université de Montréal/Canadá. As propriedades intrínsecas de membrana dos neurônios (N=48; D=42) localizados na parte dorsal do núcleo sensorial principal do trigêmeo (NVsnpr) foram avaliadas usando o registro com a técnica de patch-clamp de células-inteiras em preparações do tronco encefálico *in vitro* de ratos com 14 a 21 dias de idade. O disparo ritmico foi induzido pela aplicação local de N-metil-D-aspartato (NMDA) à concentração de 1mM. Ratos foram decaptados e o cérebro foi cuidadosamente dissecado e colocado em fluido cerebroespinal artificial baseado em sacarose resfriado a 4°C (composição do ACSF em mM: 225 sacarose; 3 KCl; 1,25 KH_2PO_4; 4 $MgSO_4$; 0,2 $CaCl_2$; 20 $NaHCO_3$; e,

10 D-glicose) e oxigenado com O2 à 95%-CO_2 à 5%, pH 7,4. No mesmo meio, cortes transversais (320µm de espessura) do NVsnpr foram preparadas usando um vibratomo (VT1000 S, Leica). Os cortes foram incubados à temperatura ambiente (21-24°C) numa câmara de conservação preenchida com ACSF normal (em mM: 125 NaCl; 3 KCl; 1,25 KH_2PO_4; 1,3 $MgSO_4$; 2,4 $CaCl_2$; 26 $NaHCO_3$; e 25 D-glicose). Os cortes foram transferidos para uma câmara de imersão do corte e perfundido em ACSF normal a uma taxa de ~ 2 ml/min. Os cortes foram mantidos em repouso por um período ≥ 1 h antes do experimento começar.

Os neurônios foram visualizados usando um microscópio (Eclipse E600FN, Nikon) acoplado com lentes de imersão em água com aumento de 40X. A imagem foi melhorada com uma câmera CCD sensível à infravermelho e apresentada em um monitor de vídeo. Registros "patch-clamp célula completa" no modo corrente-clamp foram realizadas em células visivelmente identificadas localizadas na parte dorsal do NVsnpr usando um amplificador Axoclamp 2B (Axon Instruments, Foster City, CA). Os eletrodos patch (6-9 MΩ) foram puxados de capilares de vidro borosilicados (1,5 mm OD, 1,12 mm ID; World Precision Instruments, Sarasota, FL) em um puxador Sutter P-97 (Sutter Instruments, Novato, CA) e preenchidos com uma solução baseada em K-gluconato (em mM: 140 K-gluconato; 5 NaCl; 2 $MgCl_2$; 10 HEPES; 0,5 EGTA; 2 ATP; e 0,4 GTP).

3.4. Aquisição dos dados

Os dados eletrofisiológicos foram adquiridos por uma interface Digidata 1322A e analisada com o software Clampex 9 (Axon Instruments). As propriedades passivas de

membrana das células foram mensuradas pela injeção de pulsos pequenos de corrente hiperpolarizante para evitar a ativação de correntes sensíveis à voltagem. Os disparos (bursts) foram definidos como uma série de 3 potenciais de ação ou mais potenciais platô. Eles foram considerados como ritmicos quando recorreram regularmente e foram separados por períodos silenciosos sem disparo.

3.5. Análise dos parâmetros comportamentais da mastigação

Animais (N=32; D=28) com 17,19 e 21 dias de idade pós-natal foram submetidos a análise dos parâmetros da mastigação. Após um período de 3 horas de privação alimentar (das 14:00 às 17:00 hs), os animais foram colocados em uma gaiola individual transparente e filmados por 10 minutos. Por meio da observação de um período de 1 minuto dos movimentos mastigatórios registrados, foi realizada a avaliação dos seguintes parâmetros: número de sequências mastigatórias (quantidade de movimentos mastigatórios realizados desde a incisão até a deglutição do alimento); duração da sequência mastigatória (duração dos movimentos mastigatórios realizados desde a incisão até a deglutição do alimento); número de ciclos mastigatórios (quantidade de movimentos mastigatórios, abertura e fechamento da mandibula, para uma deglutição); duração do ciclo mastigatório (tempo de realização dos movimentos mastigatórios, abertura e fechamento da mandíbula, para uma deglutição); número de incisões (quantidade de movimentos de incisão do alimento); e, duração das incisões (duração dos movimentos de incisão do alimento) (Adaptado de MOSTAFEEZUR et al., 2012).

3.6. Preparação do músculo

Aos 25 dias de idade, os ratos foram decaptados. Após uma incisão longitudinal no pescoço, o músculo masseter foi dissecado. Para análise histológica, o músculo foi removido, imerso em n-hexano à baixa temperatura e armazenado à -80°C até ser processado.

3.7. Análise histoquímica

Secções tranversais (10µm) foram cortadas com um criostato mantido a -20°C e coradas por ATPase miofibrilar (BROOKE, KAISER, 1970). Os cortes foram mantidos à temperatura ambiente. Em seguida, foram incubados por 30 s à temperatura ambiente em 20 mM de glicina tamponada (pH 9,4), contendo 20 mM CaCl2. Depois, foram incubados por 30 mim (KCl), à 23-25°C, em uma solução de 20 mM de $CaCl_{2;}$ e, 2,5 mM de sal de ATP disódio em 40 mM de glicina tamponada (pH 9,4). Em seguida, os cortes foram lavados em água destilada (3x30s); revelados em sulfeto de amônio a 1,5% por 3 min; lavados em água destilada; desidratados em uma bateria crescente de álcools e montados em entellan.

Os cortes foram analisados com um microscópio (Olympus Optical U-CMAD-2, Tokyo, Japan; objetiva das lentes de 109) conectado a um computador (TV TUNER APPLICATION— TelSignal Company Limited, Taiwan, software de captura de imagem). As imagens das secções histológicas do masseter superficial foram capturadas para posterior análise. As fibras musculares foram coradas com relação aos três tipos principais de fibras (I, IIa, IIb), com base nas diferenças da intensidade da coloração por ATPase após a pré-incubação ácida (pH 4,4 e 4,7) (Cobos et al., 2001). Conforme as

diferentes intensidades de coloração, a seguinte classificação foi usada para o masseter: pH 4,4 (tipo I, mais escura e tipo II, mais clara) e pH 4,7 (tipo I, mais escura; tipo IIa, mais clara; e, tipo IIb, cinza).

A análise histoquímica foi realizada usando imagem computadorizada do software Mesurim PRO 3.2 (desenvolvido por Jean-François Madre-Amiens, França). A composição do tipo de fibra muscular foi determinada pela contagem de aproximadamente 600 fibras em 10 campos que foram igualmente distribuídos sobre a amostra de 4,7 (tipo I, mais escura; tipo IIa, mais clara; e, tipo IIb, cinza). Para avaliar a área e o perímetro das células musculares, campos microscópios de cada secção foram analisadas sob microscópio óptico (Leica, objetiva de 40x). Imagens de cinquenta células musculares de cada animal (N=4; D=5) foram feitas para cada preparação para posterior análise no software Scion Image Beta 4.0.2.

3.8. Análise estatística

Os dados estão apresentados como média±DP. Um ANOVA two-way foi realizada para o peso corporal, propriedades intrínsecas de membrana de neurônios localizados na parte dorsal do NVsnpr, e parâmetros da mastigação. Os dados foram analisados por ANOVA two-way, com os fatores dieta materna (N, D) e idade. O teste post hoc Bonferroni foi usado. Quanto à avaliação dos tipos de fibra e morfologia, comparações entre os grupos nutrido e desnutrido foram realizadas usando o teste t-student. A significância estatística considerada foi $p<0,05$. A análise dos dados foi realizada usando o programa estatístico Sigma Stat 3.5.

3.9. Considerações éticas

O protocolo experimental usado seguiu as normas das diretrizes dos Institutos Canadenses de Pesquisa em Saúde e foi aprovado pelo Comitê de uso e cuidado animal da Université de Montréal/Canadá (número do protocolo: 10-165); e, pelo Comitê de ética em experimentação animal da Universidade Federal de Pernambuco, conforme as normas do COBEA (número do protocolo: 23076.001483/2010-61).

4.Resultados

Os resultados da presente tese estão apresentados no formato de dois artigos originais apresentados no Apêndice B e no Apêndice C.

4.1.Artigo original 1

Primeiro artigo original, intitulado “Does neonatal low-protein diet reduces the rhythm of chewing in rats?” (Apêndice B).

4.2. Artigo original 2

Segundo artigo original, intitulado “A low-protein diet during lactation changes the phenotype of the fibers and alters the morphology of the masseter muscle in rats” (Apêndice C).

5. Discussão:

O principal objetivo da tese foi avaliar os efeitos da desnutrição protéica neonatal sobre a morfofisiologia da mastigação em ratos lactentes. Em particular, observou-se o impacto da desnutrição sobre os parâmetros intrínsecos e de disparo de neurônios localizados na parte dorsal do núcleo sensorial principal do trigêmeo, responsável pelo controle do ritmo da mastigação; sobre os movimentos mandibulares realizados durante a sequência mastigatória; bem como, sobre o fenótipo das fibras e a morfologia do musculo masseter. Com isso, tais avaliações nos possibilitaram investigar as consequências precoces da restrição protéica neonatal nos parâmetros neuromusculares e comportamentais, que sao fundamentais para o controle dos movimentos mandibulares necessários para a degradação do alimento durante a ingestão alimentar.

No presente estudo, observamos que a desnutricão durante o período crítico do desenvolvimento reduziu o peso corporal. Nossos resultados estão em conformidade com outros estudos os quais demonstraram que a desnutrição protéica afeta a ingestão alimentar e o ganho de peso (POWER, SCHULKIN, 2008; SAKATA et al., 2003). A deficiência de proteína pode causar alterações na qualidade do leite materno (ZAFRA et al., 2006; MOYERS, CARLSON, 1993), o que induz danos no crescimento corporal em várias espécies de mamíferos.

Quanto aos parâmetros neuromusculares e comportamentais, o presente estudo demonstrou que filhotes de mães que foram submetidas à desnutrição protéica durante a lactação apresentam retardo no desenvolvimento morfológico e fisiológico da mastigação. Em relação ao impacto da desnutrição protéica neonatal sobre o ritmo da mastigação, não foi verificada alteração nas propriedades intrínsecas de membrana de

neurônios localizados na região dorsal do núcleo sensorial principal do trigêmeo. Contudo, o estudo mostrou que algumas propriedades de disparo do neurônios NVsnpr, em particular, o número de células que disparam e a frequência de disparo, se encontraram reduzidas. Tais alterações indicam que os animais que sofreram desnutrição durante a lactação apresentaram um ritmo de mastigação mais lentificado. Ademais, os animais desnutridos demonstraram um potencial de membrana para geração de disparo mais despolarizado. Contudo, apresentaram menor número de sequências e ciclos mastigatórios. Mais ainda, no que concerne o impacto da desnutrição protéica neonatal sobre a musculatura mastigatória, verificamos um aumentou na proporção de fibras do tipo IIa e uma redução na distribuicão de fibras do tipo IIb, bem como uma diminuição da área e do perímetro do músculo masseter, sugerindo que a restrição de proteína demonstrou um atraso no desenvolvimento muscular, sobretudo, na maturação fenotípica e morfológica do feixe superficial do músculo masseter.

Em relação ao impacto da restrição protéica sobre as propriedades intrínsecas da membrana, estes dados estão em conformidade com o estudo realizado por Rushmore et al., (1998), que examinaram os efeitos da desnutrição pré-natal sobre as características de membrana intrínseca e potencial de ação nas células do grânulo dentado e nas células piramidais CA1. Os autores observaram que as propriedades de membrana, as características de disparo do potencial de ação e as respostas sinápticas dos campos de ratos sumetidos à desnutrição protéica durante a gestação estavam inalteradas comparadas com os animais controle (RUSHMORE et al., 1998). Ademais, os achados do presente estudo também podem ser justificados com base no estudo de Navarrete et al., 2011. Neste estudo, os autores discorreram sobre o possível efeito poupador dos

insultos incidentes durante o período crítico do desenvolvimento do sistema nervoso sobre o tecido cerebral (NAVARRETE et al., 2011). Em suma, para poupar o cérebro de possíveis alterações que possam comprometer a sobrevivência, outros tecidos do corpo, a exemplo do tecido adiposo e muscular, sofrem alteraçães mais significativas (NAVARRETE et al., 2011).

Todavia, os achados supracitados são contrastantes com os estudos que relatam alterações na maturação morfológica, bioquímica e funcional do sistema nervoso central como resultado da desnutrição protéica (ROTTA et al., 2002). Estudos em modelos animais relatam que a restrição de proteína precoce pode levar a modificações nas etapas ontogenéticas do desenvolvimento cerebral, como a proliferação, migração e mielinização (GALLER et al., 1997). Ademais, diversos parâmetros da plasticidade sináptica, a exemplo da fosforilação das proteínas de membrana sináptica e conexões neuronais, assim como as atividades dos sistemas de neurotransmissores catecolaminérgicos, colinérgicos, serotoninérgicos, GABAérgicos, e opióides podem sofrer a influência da desnutrição durante o período crítico do desenvolvimento cerebral (GRESSENS et al., 1997).

Ademais, observamos que algumas propriedades de disparo de neurônios localizados na parte dorsal do núcleo sensorial principal do trigêmeo, em especial, o número de células que dispara e a frequência de disparo, encontraram-se reduzidas em filhotes de mães que sofreram desnutrição protéica durante o período de aleitamento. Tais alterações são indicativas de que animais que sofreram desnutrição protéica durante o período de aleitamento apresentam um ritmo de mastigação mais lento. Estudos relatam

que alterações significantes na morfologia dos neurônios podem resultar em mudanças nas propriedades de membrana intrínsecas, como a resistência de entrada e a constante de tempo da membrana, assim como nos parâmetros caracteristicos do potencial de ação, que são pelo menos em parte dependentes da estrutura das células (RUSHMORE et al., 1998). Dessa forma, uma redução no tamanho do soma resultaria em um aumento na resistência de entrada e um decréscimo na constante de tempo da membrana de uma determinada célula (RUSHMORE et al., 1998). Ademais, se esta redução for suficientemente significante, modificações na forma da onda dos potenciais de ação pode ocorrer, devido a mudanças no número de canais iônicos presentes na membrana (RUSHMORE et al., 1998). Além do mais, podemos justificar a redução das supracitadas propriedades de disparo de burst pela interferência da desnutrição nos níveis e funções dos receptores de neurotransmissores (RUSHMORE et al., 1998). No nosso estudo, os neurônios da região dorsal do NVsnpr foram estimulados por meio da aplicação de NMDA. Então, provavelmente, a desnutrição protéica neonatal pode ter gerado alterações na atividade do sistema glutamatérgico. Estudos demonstram mudanças no sistema de receptor glutamatérgico em resposta à desnutrição pré-natal (RUSHMORE et al., 1998). Trabalhos que utilizaram ligante do receptor de NMDA relataram uma redução na ligação deste em todos os subcampos do hipocampo em animais submetidos à desnutrição protéica (RUSHMORE et al., 1998). Assim, a alteração da proteína pode afetar severamente a transmissão do aminoácido excitatório (MELDRUM, 2000).

Um outro ponto importante a ser discutido no presente estudo é a maior despolarização das células que disparam em animais desnutridos. Acreditamos que este fato ocorreu como um mecanismo adaptativo dos animais desnutridos em detrimento do

menor número de células que são capazes de gerar disparos ritmicos. Ademais, estes resultados podem ser associados com os dados relacionados aos parâmetros da mastigação. No presente estudo, não observamos diferenças entre os grupos experimentais nos movimentos mandibulares realizados durante a sequência mastigatória. Não há estudos na literatura que descrevam os efeitos deste insulto nas sequências de movimentos mastigatórios. Contudo, podemos sugerir que o potencial de membrana mais depolarizado em animais desnutridos pode auxiliar na manutenção dos movimentos mandibulares durante a sequência mastigatória. Assim, estes animais são capazes de adaptar o menor número de células que disparam, tornando-a mais despolarizada. Dessa forma, elas serão capazes de gerarem potenciais de ação, estimulando, por conseguinte, os músculos mastigatórios a realizarem os movimentos mandibulares durante a ingestão alimentar.

Ademais, também podemos sugerir que estes resultados estejam relacionados com o aspecto hedônico do comportamento alimentar. Como os animais foram submetidos a um período de privação alimentar, os animais estavam com fome e isto pode ter levado à manutenção dos movimentos da mandíbula durante as sequências mastigatórias. Tal associação foi relatada no estudo realizado por Yamamoto et al., (1982). Os autores verificaram a relação entre a atividade EMG dos músculos mastigatórios com o aspecto hedônico (YAMAMOTO et al., 1982).

Quanto ao efeito da desnutrição precoce na musculatura mastigatória, observamos que a manipulação nutricional aumentou a proporção de fibras do tipo IIa e reduziu a distribuição das fibras do tipo IIb, bem como diminuiu a área e o perímetro das fibras do

feixe superficial do musculo masseter. Estes dados sugerem que os animais desnutridos demonstraram um atraso no desenvolvimento muscular, sobretudo na maturação fenotípica e morfológica do músculo masseter, o que pode comprometer sua função. Estes dados estão de acordo com os estudos que demonstraram que a desnutrição perinatal pode induzir atrofia muscular e modificar irreversivelmente a morfologia do músculo (PARK et al., 2003; BAYOL et al., 2004). Em adição, estudos demonstram que a restrição protéica materna pode reduzir a proliferação celular, que compromete o número de fibras musculares (TOSCANO et al., 2008). Ademais, os dados supacitados estão de acordo com um modelo de desnutrição, que encontrou aumento na proporção das fibras do tipo I e uma redução na proporção das fibras do tipo II em filhotes jovens, devido à diminuição no número de fibras rápidas formadas (WHITE et al., 2000).

Portanto, podemos sugerir que a menor formação das fibras do tipo IIb levou, provavelmente, a maior distribuição das fibras do tipo IIa, o que demonstra um atraso na maturação do músculo masseter. Esta conclusão foi baseada no processo de desenvolvimento do mencionado músculo. Estudos em ratos reportam que mecanismos importantes para a maturação da musculatura mastigatória, em especial, do músculo masseter ocorre durante o período embrionário (YAMANE et al., 2000a,b; SAITO et al., 2002a,b). Contudo, o fenótipo das fibras musculares só são estabelecidas após a mudança do padrão motor de ingestão alimentar de sucção para mastigação (YAMANE, 2005). Em roedores, por volta das duas semanas de idade, há uma predominância de fibras do tipo IIa (SHIDAA et al., 2005). No entanto, após as 2 semanas de idade, observa-se um aumento na proporção das fibras do tipo IIb (SHIDAA et al., 2005). Assim, com a

maturação funcional, ocorre um aumento na distribução das fibras do tipo IIb e uma redução nas fibras IIa (SHIDAA et al., 2005).

Além do mais, sob um ponto de vista funcional, estudos têm reportado que a quantidade de força que um músculo pode produzir depende não somente do tipo de isoforma de miosina, mas também de sua área (MAUGHAN et al., 1983). Logo, a área aumenta com a quantidade de resistência durante a contração (MCCALL et al., 2004). Portanto, a determinação da composição do tipo de fibra e a área pode ser usada para caracterizar as propriedades funcionais e as necessidades de um músculo (SANO et al., 2007). Diante do exposto, sugerimos que os animais desnutridos exibiram um decréscimo da força de contração, uma vez que ambos os tipos de fibras mostraram menor área.

Em resumo, podemos constatar que os animais desnutridos apresentam uma menor capacidade de gerar disparos ritmicos, bem como uma redução na quantidade de fibras do tipo IIb e na área e perímetro das fibras musculares. Tais resultados são indicativos de que os animais desnutridos apresentam uma mastigação mais lentificada e com menor força de contração muscular. Contudo, como a função mastigatória está relacionada com a sobrevivência, haja vista que consiste no primeiro comportamento motor realizado durante a ingestão alimentar, acreditamos que, para compensar estas alterações, as células que são capazes de gerar disparos se tornam mais despolarizadas e ocorre o aumento da distruição das fibras do tipo IIa, que são mais resistentes à fadiga e mais facilmente recrutadas. Dessa forma, os animais desnutridos conseguem manter os movimentos mandibulares imprescindíveis para a redução das partículas de alimento durante o comportamento alimentar.

6. Considerações finais e Perspectivas

O presente estudo possibilitou testar a hipótese de que filhotes de mães submetidas à restrição de proteína durante o período de lactação apresentam atraso morfológico e funcional da mastigação. Todavia, os parâmetros comportamentais são capazes de se adaptarem às alterações funcionais e estruturais observadas, mantendo os movimentos mandibulares durante a sequência mastigatória. Dessa forma, mais estudos são necessários a fim de verificar os mecanismos subjacentes ao controle neuromuscular e comportamental da mastigação. Ademais, como os achados foram avaliados em animais jovens, seria interessante também investigar os efeitos à longo prazo do mencionado insulto e sua possível relação com a obesidade.

Diante do exposto, tem-se como perspectivas investigar o efeito à longo prazo da desnutrição protéica neonatal sobre a morfofisiologia da mastigação; avaliar o efeito da desnutrição protéica pré e/ou pós-natal na expressão de neurotransmissores, em particular, da serotonina e do glutamato nos núcleos trigeminais; analisar o efeito da desnutrição precoce sobre as propriedades contratéis e elásticas dos músculos mastigatórios; investigar o impacto da desnutrição pré e/ou pós-natal sobre a resposta à mastigação de hormônios de ação periférica responsáveis pelo controle do apetite; realizar o estudo da preferência da consistência alimentar após período de restrição nutricional; e, por fim, investigar o efeito à curto e longo prazo do sobrepeso na morfofisiologia da mastigação.

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APÊNDICE A

Artigo de revisão da literatura

Title: Effect of early undernutrition on masticatory morphophysiology: review of the literature

Running title: Perinatal undernutrition and mastication.

Ferraz-Pereira, KN; Toscano, AE; Manhães-de-Castro, R.

Kelli Nogueira Ferraz Pereira. Ph.D., Nutrition, Federal University of Pernambuco. Mailing address: Federal University of Pernambuco. Department of Nutrition. Av. Moraes Rego, 1235. Cidade Universitária. CEP: 50670-901. Recife/Brazil. (kelliferraz@hotmail.com).

Ana Elisa Toscano. Ph.D. Ph.D., Nutrition, Federal University of Pernambuco. Mailing address: Federal University of Pernambuco. Department of Nutrition. Av. Moraes Rego, 1235. Cidade Universitária. CEP: 50670-901. Recife/Brazil. (aetoscano@hotmail.com)

Raul Manhães de Castro. Ph.D., Life Sciences, Université de Paris VI (Pierre et Marie Curie). Associate Professor of Nutrition Department, Federal University of Pernambuco. Mailing address: Federal University of Pernambuco. Department of Nutrition. Av. Moraes Rego, 1235. Cidade Universitária. CEP: 50670-901. Recife/Brazil. (manhaesdecastroraul@gmail.com)

Correspondence: Raul Manhães de Castro. Federal University of Pernambuco. Department of Nutrition. Av. Moraes Rego, 1235. Cidade Universitária. CEP: 50670-901. Recife/Brazil. (manhaesdecastroraul@gmail.com).

ABSTRACT:

Introduction: Certain periods of development of the nervous system are critically vulnerable to environmental insults because of the processes involved that cycle very quickly. Morphologic and functional development of mastication occurs coincidently during these stages. Early environmental insults during critical periods can cause permanent effects on both structures and functions of organic systems that can have lasting repercussions in adulthood. **Objective:** In this study, we investigated, through a literature review, the possible effects of perinatal calorie and/or protein low diet on structural and physiological development of mastication. **Design:** A systematic literature search was conducted from in the PUBMED electronic database. In collecting literature we used the keywords: "undernutrition" and "stomatognathic system". Criteria used in the selection of articles for inclusion were: studies evaluating the effects of perinatal calorie and/or protein low diet on masticatory morphology and function. Exclusion criteria included, short communications and nonavailability in full text format. **Conclusion:** Undernutrition during critical periods of life causes changes in the key structures of masticatory function. This fact can affect the selection of essential nutrients, thereby interfering with the process of satiation.

Keywords: Undernutrition; Mastication; Development; Morphology; Physiology.

INTRODUCTION:

There are critical periods of development of the nervous system that are vulnerable to environmental insults[1]. In mammals, these periods also coincide with the structural and functional development of mastication[2,3]. During these steps, early stimuli or insults may result in persistent changes in the structure and function of the organism [4,5,6]. Neonatal undernutrition can lead to permanent damage to organs and body systems[5,7,8,9]. Furthermore, nutritional restriction in pre and postnatal periods affects neurotransmitter systems, among them the serotonergic system[10]. Moreover, perinatal calorie and/or protein low diet can cause delay in the morphologic and functional development of mastication [11,12,13].

Given the above, nutrition may interfere with the maturation of key structures of masticatory function, as well as the neural components responsible for their motor control. It is likely that environmental insults in the neonatal period may interfere with the acquisition of mature structures essential to the functioning of the masticatory system. The present study investigated, through a review of scientific literature, the possible effects of perinatal calorie and/or protein low diet on structural and physiological development of mastication.

MATERIALS AND METHODS:

A literature review was conducted using the PUBMED electronic database. This search prioritized studies that addressed the possible consequences of calorie and/or protein low diet on structural and functional development of mastication. For the bibliographic search, the authors initially used the intersection of keywords

"undernutrition" and "mastication". However, in order to try to expand the search, we used the keywords "undernutrition" and "stomathognatic system".

Inclusion criteria used in the selection of articles was studies evaluating the effects of perinatal calorie and/or protein low diet on masticatory morphology and function. For selection of articles related to masticatory morphology were chosen who reported the effect of nutritional insult mentioned on structures important for masticatory act, like the teeth, lips, tongue, jaw, masticatory muscles, among other. While the articles related to masticatory function were selected that assessed the impact of undernutrition on the different phases of mastication (incision, crushing and pulverizing food). In particular, behavioral parameters, such as the number and duration of the masticatory sequence and cycle. And as exclusion criteria included: short communications and non-availability in full text format.

This article is divided into two parts. In the first, a discussion was held regarding the mastication morphophysiology. And, in the second part, the authors spoke about the effects of calorie and/or protein undernutrition on the structural parameters of mastication.

MORPHOPHYSIOLOGY OF MASTICATION:

Mastication is one of the essential functions of the oral cavity, given that it breaks the food eaten to promote digestion [1,2]. The sensory stimulation, triggered by contact of food with the oral cavity, can promote the release of appetite hormones, such as insulin [14], ghrelin [15,16,17], pancreatic peptide (PP) [16,17], cholecystokinin (CCK) [18], peptide YY (PYY)

[19], and glucagon-like peptide (GLP-1) [20]. Therefore, the aforementioned motor function appears to stimulate neuroendocrine cascades in order to optimize the efficiency of digestion and metabolism [16,17,21,22]. Mastication also directly and indirectly controls mechanisms of appetite and satiety, and thus, the size and duration of meals [23]. Furthermore, mastication appears to regulate energy balance, which is extremely important in maintaining body weight [24,25].

The maturation of key structures for mastication occurs concurrently with the critical stages of central nervous system development [2,3]. In humans, these critical stages stand out in embryogenesis, in the third trimester of pregnancy, and extend until the end of the first two to four years of postnatal life [1]. In the rat, these stages present in embryogenesis, and particularly after birth through the period of weaning or at about 21 days of postnatal life [1]. Therefore, maturation of mastication is determined by the growth of skeletal muscle, neural development, peripheral afferent input, and motor learning [26].

Early stages of oral motor development are characterized by progressive formation of neuromuscular connections and the central nervous system [27]. During this stage, the patterns of muscle activation have been correlated with the ontogenetic changes in size and geometry of the skull and mandible [28,29]. Simultaneously with morphological development, there are observable changes in motor patterns of food intake [2]. At first, the newborn mammals acquire all the nutrients necessary for survival through suckling behavior [2]. As mature mammals, sucking gives way to mastication behavior [2,30]. In humans, the initial phase of development of mastication occurs between 6 and 9 months postnatal[30]. While in rats, the first masticatory movements appear around the 12th postnatal day, and the adult pattern is reached after 18 to 21 days [2].

The adult pattern of masticatory movements, jaw opening and closing, can only be established with the maturation of the craniofacial complex [28]. The components that form the stomatognathic system include of nerves, vessels and muscles[28]. These are the active elements of the stomatognathic system because they move the jaw in different directions according to their insertion[31]. In mammals, the temporalis, masseter and medial pterygoid act on mandibular closing movements, while the anterior belly of the digastric, lateral pterygoid and mylohyoid perform the function of opening[26] Oral muscles (masticatory and suprahyoid muscles), with weaning, show functional changes during development [32]. A number of studies have demonstrated changes in the properties of the fibers of the masticatory muscles during muscular growth and development [33]. Composition ratio of MyHC-2b (fast contraction and high contractile force) increased during weaning in the masseter muscle [33]. Moreover, studies also show that after weaning (at 4 and 9 weeks of postnatal life), the proportion of MyHC-2b-positive muscle fibers, as well as the expression of MyHC-2b mRNA in the anterior and posterior bundles of the digastric muscle, increases with age, and this increase is a rapid adaptation to changes resulting from mastication [32].Thus, masticatory function requires sufficient muscle activity to perform jaw movements and thereby generate force to reduce the size of the particles of food that will be swallowed[28].

The basic pattern of jaw movements during mastication are controlled by a central pattern generator (CPG)[34] located in the brainstem, between the rostral poles of the trigeminal motor nuclei (NVmot) and facial (NVII) [35,36,37]. This area includes the trigeminal main sensory nucleus (NVsnpr), traditionally known as a relay station to the thalamus and other regions of the somatosensory system [3], and that is thought to be

involved in the control the masticatory rhythm [38], particularly in its dorsal part [39]. The NVsnpr receives stimuli from the cortical masticatory area and from trigeminal sensory afferents, and neurons of its dorsal part project directly to the trigeminal motor nucleus [40,41,42]. Studies report that about one third of the neurons in this region fire rhythmically during fictive mastication [38]. Moreover, there was also a higher expression of c-fos protein in neurons of the dorsal NVsnpr after fictive mastication [43]. Therefore, this modulation influences the characteristics of electromyographic burst of muscles controlling mastication movements and durations of constituent phases of the cycles [44].

Furthermore, the regulation of rhythmic movements of the jaw during mastication involves neurotransmitter systems, particularly serotonin (5-HT) [45]. Experimental studies suggest the involvement of serotonin in controlling feeding behavior [46,47,48,49] and modulation of hunger and satiety [50], depending on the receptor subtype involved [51,52,53]. Serotonin participates in the regulation of mastication [45,54] through interaction between the trigeminal sensory-motor complex [32,52] and the caudal raphe nucleus [45,54]. The 5-HT system in the brain facilitate the motor output and inhibit the sensory information processing [55]. Studies in animal models involve the activity of brain serotonergic neurons with motor activity, especially tonic or repetitive character [56]. In the caudal raphe nucleus, pallidus and obscurus, the activity of serotonergic neurons is associated with mastication and other orofacial behaviors [45,57]. Pallidus and obscurus nuclei send serotonergic projections to the trigeminal motor nuclei [58], which have their function facilitated by serotonergic neurons [45,53]. The 5-HT enhance the excitability and discharge of trigeminal motoneurons during rhythmic jaw movements, as well as increases membrane excitability through effects on ion channels [59,60]. Therefore, the 5-HT

contributes to maintenance of the position of the mandible and facilitation of jaw movements during ingestion of food [45].

Given the above, early environmental insults, as undernutrition, may interfere with the acquisition of mature structures essential to the functioning of the masticatory system. Thus, it is suggested that possible morphological changes can lead to functional impairment causing modifications in the pattern of food intake.

UNDERNUTRITION AND MASTICATORY MORPHOPHYSIOLOGY:

The undernutrition is a multifactorial disease that can have an early onset during intrauterine or childhood life or may occur during an individual's lifetime as a result of poor nutrition and/or repeated episodes of infectious or chronic diseases [61]. High incidence of protein-energy malnutrition are registered in developing countries and is associated with economic restrictions, considering that is an important indicator of the life's quality of the population [62].

In mammals, maternal nutritional status during pregnancy and lactation periods is essential for normal growth and development of different organs and body systems [63,64]. Neonatal maternal protein restriction is associated with a lower store of mother's nutrients and, subsequently, less transfer of nutrients to the offspring, which is related to decreased postnatal growth[65,66]. Thus, the quantity or quality of nutrition during critical periods of development may have permanent consequences for the lives of people affected [67].

Epidemiological and animal studies have shown deleterious consequences in adulthood of environmental insults during the fetal, neonatal or childhood periods [5,6,9,68].

Early undernutrition may induce lasting or permanent consequences in different structures and functions of the body [15]. Studies report that pre and/or postnatal undernutrition in rats can influence brain growth [16], feeding behavior [17], the mechanical properties of skeletal muscle [18], and locomotor activity [18]. Furthermore, the low protein diet can cause alterations in the morphological maturation, biochemical and functional central nervous system [69]. Animal model studies have reported that early protein restriction can lead to changes in ontogenetic stages of brain development, such as cell proliferation, migration and myelination [70]. With relation to the serotonergic system, experimental studies suggest that postnatal undernutrition promotes lasting increases in the concentrations of serotonin in the central nervous system (CNS) [71,72].

Particularly with relation to the effects of early caloric and/or protein undernutrition on different body structures, a variety of studies showed retarded growth and development of the heart [73], larynx [74], and skeletal muscles of locomotion [75]. Moreover, studies showed that nutritional status is also associated with various oral health problems [12,13]. Perinatal protein low diet appears to result in delayed craniofacial maturation, causing muscle atrophy, changes in the size of the jaw and their biomechanical properties [12,13]. Also are observed changes in teeth including delayed tooth eruption, size and morphology, and increased susceptibility to decay [11].

According to the literature review in this study, after the crossing of the keywords above 999 articles were presented. However, after reading the title, 42 works were selected according to the inclusion criteria. Thus, after full reading of the articles, 09 papers were selected. Of these last, all articles (09 papers) studied the impact of energy

and/or protein undernutrition on the masticatory structure. Thus, all selected articles showed the effect of the mentioned insult on the keys structures to masticatory function, like the teeth and jaw. No article investigated the impact of malnutrition on the masticatory sequences and cycles. In addition, 02 studies investigated the effect of early nutritional manipulation in children, and 07, evaluated the impact of that insult, using the rat as experimental model (Table 1).

Studies of children have shown a strong correlation between anthropometric parameters at birth and the time of tooth eruption[76,77]. Delgado et al. (1975)[76] observed that the nutritional status at birth, whether expressed as full-term birth weight or as maternal caloric supplementation during pregnancy, influences the timing of deciduous tooth eruption. However, the authors found that the timing of deciduous tooth eruption seems more closely associated with postnatal weight than with birth weight. Gaur, Kumar (2012)[77] indicate a relationship between stature, weight, and the emergence of deciduous dentition. However, they found that the number of emerged deciduous teeth is better correlated with stature than weight. So, the early nutritional status may be an important factor that can influence the timing of deciduous tooth emergence, extremely important for the maturation of the masticatory function, as well as the phases of incision, crushing and pulverization of the food bolus.

The delay in tooth eruption was also observed in studies using the rat as an experimental model[11,78]. Menaker, Navia (1973)[11] reported delayed tooth eruption in pups whose mothers were submitted to protein malnutrition during gestation and lactation. However, Diorio et al., (1973)[78] indicated a limited growth of incisors and molars,

delayed eruption and increased caries susceptibility resulting from protein-caloric malnutrition during sucking.

The other articles, which used the rat as an experimental model, demonstrated the effect of early nutritional manipulation in the growth and development of the mandible[13,67,79,80,81,82]. Nakamoto, Miller (1977; 1979)[79,80] reported reduction in the deposit of calcium, as well as in the protein and collagen synthesis of the jaw in pups whose mothers were fed with a low protein diet during lactation. However, Nakamoto, Porter, Winkler (1983)[81] presented modifications on growth and metabolism of the jaw as a result of protein-energy malnutrition during gestation. Degani Junior et al. (2011)[67] showed retarded growth of the mandible in adult animals (90 days old) resulting from protein-caloric malnutrition during lactation. While, Alippi et al. (2002)[13] demonstrated reduction in mandibular size in animals whose mothers were fed with a calorie and protein low diet during pregnancy and lactation.

Thus, it is suggested that different structures affected, for example the jaw and teeth can influence the phases of incision, crushing and pulverization of food during a sequence masticatory (Figure 1). Studies report that dysfunction in chewing leads to greater difficulty to degrade the food[45,46]. Therefore, it would take a greater amount of masticatory sequences and cycles, as well as masticatory sequence longer to break foods so that they are safely swallowed.

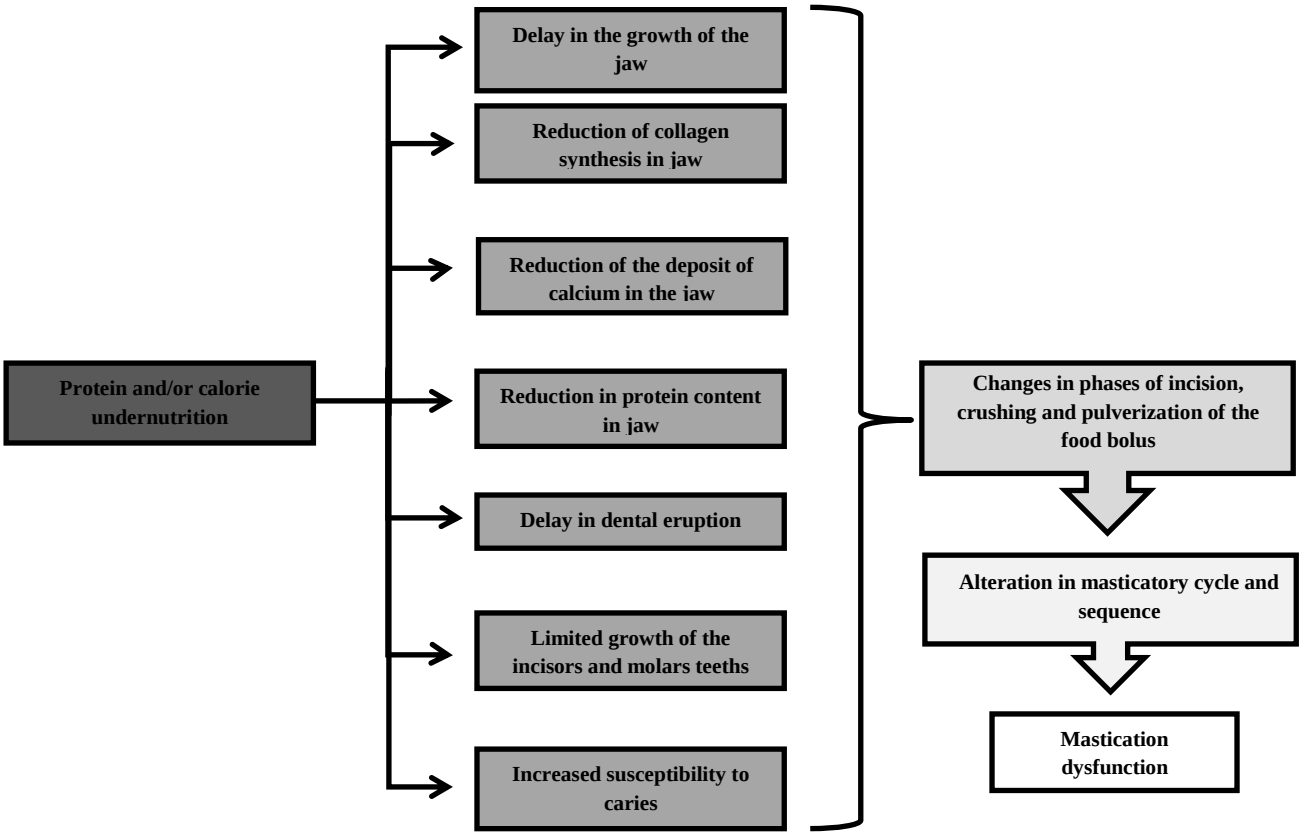

Figure 1 – Effects of early protein-calorie undernutrition on masticatory structures and function.

Therefore, mastication is completely related to nutrition, given that to obtain a proper nutrition, besides eating foods rich in essential nutrients, we need that food can be degraded properly. Thus, studies show that the impairment of mastication seems to lead to changes in the pattern of food intake, which may cause a deficit in nutrient intake, or even increase the likelihood of digestive diseases and reduce intestinal absorption[45]. Dysfunction in mastication can lead to inappropriate selection of food[46,47], ie. there is an increase in consumption of soft and easy to chew food and a decrease in the intake of

hard foods, like vegetables and raw nuts, fibrous foods like meats, and dry foods like breads[48]. Studies in humans with damage mastication report a preference for processed foods over natural foods[48]. This fact may favor the absorption of fat and markedly increased levels of cholesterol and saturated fatty acids[49]. Given the above, there is a greater predisposition to obesity[45].

CONCLUSION:

Neonatal malnutrition seems to schedule changes in the structures and functions belonging to the stomatognathic system, especially related to mastication. These changes caused by nutritional restriction seem to interfere with the process of food intake and in selection of essential nutrients.

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Table 1 - Effects of calorie and/or protein low diet on masticatory structures.

Reference	Objectives	Dietary manipulation	Result
Delgado et al., 1975	To investigate the influence of maternal nutrition status on eruption of deciduous dentition in a population living under conditions of mild-to-moderate protein-calorie malnutrition.	The study sample includes 273 of a total of 470 live births, from the end of 1968 through May, 1972, for whom body weight was measured by standardized methods within 24 hours of birth. Dental eruption status was assessed every 3 months between birth and 24 months of age *(*±5 days), and at 6-month intervals between 24 and 36 months (±7days). Using birth weight and the amount of calories ingested during pregnancy as indicators of the nutritional status of the child and the mother, the following subgroups were established: *a)* birth weight: >3 kg and ı3 kg; and ***b)*** supplementation levels: high caloric supplementation during pregnancy (>20,000 total kcalories during the whole of pregnancy) and low caloric supplementation during pregnancy (<10,000 kcal).	1.Nutritional status at birth, whether expressed as full-term birth weight or as maternal caloric supplementation during pregnancy, influences the timing of deciduous tooth eruption. 2.The timing of deciduous tooth eruption seems more closely associated with postnatal weight than with birth weight.
Gaur, Kumar, 2012	To examine a sample of Rajput children from the state of Himachal Pradesh of India to assess the influence of undernutrition on the emergence of deciduous	The children (age from birth to 48 months) were collected from the villages of Jubbal and Kotkhai Tehsils of the Shimla District of Himachal Pradesh. The dates of birth of the subjects were taken either from the birth certificates or from the parents.	1.The results indicate a relationship between stature, weight, and the emergence of deciduous dentition. 2.The number of emerged deciduous teeth is better correlated with stature than weight. 3.The nutritional status may be an

	dentition.		important factor that can influence the timing of deciduous tooth emergence. 4.Delayed deciduous tooth emergence is more pronounced in stunted children. 5.Measures of linear growth status are, thus, more strongly correlated with dental development than measures of growth in mass/weight. 6.Emergence can be delayed in children with even moderate undernutrition.
Menaker, Navia, 1973	To evaluate the contribution of differential eruption patterns to the subsequent increased caries experience observed previously in malnourished rats.	During gestation and lactation dams were pair-fed an isocaloric, agar-gel diet that contained either 25 or 8% protein.	1.Delayed teeth eruption
Diorio et al., 1973	To study the effects of specific protein malnutrition on the development and growth of teeth, as well as bones, during the suckling period.	Rat dams were fed a low protein (7%) diet so as to induce a protein-calorie deficiency in the suckling pups. Control rats were offered a diet containing 25% casein.	1.Limited the growth of incisors and molars and delayed eruption, but had no effect on tooth mineral composition. 2.Increased caries susceptibility
Nakamoto, Miller, 1979	To investigate what effect protein-energy malnutrition on collagen synthesis in bones and its relation to calcium deposition.	Control dams were fed a 25% protein diet. Isoenergetic 6% protein diets were fed to the malnourished group. On days 1, 5, 10, 15 and 20, mandibles and long bones were examined.	1.Dams fed with a low-protein diet during the lactation period, the hard tissue of their offspring is greatly affected. 2.Collagen synthesis in both mandibles and long bones of malnourished rat pups

			decreased by day 5 compared with controls, suggesting that collagen synthesis was impaired in the malnourished group at an early -age. 3.The calcium complex fraction, collagen synthesis and total bone protein were affected in the malnourished groups 4.The ratio of calcification per unit of matrix was constant in both bones in both the control and malnourished groups, indicating that the organic phase of bone is functionally related to mineralization.
Nakamoto, Miller 1977	To determine what effects protein malnutrition imposes on the growth and development of the mandible and long bone of the newborn rat pup and to determine whether these effects differ between male and female.	Control dams were 25% protein diets; the source of the protein was casein. Diet of malnourished rats were isoenergetic but provided only 6% protein. The difference in energy was made up by an addition of dextrose and dextrin.	1.The protein content of mandibles from the malnourished group was depleted at day 5. 2.The size of cells in mandibles from the malnourished group appeared to be unaffected, whereas the cells in long bones were smaller. 3.At day 20, the weight and calcium content of mandibles from the malnourished group were about half those of controls. 4.Malnutrition had no effect on calcification in the mandible. 5.The ratio of calcium in mandibles from the malnourished group was similar to that found in controls, but in long bones this ratio was smaller. When these

			results are related to cell size, the mandibles from the malnourished group were smaller because they had fewer cells of normal size.
Nakamoto, Porter, Winkler, 1983	To evaluate the role of gestational protein-energy malnutrition on fetal hard-tissue growth and metabolism.	Eighteen pregnant Sprague-Dawley rats were given a standard stock diet until day 13 of gestation. Nine control dams were given 250 g protein/kg diet from the 13th day of gestation until birth. The diet of the nine malnourished rats was isoenergetic, but provided only 60 g protein/kg. Within 8 h of delivery, all pups, both male and female, were combined and weighed, since no weight difference is attributable to sex in early ages. Then, the same number of pups from each dam in respective groups were killed by cutting the carotid artery.	1. The malnourished group had more cells in the mandible although cell size was the same as that of controls. 2. The malnourished group had fewer cells than did controls whereas cell size was unchanged. 3. Calcium content was the same in long bones of both groups, but was less in the mandibles of pups from malnourished dams. 4. Ca metabolism was unchanged in the long bones, but was increased in the mandibles of the malnourished group shortly after birth. 5. Prenatal nutritional stress resulted in a disturbance of the pituitary adrenal system. Increased adrenal corticosterone could possibly be related to the different observed changes in bone metabolism.
Degani Junior et al., 2011	To examine the effect of maternal protein and energy malnutrition during lactation on mandible growth of the female offspring in the adulthood.	Pregnant Wistar rats were separated at delivery into three groups: 1) control group (C) – with free access to a standard laboratory diet containing 23% protein, 68% carbohydrate, 5% lipid, 4% salts and 0.4% vitamins, 17,038.7 total energy (kJ/kg); 2) protein-energy-restricted group (PER) – with free access to an	1.Delay in mandible growth; 2.Abnormalities remain until adulthood (90 days), even after reestablishing normal diet (control) for study groups (ER and PER) at weaning period.

		isoenergetic, protein-restricted diet containing 8% protein, and 3) energy-restricted group (ER) – fed with a standard laboratory diet in restricted quantities that was calculated based on mean ingestion of the PER group.	
Alippi et al., 2002	To investigate, in neonatal rats nursed by dams put on a protein-free diet to depress milk production and thus create a state of protein-energy malnutrition in the offspring, subsequent growth and long-term effects by analyzing mandibular dimensions and bone quality in adulthood.	Pregnant Wistar rats were fed a 20% protein diet (control) or a protein-free diet (malnourished) to obtain normal or subnormal milk production, respectively. After weaning, the offspring (males) were fed a 20% protein diet for 70 days. Control dams were fed a 20% protein diet, with casein the source of protein. The diet of malnourished dams was isoenergetic but provided 0% protein. The difference in energy was made up by the addition of dextrin.	1.Smaller mandibular base length, height and area (an index of mandibular size) in malnourished group

APÊNDICE B

Artigo original 1

TITLE: DOES NEONATAL LOW-PROTEIN DIET REDUCES THE RHYTHM OF CHEWING IN RATS?

Ferraz-Pereira, KN; Aragao, RS; Verdier, D; Toscano, AE; Manhães-de-Castro, R; Kolta, A.

Kelli Nogueira Ferraz-Pereira – Department of Nutrition, Federal University of Pernambuco, Recife, PE, Brasil. kelliferraz@hotmail.com

Raquel da Silva Aragão - Department of Nutrition, Federal University of Pernambuco; Recife, PE, Brasil. raquel.aragao@gmail.com

Dorly Verdier - Research Group on Central Nervous System, and Faculty of Dentistry, University of Montreal, Montreal, Quebec, Canada; dorly.verdier@umontreal.ca .

Ana Elisa Toscano - Department of Nutrition, Federal University of Pernambuco; Recife, PE, Brasil. aetoscano@hotmail.com

Raul Manhães-de-Castro - Department of Nutrition, Federal University of Pernambuco; Recife, PE, Brasil. manhaesdecastroraul@gmail.com

Arlette Kolta - Research Group on Central Nervous System, and Faculty of Dentistry, University of Montreal, Montreal, Quebec, Canada; arlette.kolta@umontreal.ca

Correspondence: Raul Manhães-de-Castro/ Department of Nutrition, Federal University of Pernambuco. Av. Morais Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brasil. Telephone: (081) 3271.8471 Fax: (081) 3271.8473 manhaesdecastroraul@gmail.com

ABSTRACT:

Objectives: To analyze in rats the effects of neonatal malnutrition on the intrinsic membrane properties and the firing patterns of neurons of the dorsal region of the trigeminal main sensory nucleus (NVsnpr), and on parameters of masticatory behavior. **Methods:** Rats were divided into experimental groups according to established dietary manipulations imposed to mothers during the lactation period. The nourished group (N, n=40), consisted of eight litters of newborn males whose mothers were fed with 17% casein (AIN-93G: REEVES, NIELSEN, FAHEY, 1993), was compared to a malnourished group (M, n=40), whose mothers were fed a diet with 8% casein. Body weight of animals was measured at P3, 8, 14, 15, 16, 17, 18, 19, 20 and 21 days of age during lactation, to establish weight gain. The intrinsic membrane properties of rhythmogenic neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr) were assessed using whole cell patch recordings in *in vitro* brainstem slice preparations (N, n=48, M n=42) taken from rats 14 - 21 days old. Rhythmic firing was induced by local application of NMDA (1mM). The analysis of the parameters of mastication was performed at 15, 17, 19 and 21 days postnatal (N, n=32; M, n=28). After a 3 hour period of food deprivation, the animals were placed in individual cages and videotaped for 10 minutes. The following parameters were assessed: number of masticatory sequences, duration of masticatory sequences; number of chewing cycles and duration of masticatory cycles, the number of incisions, and, duration of incisions. All surgical and experimental procedures conformed to guidelines of the Canadian Institutes of Health Research and were approved by the University Animal Care and Use Committee and Committee of Ethics in Animal Experimentation of the Federal University of Pernambuco. **Results:** Early protein restriction during lactation was associated to a reduction in body weight ($F(9.481)=7,053$, $p<0.001$), but did not have a significant effect on a number of basic electrophysiological parameters measured which included the resting membrane potential ($F(1.72)=0.174$, $p=0.678$), input resistance ($F(1.72)=0.0185$, $p=0.892$) and firing threshold ($F(1.72)=0.0153$, $p=0.902$). Local application of NMDA elicited rhythmic bursting in 33/48 neurons and 24/42 neurons of the nourished and malnourished groups respectively. Bursts were defined as series of 3 action potentials or more overriding plateau potentials. They were considered as rhythmic when they recurred regularly and were separated by silent periods without firing. Neonatal malnutrition had no effect on the intraburst firing frequency ($F(1.29)=0.200$, $p=0.658$); nor on the amplitude ($F(1.29)=0.906$, $p=0.349$) or the duration of the plateau ($F(1.29)=0.427$, $p=0.519$). However, it was associated to a reduction of the bursts frequency ($F(1.29)=4.667$, $p=0.039$) and the membrane potential at which bursting was triggered ($F(1.29)=5.151$, $p=0.031$). With regard to masticatory movements, a number of parameters were similar between the two groups. These included the duration of the masticatory sequences ($F1.54=0.0627$, $p=0.803$), cycles ($F1.54=0.0165$, $p=0.898$), and incisions ($F1.54=1.578$, $p=0.215$), as well as the number of incisions ($F1.54=3.929$, $p=0.053$). However, the pups whose mothers were submitted to protein malnutrition had a lower number of masticatory sequences ($F1.54=4,659$, $p=0,035$) and cycles per sequence ($F1.54=4,659$, $p=0,035$). **Conclusions:** Bursting properties are altered in puppies of mothers undergoing protein malnutrition during lactation have a lower capacity to generate rhythmic bursts. However, they are able to adapt this capability

becomes more depolarized, which can assist in the maintenance of mandibular movements during the masticatory sequence.

Keywords: Malnutrition; Trigeminal main sensory nucleus; Mastication; Development.

INTRODUCTION:

Chewing is one of the essential functions of the oral cavity, given that it breaks the food eaten to promote digestion. The maturation of this motor function occurs during the critical period of development of the central nervous system concomitant with the morphological and functional maturation of the craniofacial complex[1,2,3]. During this period, changes in food intake patterns are also observed[1]. Initially, the mammalian newborns acquire all the necessary nutrients for survival through suckling movements[1]. However, in mature mammals, suckling gives rise to mastication[1]. In rats, the first chewing movements are observed around the 12th postnatal day and mature chewing occurs at about 18 - 21 days[1].

The basic pattern of jaw movements during mastication are controlled by a central pattern generator (CPG)[4] located in the brainstem, between the rostral poles of the trigeminal motor (NVmot) and facial (NVII) nuclei[5,6,7]. This area includes the trigeminal main sensory nucleus (NVsnpr), traditionally known as a relay station to the thalamus[8] and other regions of the somatosensory system[9,10,11], and that is thought to be involved in the control of the masticatory rhythm[12], particularly in its dorsal part[13]. The NVsnpr receives stimuli from the cortical masticatory area and from trigeminal sensory afferents, and neurons of its dorsal part project directly to the trigeminal motor nucleus[14,15,16]. Studies report that about one third of the neurons in this region fire rhythmically during

fictive mastication[12]. Moreover, there was also a higher expression of c-fos protein in neurons of the dorsal NVsnpr after fictive mastication[17]. Therefore, this modulation influences the characteristics of electromyographic burst of muscles controlling chewing movements and durations of constituent phases of the cycles[18].

Membrane properties of neurons located in the dorsal part of the NVsnpr undergo changes in the first three weeks of postnatal life, precisely the period in which occurs the transition from sucking to chewing behaviors[1]. During this phase, there is also a change in the neuronal firing patterns[3]. Immature cells tend to adapt quickly, and the ability to fire repeatedly appears during the first two weeks of postnatal life[3]. In summary, the incidence of rhythmic bursts is rare or absent before P12, the age at which the first masticatory movements appear[1], but becomes common with the development[3].

Proper nutrition is essential for normal growth and development of different organs and body systems[19]. Therefore, early malnutrition may induce lasting or permanent consequences in different structures and functions of the body[20,21]. Studies report that pre and/or postnatal malnutrition in rats can influence brain growth[22], feeding behavior[23], the mechanical properties of skeletal muscle[24], and locomotor activity[24]. Moreover, in relation to the masticatory system, experimental studies suggest that perinatal malnutrition appears to result in delayed craniofacial maturation, causing muscle atrophy, changes in the size of the jaw and their biomechanical properties[25,26]. Also observed are changes in teeth including delayed tooth eruption, size and morphology, and increased susceptibility to decay[27].

Thus, the present study aimed to analyze in rats the effects of neonatal malnutrition on the intrinsic membrane properties and the firing patterns of neurons of the dorsal region of the NVsnpr. In addition, to complement the results observed with electrophysiology, another analysis was conducted to verify the effects of malnutrition on neonatal parameters of mastication, i.e. on jaw movements made during the masticatory sequence. The observations suggest that offspring of mothers fed a low protein diet during the period of lactation have a slower masticatory rhythm, requiring more chewing cycles and longer time to degrade food during food intake.

MATERIALS AND METHODS:

Animals and nutritional manipulation

All surgical and experimental procedures conformed to guidelines of the Canadian Institutes of Health Research and were approved by the University Animal Care and Use Committee (Protocol number: 10-165) and the Committee of Ethics in Animal Experimentation of the Federal University of Pernambuco (Protocol number: 23076.001483/2010-61).

Albino rats were mated. Within 24 hours of birth, litters were adjusted to eight male newborns from mothers that were chosen at random. The animals were kept in a controlled environment with a temperature of 23±1°C and a constant cycle of light (6:00 a.m. to 6:00 p.m.) and darkness (6:00 p.m. to 6:00 a.m.).

The rats were divided into two groups according to their mother's diet during lactation: nourished and malnourished. The nourished group (N) consisted of eight litters

of newborn males whose mothers were fed with 17% casein (AIN-93G: REEVES, NIELSEN, FAHEY, 1993). While the malnourished group (M) consisted of eight litters of newborn males whose mothers were fed with 8% casein (Table 1).

Table 1: Diets composition

Ingredients	Amount for 1 Kg of Casein	
	Low-protein (8%)	Control (17%)
Casein	79.3g	179.3g
Vitamin mix[1]	10g	10g
Mineral mixture[2]	35 g	35 g
Cellulose	50 g	50 g
Bitartrate of choline	2.5 g	2.5 g
α-methionine	3.0 g	3.0 g
Soya oil	70 mL	70 mL
Corn starch	750.2 g	650.2 g

[1]The vitamin mixture contained (milligrams per kilogram of diet): retinol 12, cholecalciferol 0.125, thiamine 40, riboflavin 30, pantothenic acid 140, pyridoxine 20, inositol 300, cyanocobalamin 0.1, menadione 80, nicotinic acid 200, choline 2720, folic acid 10, p-aminobenzoic acid 100, biotin 0.6.

[2]The mineral mixture contained (milligrams per kilogram of diet): CaHPO4 17 200, KCl 4000, NaCl 4000, MgO 420, MgSO4 2000, Fe2O2 120, FeSO4.7H2O 200, trace elements 400 (MnSO4.H2O 98, CuSO4.5H2O 20, ZnSO4.7H2O 80, CoSO4.7H2O 0.16, KI 0.32, sufficient starch to bring to 40 g/Kg of diet).

Offspring body weight

The animals were weighed on a digital electronic scale (Marte AS 1000C). Body weight of animals was measured at P3, 8, 14, 15, 16, 17, 18, 19, 20 and 21 days of age during lactation, to establish weight gain.

Electrophysiological recordings

The intrinsic membrane properties of neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr) were assessed using whole cell patch recordings in *in vitro* brainstem slice preparations (N, n=48; M, n=42) from 14- to 21-day old rats. Rhythmic firing was induced by local application of NMDA (1mM). Rats were decapitated and the brain was quickly taken out and placed in cold (4°C) sucrose-based artificial cerebrospinal fluid (ACSF, composition in mM: 225 sucrose, 3 KCl, 1.25 KH_2PO_4, 4 $MgSO_4$, 0.2 $CaCl_2$, 20 $NaHCO_3$, and 10 D-glucose) bubbled with 95% O_2-5% CO_2, pH 7.4. In the same medium, transverse slices (320 μm thick) through the NVsnpr were prepared using a Vibratome (VT1000 S, Leica). Slices were incubated at room temperature (21–24°C) in a holding chamber filled with normal ACSF (in mM: 125 NaCl, 3 KCl, 1.25 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 25 D-glucose). The slices were transferred to an immersion chamber, perfused with normal ACSF at a rate of ~ 2 ml/min and allowed to rest ≥ 1 h before the experiment was started.

Neurons were visualized using a fixed stage microscope (Eclipse E600FN, Nikon) coupled with a 40X water immersion lens. The image was enhanced with an infrared-sensitive CCD camera and displayed on a video monitor. Whole cell patch-clamp recordings in current-clamp mode were performed from visually identified cells located in the dorsal part of the NVsnpr using an axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Patch electrodes (6–9 MΩ) were pulled from borosilicate glass capillaries (1.5 mm OD, 1.12 mm ID; World Precision Instruments, Sarasota, FL) on a Sutter P-97 puller (Sutter Instruments, Novato, CA) and filled with a K-gluconate based

solution (in mM: 140 K-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 2 ATP, and 0.4 GTP).

Data acquisition

Electrophysiological data were acquired through a Digidata 1322A interface and analyzed with Clampex 9 software (Axon Instruments). Passive membranes properties of cells were measured by injecting small hyperpolarizing currents pulses to avoid the activation of voltage-sensitive currents. Bursts were defined as series of 3 action potentials or more overriding plateau potentials. They were considered as rhythmic when they recurred regularly and were separated by silent periods without firing.

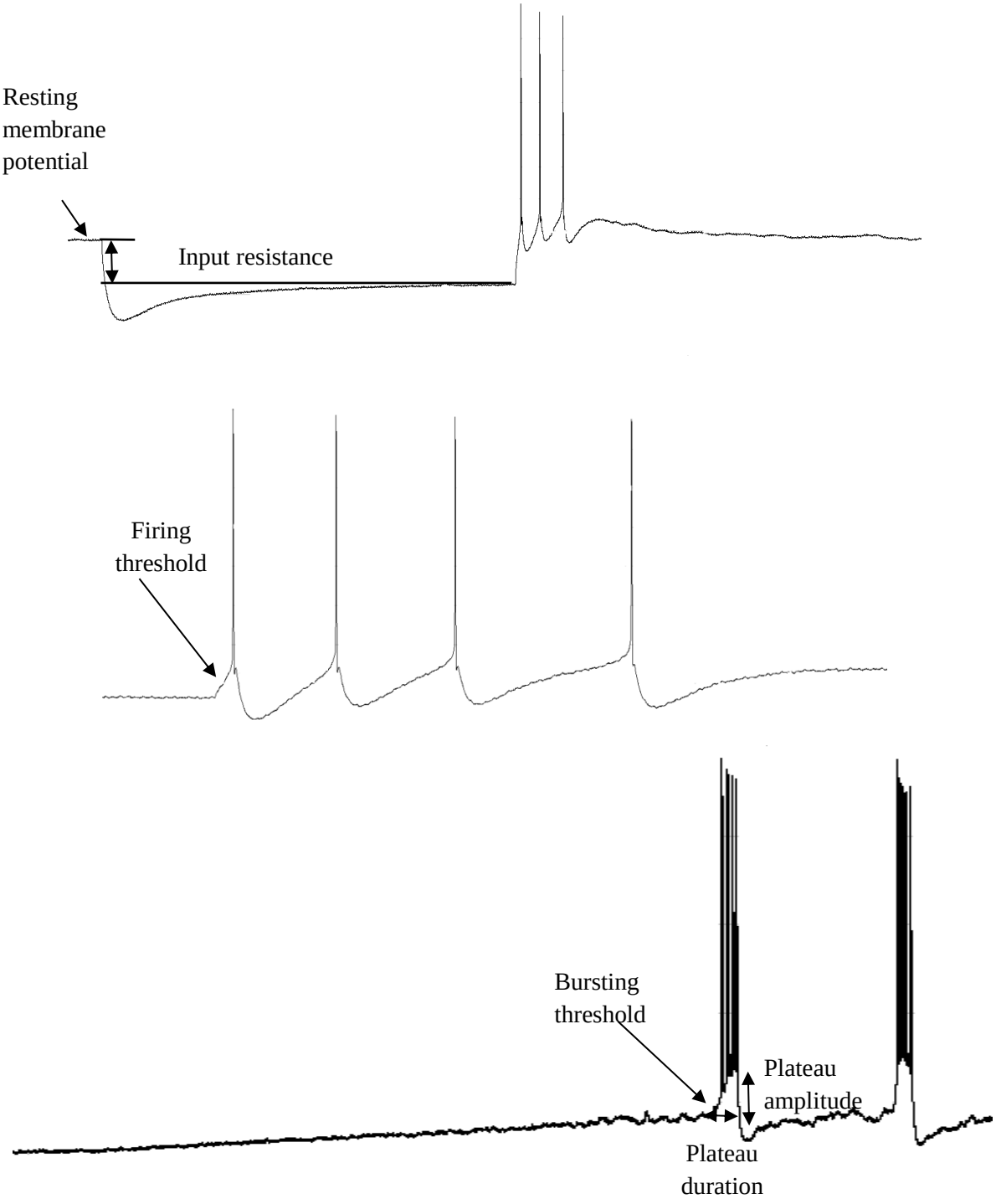

Figure 1. Intrinsic membrane properties of neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr) assessed using whole cell patch recordings in *in vitro* brainstem slice preparations in rats taken from rats 14 - 21 days old.

Analysis of the mastication parameters

Animals (N, n = 32: M, n = 28) aged 15, 17, 19 and 21 days postnatal were subjected to analysis of mastication parameters. After a period of 3 hours of food deprivation (from 14:00 to 17:00), the animals were placed in transparent individual cages and filmed for 10 minutes. The following parameters were assessed in one minute periods of recorded chewing movements: number of masticatory sequences (amount of chewing movements made since the incision until the swallowing of food); duration of masticatory sequences (duration of chewing movements made since the incision until the swallowing of food); number of masticatory cycles (amount of chewing movements, jaw opening and closing, for a swallowing); duration of masticatory cycles (time of realization of chewing movements, jaw opening and closing, for a swallowing); number of incisions (amount of movement of the food incision); and, duration of incisions (duration of the movement of food incision) (Adapted Mostafeezur et al., 2012) [24].

Analysis

Data are presented in the text and in the tables as mean ±SEM. A two-way ANOVA was performed for body weight, intrinsic membrane properties of neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr), and mastication parameters. In addition, a post-hoc Bonferroni t-test was used. Statistical significance was defined as $p<0.05$ in all cases.

RESULTS:

The body weight of both nourished and malnourished animals increased linearly with age but in a biphasic manner with a steeper relationship in the two first postnatal weeks. A clear difference appeared between nourished and malnourished at P14 and was maintained until P21at which point the experiment ended (Fig 2).

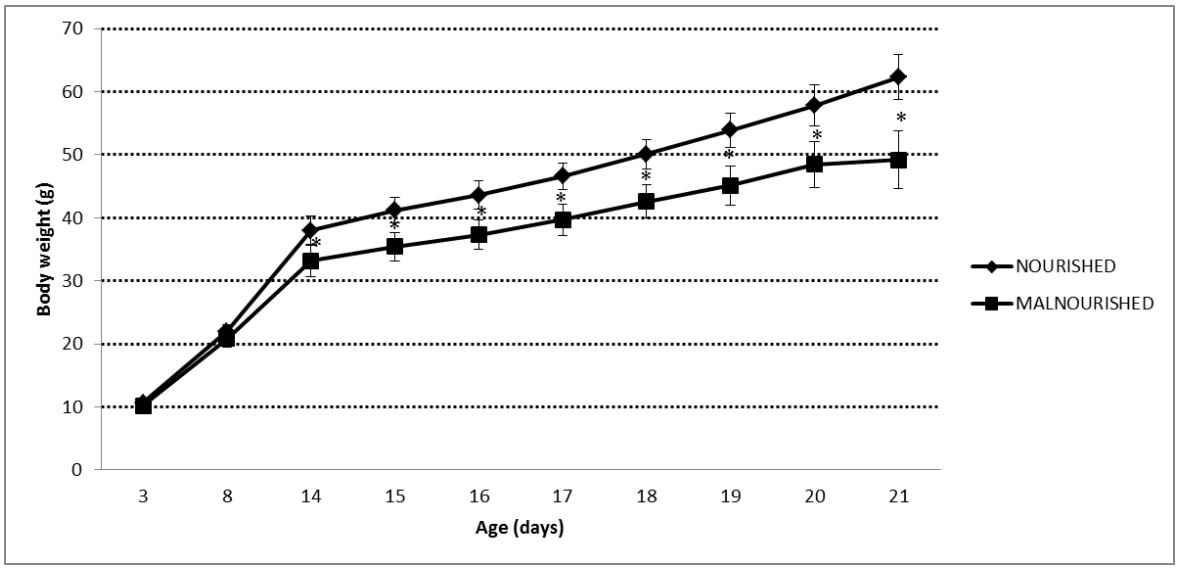

Figure 2. Effect of malnutrition on the body weight curve. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period (two-way ANOVA). Data are represented as mean ± SEM. Multiple comparisons (Bonferroni t-test) - $p<0.05$: * Nourished vs. Malnourished.

Electrophysiological properties of NVsnpr neurons

Forty eight neurons were recorded from 24 normally nourished rats aged between 14 and 21 days. Seventeen of these neurons were spontaneously active. Their membrane potential ranged from -48 to -63 mV, while their input resistance ranged from 52 to 330

MΩ. Their firing threshold, defined as the potential at which appeared the first action potential, varied between -33 and -52 mV.

Local NMDA applications (1mM) elicited bursting in 69% of neurons (n=33/48) (Fig 3B) and a tonic excitation in the remaining 31%. These bursts consisted in plateaus that lasted 0.18 ms overridden by short trains of action potentials that occurred at an average frequency of 60 Hz (±7.78). The plateaus were triggered at potentials more hyperpolarized than firing threshold, around -57mV (+0.76 mV) and reached an amplitude of 23 mV (+1.67 mV). They recurred regularly at an average frequency of 2.7 Hz (+0.41).

These basic electrophysiological characteristics of NVsnpr neurons obtained from nourished and malnourished animals are listed in Table 2. Most values obtained from malnourished animals were similar to those obtained from nourished animals. Significant differences were found only in the proportion of cells in which bursting could be induced with NMDA (57% in malnourished animals vs 69% in nourished animals and in some of the bursting parameters. Neonatal malnutrition had no effect on the intraburst firing frequency ($F(1.29)=0.200$, $p=0.658$); nor on the amplitude ($F(1.29)=0.906$, $p=0.349$) or the duration of the plateaus ($F(1.29)=0.427$, $p=0.519$) (Table 2). However, it was associated to a reduction of the bursts frequency ($F(1.29)=4.667$, $p=0.039$) and the membrane potential at which bursting was triggered ($F(1.29)=5.151$, $p=0.031$) (Table 2; Figure 2).

Table 2. Effect of malnutrition on intrinsic membrane properties of neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr).

	EXPERIMENTAL GROUPS		**ANOVA**	
PARAMETERS	**NOURISHED (N)**	**MALNOURISHED (M)**	**F**	**P**
RESTING MEMBRANE POTENTIAL (mV)	-53.22±0.68 (n=48)	-53.63±0.70 (n=42)	F(1.72)=0.174	= 0.678
INPUT RESISTENCE (MΩ)	143.28±8.73 (n=48)	144.98±8.94 (n=42)	F(1.72)=0.0185	= 0.892
FIRING THRESHOULD (mV)	-44.54±0.76 (n=48)	44.41±0.78 (n=42)	F(1.72)=0.0153	= 0.902
NUMBER BURST	33/48	24/42		
BURST FREQUENCY	2.69±0.41 (n=48)	1.14±0.59 (n=42)	F(1.29)=4.667	= 0.039*
INTRABURST FIRING FREQUENCY	60.20±7.78 (n=48)	54.12±11.13 (n=42)	F(1.29)=0.200	= 0.658
MEMBRANE POTENTIAL BURST (mV)	-56.85±0.76 (n=48)	-53.84±1.08 (n=42)	F(1.29)=5.151	= 0.031*
AMPLITUDE OF THE PLATEAU (mV)	23.03±1.67 (n=48)	20.24±2.40 (n=42)	F(1.29)=0.906	= 0.349
DURATION OF THE PLATEAU (ms)	0.18±0.06 (n=48)	0.25±0.08 (n=42)	F(1.29)=0.427	= 0.519

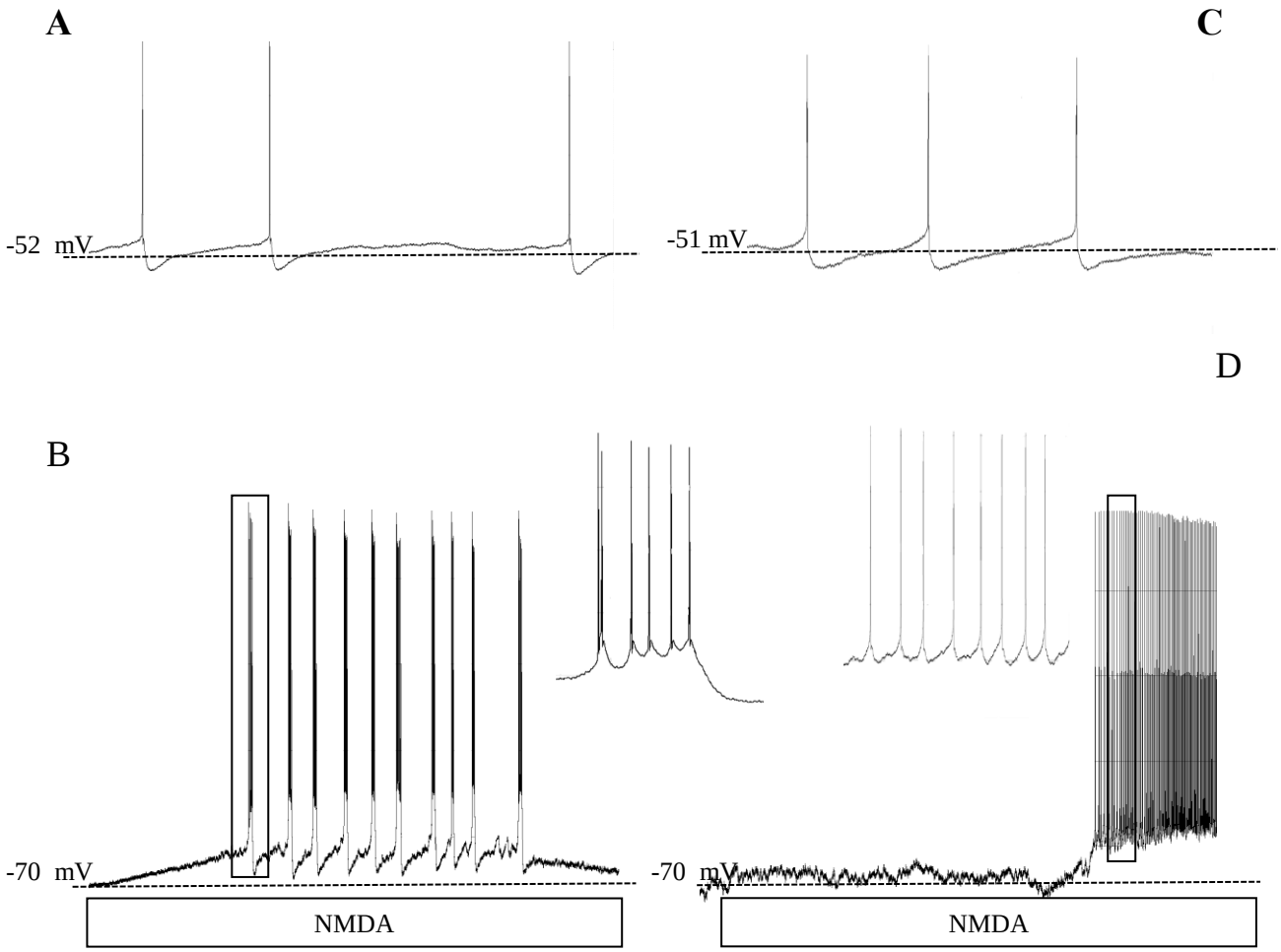

Figure 3. Effect of malnutrition on intrinsic membrane properties of neurons located in the dorsal part of the trigeminal principal sensory nucleus (NVsnpr) assessed using whole cell patch recordings in *in vitro* brainstem slice preparations in rats. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period. A and B – Nourished Group; C and D – Malnourished Group.

Mastication parameters

Generally, the parameters of mastication showed no difference between the two experimental groups (Table 3). The malnourished animals showed similarities between puppies nourished with relation to the duration of masticatory sequence (F1.54=0.0627, p=0.803); to duration of masticatory cycles (F1.54=0.0165, p=0.898); to number of

incisions (F1.54=3.929, p=0.053); and, to duration of incisions (F1.54=1.578, p=0.215) (Table 3). However, the pups whose mothers were submitted to protein malnutrition had lower neonatal number of masticatory sequences (F1.54=4,659, p=0,035) and masticatory cycles (F1.54=4,659, p=0,035) (Table 3).

Table 3. Effect of malnutrition on mastication parameters from rats 17, 19 and 21 days old. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period (two-way ANOVA). Data are represented as mean ± SEM.

	EXPERIMENTAL GROUPS		**ANOVA**	
MASTICATION PARAMETERS	**NOURISHED (N)**	**MALNOURISHED (M)**	**F**	**P**
NUMBER OF MASTICATORY SEQUENCES	9.824±0.408 (n=32)	8.536±0.435 (n=28)	F(1.59)= 4.659	= 0.035*
DURATION OF MASTICATORY SEQUENCES (s)	5.545±0.256 (n=32)	5.639±0.273 (n=28)	F(1.59)=0.0627	= 0,803
NUMBER OF MASTICATORY CICLES	9.824±0.408 (n=32)	8.536±0.435 (n=28)	F(1.59)= 4,659	= 0.035*
DURATION OF MASTICATORY CICLES (s)	1.567±0.178 (n=32)	1.601±0.189 (n=28)	F(1.59)= 0,0165	=0,898
NUMBER OF INCISIONS	10.989±0.415 (n=32)	9.780±0.443 (n=28)	F(1.59)= 3.929	= 0,053
DURATION OF INCISIONS (s)	3.637±0.225 (n=32)	4.050±0.239 (n=28)	F(1.59)= 1.578	= 0.215

Multiple comparisons (Bonferroni test) - p<0.05: * Nourished vs. Malnourished.

DISCUSSION:

The aim of this study was to investigate the effect of protein restriction during lactation on the masticatory pattern and rhythm in rats through electrophysiological recording of rythmogenic neurons located in the dorsal region of the main sensory trigeminal nucleus on one hand and masticatory movements on the other.

In agreement with previous reports, the malnutrition produced during this critical period reduced body weight[29,30]. This effect results presumably from the protein deficiency imposed to the mothers, which has been shown to alter the quality of breast milk[31,32] and affect body growth in several mammal species[33] .

However, protein deficiency during lactation did not seem to have a major effect on the membrane properties of NVsnpr neurons recorded from animals aged of 14 to 21 postnatal days. These data are in accordance with the study of Rushmore et al., (1998)[34], that examined the effects of prenatal malnutrition on the intrinsic membrane properties and action potential characteristics of dentate granule and CA1 pyramidal cells. The authors noted that the neuronal membrane properties and firing characteristics of hippocampal cells of rats submitted to protein malnutrition during gestation were unchanged compared with control animals[34]. This lack of change in membrane properties is surprising in regard to the abundance of studies reporting alterations in central nervous system morphological, biochemical and functional maturation as a result of protein malnutrition (for review[35,36)]. Those numerous reports of alterations lead to believe that, as a result of malnutrion, neurons undergoes important structural changes and, it is known that significant changes in the morphology of neurons can result in changes in

membrane intrinsic properties, such as input resistance and the membrane time constant, as well as the action potential characteristic parameters, which are at least partly dependent on the cells structure. The absence of change in basic membrane properties in our study suggest that the morphology and integrity of NVsnpr neurons are both well preserved in the malnourished rats. In this regard, Gressens et al. (1997)[37] reported that, in spite of the observance of numerous abnormalities in brain development, brain weight and cytoarchitecture in postnatal animals submitted to protein malnutrition during gestation were unchanged compared with control animals. This relative preservation of brain tissue integrity may repose on the existence of a possible sparing effect, brought by evolution[38], of insults incidents on brain tissue during the critical period of development of the nervous system. In short, to save the brain of possible alterations that may compromise survival, other body tissues, such as adipose tissue and muscle, suffer more significant alterations.

Our study has shown that some firing properties of neurons located in the dorsal part of the trigeminal main sensory nucleus, in particular, in the number of cells that burst and burst frequency, are reduced in offspring of mothers who suffered protein malnutrition during period of lactation. Such electrophysiological alterations suggest that animal that suffered protein malnutrition during the period of lactation may show decreased occurence of chewing episodes with normally effective stimuli concomitant with a slowing of chewing. These electrophysiological results do not seem to relate with the data obtained from analysis of mastication parameters. In the present study, we observed only slight differences in mandibular movements during the masticatory sequences between the different experimental groups. Studies report that dysfunction in

chewing leads to greater difficulty to degrade food[39-42]. Therefore, it would take a greater amount of masticatory sequences and cycles, as well as longer masticatory sequence to break foods so that they are safely swallowed. Incidentally, we may state that our behavioral data do not indicate impairment of chewing habilities in the malnourished rats.

Previous works from our lab have shown that intrinsic bursting in NVsnpr neurons relies on a voltage-dependant sodium persistent current (I_{NaP}) whose magnitude is modulated by external calcium concentration[3]. The amplitude and duration of the plateaus generated by I_{NaP} activation are voltage-dependant and inversely related to external Ca^{2+} concentration. The voltage dependency of the plateau (between −60 mV and −50 mV) corresponds to I_{NaP} activation range[3, 43]. In the same study, we also showed that plateaus duration depends upon activation of K^+ current. In the present study, bursting was produced by local applications of NMDA. Recent evidences from our works suggested that NMDA-induced bursting in NVsnpr neurons relies on I_{NaP} since it can be abolished by Riluzole, a blocker of I_{NaP}, and it is produced only when membrane potential was maintained within I_{NaP} activation range[44]. These results suggest that NMDA-induced bursting relies on the same conductances that produce bursting in low calcium conditions. In the present study, amplitude and duration of the NMDA-induced plateaus were not affected by the low protein diet suggesting that malnutrition does not seem to alter the conductances supporting bursting habilities in NVsnpr neurons. Malnutrition may have only caused a slight shift in the activation curve of I_{NaP}, which could account for the more depolarized potentials at which bursting occurs. No evidence of such an effect of malnutrition on sodium channels has never been reported but, a recent study using neonatal maternal deprivation to induce chronic visceral hyperalgesia

showed that this kind of stressful manipulation can effectively modulate voltage-gated sodium channels sensitivity[45]. Another current that has been shown to contribute importantly to pacemaking properties is the hyperpolarization activated current (I_h). NVsnpr neurons do express an I_h current that could contribute to bursting by helping neurons get in the activation range of I_{NaP} in order to generate a subsequent burst. However, Brocard et al. (2006)[3] showed that bath application of ZD 7288, a specific I_h blocker, decreases the plateau duration and amplitude besides changing bursts frequency. In regard of this dual effect of ZD 7288 and considering that burst duration and amplitude were unchanged in our study, we cannot speculate on a clear effect of malnutrition on I_h current in NVsnpr neurons.

Since rhythm generation does not rely exclusively on the membrane properties of the instrinsic bursters but on an interplay between these properties and synaptic connections, we can most likely explain the reduction of the above mentioned properties of burst firing by interference of malnutrition at the levels and functions of neurotransmitter receptors. Indeed, malnutrition has been shown to have an effect on several neurotranmitters systems[46-49] and to alter their receptors number and distribution[50]. Since in our study, bursting in NVsnpr neurons was elicited by NMDA applications, we are interested in changes relative to the glutamatergic system but more specifically in relation to rhythm generation. In a study done by Rotta et al (2003)[47], in which they tested the effect of undernutrition on this neurotransmitter system, they noticed changes in biding and release of glutamate as well as changes in the sensitivity of a sub-type of glutamate receptors, but in spite of these alterations, they reported that behavioral parameters related to locomotion were not affected. On the other hand,

malnutrition has been shown to affect the GABAergic system in several ways (i.e. it causes changes in the number of GABAergic neurons[51], in the composition and sensitivity of GABAergic receptors[52,53] and it affects the frequency of miniature inhibitory postsynaptic currents[54]), and this neurotransmitter system is well known for his modulatory action on burst frequency in locomotion[55,56] . In a study made by Tegnér et al. (1993)[56], it was shown that agonists of GABAA and GABAB receptors applications reduced the frequency of bursting and that GABA uptake blockers (nipecotic acid) and a benzodiazepine receptor agonist induced a pronounced slowing of the (NMDA)-induced fictive locomotion. In regard of all these data, we propose that in our study, malnutrition may have caused similar changes in the GABAergic trigeminal system and it is likely that the decreased frequency and occurrence of bursting observed could be due to a more powerful action of GABA on the NVspr neurons.

In summary, NVsnpr neurons of pups of mothers undergoing neonatal protein malnutrition have a lower capacity to generate rhythmic bursts and show a slowing down of the rhythm. However, these changes are not reflected by the behavioral masticatory parameters, suggesting that at one level or another the trigeminal system is able to adapt is output in order to sustain the maintenance of efficient mandibular movements during masticatory sequences. These findings may result from the fact that chewing is related to survival, given that mastication is the first motor behavior performed during food intake. Thus, to obtain a proper nutrition, besides eating foods rich in essential nutrients, we need food to be degraded properly. Studies show that the impairment of mastication seems to lead to changes in the pattern of food intake, which may cause a deficit in nutrient intake, or even increase the likelihood of digestive diseases and reduce intestinal absorption[39].

Dysfunction in mastication can lead to inappropriate selection of food[40,41], ie. there is an increase in consumption of soft and easy to chew food and a decrease in the intake of hard foods, like vegetables and raw nuts, fibrous foods like meats, and dry foods like breads[42]. Studies in humans with altered mastication report a preference for processed foods over natural foods[42]. This fact may favor the absorption of fat and markedly increased levels of cholesterol and saturated fatty acids. Given the above, there is a greater predisposition to obesity[39]. Therefore, mastication is completely related to nutrition.

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APÊNDICE C

Artigo original 2

TITLE: A low-protein diet during lactation changes the phenotype of the fibers and alters the morphology of the masseter muscle in rats

Kelli Nogueira Ferraz-Pereira[1]; Raquel da Silva Aragao[1]; Carol Góis Leandro[1]; Ana Elisa Toscano[1]; Raul Manhães-de-Castro[1]

[1]Department of Nutrition, Federal University of Pernambuco, Recife, PE, Brasil.

Correspondence: Raul Manhães-de-Castro

Department of Nutrition, Federal University of Pernambuco

Av. Morais Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brasil.

Telephone: (081) 3271.8471 Fax: (081) 3271.8473 manhaesdecastroraul@gmail.com

ABSTRACT:

Objectives: The present study aimed to analyze the effects of neonatal low-protein diet on the proportion, area and perimeter of fiber types of the superficial masseter muscle in rats. **Methods:** Male Wistar rats were divided according to their mother diet during lactation: nourished group (N, n=4 offsprings) whose mothers fed a 17% casein diet, and malnourished group (M, n=5 offsprings), whose mothers fed a 8% casein diet. Body weight and body weight gain were measured during lactation. At weaning (25 d old), the rats were killed and the superficial masseter muscle was dissected. Serial cross sections were stained for myofibrillar ATPase. **Results:** M group had reduced body weight since 14^{th} day. M group showed an increased proportion of fiber type IIa and a reduction of fiber type IIb when compared to N group. Moreover, malnourished animals showed lower area and perimeter in relation to the N group in both fiber type IIa and the type IIb. **Conclusions:** The neonatal malnutrition led to early change in phenotype of the fibers, as well as the morphology of the superficial masseter muscle.

Keywords: Malnutrition; Mastication; Fiber type; Masseter; Development.

1.INTRODUCTION:

The adult pattern of masticatory movements, jaw opening and closing, can only be established with the maturation of the craniofacial complex[1]. The components that form the stomatognathic system include of nerves, vessels and muscles[1]. These are the active elements of the stomatognathic system because they move the jaw in different directions according to their insertion[2]. In mammals, the temporalis, masseter and medial pterygoid act on mandibular closing movements, while the anterior belly of the digastric, lateral pterygoid and myloiody perform the function of opening[3]. Thus, chewing requires sufficient muscle activity to perform jaw movements and thereby generate force to reduce the size of the particles of food that will be swallowed[1]. During mastication, the most important masticatory muscle is masseter[1].

The skeletal muscles possess a variety of contractile muscle fibers with different properties, such as maximal strength, contraction speed and fatigability[4]. According to the contents of myosin heavy chain (MyHC), the skeletal muscle fibers can be classified into several types, such as I, IIa, IIx, and IIb[5]. The muscles containing predominantly type I fibers are easily ordered, contraction of slow and fatigue resistant[6]. While, the muscles fast-contracting, fatigable fibers are categorized as type II fibers[6].

The skeletal muscle undergoes rapid changes in the composition of their contractile systems, regulatory and energetic during growth, particularly before birth and during early postnatal life[7]. However, in relation to masticatory muscles is observed modifications of most importance after birth[8], considering that the first chewing movements in rats are observed around the 12th day of postnatal life and mature chewing

occurs about 18 - 21 days old[9]. Therefore, after the period of the change pattern of food intake suction for chewing, the masticatory muscles are able to modify your muscle phenotype in order to adapt to rapidly changing functional[8].In summary, during the late postnatal development, the masseter acquires increased amount of fiber type fast and also in daily activity[4].

Proper nutrition is essential for normal growth and development of different organs and body systems[10]. Therefore, early malnutrition may induce lasting or permanent consequences in different structures and functions of the body[11,12]. Regarding the muscular system, studies of the muscles of the locomotor system have shown that neonatal malnutrition can irreversibly damage the muscle structure[13]. It can also reduce cell multiplication, thereby reducing the number of muscle fibers and nuclei in the offspring[13,14]. Moreover, changes in the proportions of fiber types after malnutrition have also been observed[15]. Toscano et al. [16] observed an increase in the IIa fibers in the soleus an increase in the IIb fibers and a diminution in the IIa fibers in the EDL in malnourished animals at 25 and 90 d whose mothers fed with 7.8% protein during pregnancy. A model of malnutrition found an increase in the proportion of type I fibers and a reduction in the proportion of type II fibers in young puppies, due to a diminution in the number of fast fibers formed[17]. However, regarding the masticatory muscles there are no reports in the literature.

Thus, the present study aimed to analyze in rats the effects of neonatal protein malnutrition on proportion of fiber types of the superficial masseter muscle, as well as the area and perimeter of each fiber type. Therefore, such evaluations possible to test the

hypothesis that offspring of mothers fed a low protein diet during the period of lactation have a delay in the development of the superficial masseter muscle. This may compromise the process of degradation during the act of chewing food, given that the superficial masseter muscle plays a key role in the strength of chewing strokes.

2.METHODS AND MATERIALS:

Animals and nutritional manipulation

Male Wistar rats (Rattus norvegicus) were obtained from the Department of Nutrition, Federal University of Pernambuco, Brazil. The rats were maintained at a room temperature of 23 ± 1°C and in a light–dark cycle (light6:00 a.m.–6:00 p.m.). Virgin female rats (aged 110 days and weighing 258.8 ± 5.7 g) were mated with male breeders (2 x 1). The day on which spermatozoa were present in a vaginal smear was designated as the day of conception, i.e., day 0 of pregnancy. On the first day after birth (24 h after delivery), litters were standardised to 8 pups, and during the suckling period, their mothers provided with a diet of either 8% casein or 17% casein. After weaning (on the 22nd day of age), only male offspring (one randomly chosen male pups from each mother) were used. Male pups were divided into two groups according to their mother's manipulations (N, n = 4; pups from control mothers; and M, n = 5; pups from mothers fed a low-protein diet) (Table 1). The offspring were housed in a collective cage and received animals' standard laboratory chow (52% carbohydrate, 21% protein, and 4% lipids—Nuvilab CR1-Nuvital) [18] ad libitum.

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

Ingredients	Amount for 1 Kg of Casein	
	8%	17%
Casein	79.3g	179.3g
Vitamin mix*	10g	10g
Mineral mixtureł	35 g	35 g
Cellulose	50 g	50 g
Bitartrate of choline	2.5 g	2.5 g
α-methionine	3.0 g	3.0 g
Soya oil	70 mL	70 mL
Corn starch	750.2 g	650.2 g

*The vitamine mixture contained (milligrams per kilogram of diet): retinol 12, cholecalciferol 0.125, thiamine 40, riboflavin 30, pantothenic acid 140, pyridoxine 20, inositol 300, cyanocobalamin 0.1, menadione 80, nicotinic acid 200, choline 2720, folic acid 10, p-aminobenzoic acid 100, biotin 0.6.

łThe mineral mixture contained (milligrams per kilogram of diet): CaHPO4 17 200, KCl 4000, NaCl 4000, MgO 420, MgSO4 2000, Fe2O2 120, FeSO4.7H2O 200, trace elements 400 (MnSO4.H2O 98, CuSO4.5H2O 20, ZnSO4.7H2O 80, CoSO4.7H2O 0.16, KI 0.32, sufficiente starch to bring to 40 g/Kg of diet).

Offspring body weight

The body weight of pups was recorded daily throughout the experiment with a Marte Scale, AS-1000, approaching 0.01 g. Percentage weight gain = [body weight (g) x 100/ weight at first day of life (g)] - 100 [14]. The growth rate was calculated by the number of grams of body weight gained per day [14].

Muscle preparation

At 25 days of age, the rats were killed by decapitation. From a longitudinal incision in the anterior neck, the superficial masseter muscle was dissected. For histological analysis, the muscle was removed, immersed in n-hexane at low temperature and stored at –80°C until required for further processing.

Histochemical analyses

Serial cross sections (10 µm) were cut with a cryostat maintained at -20 C and stained for myofibrillar ATPase[19]. The sections were left at room temperature and were incubated for 30 s at room temperature in a 20 mm glycine buffer (pH 9.4) containing 20 mM CaCl2; sections incubated for 30 min (KCl) at 23-25 °C in a solution of 20 mM CaCl2 and 2.5 mM ATP disodium salt in 40 mM glycine buffer (pH 9.4); sections washed in 1% CaCl2 (3x30 s); sections immersed in 2% CoCl2 for 3 min; sections washed in distilled water (3x30 s); sections revealed in 1.5% yellow ammonium sulphide for 3 min; sections washed in distilled water and mounted in Entellan.

The sections were analysed with a light microscope (Olympus Optical U-CMAD-2, Tokyo, Japan; 109 objective lens) connected to a computer (TV TUNER APPLICATION— TelSignal Company Limited, Taiwan, image capture software). The images of the histological cross sections of the superficial masseter were captured for further analysis. Muscle fibres were labelled with respect to the three major types (I, IIa, IIb) of fibres on the basis of differences in the staining intensity for ATPase after acid pre-incubation (pH 4.4 and 4.7)[20]. According to the different staining intensities, the following classification was used for masseter: pH 4.4 (type I, darkest and typeII, lightest) and pH 4.7 (type I, darkest; type IIa, lightest and type IIb, grey). Histochemical analysis was performed using computerised image analysis from Mesurim PRO 3.2 software (developed by Jean-François Madre-Amiens, France). The muscle fibre type composition was determined by counting approximately 600 fibres in ten fields that were equally distributed over the sample 4.7 (type I, darkest; type IIa, lightest and IIb, grey).

To evaluate the area and perimeter of the muscle cells, microscope fields from each section were analyzed under the optical microscope (Leica, 40x objective). Images of fifty muscle cells de cada animal were taken from each preparation for later analysis with Scion Image Beta 4.0.2 software.

Statistical analysis

Results are presented as the mean ± SEM. Comparisons between the control and the lowprotein groups were performed using Student's t test. Data were analysed by two-way ANOVA, with the mother's diet (N, M) and age as factors. Bonferroni's post hoc test was used. Significance was set at $p<0.05$. Data analysis was performed using the statistical program Graphpad Sigma Stat 3.5.

Ethical considerations

The experimental protocol used in this article was approved by the Ethics Committee for Animal Experimentation at the Federal University of Pernambuco, according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Protocol number: 23076.001483/2010-61).

RESULTS:

Comparisons across nutritional manipulation ($F(1.481)= 164.739$, $p<0.001$) and the interaction of manipulation with age ($F(9.481)=7,053$, $p<0.001$) all showed differences in body weight. The rats in the M group (36.212±0.342) had lower body weights beginning on the 14^{th} day ($t=4.518$, $p<0.001$) compared with rats in the N group (42.623±0.364) (Figure 1).

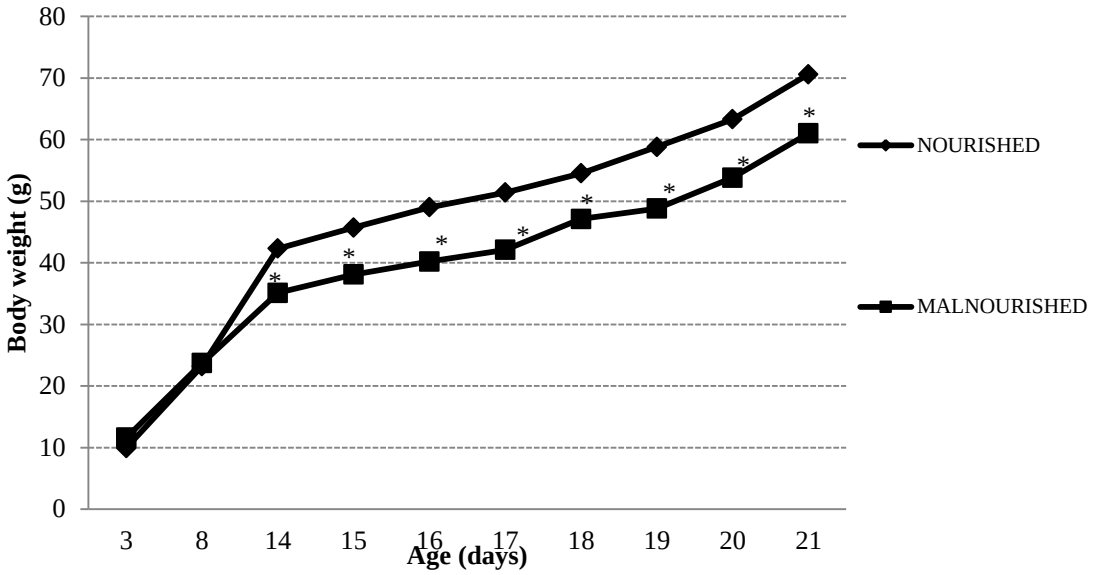

Figure 1. Effect of malnutrition on the body weight curve. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period (two-way ANOVA). Data are represented as mean ± SEM. Multiple comparisons (Bonferroni t-test) - $p<0.05$: * Nourished vs. Malnourished.

With relation to the proportion of muscle fiber types, the malnourished animals (71.96±5.78;p=0,03) had a higher amount of type IIA fibers compared with the nourished group (60.65±7.54) (Figure 2 and 3). Whereas type IIB fibers of the malnourished group (27.84±6,00; p=0.04) showed a lower proportion in relation to control animals (39.15±7.66) (Figure 2 and 3).

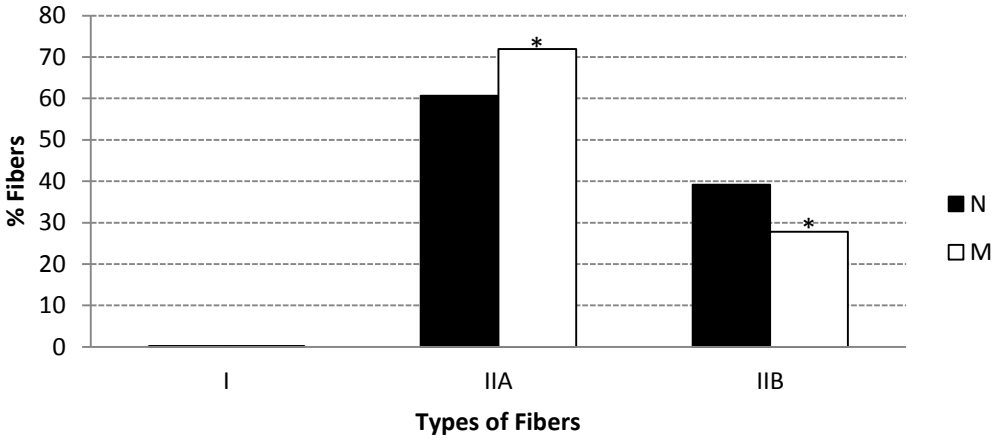

Figure 2. Effect of malnutrition on percentage of different types of fibers in the superficial masseter muscle at 25 d of life. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period (t-test). Data are represented as mean ± SEM.

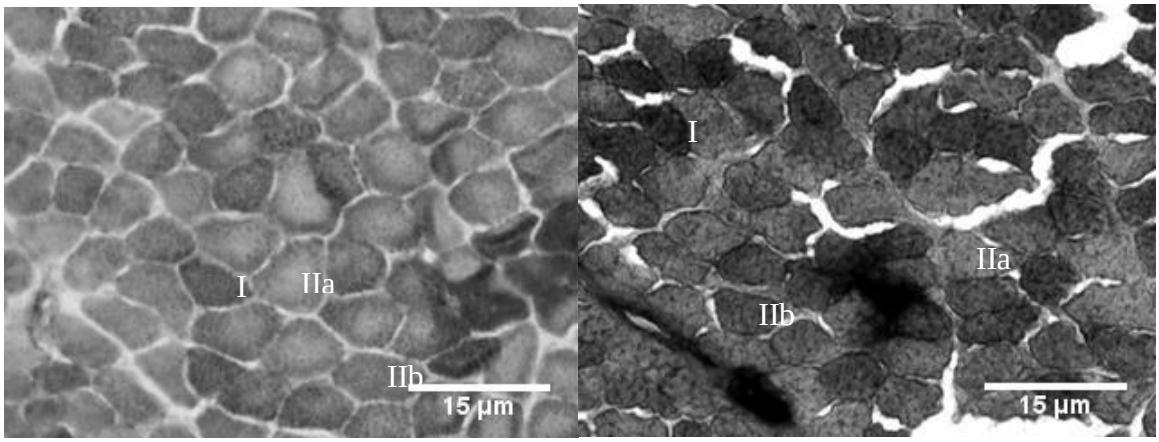

Figure 3. Rat superficial masseter muscle sections stained for myofibrillar ATPase activity after pre-incubation in buffer at pH 4.7 showing the variability in fibre type content between each experimental group. At this pH the type I fibres are stained dark; the type Iia fibres are stained light and the type IIb are stained grey. Panel A is of nourished group (N, n=4); and, panel B is of malnourished group (M, n=5).

Regarding the effect of protein malnutrition on neonatal morphometric parameters of the superficial masseter muscle was observed that malnourished animals showed lower

area (IIa - 90.19±13.86, p<0.001; IIb - 122.05±21.32, p<0.01) and perimeter (IIa - 37.79±3.03, p<0.001; IIb - 44.42±4.48, p<0.001) in relation to the control group both fiber type IIA and the type IIB (Table 2).

Table 2. Effect of malnutrition on morphometric parameters of different types of fibers in the superficial masseter muscle at 25 d of life. Rats were subjected to nutritional manipulation (nourished or malnourished) during the lactation period (t-test). Data are represented as mean ± SEM.

	EXPERIMENTAL GROUP		**t-TEST**
MORPHOMETRIC PARAMETERS	**NOURISHED (N)**	**MALNOURISHED (M)**	**P**
AREA OF MUSCLE FIBER TYPE IIA (µm)	126.66±27.05 (n=200)	90.19±13.86* (n=250)	< 0.001
AREA OF MUSCLE FIBER TYPE IIB (µm)	151.19±30.73 (n=200)	122.05±21.32* (n=250)	< 0.001
PERIMETER OF MUSCLE FIBER TYPE IIA (µm)	44.87±5.38 (n=200)	37.79±3.03* (n=250)	< 0.001
PERIMETER OF MUSCLE FIBER TYPE IIB (µm)	49.53±5.48 (n=200)	44.42±4.48* (n=250)	< 0.001

Comparisons (Mann-Whitney Test) - p<0.05: * Nourished vs. Malnourished.

4.DISCUSSION:

The focus of this study was to analyze the effect of neonatal malnutrition on the distribution of fiber types of the superficial masseter muscle, as well as on the morphology of these fibers. The above evaluations have investigated the impact of protein restriction during lactation in the development of masseter muscle, which is

extremely important for closing mandibular movements during the masticatory sequence, that are required for the degradation of the food during the food intake. Thus, changes in muscle mentioned can compromise the mechanism of degradation of the food, thus influencing, feeding behavior and therefore the proper nutrition.

In this study, we observed that malnutrition during the critical period of development reduced body weight. Our results support studies showing that protein malnutrition affect food intake and weight gain[21,22]. This effect may be a consequence of the protein deficiency that was imposed on the mothers during the suckling period. At this stage, the protein deficiency causes alterations in the quality of breast milk[23,24] that induces damage to body growth in several mammal species.

Moreover, we also observed that protein malnutrition during lactation increased the proportion of type IIA fibers and reduced the distribution of type IIB fibers in the bundle superficial masseter muscle. These data suggest that malnourished animals showed a delay in muscle development, particularly in phenotypic and morphologic maturation of the masseter muscle, which may compromise its function. This conclusion was based on the maturation of the masseter muscle. Studies in rats report that the differentiation of myoblasts of the masseter muscle occurs most actively between embryonic day 13 (E13) and the birth and maturation of myofibers get active with about E15 and continues after the change of feeding behavior of sucking to chewing with about 4 weeks of age[25,26,27,28]. When compared to other skeletal muscles, the maturation of myofibers and synaptogenesis of the masseter muscle presents retarded, considering what happens with approximately 3 weeks of age[29]. This process of developing differentiated skeletal

muscle depends on the functional demand[29]. As the masseter muscle acts mainly in the movements of bite, complete myogenesis and synaptogenesis in the masseter muscle is probably unnecessary until closing mandibular movements begin[29].

Furthermore, studies report that the surface region of the masseter muscle of mice is composed primarily of type II muscle fibers quickly[30,31]. However, initially, until 2 weeks of age, the superficial muscle is composed largely of type IIA fast fibers of oxidative-glycolytic metabolism[8]. However, with the functional maturation, ie by changing the pattern of food intake engine sucking to chewing, there is an increase in the distribution of type IIB fibers[8], glycolytic metabolism, with faster contraction speed and less fatigue resistant than the type IIA[9]. Therefore, after 2 weeks of age, muscle mentioned has an increased amount of fiber type IIB[8]. Thus, as in our study assessments were performed in animals at 25 days of age, or soon after weaning, it was expected that there would be a larger number of type IIB fibers. These data are in accordance with a model of malnutrition, which found an increase in the proportion of type I fibers and a reduction in the proportion of type II fibers in young pups, due to a diminution in the number of fast fibers formed[17]. Therefore, we suggest that the lower formation was observed in type IIB fibers, so that probably found a greater distribution of type IIA fibers, which shows the delay in the maturation of the masseter muscle.

Moreover, we can also suggest that neonatal protein restriction promoted a reduction in function of the superficial masseter muscle, given that he has less need to force contraction for the realization of the masticatory stroke, leading to increased expression of type IIA fibers. Because of IIA fast fibers are more resistant to fatigue[4], probably

malnourished animals have developed an adaptive mechanism to keep the jaw movements during masticatory sequence.

Regarding the effects of protein malnutrition on the area and perimeter of the masseter muscle fibers, we found that nutritional restriction led to reduction in both the area as the perimeter of the muscle fibers of type IIA and IIB. These results are in agreement with studies showing that perinatal malnutrition can induce muscle atrophy and may irreversibly change muscle morphology[13,14]. In addition, studies show that perinatal malnutrition can reduce cell proliferation, which compromises the number of muscle fibers and nuclei in the offspring[16]. This fact can be explained in reason for the restriction of maternal protein cause changes in the composition and volume of breast milk[32]. Studies report that neonatal maternal malnutrition is associated with lower maternal nutrient stocks and subsequently lower the transfer of nutrients to the baby, which is related to the decrease in the postnatal growth of the different organs and tissues of the body[32].

Moreover, under a functional point of view, studies have reported that the amount of strength that a muscle can produce depends not only on the type of myosin isoform, but also its area[33]. Thus, the area increases with the amount of resistance during contraction[34]. Accordingly, the composition determination fiber type and area can be used to characterize the functional properties and the needs of a muscle[35]. Given the above, we suggest that the malnourished animals exhibit a decreased force of contraction, since both fast fibers showed smaller area.

In summary, the neonatal malnutrition led to early change in phenotype of the fibers, as well as in the morphology of the superficial masseter muscle. So we can prove the importance of the postnatal period in the establishment of the phenotypic properties of muscle, which undergo adaptation by changing the pattern of food intake of sucking to chewing. Furthermore, we suggest that protein malnutrition may impair neonatal force generated during the mastication essential for degradation of food.

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ANEXO A

Archives of Oral Biology
Title: Effect of early malnutrition on masticatory function: review of the literature
Authors: Kelli Nogueira Ferraz-Pereira, MD; Raul Manhães-de-Castro, Ph.D.
Article Type: Review Article

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Archives of Oral Biology

ANEXO B

Ms. Ref. No.: NR-12-635
Title: A low-protein diet during lactation changes the phenotype of the fibers and alters the morphology of the masseter muscle in rats
Nutrition Research

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ANEXO C

Ms. Ref. No.: NR-12-634
Title: NEONATAL LOW-PROTEIN DIET REDUCES THE RHYTHM OF CHEWING IN RATS?
Nutrition Research

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XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental – FeSBE, realizada na cidade de Águas de Lindóia – SP, de 22 a 25 de agosto de 2012.

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na modalidade de Poster tendo como autores **LUANNA MOITA, Camila Tavares de Souza, Diego Lacerda Cabral, Tomas Ferreira Xavier, Kelli Nogueira Ferraz Pereira, Ana Elisa Toscano, Raul Manhães de Castro** foi apresentado durante o **XXV Congresso Brasileiro de Anatomia, XIV Congreso de Anatomia Del Cono Sur, XXXIII Congreso Chileno de Anatomia e I Encontro de Ligas Estudantis de Morfologia** realizados no período de 03 a 06 de outubro de 2012 no Centro de Convenções da Bahia.

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