FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

RAQUEL DA LUZ DIAS

EFEITO DA SUPLEMENTAÇÃO DE LEUCINA SOBRE A PROLIFERAÇÃO DE PRÉ-OSTEOBLASTOS DA LINHAGEM MC3T3-E1

Porto Alegre, junho de 2016

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

EFEITO DA SUPLEMENTAÇÃO DE LEUCINA SOBRE A PROLIFERAÇÃO DE PRÉ-OSTEOBLASTOS DA LINHAGEM MC3T3-E1

Autora: Raquel da Luz Dias

Tese de Doutorado apresentada ao Programa de Pós-graduação Medicina e Ciências da Saúde da Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS, como parte dos requisitos necessários à obtenção do título de Doutor.

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

Porto Alegre, junho de 2016.

Dados Internacionais de Catalogação na Publicação (CIP)

D541e Dias, Raquel da Luz

Efeito da suplementação de leucina sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1. / Raquel da Luz Dias. – Porto Alegre, 2016.

85f.: il. tab. Inclui dois artigos a serem submetidos à publicação em periódico científico.

Tese (Doutorado em Medicina e Ciências da Saúde) – Faculdade de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS.

Orientador: Prof. Dr. Jarbas Rodrigues Oliveira

1. Dietoterapia. 2. Suplementação Alimentar. 3. Leucina Metabolismo. 4. Proliferação de Células. I. Oliveira, Jarbas Rodrigues. II. Título.

CDD 613.2

Bibliotecária Responsável: Elisete Sales de Souza - CRB 10/1441

“The greatest enemy of knowledge is not the ignorance, it is the ilusion of knowlodge.” – Stephen Hawking

Aos meus pais, Telmo e Laura,
por me ensinarem a maior das virtudes: a humildade.
Ao meu marido Giovane,
meu ponto de equilíbrio constante.
E ao meu filho, Luca Toni,
pelo amor incondicional.

AGRADECIMENTOS

Todos dizem que a tese de doutorado é como um filho. No meu caso, esta afirmação tem um significado especial. Em busca do título de doutora, iniciei a jornada pelo caminho da pesquisa quando meu filho tinha apenas 1 ano e 5 meses, e ao longo destes 4 anos de caminhada, pude perceber que existem muitas semelhanças entre uma tese e um filho.

Assim como um filho, a tese demanda tempo e dedicação. Assim como um filho, a tese não vem com manual de instruções. Os já doutores, assim como as mães, não fazem questão de falar das noites mal dormidas ou das preocupações a respeito do desconhecido, inerentes a missões tão semelhantes.

São muitas as semelhanças encontradas entre o processo de pesquisa do doutorado e a criação de um filho, mas, na minha opinião, a característica que mais aproxima ambas as situações é a necessidade de uma rede de apoio formada por pessoas dispostas a ajudar incondicionalmente nas mais diversas situações.

Por este motivo, tenho muito a agradecer a todos os que estiveram presentes durante este período de “criação” (do meu filho e da minha tese), segurando algum ponto desta rede invisível, que sempre me sustentou e me fez acreditar que superaria cada etapa que viria pela frente.

Ao meu orientador, Prof. Dr. Jarbas Rodrigues de Oliveira, por ter aceitado o desafio de orientar uma novata na área da pesquisa básica, por toda a paciência, disponibilidade, respeito e ensinamentos proporcionados.

Aos colegas, professores do Laboratório de Biofisica Celular e Inflamação da PUCRS, Profa. Dr. Fernanda Bordignon, Prof. Dr. Denizar Alberto da Silva Melo e Prof. Dr. Marcio Vinícius Donádio, pelas contribuições constantes durante todo o processo de construção da tese.

De forma especial, agradeço aos bolsistas de iniciação científica e aos colegas mestrandos e doutorandos, Bruno Basso, João Tassinary, Paula Caruso, Gabriele Krause, Henrique Dias, Renan, Luisa e Leonardo Pedraza por serem meus grandes parceiros de laboratório, por dividirem o conhecimento comigo e por terem dedicado tempo e trabalho em prol desta pesquisa.

Aos colegas de outros laboratórios, Cassiana Viau (Laboratório de Genética Toxicológica da UFCSPA), Nathália Jaeger (Laboratório de Imunologia da PUCRS) e à equipe do Laboratório de Microscopia da PUCRS por fornecerem estrutura e suporte para a realização de alguns dos experimentos desta pesquisa.

Agradeço à CAPES, pelo apoio financeiro durante o período de doutorado sanduíche, e aos professores Francesc Ventura e José Luís Rosa, bem como os demais colegas da Unidade de Bioquímica da Universidade de Barcelona.

Aos colegas da FAENFI, em especial, a Profa. Dra. Beatriz Sebben Ojeda e à Profa. Dra. Ana Maria Pandolfo Feoli, pelo incentivo à realização do doutorado e às demais professoras do curso de Nutrição da PUCRS, pelo apoio nas atividades acadêmicas.

Por fim, agradeço à minha família: aos meus pais, Telmo e Laura e ao meu marido, Giovane, por estarem sempre presentes, em todos os momentos.

Ao meu filho, Luca Toni, que com a simplicidade do seu sorriso, me ensina a cada dia o significado de um amor incondicional.

A todos vocês, meu sincero reconhecimento. Espero que este trabalho, tal qual a esperança depositada num filho que se cria para formar novas gerações, possa auxiliar a ciência na construção de um futuro melhor.

EFEITO DA SUPLEMENTAÇÃO DE LEUCINA SOBRE A PROLIFERAÇÃO DE PRÉ-OSTEOBLASTOS DA LINHAGEM MC3T3-E1

RESUMO

Introdução: a leucina (Leu) é um aminoácido (AA) essencial, de cadeia ramificada, presente na alimentação humana, principalmente no leite e seus derivados, que tem sido investigado por exercer um importante papel na sinalização celular. Contudo, a maioria dos estudos que avalia as respostas celulares mediadas pela Leu, trabalha dentro de uma perspectiva de normalidade na oferta do AA e pouco se sabe sobre os efeitos que a suplementação pode gerar sobre os mecanismos de proliferação celular. Os efeitos do excesso deste aminoácido, têm sido extensivamente estudado em diversos tipos de células, entretanto existe uma limitação importante na quantidade de informações disponíveis na literatura científica em relação as suas ações em células do tecido ósseo.

Objetivo: este estudo teve como objetivo analisar os efeitos da suplementação de leucina sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1.

Métodos: a cultura dos pré-osteoblastos da linhagem MC3T3-E1, foi realizada com α-MEM, suplementado com 10% de soro fetal bovino e 1% de antibiótico. Após determinação de concentrações, o tratamento foi feito com a adição de Leu, diluída ao meio de cultura nas concentrações de 50 μM, o que corresponde a um acréscimo percentual de 12.5% a mais do aminoácido ao meio de cultura, por 48 horas. A viabilidade e a proliferação celular foram avaliadas pela técnica do *Trypan Blue*. Para a identificação dos mecanismos relacionados a inibição da proliferação celular, foram realizados ensaios que avaliaram a citotoxicidade (LDH); apoptose (Anexina V); estresse oxidativo (TBARS e DCFH); perfil inflamatório (TGF-β 1 e CBA); autofagia (laranja de acridina e citometria de fluxo); senescência (DAPI) e dano ao DNA (teste cometa).

Resultados e conclusões: a suplementação de Leu (50 μM) inibe a proliferação celular em 40%, com causas não relacionadas a necrose celular, apoptose, estresse oxidativo, inflamação ou autofagia. A suplementação de Leu provocou dano ao DNA, com consequente senescência e diminuição da proliferação celular de pré-osteoblastos da linhagem MC3T3-E1.

EFFECT OF LEUCINE SUPPLEMENTATION ON PRE-OSTEOBLASTS MC3T3-E1 CELL LINEAGE PROLIFERATION

ABSTRACT

Introduction: leucine (Leu) is an essential branched-chain amino acid, present in dairy products, which has been investigated for exert an important role in cell signaling. However, most studies evaluating cellular responses mediated by Leu, works within a normal perspective in AA supply and little is known about the effects that supplementation can generate on the cell proliferation mechanisms. The effects of excess of this amino acid, have been extensively studied in many cell types, but there is an important limitation on the amount of information available in the scientific literature regarding their actions in bone cells.

Objective: the aim of this study to determine the effects of leucine supplementation on proliferation of pre-osteoblasts of the MC3T3-E1 lineage.

Methods: the MC3T3-E1 cells were kept in α-MEM supplemented with 10% fetal bovine serum and 1% antibiotic. After initial determination of concentrations, the cells were treated during 48 hours, by the addition of 50 μM Leu, which corresponds to 12,5% in addition of the amino acid to the culture medium. Untreated cells represented the control group. The evaluation of the viability and proliferation of cultured cells was performed with Trypan Blue dye (0.4%). To identify the mechanisms related to decreased cellular proliferation, assays were performed to verify cytotoxicity (LDH); apotosis (Annexin V); oxidative stress (TBARS and DCFH); inflammation (TGF-β 1 and CBA); autophagy (acridine orange and flow cytometry); senescence (DAPI and flow cytometry); and DNA damage (alkaline comet assay).

Results and conclusions: Leu supplementation (50 μM) decreases cell proliferation by 40% with causes not related to cell necrosis, apoptosis, oxidative stress, inflammation or autophagy. The Leu supplementation caused DNA damage, with consequent increase in senescence and decrease of proliferation of MC3T3-E1 cells.

LISTA DE FIGURAS

CAPÍTULO I:

CAPÍTULO II:

CAPÍTULO III:

CAPITULO IV:

LISTA DE TABELAS

LISTA DE SIGLAS E ABREVIATURAS

AA – aminoácidos

AAE – aminoácidos essenciais

ACR – aminoácidos de cadeia ramificada

AI – *Adequate Intake*

DM2 – Diabetes Mellitus tipo 2

DNA – *Deoxyribonucleic acid*

K+ - potássio

Leu - Leucina

MSUD - *Maple Syrup Urine Disease*

mTOR – M*ammalian Target of Rapamycin*

mTORC1 – *Complex 1 of Mammalian Target of Rapamycin*

mTORC2 – C*omplex 2 of Mammalian Target of Rapamycin*

Na^+ - sódio

PI3K – *Phosphatidylinositol-3-kinases*

Rapa – rapamicina

RDA – *Recommended Dietary Allowances*

S6K – S6 *Kinases*

eIF4E- *Eukaryotic translation initiation factor 4E* **Eukaryotic translation initiation factor 4E-binding protein 1**

SGK1 – *Serum and Glucocorticoid-regulated Kinase 1*

PKC – *Proteína quinase C*

Raptor – *Regulatory-associated protein of mTOR*

Rictor - R*apamycin-insentive companion of mTOR*

HIF-1 (hypoxia-inducible factor de 1

SUMÁRIO

CAPÍTULO I

APRESENTAÇÃO

1 FUNDAMENTAÇÃO TEÓRICA

1.1 LEUCINA

1.1.1 ESTRUTURA, ABSORÇÃO E FUNÇÕES

A leucina (Leu) é um α-aminoácido, apolar em pH fisiológico, classificado como um aminoácido de cadeia ramificada (ACR), por apresentar em sua composição uma cadeia lateral alifática ramificada, além de um grupo α-amino e um grupo de ácido α-carboxílico (Figura 1). Por ser um aminoácido (AA) cetogênico, ao passar pelo processo de desaminação, durante o catabolismo, é capaz de converter seu esqueleto de carbono em acetoacetato ou Acetil-CoA, que podem ser utilizados para a obtenção de energia. É um dos 20 aminoácidos essenciais (AAE) e, por não poder ser sintetizado pelo organismo humano, deve ser obtido pela dieta (1).

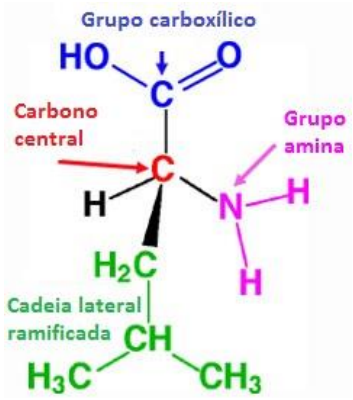

Figura 1: Estrutura química da leucina
Nota: modificado de Marks, Allan D. 2005 (1)

A absorção da Leu pelo trato gastrointestinal se dá após a completa digestão das proteínas ingeridas. Neste processo, participam enzimas proteolíticas que agem sobre as ligações peptídicas hidrolizando-as em peptídeos com tamanho cada vez menor, chegando até os aminoácidos livres. Nos enterócitos, a Leu é absorvida no pólo apical através de simporters, que permitem o transporte ativo secundário, juntamente com o Na^{+}. Esta ação é dependente de ATPase, Na+/K+, pois a energia envolvida no processo é o potencial elétrico negativo no interior das células. No pólo basal dos enterócitos os aminoácidos são absorvidos pelos uniporters, não envolvendo co-transporte de ions inorgânicos (2).

A Leu e os demais AA absorvidos pelos enterócitos entram na corrente sanguínea através do sistema porta hepático. Por se tratar de um L-aminoácido, a entrada de Leu para o interior das células através da membrana plasmática é feita pelos *L-type amino acid transporter-1* (LAT-1) (3, 4).

Como funções básicas e por ser um nutriente estrutural, a Leu, em conjunto com os demais AA, é responsável pelos processos anabólicos corporais, o que inclui a construção e manutenção dos tecidos; a formação de enzimas, de hormônios e de anticorpos, além de ser uma importante fonte de energia. Recentemente, a Leu tem sido investigada por exercer um importante papel na sinalização celular, regulando múltiplas funções biológicas, envolvidas em rotas de proliferação celular, de transcrição de genes e de homeostase da glicose (5). Algumas das funções relacionadas a estas vias serão destacadas nas sessões a seguir.

1.1.2 A LEUCINA NA ALIMENTAÇÃO E NA SAÚDE HUMANA

O fornecimento de Leu através da alimentação inicia logo após o nascimento. O leite humano fornece 104 mg de Leu a cada 100 mL e, dentre os leites dos mamíferos, é o que possui a menor quantidade de proteína total, que varia entre 1,14 a 1,21 g /100 mL. Após o período do desmame, que deve ocorrer por volta dos 6 meses de vida, os seres humanos mantém o fornecimento de Leu ao organismo através da inclusão de alimentos de origem animal à dieta. Como uma exceção aos mamíferos, os seres humanos continuam a ingerir leite ao longo de toda a vida e desta forma, o leite de vaca passa a ser uma das principais fontes proteicas durante a infância e também na vida adulta (6, 7).

O perfil proteico do leite de vaca é caracterizado por suas proteínas solúveis, também conhecidas como proteínas do soro do leite, ou *whey protein* e, por proteínas insolúveis, denominadas de caseína. Estas compõem 20% e 80% do total de proteínas deste alimento, respectivamente e são responsáveis por fornecerem quantidades expressivas de Leu, contendo, em média 333 mg do aminoácido a cada 100 mL (8-10) .

As necessidades de Leu variam de acordo com a faixa etária, condições fisiológicas e nível de atividade física, sendo que a média do consumo diário deve corresponder a 42 mg/Kg para os adultos. O consumo humano de proteínas possui uma grande variabilidade, dependendo da dieta estabelecida. Em estado alimentado, as concentrações de AA estão aumentadas, assim como as substâncias resultantes de seu catabolismo. Tais alterações, fazem parte da regulação normal de AA e nitrogênio e, aparentemente, não representam um perigo quando a ingestão proteica permanece dentro de uma faixa de ingestão recomendada. Não há níveis máximos estabelecidos para o consumo de Leu, porém, existem recomendações a respeito do aporte de proteínas totais (11, 12). A tabela 1 apresenta os valores recomendados de Leu e proteínas para diferentes faixas etárias.

Tabela 1 – Valores diários recomendados para o consumo de proteínas totais e leucina através da dieta.

Faixa etária	Leucina mg/Kg	Proteína g/Kg
Bebês		
0 – 6 meses	156*	1,52*
7 – 12 meses	93	1,2
Crianças		
1 – 3 anos	63	1,05
4 – 8 anos	49	0,95
Homens		
9 – 13 anos	49	0,95
14 – 18 anos	47	0,85
19 – >70 anos	42	0,8
Mulheres		
9 – 13 anos	47	0,95
14 – 18 anos	44	0,85
19 - >70 anos	42	0,8
Gestantes	56	1,1 +25 g adicional
Lactantes	62	1,3 + 25 g adicional

*Recommended Dietary Allowances (RDA). *Adequate Intake (AI).*
Nota: modificado de *Institute of Medicine*, 2005 (12)

Por outro lado, o consumo de proteína em excesso pode causar possíveis efeitos deletérios ao organismo humano. No sistema renal, o excesso de proteínas pode causar uma sobrecarga, já que o rim é o órgão responsável por excretar a uréia, um dos principais metabólitos dos AA, através da urina. No tecido ósseo, excesso de AA pode acarretar numa diminuição da massa

óssea. O possível mecanismo envolvido é chamado de hipótese da carga ácida, situação causada pelo excesso de prótons (íons hidrogênio) provenientes dos AA. Tal hipótese é utilizada para explicar o processo de redução de massa óssea em indivíduos que realizam dietas hiperproteicas. Como o osso armazena elevadas quantidades de cálcio, em situações de estresse ácido, liberaria este mineral para promover a homeostase do pH sanguíneo, gerando assim a osteoporose (13, 14).

O excesso de proteínas na alimentação também pode estar relacionado ao aumento da adiposidade corporal. A energia em excesso é obtida através dos esqueletos de carbono dos AA e caso não haja demanda energética suficiente, a energia é armazenada na forma de triglicerídios no tecido adiposo. Este efeito adipogênico também tem origem em mecanismos de sinalização e proliferação celular sensíveis aos AA (em especial à Leu), que compõem as bases moleculares de doenças como o Diabetes Tipo 2 (DM2) e o câncer (15).

1.1.3 LEUCINA E SINALIZAÇÃO CELULAR

Dentre as principais vias de sinalização celular sensíveis à Leu, está o *mammalian Target of Rapamycin* (mTOR), um fator de trascrição, agonista de 289 kDa, de uma serina/treonina quinase pertencente à família da fosfatidilinositol 3-quinase (PI3K). A identificação e o nome deste complexo proteico, responsável por regular os mecanismos de proliferação celular, deu-se a partir da descoberta de uma substância produzida por bactérias (*Streptomyces hygroscopicus*) no solo da na ilha de Páscoa, denominada de Rapamicina (Rapa) como uma alusão ao nome da ilha (Rapa Nui) (16). Este macrolídeo ficou conhecido por atuar em seu alvo específico, inibindo a atividade proliferativa celular de mamíferos.

O mTOR está presente nas células de mamíferos em dois complexos, denominados complexo 1 (mTORC1) e complexo 2 (mTORC2). Estes complexos proteicos fosforilam diferentes substratos para regular funções celulares distintas. O mTORC1 estimula a proliferação celular pelo aumento da iniciação da tradução cap-dependente, processo mediado pela eIF4E-*binding proteins* e pelas S6 *Kinases* (S6K1 e S6K2) e é sensível às ações da Rapa. Por outro lado, o mTORC2 fosforila AKT, SGK1 e PKC (membros da

família AGC kinase), que controla a sobrevivência da célula e a organização do citoesqueleto, sendo insensível à Rapa. A sensibilidade do mTORC1 à Rapa se dá pela presença da proteína regulatória *Raptor* (*regulatory-associated protein of mTOR*), que no mTORC2 é substituída pela proteína Rictor (*rapamycin-insentive companion of mTOR)*.

Se por um lado a Rapa é capaz de inibir o mTORC1 e, consequentemente, a proliferação celular, por outro, a presença de nutrientes, é capaz de ativar os mecanismos envolvidos nesta ação, promovendo a proliferação e a regulação de insulina na célula. Dentre os nutrientes capazes de sensibilizar esta via, estão os AA e, nos últimos anos, a Leu ganhou destaque, por ser o principal AA capaz de ativar a rota da mTOR e promover proliferação celular. A Figura 2 demonstra de forma resumida a via de sinalização do mTOR.

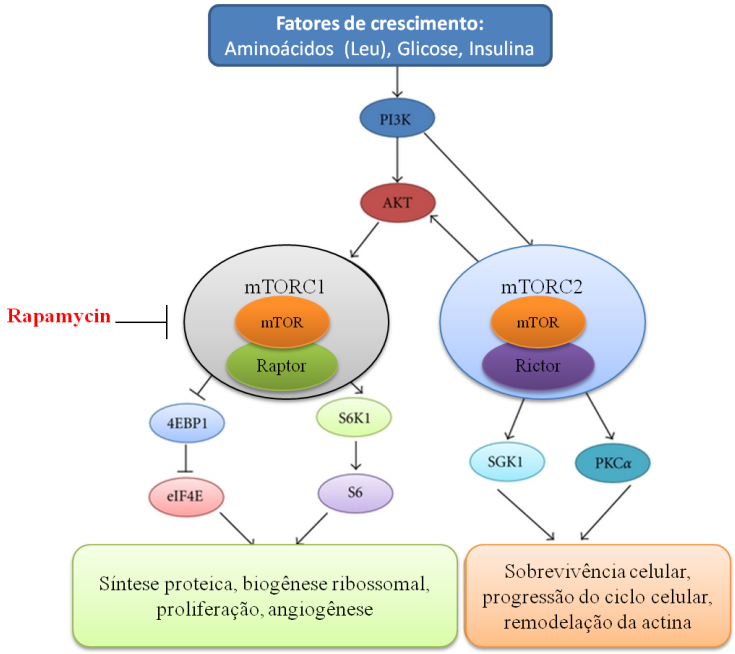

Figura 2: modelo esquemático das vias de ativação e inibição de proliferação celular pelo mTOR. A figura demonstra de ofrma esquemática a ação dos fatores de crescimento (dentre eles a Leu) atuando sobre as vias PI3K e AKT, que ativam diretamente o mTORC1, que é sensível a ação inibitória da Rapamicina, pela presença da proteína regulatória Raptor. A ativação do mTORC1 pela iniciação da tradução e fosforilação da 4EBP1 e S6K, que regulam todo o processo de proliferação celular. Por outro lado, o mTORC2, não possui sensibilidade à rapamicina, com a proteína regulatória Rictor, mas quando ativado pela PI3K, fosforila proteínas que controlam a sobrevivência celular e a organização do citoesqueleto.
Nota: modificado de Dann, Stephen G. *et al*. *Trends in Molecular Medicine*, 2007 (17)

Contudo, a maioria dos estudos que avalia as respostas celulares mediadas pela Leu, trabalha dentro de uma perspectiva de normalidade na oferta do AA. Sendo assim, pouco se sabe sobre os efeitos que uma suplementação de Leu pode gerar nos mecanismos de proliferação celular sensíveis ao AA (18).

A teoria de que, quando em excesso, a Leu poderia gerar uma superativação da via mTORC1 tem sido demonstrada em alguns estudos *in vitro,* com diferentes tipos de células (19-22). Os efeitos vão desde a síntese proteica desordenada em células tumorais, até a lipogênese excessiva em adipócitos (23). *In vivo*, a suplementação da dieta de ratas *Wistar* em período gestacional com Leu, aumentou siginificativamente o peso corporal fetal, causou hiperglicemia e hipoinsulinemia nos fetos e diminuiu a área das ilhotas pancreáticas. A fim de elucidar os mecanismos da ação da Leu no desenvolvimento das células β-pancreáticas, seguiu-se o estudo com a cultura das células pancreáticas dos embriões. A presença de Leu (10 mMol/L), provocou efeitos negativos na regulação da massa de células β-pancreáticas durante o desenvolvimento pancreático, impedindo o desenvolvimento das mesmas, através do aumento da expressão do HIF-1 (hypoxia-inducible factor de 1), um repressor do desenvolvimento de células endócrino progenitoras (24). Estes achados indicam que a suplementação de Leu durante a gestação pode potencialmente aumentar o risco de DM2 por inbibir a diferenciação de células progenitoras endócrinas durante o período suscetível da vida fetal (18, 23-25).

O excesso de Leu também tem sido investigado em situações patológicas, como por exemplo, na *Maple Syrup Urine Disease* (MSUD), conhecida como a doença do xarope de bordo. A MSUD é um erro inato do metabolismo, em que o indivíduo portador apresenta uma deficiência grave na atividade do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada, que incluem a Leu. A incapacidade deste complexo enzimático em descarboxilar os α-cetoácidos de cadeia ramificada, leva a um acúmulo tecidual de destes metabólitos e de seus AA, causando o sintoma de odor de xarope de bordo, com cheiro adocicado, semelhante a açúcar queimado, que dá nome à doença. Este excesso provoca sinais e sintomas clínicos, sendo

característicos as alterações neurológicas, podendo chegar ao dano permanente das células nervosas, o que revela o potencial efeito tóxico da Leu e de outros ACR nestas condições (27). Estudos *in vitro* em condições que mimetizam o ambiente tóxico pelo escesso de leucina, assim como em indivíduos portadores de MSUD, demonstram que este excesso pode provocar um aumento do estresse oxidativo celular e até mesmo dano celular por quebra de DNA (28-31).

Apesar de já ser possível encontrar na literatura científica algumas condições que evidenciam o potencial efeito nocivo da Leu, observa-se ainda, que não existem dados que avaliam este efeito sobre as células do tecido ósseo. O interesse neste foco se deu pelo fato de que a Leu é o principal AA do leite, alimento que está diretamente relacionado à saúde óssea. As próximas sessões abordarão aspectos relacionados à estrutura e função do tecido ósseo, esclarecer como estas células se desenvolvem, para, posteriormente compreender como a Leu pode atuar neste processo.

1.2 TECIDO ÓSSEO

O tecido ósseo é composto por um conjunto de componentes altamente especializados e organizados, responsáveis pela função estrutural do organismo humano. A matriz óssea é constituída por uma parte orgânica, com a presença de elementos proteicos como o colágeno, os mucopolissacarídeos, as glicosaminas e as proteínas não colágenas e, por uma parte inorgânica, composta principalmente pelos minerais cálcio e fósforo, presentes na estrutura sob a forma de hidroxiapatita. Enquanto a matriz orgânica desempenha um papel importante na organização e sinalização celular, conferindo ao osso uma grande capacidade de resistência às forças de tensão, a matriz inorgânica atribui ao esqueleto uma maior resistência às forças de compressão (32).

O tecido ósseo é formado a partir das células tronco mesenquimais, pluripotentes, ainda indiferenciadas e que permanecem na medula óssea. Dependendo da sinalização celular, desencadeada pela ativação de genes específicos, estas células podem se diferenciar em diferentes tipos de

tecidos, como tecido muscular, adiposo ou cartilaginoso (Figura 4). Estes genes também são conhecidos como *master genes*, que assinalam o ponto de “virada”, teoricamente irreversível, comprometendo o destino destas células em formar os tecidos específicos. Além do controle genético, fatores de crescimento, conhecidos como proteínas morfogênicas ósseas, também contribuem para o processo de diferenciação e formação da matriz óssea. Uma vez comprometidas, as células tronco mesenquimais transformam-se em osteoblastos transitórios, ou pré-osteoblastos, que ainda necessitam passar pelo processo de diferenciação para tornarem-se osteoblastos, que, efetivamente, são as células responsáveis pela formação óssea. O tecido ósseo renova-se constantemente e para isso outras células, denominadas osteoclastos, desempenham um papel fundamental no processo de remodelação, permitindo que, em média, a cada 10 anos, tenhamos um novo esqueleto. Apesar de aparentemente inerte, o tecido ósseo possui uma incrível dinamicidade entre suas estruturas celulares, com vias de sinalização muito bem reguladas entre as células osteoprogenitoras, osteoblastos, osteócitos e osteoclastos (32, 33).

O osso também desempenha uma função essencial no equilíbrio do metabolismo mineral, fornecendo cálcio para inúmeras funções celulares, sendo este tecido, uma importante reserva para o organismo. Porém, além da função estrutural e da função reguladora dos minerais, nos últimos anos, têm-se investigado as possíveis interações do osso com outros órgãos e algumas evidências indicam que este tecido produz substâncias que estão envolvidas no controle endócrino do organismo (34, 35).

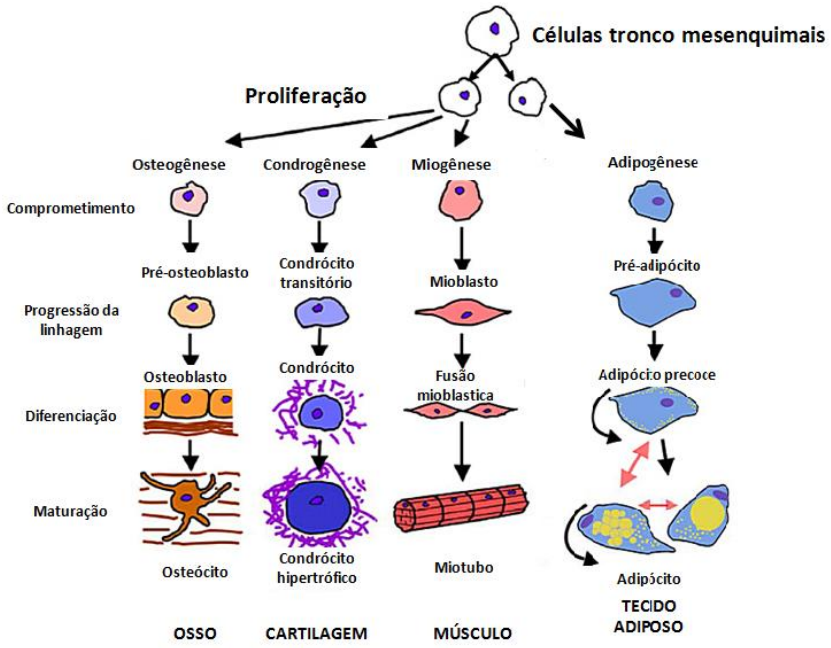

Figura 4: Sequência de eventos envolvidos nas fases de proliferação, diferenciação e maturação de diferentes tecidos a partir de células tronco mesenquimais. No tecido ósseo, a fase de proliferação acompanha a célula osteoprogenitora nas fases de comprometimento e progressão da linhagem, quando finalmente os pré-osteoblastos se diferenciam em osteoblastos, osteoblastos maduros e por fim em osteócitos.
Nota: modificado de Bonfield, TL. *Discovery Medicine*, 2010 (33)

O processo de remodelação óssea é altamente dependente do status energético do organismo, e estudos em animais já demonstramram isso claramente. Ratos deficientes em leptina, um hormônio produzido pelos adipócitos, apresentam um fenótipo clássico de aumento do apetite, da adiposidade periférica e da massa óssea. Um fenótipo semelhante também é encontrado em ratos deficientes em osteocalcina, uma das proteínas não-colágenas, que faz parte da matriz orgânica do osso, produzida pelos osteoblastos. Eles apresentam intolerância à glicose, diminuição na produção e secreção de insulina e diminuição da produção de adiponectinas, indicando que esta proteína, atua como um hormônio, regulando o metabolismo energético e da glicose (35).

A perspectiva de investigar o tecido ósseo como um órgão endócrino, a partir das evidências sobre a ação coordenada entre o osso e o metabolismo energético, coincidem com dados relacionados a aspectos nutricionais da população. Como já citado anteriormente, um número

expressivo de estudos apontam para a influência do excesso de nutrientes na etiologia de doenças crônicas, relacionadas a diferentes órgãos e sistemas do organismo, mas poucos avaliaram os efeitos desta condição sobre o tecido ósseo, com os mecanismos celulares ainda desconhecidos (36-38).

Neste sentido, estudos *in vitro*, conduzidos com linhagens celulares são vantajosos, pois permitem a análise de mecanismos celulares, com relativa estabilidade, facilidade de manutenção, número ilimitado de células e sem a necessidade de isolamento fenotípico.

1.3 CÉLULAS MC3T3-E1

As MC3T3-E1 são uma linhagem de células osteoblásticas estabelecidas a partir da calota craniana de camundongos C57BL/6 e selecionadas com base na elevada atividade da fosfatase alcalina no estado de repouso. Estas células têm a capacidade de se diferenciarem em osteoblastos e osteócitos e, ainda, depositarem hidroxiapatita na matriz óssea desenvolvida a partir da cultura. O processo de diferenciação observado em culturas de células MC3T3-E1 é muito semelhante ao observado na osteogênese intermembrana *in vivo* (39).

O padrão de crescimento desta linhagem celular, apresenta um ritmo logarítmico, com morfologia fibroblástica, contendo abundância de microtúbulos e microfilamentos no citoplasma. Cerca de 18 horas após o início da cultura, as células dobram a sua população. Com 4 dias de cultura, as células atingem confluência (5-6 x 10^4 células/cm^2) com contornos celulares com uma aparência de mosaico. As células crescem lentamente, em múltiplas camadas, formando fibras de colágenos com cerca de 18 dias de cultura. O crescimento das células pode ser alterado pela limitada quantidade de colágeno produzido, principalmente quando o meio não é suplementado com ácido ascórbico. Com 21 dias, pequenas regiões nodulares osmiofílicas começam a se desenvolver. Na região nodular, aglomerados de pequenas células são cercados de uma matriz densa e fibrosa. As células exibem características ultraestruturais de osteoblastos. Os nódulos aumentam em número e tamanho, conforme o tempo de incubação.

Na parte central da placa, se concentra uma maior quantidade de nódulos, observando-se a presença de osteócitos e deposição mineral (34). As características morfológicas das células MC3T3-E1 podem ser visualizadas na figura 5.

Figura 5: Aspecto morfológico das células MC3T3-E1.
Imagem obtida através de microscópio eletrônico.
Nota: imagem cedida por Tassinary, João. (2015)

Os parâmetros morfológicos e observações citoquímicas da células MC3T3-E1, sugerem que a deposição mineral neste sistema, procede de maneira similar ao observado em sistemas *in vivo* e que osteoblastos, osteócitos jovens e vesículas de matriz desempenham funções importantes no processo de mineralização. Assim, a linhagem MC3T3-E1 pode ser utilizada como um modelo para estudos de proliferação, diferenciação e mineralização de células ósseas e de mecanismos biológicos de calcificação, bem como, para avaliar substâncias como citocinas, genes e hormônios relacionados ao metabolismo ósseo (40, 41).

2 JUSTIFICATIVA

A Leu é um aminoácido essencial e de cadeia ramificada e vem ganhando destaque por exercer um importante papel na sinalização de rotas metabólicas responsáveis pela proliferação celular. Entretanto, especula-se que o consumo excessivo deste aminoácido, na forma de alimentos ou suplementos, possa exercer um efeito negativo.

O interesse por analisar os efeitos da leucina em uma linhagem celular de pré-osteoblastos, surgiu, em primeiro lugar, porque este é o principal aminoácido do leite, alimento que tem uma relação direta com a saúde óssea. Além disso, o tecido ósseo, tem sido considerado um importante órgão endócrino, e por este motivo, sofre os efeitos do excesso de aminoácidos, que são capazes de ativar rotas sensíveis a nutrientes, que atuam na regulação do crescimento, biossíntese e metabolismo celular.

Neste sentido, observa-se que os efeitos do excesso deste aminoácido têm sido extensivamente estudados em diversos tipos de células. Surpreendentemente, existe uma limitação importante na quantidade de informações disponíveis na literatura científica em relação as suas ações em células do tecido ósseo.

Tendo em vista a busca de uma melhor compreensão dos efeitos que o excesso de leucina, além de níveis fisiológicos para o meio de cultura, possa exercer no tecido ósseo, o objetivo desta pesquisa foi o de analisar os efeitos da suplementação de leucina sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1.

3 OBJETIVOS

3.1 Objetivo geral

Analisar os efeitos da suplementação de leucina sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1.

3.2 Objetivos específicos

- Analisar os efeitos da suplementação de leucina sobre a proliferação e viabilidade celular, apoptose, dano oxidativo, diferenciação e rota do mTOR de pré-osteoblastos da linhagem MC3T3-E1;
- Analisar o estresse oxidativo, perfil inflamatório, autofagia, senescência e dano ao DNA em pré-osteoblastos da linhagem MC3T3-E1 suplementados com leucina *in vitro*.

REFERÊNCIAS

1. Marks AD, Lieberman M. Amino acids in proteins. In: Wilkens LW, editor. Mark`s Basic Medical Biochemistry: a clinical aproach. Philadelphia, USA. 2005. p. 73-91.
2. Conti e Silva AC, Frota KdMG, Arêas JAG. Funções plenamente reconhecidas de nutrientes: proteína. Série de Publicações ILSI Brasil [Internet]. 2012; 20.
3. Poncet N, Taylor PM. The role of amino acid transporters in nutrition. Current Opinion in Clinical Nutrition & Metabolic Care. 2013;16(1):57-65.
4. Taylor PM. Role of amino acid transporters in amino acid sensing. The American journal of clinical nutrition. 2014;99(1):223S-30S.
5. Torres-Leal FL, Vianna D, Teodoro GFR, Capitani MD, Tirapegui J. Aspectos atuais do efeito da leucina sobre o controle glicêmico e a resistência à insulina. Nutrire Rev Soc Bras Aliment Nutr. 2010;35(2).
6. Prentice AM. Dairy products in global public health. The American journal of clinical nutrition. 2014;99(5):1212S-6S.
7. Weaver CM. How sound is the science behind the dietary recommendations for dairy? The American journal of clinical nutrition. 2014;99(5):1217S-22S.
8. Pereira PC. Milk nutritional composition and its role in human health. Nutrition. 2014;30(6):619-27.
9. Melnik BC. Excessive leucine-mTORC1-signalling of cow milk-based infant formula: the missing link to understand early childhood obesity. Journal of obesity. 2012.
10. Sánchez LC, Hernandez A, Rodriguez A, Rivero M, Barriga C, Cubero J. [Nitrogen and protein content analysis of human milk, diurnality vs nocturnality]. Nutricion hospitalaria; 2010;26(3):511-4.
11. Padovani RM, Amaya-Farfán J, Colugnati FAB, Domene SMA. Dietary reference intakes: aplicabilidade das tabelas em estudos nutricionais. Rev nutr. 2006;19(6):741-60.
12. Institute of Medicine. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids Washington (DC): National Academy Press. 2005.
13. Nicoll R, Howard JM. The acid–ash hypothesis revisited: a reassessment of the impact of dietary acidity on bone. Journal of bone and mineral metabolism. 2014;32(5):469-75.
14. Conigrave A, Brown E, Rizzoli R. Dietary protein and bone health: roles of amino acid-sensing receptors in the control of calcium metabolism and bone homeostasis. Annu Rev Nutr. 2008;28:131-55.
15. Hirahatake KM, Slavin JL, Maki KC, Adams SH. Associations between dairy foods, diabetes, and metabolic health: potential mechanisms and future directions. Metabolism. 2014;63(5):618-27.

16. Tato I, Bartrons R, Ventura F, Rosa JL. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. Journal of Biological Chemistry. 2011;286(8):6128-42.
17. Dann SG, Selvaraj A, Thomas G. mTOR Complex1–S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends in molecular medicine. 2007;13(6):252-9.
18. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell metabolism. 2006;3(6):393-402.
19. Vianna D, Teodoro GFR, Torres-Leal FL, Tirapegui J. Protein synthesis regulation by leucine. Brazilian Journal of Pharmaceutical Sciences. 2010;46(1):29-36.
20. Chong ZZ, Maiese K. Mammalian target of rapamycin signaling in diabetic cardiovascular disease. Cardiovasc Diabetol. 2012;11(1):45.
21. Luo Z, Saha AK, Xiang X, Ruderman NB. AMPK, the metabolic syndrome and cancer. Trends in pharmacological sciences. 2005;26(2):69-76.
22. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology. 2011;12(1):21-35.
23. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146(3):1328-37.
24. Rachdi L, Aïello V, Duvillié B, Scharfmann R. L-leucine alters pancreatic β-cell differentiation and function via the mTor signaling pathway. Diabetes. 2012;61(2):409-17.
25. Nicastro H, Zanchi NE, da Luz CR, de Moraes WM, Ramona P, de Siqueira Filho MA, et al. Effects of leucine supplementation and resistance exercise on dexamethasone-induced muscle atrophy and insulin resistance in rats. Nutrition. 2012;28(4):465-71.
26. Melnik BC. Leucine signaling in the pathogenesis of type 2 diabetes and obesity. World J Diabetes. 2012;3(3):38-53.
27. Strauss KA, Puffenberger EG, Morton DH. Maple syrup urine disease. 2013.
28. Guerreiro G, Mescka CP, Sitta A, Donida B, Marchetti D, Hammerschmidt T, et al. Urinary biomarkers of oxidative damage in Maple syrup urine disease: the l-carnitine role. International Journal of Developmental Neuroscience. 2015;42:10-4.
29. Sitta A, Ribas GS, Mescka CP, Barschak AG, Wajner M, Vargas CR. Neurological damage in MSUD: the role of oxidative stress. Cellular and molecular neurobiology. 2014;34(2):157-65.
30. Mescka CP, Guerreiro G, Hammerschmidt T, Faverzani J, de Moura Coelho D, Mandredini V, et al. l-Carnitine supplementation decreases DNA damage in treated MSUD patients. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2015;775:43-7.

31. Mescka CP, Wayhs CAY, Guerreiro G, Manfredini V, Dutra-Filho CS, Vargas CR. Prevention of DNA damage by L-carnitine induced by metabolites accumulated in maple syrup urine disease in human peripheral leukocytes in vitro. Gene. 2014;548(2):294-8.
32. Judas F, Palma P, Falacho RI, Figueiredo H. Estrutura e dinâmica do tecido ósseo. 2012.
33. Bonfield TL, Caplan AI. Adult mesenchymal stem cells: an innovative therapeutic for lung diseases. Discovery medicine. 2010;9(47):337-45.
34. Shapses SA, Sukumar D. Bone metabolism in obesity and weight loss. Annual review of nutrition. 2012;32:287.
35. Schwetz V, Pieber T, Obermayer-Pietsch B. Mechanisms In Endocrinology: The endocrine role of the skeleton: background and clinical evidence. European Journal of Endocrinology. 2012;166(6):959-67.
36. Michaelsen KF, Larnkjær A, Mølgaard C. Amount and quality of dietary proteins during the first two years of life in relation to NCD risk in adulthood. Nutrition, Metabolism and Cardiovascular Diseases. 2012;22(10):781-6.
37. Yang YH, Li B, Zheng XF, Chen JW, Chen K, Jiang SD, et al. Oxidative damage to osteoblasts can be alleviated by early autophagy through the endoplasmic reticulum stress pathway—implications for the treatment of osteoporosis. Free Radical Biology and Medicine. 2014;77:10-20.
38. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. The Journal of Clinical Endocrinology & Metabolism. 2013;98(6):E1060-E5.
39. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. The Journal of cell biology. 1983;96(1):191-8.
40. Raouf A, Seth A. Ets transcription factors and targets in osteogenesis. Oncogene. 2000;19(55):6455-63.
41. Czekanska E, Stoddart M, Richards R, Hayes J. In search of an osteoblast cell model for in vitro research. Eur Cell Mater. 2012;24(4):1-17.

CAPÍTULO 2

ARTIGO ORIGINAL

Leucine reduces the proliferation of MC3T3-E1 cells through mTOR-independent pathway[ab]

[a] Este artigo foi submetido ao *Journal of Bone and Metabolism* e está formatado de acordo com as normas do periódico.
[b] O comprovante de submissão do manuscrito encontra-se no Apêndice A.

TITLE: Leucine reduces the proliferation of MC3T3-E1 cells through mTOR-independent pathway

AUTHORS:

Raquel da Luz Dias

Doctoral student of the Graduate Program in Medicine and Health Sciences of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Address: Rua Itaboraí, 840/502. Jardim Botânico, Porto Alegre-RS. CEP 90670-030

Bruno Basso

Master´s student of the Graduate Program in Molecular and Cellular Biology of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Dr. Márcio Vinícius Donadio

Full Professor at the Laboratory of Biophysics and Inflammation of the of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Dr. Francesc Ventura Pujol

Full Professor at the Department of Physiological Sciences II of the University of Barcelona - Spain

Dr. José Luis Rosa

Full Professor at the Department of Physiological Sciences II of the University of Barcelona - Spain

Dr. Ramón Bartrons

Full Professor at the Department of Physiological Sciences II of the University of Barcelona - Spain

Dr. Jarbas Rodrigues de Oliveira

Full Professor at the Laboratory of Biophysics and Inflammation of the of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

AUTHOR FOR CONTACT:

Raquel da Luz Dias[1]

e-mail: raquel.dias@pucrs.br

Phone number: +55 5133203646

Fax number: +55 5133203647

Conflict of interest: the authors declare that they have no conflicts of interest.

Research involving Human Participants and/or Animals: the authors state that the research does not involve human participants or animals.

ABSTRACT

Purpose: The effects of Leucine on several types of cells have been studied, yet little is known about its action upon bone cells. In this study we analysed the effects of Leucine supplementation on the proliferation and viability, apoptosis, oxidative damage, cell diferentiation and mTOR pathway, in MC3T3-E1 pre-osteoblast cells.

Materials and methods: The MC3T3-E1 cells were treated during 48 hours with Leucine 50 μM, 100 μM and 200 μM. The proliferation and cellular viability were assessed by using Trypan Blue. The evaluation of the Leucine cytotoxicity was performed by determining Lactate Dehydrogenase (LDH) content in the supernatant of the cell culture. The presence of apoptosis was evaluated with the use of Propidium Iodide and Annexin V through an Apoptosis Detection Kit I (Pharmigen). Oxidative stress was evaluated through the measurement of thiobarbituric acid reactive substances (TBARS). TGF-β was assessed through an immunoenzymatic assay and collagen content in MC3T3-E1 cells was measured using Picro-Sirius Red. For the alkaline phosphatase evaluation, p-nitrophenyl phosphate substrate was used.

Results: The cells treated with Leucine showed reduced proliferation when compared to the control, as well as to other amino acids. This effect seems not be related to cellular necrosis, apoptosis or oxidative stress. When Leucine was associated with insulin (200nM), which activates mTOR, it was not able to block the insulin proliferative effect. TGF-β presented no changes in cells treated with Leucine, whereas increased levels were seen in cells treated with rapamycin (10nM), a potent mTORC1 inhibitor .

Conclusions: These results suggest that the proliferative reduction caused by Leucine on pre-osteoblasts occurs through a mTORC1 independent pathway.

Key-words: Leucine, osteoblasts, cell proliferation, m-TOR

1 INTRODUCTION

A significant number of studies have evidenced the importance of nutrients in the etiology of chronic diseases related to different organs and human body systems (1-4). Some studies indicate that the increased prevalence of these diseases is related to the excess of proteins obtained from dairy products or from dietary supplementation with soluble proteins of milk (whey protein), which are frequently present in modern diet. These foods and supplements are rich in Leucine (Leu), a branched-chain amino acid (BCAA) that acts on growth regulation, protein biosynthesis and cellular metabolism (5).

In this way, Leu has been gaining importance for playing a key role in signaling metabolic pathways that are nutrient-sensitive, such as mTOR (*mammalian target of rapamycin*) (6, 7). However, studies show that the excess of Leucine may cause a superactivation of this pathway, leading to the senescence of β-pancreatic cells, excess of adipose tissue and disordered protein synthesis. These conditions, observed *in vitro and in vivo*, mimic clinical metabolic alterations related to the insulin signaling and to the cellular growth, which in humans can lead to both type 2 diabetes mellitus and cancer (8-10).

The Leu effects have been thoroughly studied in several types of cells. Leu has many functions, such as stimulating insulin release and regulating gene expression and protein sintesis. Studies in vitro and in vivo, have shown that Leu is capable to estimulate the proliferation and diferentiation of liver, muscle, pancreatic and adipose cells. However, most studies analyze the effects of this amino acid at basal conditions, and the effects of excess are still unclear (8-11).

Analyse the effects of this nutrient on the bone cells is important, because Leu is the main amino acid present in the from dairy products, which constitutes an important group of foods related to bone health (12, 13). Beside of this, surprisingly, there is a limited evidence about the effects of Leu supplementation on bone tissue cells. In this way, studies in vitro, with cell

lineages, as a MC3T3-E1 cells, are usefull to understand the underlying mechanisms in this process (14).

Thus, considering the role of Leu as an important nutrient for cell growth and due to an unknown effects on bone tissue, in the present work, we aim analyze the effects of Leu supplementation on the proliferation of pre-osteoblasts MC3T3-E1 cell *in vitro*.

2 MATERIALS AND METHODS

2.1 Cell culture

The MC3T3-E1 cells consist of a pre-osteoblastic cell lineage, which has been established from C57BL/6 mouse calvaria (14). The cells were kindly donated by the University of Barcelona. MC3T3-E1 cells were kept in Alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimitotic (penicillin + streptomycin). The pre-osteoblasts were kept in an incubation cabinet at 37ºC in a humid atmosphere with 5% CO2. The culture was performed in 24-well plates, with 15.6mm diameter, 1.900mm^2 area, 600µL of medium and 20.000 cells/plate. All experiments were performed at least three times and with triplicate samples.

2.2 MC3T3-E1cell treatment

Initially, the cells were treated during 48 hours, after the complete cellular adhesion to the culture plate, by the addition of 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 µM Leu (All Chemistry / Brazil) diluted in Alpha medium (Gibco) containing 10% FBS, which corresponds to 1.56 at 50% in addition of the amino acid to the culture medium, considering that the Leucine concentration in the α-MEM is 396µM. Untreated cells represented the control group. Also, the cells were treated with other amino acids (L-Valine, L-Glutamine, L-Glycine) at a concentration of 50 µM for 48 hours. The effect of the Leucine supplementation on the proliferation of MC3T3-E1 cells was compared to the well-known effect of other substances in reducing or increasing cellular growth, specifically through the mTOR pathway. Thus, insulin (200nM) has been used as a positive control of growth and rapamycin (10nM) as a negative control (7).

2.3 Evaluation of proliferation and cellular viability

The evaluation of the viability and proliferation of cultured cells was analyzed by means of vital dye Trypan Blue (0.4%). For the essays, freshly obtained cells with viability higher than 95% were used. The number of viable cells was determined by using a Neubauer chamber (15).

2.4. Analysis of cytotoxicity

The assessement of a potential cytotoxicity of the Leucine was performed by determining Lactate Dehydrogenase (LDH) content in the supernatant of the cell culture (LDH/SigmaPharma). The LDH is an oxidoreductase enzyme that catalyzes the interconversion of lactate and pyruvate, a cytotoxic compound that compromises cell membrane integrity by inducing necrosis. When the treatment used induces damage to the membrane, LDH is released into the cell medium, so the enzyme is used as a marker to evaluate the presence of tissue or cell damage (16).

2.5 Analysis of apoptosis

MC3T3-E1 cells were incubated in the presence of Leu (50 μM) and Rapa (10nM) for 48 h. Untreated cells represented the control group. Apoptosis was evaluated by flow cytometry using Annexin-V Kit assay. Annexin-V and 7-AAD were added to 1 × 105 cell suspension according to the manufacturer's instructions (BD Biosciences) and then incubated for 15 min at room temperature in the dark. Subsequently 2 × 104 cells were analyzed by flow cytometry within 1 h. Early apoptotic cells were stained with Annexin V alone, whereas necrotic cells and late apoptotic cells were stained with both Annexin V and 7-AAD (17). Data were analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA).

2.6 Analysis of the oxidative stress

The evaluation of the cell oxidative stress was performed through the measurement of thiobarbituric acid reactive substances (TBARS). The supernatant was used to determine the concentration of TBARS. This method evaluates the oxidative damage on the cell membrane and is based on the reaction of two thiobarbituric acid molecules with one of malondialdehyde, quantitated by a spectrophotometer reading at 535nm. The results were

adjusted for the protein concentration in each sample and presented in nM by mg of protein. As a positive control for the oxidative damage, a 100μM hydrogen peroxide (H_2O_2) was used (18).

2.7 *Analysis of the Transforming Growth Factor-β1*

The transforming growth factor-beta (TGF-β1) constitutes in a molecule with potent immunoregulatory properties involved in several physiologic and pathophysiologic functions, including its involvement in the mTOR pathway (19). TGF-β1 was evaluated through an immunoenzymatic assay (ELISA kit R&D Systems, USA), measured in the cell supernatant (20). The kit contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings to remove unbound substances and antibodies, a substrate solution was added to the wells. Color development was stopped by sulfuric acid and optical density was determined at 540 nm with the correction wavelength set at 570 nm in an ELISA plate reader. Results were calculated on a standard concentration curve and TGF-β1 levels were expressed as picograms per milliliter.

2.8 *Evaluation of cell differentiation markers*

Collagen and alkaline phosphatase were used as cell differentiation markers of preosteoblasts (21). Collagen content in MC3T3-E1 cells was measured using Picro-Sirius Red. Briefly, Picro-Sirius Red was added to cell supernatant to form a collagen–dye complex. After centrifugation, unbound dye was removed, and the collagen–dye complex was dissolved in NaOH. The absorbance was measured at 540nm in an ELISA plate reader. Each sample was normalized to the relative amount of total protein measured by the Bradford method (22). Results were expressed as percentage of control.

The analysis of ALP activity was performed using a commercial method (Labtest Diagnóstica) and US-treated group was compared to controls after 48, 96 and 192 hours. Cell lysates were incubated in a buffer glycylglycine, p-nitrophenyl phosphate, at 37ºC for 2 hours. This methodology is based on the hydrolysis of p-nitrophenyl that releases p-nitrophenol and inorganic

phosphate. Data obtained were expressed as ALP activity normalized by total protein content. (23).

2.9 Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to determine the normality of the data. Differences between samples were analyzed by using a one way analysis of variance (ANOVA), followed by the Tukey post-hoc test. Student's t-test was used for group comparisons. Statistical analyses were performed using *Prism GraphPad 5.0 (United States)*. The level of significance was set at 5%.

3 RESULTS

3.1 Leucine supplementation reduces the proliferation of pre-osteoblasts MC3T3-E1 cells

Leu is an essencial amino acid, known as an important cellular growth agent, which acts on the activation of proliferative pathways, such as the mTOR pathway, in different types of cells (11, 24, 25). However, when Leu was added to the culture medium of MC3T3-E1 cells for 48 hours it induced a reduction in the cell proliferation (Fig.1). When compared to the control, we have observed a decrease in the growth of cells treated with Leu at concentrations of 50, 100 and 200μM and no significant changes were observed at lower concentrations. These results indicate that the excess of Leu (values higher than 12.5% above basal levels) may reduce the pre-osteoblasts cell proliferation.

Aiming at identifying if the reduction on cell proliferation was attributed only to Leu, cells were treated with other amino acids, during the same period, with the same minimum dose in which the anti-proliferative effect occured (50μM). The results showed that the reduction on cell proliferation only occurred when cells were treated with Leu (Fig.2).

3.2 Cell proliferation reduction caused by Leucine supplentation is not related to necrosis, apoptosis or oxidative stress

The supplementation of Leu into the culture medium did not present cytotoxicity at concentrations of 50 μM and 100 μM. However, the percentage

of LDH produced by the cells supplemented with 200 μM of the amino acid was higher, indicating that the decrease of the proliferative activity was due to the increase of cell death, as a result of necrosis (Fig.3).

Once the possibility of the Leucine supplementation being toxic at concentrations of 50 μM and 100 μM was ruled out, we seeked to test other hypothesis to explain the cell proliferation decrease. By quantitatively determining the percentage of cells marked with Annexin V, evaluated through flow cytometry, no apoptosis increase in cells treated with Leu was identified (Fig.4). The flow cytometry diagrams also confirm the previous finding indicating that the cells did not die because of necrosis, once the percentage of marked cells in the left upper quadrant represents an average of just 1.36% of the total cell population. Based on these results and after checked (with pH tape) that Leu supplementation did not alter the pH of the culture medium, the concentration of 50 μM was chosen in order to perform further experiments with Leu supplementation.

Aiming at verifying if the decrease of MC3T3-E1 cell proliferation was caused by the increase in the oxidative stress, the TBARS levels were quantified, since it is an important marker that evaluates the lipid peroxidation levels and the subsequent cellular damage as a consequence of reactive oxygen species. As a positive control for the oxidative stress, we have used hydrogen peroxide (H_2O_2) for the same period of treatment (48 hours). As shown, Leucine at 50μM concentration did not increase the TBARS levels, a result opposing to that observed when cells were treated with H_2O_2 , which presented significantly high oxidative stress levels (Fig.5).

3.3 Leucine reduces pre-osteoblast proliferation through a mTOR-independent pathway

Once the possibilities of cellular proliferation reduction by cell death as a result of necrosis, apopotosis and oxidative stress were discarded, other mechanisms involving pathways of cell growth inhibition were investigated. Rapamycin (Rapa) (10 nM) was used considering its well-known effects on the decrease of cell proliferation, which occurs through the inhibition of the mTOR pathway (11, 26). We have also used insulin (Ins) (200 nM) as a

positive control for both cell growth and mTOR activation. As shown in Fig.6, the Leu supplementation (50 μM) alone could suppress cell proliferation at the same level of Rapa. However, this effect was not potencialized when Leu was administered together with Rapa, as only a difference in comparison to the control group was seen. The same inhibitory effect on cell proliferation was observed in the cells treated with Rapa associated with Ins, indicating that even in the presence of a potent mTOR activator (insulin), the suppressing effect of Rapa is more robust on this pathway, decreasing cell proliferation. On the other hand, the suppressing effect of Leu on cell proliferation was not maintained when associated to Ins. As expected, Ins alone increased cell proliferation. Based on these results, we can infer that the antiproliferative Leu effect on pre-osteoblast, seems not to involve the mTOR inhibition, once the amino acid was not able to block the proliferative effect of Ins, as demonstrated for Rapa.

Indeed, Fig.7 presents data on TGF-β1 quantification, an anti-inflammatory marker involved in the mTOR pathway. A significant TGF-β1 increase was demonstrated in cells treated with Rapa, a positive control for the technique. However, when the pre-osteoblast cells were treated with Leu, the production of TGF-β1 did not change as compared to the control group.

3.4 Leucine supplementation does not change differentiation markers in pre-osteoblasts

The collagen is an important marker of pre-osteoblasts proliferation and differentiation phases. In the initial phases of proliferation, collagen is produced in great quantities, but when the cells start the differentiation activity, collagen is produced in smaller quantities, reaching minimum levels in the mineralization phase. The results of present study indicate that Leu was not able to increase collagen production, unlike Rapa, which induced a significant increase (Fig.8).

In order to identifying if the early differentiation can decreased the proliferation of cells treated with Leu, we have also evaluated the alkaline phosphatase production, a differentiation marker of pre-osteoblasts.

Nevertheless, there was no significant difference between the Leu group and intreated group (Fig. 9).

4 DISCUSSION

In this study, we have showed that Leu supplementation reduces the proliferation of MC3T3-E1 pre-osteoblast cells. The initial hypothesis of the study, that Leucine would increase cell proliferation through the activation of mTOR (5, 11, 25), was not confirmed. On the other hand, the antiproliferative effect observed was seen exclusively in cells treated with Leu, indicating that this amino acid is involved in some specific mechanism of cell growth inhibition. Aiming to identify the potential causes of the decreased cell proliferation, we have investigated the toxicity levels of Leu supplementation on these cells, as well as the apopptosis percentage and the cell damage caused by oxidative stress.

In order to assess cellular necrosis through cytotoxicity, we evaluated the cellular release of LDH into the supernatant. We have observed that the dose of 200μM (which represents more than 50% above normal levels) increased significantly the concentration of this enzyme in the supernatant, indicating that at doses of 50μM and 100 μM, the antiproliferative effect was not associated to cytotoxicity. It is possible that the cytotoxic effect observed at 200μM is associated to a decrease of ph in the culture medium. The acidification of the culture medium, caused by the excess of amino acids protons (hydrogen ions), can reduce osteoblast proliferation, differentiation and mineralization. This effect is related to an attempt of the cells to buffer the acid pH, releasing minerals such as sodium, potassium, bicarbonate and calcium into the culture medium, which play an important role in these processes (27).

One of the most important effects that can reduce cell proliferation was also investigated. The programmed cellular death or apoptosis is a process genetically controlled that plays an important role in cellular homeostasis, being an important defense mechanism to remove cells that have been infected, damaged or mutated (28, 29). Even so, apoptosis seems not to be related to the cell proliferation decrease after treatment with Leu, considering

that there was no statistically significant differences in the percentage of apoptotic cells between the groups.

Another hypothesis that could possibly explain the cell proliferation decrease would be related to an oxidative stress increase. During oxidative stress, the cellular production of free radicals and reactive oxygen species exceeds the physiologic capacity of the antioxidant defense system in removing these compounds (30). A greater quantity of amino acids in the culture medium can increase the expression of L-type amino acid transporter-1 (LAT-1), responsible for the entry of Leu into the cells. The increased expression of these transporters enhances the influx of this amino acid into the cell and, consequently, increases the glutamate efflux out of the cell (31-33). A lower concentration of intracellular glutamate reduces the production of glutathione, what impacts negatively the antioxidant protection of the cell. These events increase the oxidative stress and reduce the proliferative activity of MC3T3-E1 cells (34-36). Even so, such hypothesis was not confirmed, considering that Leu was not capable of increasing TBARS production, unlike demonstrated for the cells treated with H_2O_2.

As previously described, Leu is able to specifically activate the complex 1 of the mammalian target of rapamycin (mTORC1) (7). mTOR pathway is activated as a response to hormonal stimuli (insulin), growth factors (IGF-1) or nutrients (glucose and amino acids). On the other hand, this pathway is inhibited through the action of Rapa, an anticancer and antidiabetes agent, known for its potent antiproliferative effect on different types of cells and also upon MC3T3-E1 cells, in which no toxicity with dosages of 0.1 to 20nM was demonstrated. This effect is directly related to the inhibitory effect of the drug upon the complex 1 of mTOR (mTORC1), which inactivates the PI3K/AKT pathway, blocking the phosphorylation of p70S6K and, consequently, of all cell growth cascade (26).

Our results showed that the decrease of pre-osteoblast cell proliferation, caused by Leu supplementation, was through a mTOR-independent pathway, once the amino acid could not block the insulin proliferative effect, as shown for Rapa. Results observed in studies with cell culture showed that treatment with Leu for over 20 minutes induces a decrease in the activity of P70S6K, a

serine/treonine kinase that indicates the activity of the mTOR (37). This indicates that in chronic conditons the Leu supplementation may not exert the same effect on the activation of mTOR and, as a consequence, on the cell proliferation. Also, another hipotesis could be related to an over-activation of mTOR (24). Leu was not capable to inhibit this pathway, as expected, but, accumuling evidence supports that this amino acid plays an important role in deseases related to mTOR, such as diabetes and cancer. Maximal mTOR stimulation leads to increased cell proliferation. However, persistently over-stimulated proliferation holds the risk of early senescence (38, 39).

We have also investigated inflammatory parameters that could be involved with cell proliferation pathways, as potential mechanisms involved in the reduction of pre-osteoblasts proliferation. The TGF-β1 is increased when the mTOR is inhibited by Rapa. Once increased, the TGF-β1 is able to stop the cell cycle, impacting negatively on the cell proliferation (19). In our study, when the mTOR was inhibited due to the Rapa action, we observed TGF-β1 levels three times higher than in those cells treated with Leu. The incapacity of Leu to increase the levels of TGF-β1 reinforce the idea that the MC3T3-E1 cell proliferation decrease is not associated to the inhibition of the mTOR.

TGF-β1 is also involved in the production of type I collagen, an important marker of the pre-osteoblats proliferation phase . In a study with MC3T3-E1 cells, an increased concentration of TGF-β1 was able to inhibit cell proliferation, differentiation and mineralization. On the other hand, it increased type I collagen levels, which seems to be a normal effect in MC3T3-E1 cells (40). In the present study, in cells treated with Leu for 48 hours, we were not able to detect a TGF-β1 production increase nor a change in collagen levels, which did not present significant differences in comparison to the control. Also, when data on alkaline phosphatase was analysed, which is another important pre-osteoblast differentiation marker, we did not observe significant differences in Leu group compared to the control group. These results suggest that, in these experimental conditions, Leu does not induce cell differentiation, which indicates that this was not responsible for the MC3T3-E1 cell proliferation decrease, as previously documented (41).

5 CONCLUSIONS

In conclusion, the results indicate that the supplementation with Leuc reduced the pre-osteoblast proliferation due to causes not related to necrosis, apoptosis or oxidative stress. Early differentiation was also ruled out as a cause for the cell proliferation deacrease, since we were not able to identify its markers in the cells treated with the amino acid. Our study showed that Leu induces a negative impact on the bone cell proliferation with mechanisms that do not involve cell proliferation pathways inhibition, as the mTORC1 pathway. These results may lead to new perspectives on the effects of the excess of nutrients on bone health. Considering the increased prevalence of conditions related to the excess of nutrients and the inadequate food intake of modern diet, further studies on the subject are still necessary.

6. REFERENCES

1. Michaelsen KF, Larnkjær A, Mølgaard C. Amount and quality of dietary proteins during the first two years of life in relation to NCD risk in adulthood. Nut, Metab Cardiovasc Dis. 2012;22(10):781-6.
2. Yang Z, Huffman SL. Nutrition in pregnancy and early childhood and associations with obesity in developing countries. Matern Child Nutr. 2013;9(S1):105-19.
3. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006;3(6):393-402.
4. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. Journal Clin Endocrinol Metab. 2013;98(6):E1060-E5.
5. Melnik BC. Excessive leucine-mTORC1-signalling of cow milk-based infant formula: the missing link to understand early childhood obesity. J Obes. 2012.
6. Torres-Leal FL, Vianna D, Teodoro GFR, Capitani MDd, Tirapegui J. Aspectos atuais do efeito da leucina sobre o controle glicêmico e a resistência à insulina. Rev Soc Bras Aliment Nutr. 2010;35(2).
7. Tato I, Bartrons R, Ventura F, Rosa JL. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. J Biol Chem. 2011;286(8):6128-42.
8. Nicastro H, Zanchi NE, da Luz CR, de Moraes WM, Ramona P, de Siqueira Filho MA, et al. Effects of leucine supplementation and resistance exercise on dexamethasone-induced muscle atrophy and insulin resistance in rats. Nutrition. 2012;28(4):465-71.
9. Rachdi L, Aïello V, Duvillié B, Scharfmann R. L-leucine alters pancreatic β-cell differentiation and function via the mTor signaling pathway. Diabetes. 2012;61(2):409-17.
10. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146(3):1328-37.
11. Chong ZZ, Maiese K. Mammalian target of rapamycin signaling in diabetic cardiovascular disease. Cardiovasc Diabetol. 2012;11(1):45.
12. Conigrave A, Brown E, Rizzoli R. Dietary protein and bone health: roles of amino acid-sensing receptors in the control of calcium metabolism and bone homeostasis. Annu Rev Nutr. 2008;28:131-55.
13. Pereira PC. Milk nutritional composition and its role in human health. Nutrition. 2014;30(6):619-27.
14. Sudo H, Kodama H-A, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 1983;96(1):191-8.

15.Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2001:A3.B.1-A3.B.

16. Chan FKM, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. Immune Homeostasis: Methods and Protocols. 2013:65-70.

17. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. J Vis Exp. 2011(50):e2597-e.

18. Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. Analyst. 2002;127(1):183-98.

19. Chatterjee A, Mukhopadhyay S, Tung K, Patel D, Foster DA. Rapamycin-induced G1 cell cycle arrest employs both TGF-β and Rb pathways. Cancer Lett. 2015;360(2):134-40.

20. Khan SA, Joyce J, Tsuda T. Quantification of active and total transforming growth factor-β levels in serum and solid organ tissues by bioassay. BMC Res Notes. 2012;5(1):636.

21. Raouf A, Seth A. Ets transcription factors and targets in osteogenesis. Oncogene. 2000;19(55):6455-63.

22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54.

23. Sabokbar A, Millett P, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone Min. 1994;27(1):57-67.

24. Melnik BC. Leucine signaling in the pathogenesis of type 2 diabetes and obesity. World J Diabetes. 2012;3(3):38-53.

25. Luo Z, Saha AK, Xiang X, Ruderman NB. AMPK, the metabolic syndrome and cancer. Trends Pharmacol Sci. 2005;26(2):69-76.

26. Singha UK, Jiang Y, Yu S, Luo M, Lu Y, Zhang J, et al. Rapamycin inhibits osteoblast proliferation and differentiation in MC3T3- E1 cells and primary mouse bone marrow stromal cells. J Cell Biochem. 2008;103(2):434-46.

27. Nicoll R, Howard JM. The acid–ash hypothesis revisited: a reassessment of the impact of dietary acidity on bone. J Bone Miner Metab. 2014;32(5):469-75.

28. Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: cytometry in studies of programmed cell death. Methods Cell Biol. 2011;103:55.

29. Smith BA, Smith BD. Biomarkers and molecular probes for cell death imaging and targeted therapeutics. Bioconjug Chem. 2012;23(10):1989-2006.

30. Bloomer RJ, Goldfarb AH. Anaerobic exercise and oxidative stress: a review. Can J Appl Physiol. 2004;29(3):245-63.

31. Poncet N, Taylor PM. The role of amino acid transporters in nutrition. Curr Opin Clin Nutr Metab Care. 2013;16(1):57-65.

32. Taylor PM. Role of amino acid transporters in amino acid sensing. Am J Clin Nutr. 2014;99(1):223S-30S.

33. Kim SG, Kim HH, Kim HK, Kim CH, Chun HS, Kanai Y, et al. Differential expression and functional characterization of system L amino acid transporters in human normal osteoblast cells and osteogenic sarcoma cells. Anticancer Res. 2006;26(3A):1989-96.

34. Uno K, Takarada T, Hinoi E, Yoneda Y. Glutamate is a determinant of cellular proliferation through modulation of nuclear factor E2 p45- related factor- 2 expression in osteoblastic MC3T3- E1 cells. J Cell Physiol. 2007;213(1):105-14.

35. Arakaki N, Yamashita A, Niimi S, Yamazaki T. Involvement of reactive oxygen species in osteoblastic differentiation of MC3T3-E1 cells accompanied by mitochondrial morphological dynamics. Biomed Res. 2013;34(3):161-6.

36. Yang Y-H, Li B, Zheng X-F, Chen J-W, Chen K, Jiang S-D, et al. Oxidative damage to osteoblasts can be alleviated by early autophagy through the endoplasmic reticulum stress pathway—implications for the treatment of osteoporosis. Free Radic Biol Med. 2014;77:10-20.

37. PEYROLLIER K, HAJDUCH E, BLAIR AS, Russell H, HUNDAL HS. L-leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. Biochem J. 2000;350(2):361-8.

38. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 2004;429(6987):41-6.

39. Teta M, Rankin MM, Long SY, Stein GM, Kushner JA. Growth and regeneration of adult β cells does not involve specialized progenitors. Dev Cell. 2007;12(5):817-26.

40. Sowa H, Kaji H, Yamaguchi T, Sugimoto T, Chihara K. Smad3 Promotes Alkaline Phosphatase Activity and Mineralization of Osteoblastic MC3T3- E1 Cells. J Bone Min Res. 2002;17(7):1190-9.

41. Yano M, Inoue Y, Tobimatsu T, Hendy GN, Canaff L, Sugimoto T, et al. Smad7 inhibits differentiation and mineralization of mouse osteoblastic cells. Endocr J. 2012;59(8):653-62.

FIGURE 1:

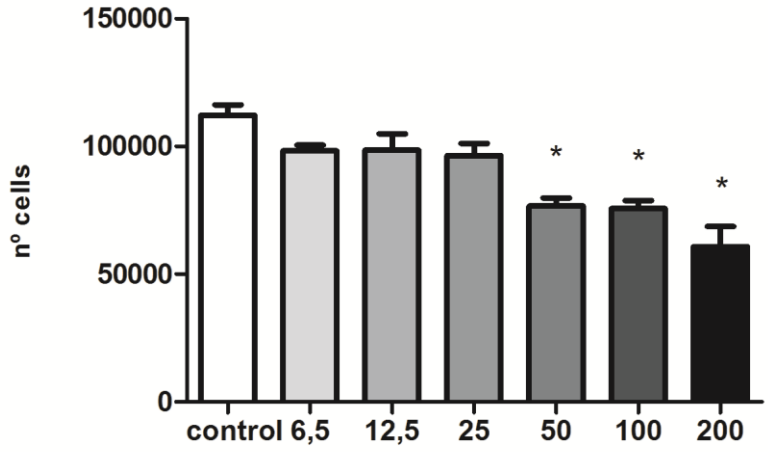

Fig. 1 Effects of Leucine supplementation on the proliferation of pre-osteoblast MC3T3-E1: cells were treated for 48 h with different concentrations of Leucine (6.5μM, 12.5μM, 25μM, 50μM, 100μM and 200μM). A plateau was seen from concentration 6.5 until 25μM. The evaluation of cell proliferation and viability was performed by Trypan Blue (0.5%) staining and counting in a Neubauer chamber. (*$p<0.05$, one-way ANOVA followed by Tukey multiple comparison test).

FIGURE 2:

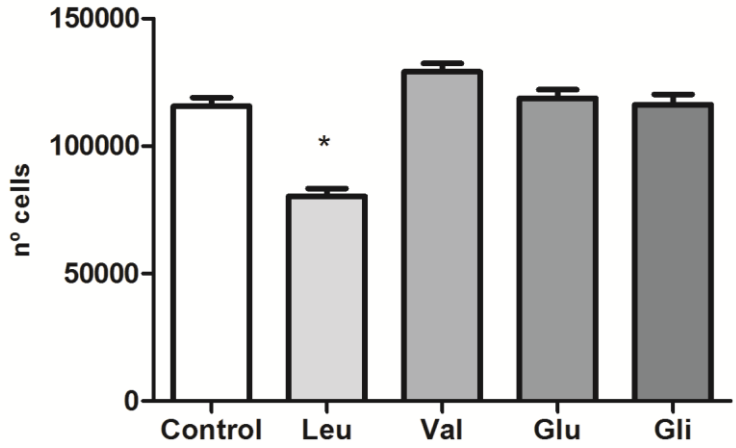

Fig. 2 Effects of supplementation with Leucine, Valine, Glutamine and Glycine on the proliferation of pre-osteoblast MC3T3-E1 lineage. Cells were treated for 48h with 50μM of each amino acid. Leucine was the only one able to decrease cell proliferation. The evaluation of cell proliferation and viability was performed by Trypan Blue (0.5%) staining and counting in a Neubauer chamber. (*$p<0.05$, one-way ANOVA followed by Tukey multiple comparison test).

FIGURE 3:

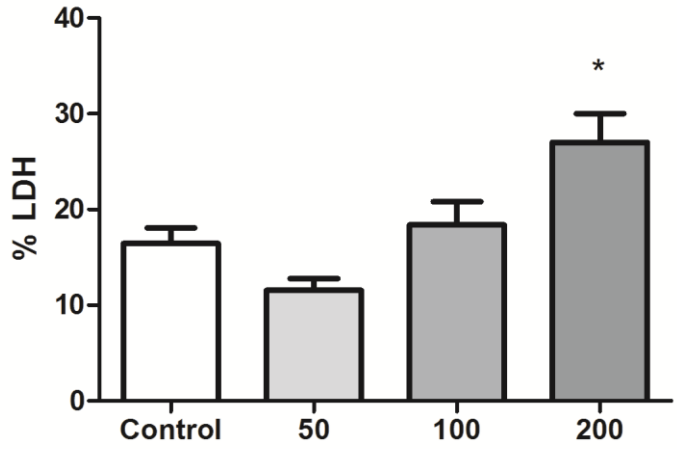

Fig. 3 Effects of Leucine supplementation on necrotic cell death: the cells supplemented with Leucine for 48 hours at concentrations of 50μM and 100μM showed no cytotoxicity according to the Lactate Dehydrogenase (LDH) levels. The difference was statistically significant only at the concentration of 200μM. (*$p<0.05$, one-way ANOVA followed by Tukey multiple comparison test).

FIGURE 4:

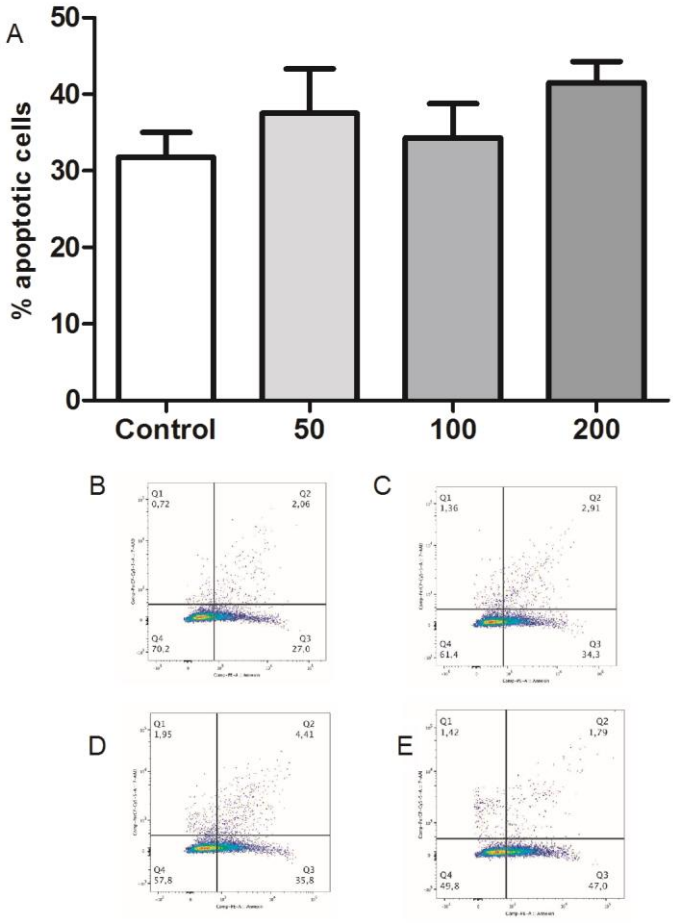

Fig. 4. Effects of Leucine supplementation on the cellular death by apoptosis: the MC3T3-E1 cells were treated for 48 hours with Leucine at concentrations of 50μM, 100μM and 200μM and showed no significant differences between groups. The evaluation of apoptotic cells was performed by flow cytometry with Propidium Iodide and Annexin V Apoptosis Detection Kit I. The cells labeled with Annexin V, visible in the lower right quadrant indicate the percentage of apoptotic cells. There was no difference between the groups as shown in the flow citometry graphs.

FIGURE 5:

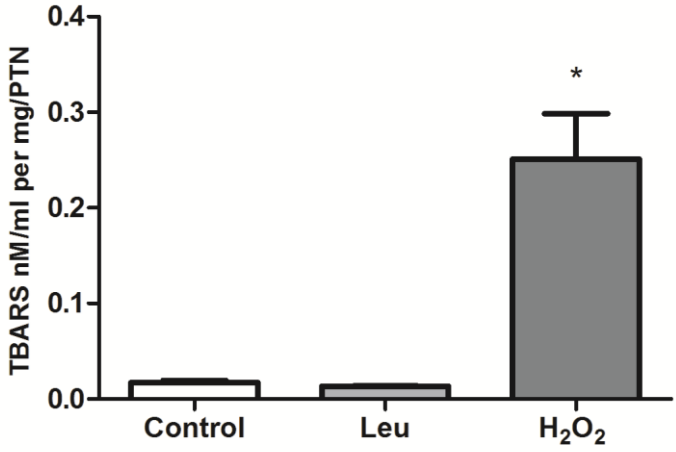

Fig. 5 Effects of Leucine supplementation on oxidative stress: MC3T3-E1 cells were treated for 48 hours with 50μM of Leucine. As a positive control to oxidative stress, H_2O_2 was used in a concentration of 100μM. When compared to the control, cells treated with Leucine showed no changes in the levels of TBARS, with the only statistically significant difference showed in the H_2O_2 treatment group. (*$p<0.05$, one-way ANOVA followed by Tukey multiple comparison test).

FIGURE 6:

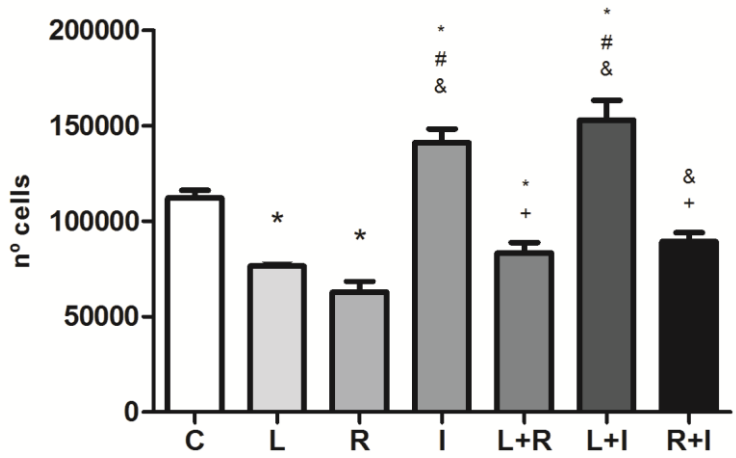

Fig. 6 Effects of rapamycin and insulin on cell proliferation of pre-osteoblasts: cells were treated for 48h with 50µM of Leucine, 10nM of rapamycin and 200nM of insulin. The evaluation of cell proliferation was performed by Trypan Blue (0.5%) staining and counting in a Neubauer chamber. ($p<0.05$ when * vs control, # vs leucine, & vs rapamycin, + vs insulin; one-way ANOVA followed by Tukey multiple comparison test). C: control; L: leucine; R: Rapamycin; I: insulin; L+R: Leucine plus Rapamycin; L+I: leucine plus insulin; R+I: rapamycin plus insulin.

FIGURE 7:

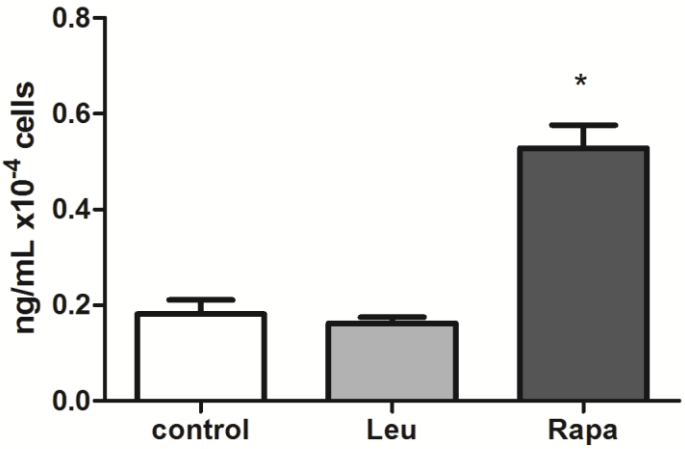

Fig. 7 Effects of Leucine supplementation and rapamycin on TGF-β production in pre-osteoblasts: MC3T3-E1 cells were treated for 48 hours with 50μM of Leucine and 10nM of rapamycin. Rapamycin treated cells showed a statistically significant difference (*$p<0.05$) in the production of TGF-β when compared to the control and the Leucine treatment group. (One-way ANOVA followed by Tukey multiple comparison test).

FIGURE 8:

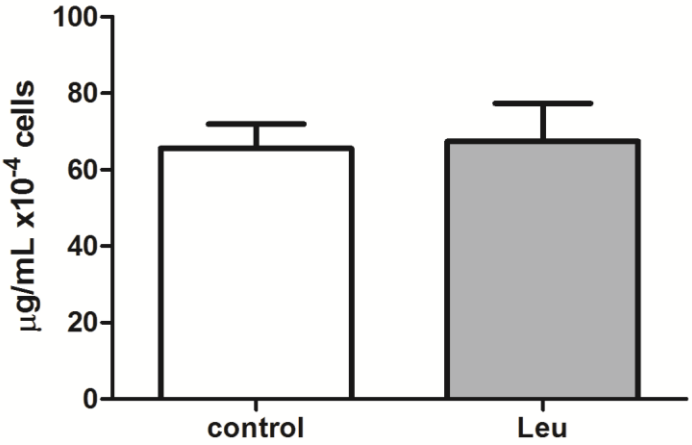

Fig. 8 Effects of Leucine supplementation on collagen production in pre-osteoblasts: MC3T3-E1 cells were treated for 48 hours with Leucine at 50µM. There was no significant difference in collagen production when cells were treated with Leucine compared to control. Student t test ($p<0.05$)

FIGURE 9:

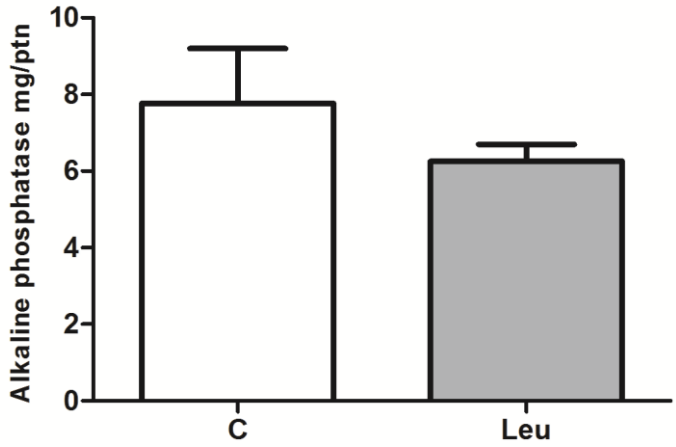

Fig. 9 Effects of Leucine supplementation on alkaline phosphatase production in pre-osteoblasts: MC3T3-E1 cells were treated for 48 hours with Leucine at 50μM. There was no statistically significant difference ($p<0.05$) in the alkaline phosphatase activity, when the treatment group was compared to the control. Student *t Test*.

CAPÍTULO III

ARTIGO ORIGINAL

Leucine supplementation promotes DNA damage and cell senescence in pre-osteoblasts[c]

[c] Artigo ainda não submetido.

TITLE: Leucine supplementation promotes DNA damage and cell senescence in pre-osteoblasts

AUTHORS:

Raquel da Luz Dias

Doctoral student of the Graduate Program in Medicine and Health Sciences of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Bruno Basso

Master´s student of the Graduate Program in Molecular and Cellular Biology of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Henrique Dias Bregolin

Doctoral student of the Graduate Program in Molecular and Cellular Biology of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Gabriele Krause

Doctoral student of the Graduate Program in Molecular and Cellular Biology of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Cassiana Viau

Doctoral student of Federal University of Health Sciences of Porto Alegre – UFCSPA/Brazil

Dra. Jenifer Saffi

Professor at the Federal University of Health Sciences of Porto Alegre – UFCSPA/Brazil

Dra. Fernanda Bordignon Nunes

Professor at the Laboratory of Biophysics and Inflammation of the of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Dr. Márcio Vinícius Donadio

Full Professor at the Laboratory of Biophysics and Inflammation of the of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Dr. Francesc Ventura Pujol

Full Professor at the Department of Physiological Sciences II of the University of Barcelona/Spain

Dr. José Luis Rosa

Full Professor at the Department of Physiological Sciences II of the University of Barcelona/Spain

Dr. Ramón Bartrons

Full Professor at the Department of Physiological Sciences II of the University of Barcelona/Spain

Dr. Jarbas Rodrigues de Oliveira

Full Professor at the Laboratory of Biophysics and Inflammation of the of the Pontifical Catholic University of Rio Grande do Sul – PUCRS/Brazil

Conflict of interest: The authors declare that they have no conflicts of interest.

Research involving Human Participants and/or Animals: The authors state that the research does not involve human participants or animals.

ABSTRACT

Purpose: The Leu effects have been studied in several types of cells, but, yet little is known about its action upon bone cells. In the present work, we extended the investigations of the effects caused by leucine supplementation in MC3T3-E1 cells, analyzing the in vitro effect on oxidative stress, inflammatory profile, autophagy, senescence and DNA damage.

Materials and methods: the MC3T3-E1 cells were kept in α-MEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimitotic (penicillin + streptomycin). The treatment was did by addition of 50 μM Leu. Untreated cells represented the control group. The evaluation of the viability and proliferation of cultured cells was performed with dye Trypan Blue (0.4%). To identify the mechanisms related to decreased cellular proliferation, assays were performed to assess oxidative stress (DCFH); inflammation (CBA); autophagy (acridine orange and flow cytometry); senescence (DAPI and flow cytometry); DNA damage (alkaline comet assay).

Results and conclusions: Leu supplementation (50 μM) decreases cell proliferation by 40% and it was not capable to induce oxidative stress, inflammation or autophagy. The Leu supplementation caused DNA damage, with consequent increase in senescence and decrease of cell proliferation in MC3T3-E1 cells.

Key-words: Leucine, osteoblasts, cell proliferation, senescence

1 INTRODUCTION

The overload of nutrients is a reality in modern society, where nutrition-related diseases such as obesity, diabetes and cancer are increasingly prevalent (1). Many times the concern about the excessive exposure relates only to carbohydrates and fats, while protein is seen only as an important nutrient for the composition of the lean body mass. High protein diets, based on animal food, have been widely used by individuals of all ages, usually with the aim of weight loss (2, 3). Another common practice is the amino acid supplementation, which it is made often without any precautionary or knowledge about the nocive effects to the human body. The most popular amino acids supplementation, among the amino acids, it is the leucine (Leu), an essential and branched chain amino acid, responsible for play an important role in signaling metabolic and proliferation pathways sensitive to nutrients, such as mammalian target of rapamycin (mTOR) pathway (4, 5).

The Leu effects have been thoroughly studied in several types of cells. In adipocytes, overactivation of these pathways, generated by excess nutrients, facilitates the differentiation and accumulation of fat, which results in increased lipid biogenesis (6). Leu supplementation in pancreatic β-cells culture, prevents their development, by increasing expression of hypoxia-related factors, which repress the development of endocrine progenitor cells. In a clinical perspective, this evidence indicates that Leu supplementation increases the risk for development of insulin resistance, diabetes and cancer (7). However, there is still limited evidence on its actions upon bone tissue cells.

Bone health depends on adequate supply of nutrients, which act in the growth and maintenance of organic and inorganic matrix. The amino acids play a crucial role in the proliferative phase of this tissue, contributing to the formation of protein network of bone organic matrix (8). Given the central role of the amino acids in proliferation process and as a structural component of bones and that leucine is the major amino acid found in dairy, which usually related to bone health, new insights into the impact of excess of this nutrient on the growth and maintenance of the skeleton arose (9). In this sense, *in*

vitro studies are useful for identifying the effects and possible mechanisms involved in this process.

Previous results of our research group (yet to be published) have showed in the, that Leu supplementation reduces the proliferation of a pre-osteoblast cells due to causes not related to necrosis, apoptosis, plasma membrane damage by oxidative stress, early differentiation and mTOR inhibition. Recent studies suggest that the excess of nutrients can decrease cell proliferation by autophagy or senescence (10, 11). Both processes can be triggered by increased oxidative stress or a change in the cellular inflammatory profile (12, 13).

Autophagy is an important mechanism of cell death and occurs when the cell needs recycles its own organelles and nonessential macromolecules, which are redundant or damaged. It is an adaptative response to stress which plays an important role in the deletion of toxic unfolded proteins, elimination of intracellular microorganisms and even in tumor suppression. Overfeeding induces autophagy by increasing the reticulum endoplasmic stress, by Unfolded Protein Response (UPR) pathway and in an attempt of the cell to correct possible errors in the tertiary or quaternary protein configuration (10, 14).

On the other hand, cell senescence occurs as a stress response, which can be activated by several mechanisms, such as oxidative stress, DNA damage and telomere shortening. In vitro, senescence process is manifested as a permanent cell cycle arrest resulting from a exhaustion of the proliferative capacity of the cells. Although decreasing the proliferative capacity, the senescent cells remain viable and metabolically active, featuring remarkable morphological changes and becoming large, flat and multinucleated (15). *In vitro* and *in vivo* studies, with cells from individuals with altered metabolism of Leu, shows that the excess of this amino acid is potentially toxic and provides DNA damage (16, 17).

In this way, in order to better understand the effects that Leu excess can exert on the bone, in the present work, we extended the investigations of the antiproliferactive effects caused by Leu supplementation in MC3T3-E1

cells, analyzing the in vitro effect on oxidative stress, inflammatory profile, autophagy, senescence and DNA damage.

2 MATERIALS AND METHODS

2.1 *Cell Culture*

The MC3T3-E1 cells consist of a pre-osteoblastic cell lineage, which has been established from C57BL/6 mouse calvaria (18). The cells were kindly donated by the University of Barcelona. MC3T3-E1 cells were kept in Alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimitotic (penicillin + streptomycin). The pre-osteoblasts were kept in an incubation cabinet at 37ºC in a humid atmosphere with 5% CO2. The culture was performed in 24-well plates, with 15.6mm diameter, 1.900mm^2 area, 600µL of medium and 20.000 cells/plate. All experiments were performed at least three times and with triplicate samples.

2.2 MC3T3-E1 cell treatment

The cells were treated during 48 hours, after the complete cellular adhesion to the culture plate, by the addition of 50 uM leucine (All Chemistry / Brazil) diluted in Alpha medium (Gibco) containing 10% FBS, which corresponds to 12.5% addition of the amino acid to the culture medium, considering that the Leucine concentration in the α-MEM is 396µM. Untreated cells represented the control group.

2.3 Evaluation of proliferation and cellular viability

The evaluation of the viability and proliferation of cultured cells was analyzed by means of vital dye Trypan Blue (0.4%). For the essays, freshly obtained cells with viability higher than 95% were used. The number of viable cells was determined by using a Neubauer chamber (19).

2.4 DCFH-DA

The generation of intracellular reactive oxygen species (ROS) of MC3T3-E1 cells was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which forms a fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Briefly, the cells

were incubated in the presence of Leu (50 µM). After 48 h of incubation, cells were washed twice with PBS and then incubated with 200 µL/well of phosphate-buffered containing 10 µM of DCFH-DA at 37 °C for 60 min. The fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor 3, PerkinElmer). As a positive control, H_2O_2 (100 µl) was used (20).

2.5 Quantification of cytokines

Multiple soluble cytokines (TNF, IL-6, MCP-1, IFɣ and IL-10) were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose, CA). Supernatants of MC3T3-E1 cells were collected and stored at -20°C for later analysis. Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences). Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The detection limit was 20–5,000 pg/mL (21). As a negative control (anti-inflammatory effect) was used 10 nM of rapamycin (Rapa).

2.6 Autophagy assay

Autophagy was performed by staining technique with Acridine Orange (AO), which under normal intracellular conditions, emits green fluorescence, but starts emitting red fluorescence during the autophagic process where the dye binds to organelles acidic vacuolar as lysosomes and autolissomes. Briefly, cells were incubated with the AO solution (1 µg/ml) diluted in culture medium for 15 minutes in low lightening conditions. The images were obtained using an inverted fluorescence microscope with green and red filters, with 40x objective.

The evaluation of positively stained cells percentage with AO was performed by flow cytometry and analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA). As positive control for autophagy, Rapa (10nM) was used (22).

2.7 Senescence assay

Senescence was evaluated by DAPI (4′,6-diamidino-2-phenylindole) staining, which analyses nucleus morphology. Briefly, cells were fixed for 15

minutes with ethanol (3%) and stained with DAPI (300nM). After, pictures were taken on an inverted fluorescence microscope, the images were treated with Image-Pro Plus (version 6.0) and analysed with NII Plug-in (23, 24).

2.8 Alkaline comet assay

The alkaline comet assay was performed as previously described (25). Briefly, 10 µL cell suspension (1×10^4 cells/mL) treated with Leu or Rapa was mixed with 90 µL LMP agarose, spread on a normal agarose precoated microscope slide, and placed at 4°C for 5 min to allow for solidification. Cells were lysed in a high-concentration salt and detergent solution (NaCl 2.5 M, Na_2EDTA 100 mM, Tris 10 mM with Triton X-100 1%, and DMSO 10% freshly added) for 2 h. Slides were removed from lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (NaOH 300 mM/ Na_2EDTA1 mM, pH >13, 30 min, 4°C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 25 min at 25 V and 300 mA (94 V/cm). After electrophoresis, the slides were neutralized and silver stained (26). One hundred cells were scored visually according to tail length and the amount of DNA present in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged) (27). Damage score was thus assigned to each sample, and ranges from 0 (completely undamaged: 100 cells X 0) to 400 (with maximum damage: 100 cells X 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis (27, 28).

2.9 Statistical analysis

The results were expressed in mean **±** standard error of the mean. The Shapiro-Wilk test was performed to determine the normality of data. The samples were submitted to a repeated measures analysis of variance (ANOVA), followed by the multiple comparison Tukey test. The t-Student test was used for the comparisons between groups. The level of significance was set at 5% for all tests described. All tests were performed by using *Prism GraphPad* software (version 5.0, United States).

3 RESULTS

We have shown in previous results of our research group that Leu supplementation reduces the proliferation of MC3T3-E1 pre-osteoblast cells. Also, we have found that this result was due to causes not related to necrosis, apoptosis, plasma membrane damage by oxidative stress and early differentiation (data not shown).

The antiproliferative effect caused by Leu supplementation in MC3T3-E1 cells, at a concentration of 50 µM, was analyzed in this study. When compared to the control, Leu reduces cell proliferation in almost 40% (Figure 1), after 48 hours of treatment.

In order to understand the antiproliferative effect caused by Leu supplementation in MC3T3-E1 cells, we extended the investigations, analyzing the mechanisms related to oxidative stress, inflammatory profile, autophagy and senescence, which could explain this result. The oxidative stress was evaluated based on the intracellular DCFH-DA which forms a fluorescent compound (DCF). Leu supplementation failed to increase the generation of intracellular ROS in MC3T3-E1 cells (Figure 2). We also investigated the effect of Leu on inflammation, analyzing soluble agent. Multiple inflammatory cytokines (TNF, IL-6, MCP-1, IFNg and IL-10) were assessed in culture supernatants by CBA and flow cytometry. All cytokines were analyzed after 48 h Leu and Rapa exposure. The results show that Leu supplementation is not able to change the inflammatory cell profile. Although not statistically significant, Rapa showed a tendency to decrease MCP-1 (Figure 3).

Next, in order to assess whether Leu supplementation would induce autophagy, we have used acridine orange (AO) staining to detect acidic vesicles. AO emits a red fluorescence in the acidic vesicles, but it is bright green in the cytoplasm and nucleus. Apparently, Leu (50 µM) and Rapa (10nM), used as positive control for autophagy, enhanced the formation of acidic vesicular organelles (red foci in the cells) in MC3T3-E1 cells compared to control cells (no apparent red staining) (Figure 4A). However, when flow cytometry was performed to quantify the percentage of autophagyc cells, stained with AO, there was no significant differences between the untreated

and the Leu groups (Figure 4B). Rapa was able to induce autophagy, confirming the results of the fluorescence microscopy images.

Thereafter, once the possibility of decreased cell proliferation as a result of autophagy was discarded, we investigated whether Leu supplementation could trigger cellular senescence. The nucleus morphology of MC3T3-E1 cells can be seen by staining with 4',6-diamidino-2-phenylindole (DAPI) and senescent cells present larger size nucleus as compared to normal cells (Figure 5A). Apparently, cells treated with Leu and Rapa showed a morphology compatible with cellular senescence. Here, again, it was possible to identify a minor number of cells per field in both treatments. In addition, the images were analyzed in order to quantify the percentage of senescent cells. The Figure 5B confirms the findings presented, indicating that Leu supplementation causes senescence in MC3T3-E1 cells, as well Rapa, when compared to the control group.

Finally, after the confirmation of senescence by morphological analysis and quantification of cells with abnormal nucleus, we further ivestigated possible causes of this process. The alkaline (pH > 13) comet assay, which detects DNA strand breaks and alkali-labile sites, showed that Leu supplementation, as well as Rapa, resulted in DNA damage, which was significantly higher than the control (Figure 6). Both treatments were performed in 6 and 12 hours. As a positive control for DNA damage, MMS (80 µM) was used.

4 DISCUSSION

As previously found in our studies, Leu supplementation (50 µM) was able to decrease cell proliferation in pre-osteoblast cultures. Some mechanisms, such as oxidative stress and high inflammatory activity, may be involved in the reduction of cell proliferation. In this study, we have shown that Leu supplementation was not capable of changing ROS release and the inflammatory profile in MC3T3-E1 cells.

The production of ROS is a common phenomenon of cellular metabolism. However, abnormal production of ROS leads to oxidative stress,

which may reduce the proliferation, differentiation and mineralization of osteoblast cells (12, 29, 30). In vivo studies with adult ovariectomized rats, demonstrate that excessive ROS can decrease bone mineral density (31-33). In the present study, the production of oxidative stress was measured by the average fluorescence intensity per cell emitted by DCFH, which was not changed by the Leu treatment. This result corroborates with previous findings from our group showing no increase in the lipid peroxidation, as measured by the TBARS levels in the supernatant of MC3T3-E1 cells treated for 48h with the same amount of amino acids. Both findings confirm that Leu supplementation did not increase oxidative stress and that this is not the cause of the decreased proliferation of these cells.

Another possible cause related to decreased cell proliferation is the inflammatory status of the cells. The inflammatory action of certain cytokines can act on cell proliferation routes (34). When the mTOR is inhibited, for instance by the action of rapamycin, TGF-β1, which has an anti-inflammatory action, is high. The relationship between high levels of TGF-β1 and cell cycle arrest has been already described in vitro (35). In order to identify whether other inflammatory cytokines (TNF, IL-6, MCP-1, IFNg and IL-10) would be involved in the decreased cell proliferation of MC3T3-E1 cells treated with Leu, we have used flow cytometry with the cytometric bead array (CBA) kit. This kit has a limit of detection for these cytokines from 20 to 5.000 pg/mL and, for this reason, only MCP-1 was sensitive to the assay (21). MCP-1 is the monomeric polypeptide member of the CC chemokine superfamily, is expressed by MC3T3-E1 cells and is involved in inflammation and bone remodeling (13). We have used ramamycin as a negative control for inflammation, as in previous studies, it has been used to promote an anti-inflammatory profile in cells (36). The data showed no significant differences between the groups, although Rapa has presented a tendency to decrease MCP-1, suggesting a mild anti-inflammatory drug action. These results complement our previous findings and, taken together, we may infer that Leu does not alter the cellular inflammatory status.

Recently, autophagy has been investigated due to its important action on bone remodeling. Although autophagy is a mechanism of programmed cell

death, in basal conditions, it may become an important process for maintaining the skeleton (37, 38). On the other hand, situations of stress may increase cellular autophagy, due to the activation or inhibition of specific routes. Inhibition of mTOR, for example, that occurs during deprivation of nutrients (glucose/amino acids) or by the action of Rapa induces autophagy (39). The results obtained in our experiments, where the rapamycin (10nM) was used as a positive control for autophagy.

Another mechanism involved in the activation of the autophagy is increased endoplasmic reticulum stress, which has been associated with pathological conditions of excessive nutrients, such as obesity. In vivo and in vitro studies indicate that excess energy and nutrients induces endoplasmic reticulum stress in adipose tissue cells, which is sufficient stimulus to promote autophagy (10, 40). In the MC3T3-E1 pre-osteoblasts, the increase in autophagy, either by endoplasmic reticulum stress or by the action of drugs, have paradoxical effects that may negatively influence cell proliferation or even to induce a protective effect against apoptosis (12, 41). When cells were treated with excess of Leu, we were not able to induce autophagy, although it was possible to identify the antiproliferative effect, since a lower number of cells per field were visible. The images of the cells stained with acridine orange obtained by fluorescence microscopy showed that, under normal intracellular conditions, it emits green fluorescence, but starts emitting red fluorescence during the autophagic process where the dye binds to organelles acidic vacuolar as lysosomes and autolissomes. While it is possible to identify small red dots on the images of cells treated with Leu, we could not find significant differences in the quantification of the percentage of autophagic cells, evaluated by flow cytometry. This result indicates that the decrease in cell proliferation caused by supplementation with Leu in MC3T3-E1 cells is not caused by increased autophagy.

The excess of nutrients has also been related to increased early senescence in several in vitro studies. The investigation of the mechanisms that lead the cells to senescence process has become a target for possible therapeutic actions, as this mechanism is involved in the proper development of healthy cells and in the control tumor cell proliferation (15). In this sense,

some studies investigating the action of amino acids, especially Leu, have been conducted in order to clarify these issues. An elegant study (7) evaluating the effect of Leu supplementation on the regulation of β-cell mass during pancreatic development, has shown that increased Leu consumption by pregnant rats resulted in a hyperglycemic and hypoinsulinemic fetuses with increased body weight, but without an adaptation to the beta cells mass. To elucidate the Leu effect on the regulation of the β-cell mass, the researchers used an in vitro bioassay that mimics the major steps that occur during β-cell development from fetal pancreatic progenitor cells. In this in vitro assay it was demonstrated that Leu increased the expression of the hypoxia-inducible factor 1-α (HIF-1α), a repressor of the development of NGN3-positive pancreatic endocrine progenitor cells. Studies in tumor liver cells (HepG2) supplemented with Leu also identified an increase of cellular senescence (11, 42).

The results presented in our study corroborate the previous findings mentioned above. Leu supplementation was able to promote senescence of pre-osteoblasts, as it is possible to identify in the cell images labeled with DAPI, obtained by fluorescence microscopy. Senescent cells, although still viable and metabolically active, lose their proliferative capacity and differentiate from the others for its increased nucleus. This result was confirmed by quantifying the percentage of senescent cells by flow cytometry. In this experiment we used Rapa in order to verify if the inhibition of mTOR in these conditions could prevent senescence, as previously described (43). Surprisingly, in these experimental conditions Rapa also induced senescence, which in this case may be related to the chronic use (48h) and the increased autophagy (39).

In general, cellular senescence is considered a programmed response to stress, that can be activated by oxidative stress, irradiation or the action of substances or drugs. These stressors cause DNA damage and, through the action of specific proteins (p53 and p21), lead to cell senescence (44). Assays that evaluate DNA damage as the cause of senescence are usually carried out in a short time, within 24 hours, because at longer periods it can activate

repair mechanisms. For this reason, the experiments were performed 6 and 12 hours, different from other study treatment times (48 hours).

Thus, DNA damage appears to be a key aspect that would justify the presence of senescence in cells supplemented with Leu. The potential toxic effect of Leu and its relation to DNA damage has mainly been studied in pathological conditions in which this amino acid is not metabolized properly and ends up accumulating in the body, as in the case of patients with maple syrup urine disease (MSUD) (45). Studies *in vitro* under conditions that mimic the toxic environment by excessive Leu, as well as in individuals with MSUD, show that excess Leu may cause cellular damage increased by DNA rupture (46). This damage can occur even in cells from healthy individuals (16). Our results indicate that Leu also has a potential toxic effect on MC3T3-E1 cells, since the addition of 12.5% more, as compared to the levels that are considered optimal for the culture medium of these cells, was able to promote DNA damage and, consequently, cellular senescence at 6 and 12 hours of treatment.

5 CONCLUSION

In conclusion, the results indicate that the antiproliferative effect caused by leucine supplementation in MC3T3-E1 cells, due to causes not related to oxidative stress or inflammation. Authoug the autophagy to be related to excess of nutrients in several studies in vitro, our findings did not show similar results. However, our study showed that the leucine supplementation was capable to induces a DNA damage which leads to cell senescence. These results may lead to new perspectives on the effects of the excess of nutrients on bone health. Considering the increased prevalence of conditions related to the excess of nutrients and the inadequate food intake of modern diet, further studies on the subject are still necessary.

REFERENCES

1. Fardet A, Rock E. The healthy core metabolism: A new paradigm for primary preventive nutrition. J Nutr Health Aging. 2015:1-9.
2. Castro MA, Baltar VT, Marchioni DM, Fisberg RM. Examining associations between dietary patterns and metabolic CVD risk factors: a novel use of structural equation modelling. Br J Nutr. 2016.p.1-12.
3. Parr EB, Coffey VG, Cato LE, Phillips SM, Burke LM, Hawley JA. A randomized trial of high-dairy-protein, variable-carbohydrate diets and exercise on body composition in adults with obesity. Obesity. 2016 May;24(5):1035-45
4. Tato I, Bartrons R, Ventura F, Rosa JL. Amino acids activate mammalian target of rapamycin complex 2 (mTORC2) via PI3K/Akt signaling. J Biol Chem. 2011;286(8):6128-42.
5. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12(1):21-35.
6. Tremblay Fdr, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology. 2005;146(3):1328-37.
7. Rachdi L, Aïello V, Duvillié B, Scharfmann R. L-leucine alters pancreatic β-cell differentiation and function via the mTor signaling pathway. Diabetes. 2012;61(2):409-17.
8. Heaney RP, Layman DK. Amount and type of protein influences bone health. Am J Clin Nutr. 2008;87(5):1567S-70S.
9. Melnik BC. Excessive leucine-mTORC1-signalling of cow milk-based infant formula: the missing link to understand early childhood obesity. J Obes. 2012;2012.
10. Zhou L, Liu M, Zhang J, Chen H, Dong LQ, Liu F. DsbA-L Alleviates Endoplasmic Reticulum Stress–Induced Adiponectin Downregulation. Diabetes. 2010;59(11):2809-16.
11. Nakano M, Nakashima A, Nagano T, Ishikawa S, Kikkawa U, Kamada S. Branched-chain amino acids enhance premature senescence through mammalian target of rapamycin complex I-mediated upregulation of p21 protein. PloS One. 2013;8(11):e80411.
12. Yang Y-H, Li B, Zheng X-F, Chen J-W, Chen K, Jiang S-D, et al. Oxidative damage to osteoblasts can be alleviated by early autophagy through the endoplasmic reticulum stress pathway—implications for the treatment of osteoporosis. Free Radic Biol Med. 2014;77:10-20.
13. Takeshita A, Hanazawa S, Amano S, Matumoto T, Kitano S. IL-1 induces expression of monocyte chemoattractant JE in clonal mouse osteoblastic cell line MC3T3-E1. J Immunol. 1993;150(4):1554-62.
14. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol: Mech Dis. 2015;10:173-94.

15. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Develop. 2010;24(22):2463-79.
16. Mescka CP, Wayhs CAY, Guerreiro G, Manfredini V, Dutra-Filho CS, Vargas CR. Prevention of DNA damage by L-carnitine induced by metabolites accumulated in maple syrup urine disease in human peripheral leukocytes in vitro. Gene. 2014;548(2):294-8.
17. Sitta A, Ribas GS, Mescka CP, Barschak AG, Wajner M, Vargas CR. Neurological damage in MSUD: the role of oxidative stress. Cell Mol Neurobiol. 2014;34(2):157-65.
18. Sudo H, Kodama H-A, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 1983;96(1):191-8.
19. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2001:A3. B.1-A3.B.
20. Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. Analyst. 2002;127(1):183-98.
21. Morgan E, Varro R, Sepulveda H, Ember JA, Apgar J, Wilson J, et al. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol. 2004;110(3):252-66.
22. Zhang X, Liu Q. Autophagy Assays (LC3B immunofluorescence, LC3B western blot, acridine orange assay). Oncol Rep. 2016 Apr;35(4):2121-30
23. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc. 2009;4(12):1798-806.
24. Filippi-Chiela EC, Oliveira MM, Jurkovski B, Callegari-Jacques SM, Da Silva VD, Lenz G. Nuclear morphometric analysis (NMA): screening of senescence, apoptosis and nuclear irregularities. PLoS One. 2012;7(8):e42522.
25. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184-91.
26. Nadin SB, Vargas–Roig LM, Ciocca DR. A silver staining method for single-cell gel assay. J Histochem Cytochem. 2001;49(9):1183-6.
27. Collins AR, Ai-Guo M, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mut Res. 1995;336(1):69-77.
28. Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, et al. Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup. Mutat Res Genet Toxicol Environ Mutagen. 2007;627(1):31-5.
29. Uno K, Takarada T, Hinoi E, Yoneda Y. Glutamate is a determinant of cellular proliferation through modulation of nuclear factor E2 p45- related

factor- 2 expression in osteoblastic MC3T3- E1 cells. J Cell Physiol. 2007;213(1):105-14.

30. Arakaki N, Yamashita A, Niimi S, Yamazaki T. Involvement of reactive oxygen species in osteoblastic differentiation of MC3T3-E1 cells accompanied by mitochondrial morphological dynamics. Biom Res. 2013;34(3):161-6.
31. Muthusami S, Ramachandran I, Muthusamy B, Vasudevan G, Prabhu V, Subramaniam V, et al. Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. Clin Chem Acta. 2005;360(1):81-6.
32. Cervellati C, Bonaccorsi G, Cremonini E, Bergamini CM, Patella A, Castaldini C, et al. Bone mass density selectively correlates with serum markers of oxidative damage in post-menopausal women. Clin Chem Lab Med. 2013;51(2):333-8.
33. Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS, et al. Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. Calcif Tissue Int. 2010;87(3):226-35.
34. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29(6):313-26.
35. Chatterjee A, Mukhopadhyay S, Tung K, Patel D, Foster DA. Rapamycin-induced G1 cell cycle arrest employs both TGF-β and Rb pathways. Cancer Lett. 2015;360(2):134-40.
36. Agrawal NK, Kant S. Targeting inflammation in diabetes: Newer therapeutic options. World J Diabetes. 2014;5(5):697-710.
37. Shapiro IM, Layfield R, Lotz M, Settembre C, Whitehouse C. Boning up on autophagy: the role of autophagy in skeletal biology. Autophagy. 2014;10(1):7-19.
38. Hocking LJ, Whitehouse C, Helfrich MH. Autophagy: a new player in skeletal maintenance? J Bone Min Res. 2012;27(7):1439-47.
39. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2016;12(1):1-222.
40. Nuñez C, Rodrigues V, Gomes F, de Moura R, Victorio S, Bombassaro B, et al. Defective regulation of adipose tissue autophagy in obesity. Int J Obes. 2013;37(11):1473-80.
41. Wei M, Duan D, Liu Y, Wang Z, Li Z. Autophagy may protect MC3T3-E1 cells from fluoride-induced apoptosis. Mol Med Rep. 2014;9(6):2309-15.
42. Nishitani S, Horie M, Ishizaki S, Yano H. Branched chain amino acid suppresses hepatocellular cancer stem cells through the activation of mammalian target of rapamycin. PloS One. 2013;8(11):e82346.
43. Walters HE, Deneka-Hannemann S, Cox LS. Reversal of phenotypes of cellular senescence by pan-mTOR inhibition. Aging 2016; Feb;8(2):231-44.

44. Di Fagagna FA. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Canc. 2008;8(7):512-22.

45. Strauss KA, Puffenberger EG, Morton DH. Maple syrup urine disease. 2013. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2016. 2006 Jan 30 [updated 2013 May 9].

46. Mescka CP, Guerreiro G, Hammerschmidt T, Faverzani J, de Moura Coelho D, Mandredini V, et al. l-Carnitine supplementation decreases DNA damage in treated MSUD patients. Mutat Res. 2015;775:43-7.

FIGURE 1:

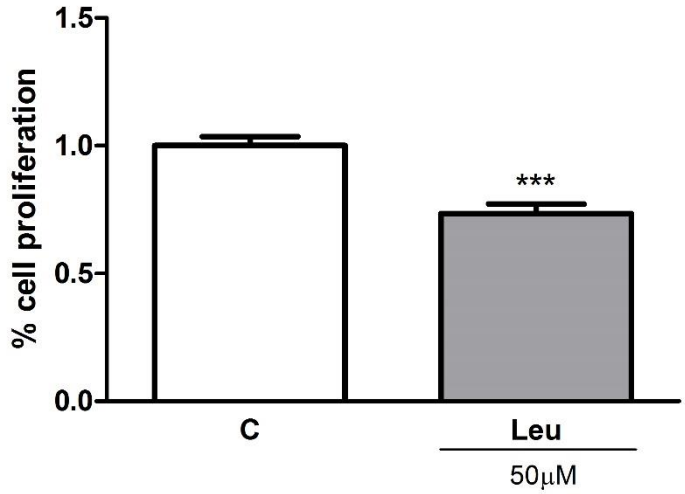

Figure 1. Effects of Leucine supplementation on the proliferation of pre-osteoblast MC3T3-E1: cells were treated for 48 h with 50μM. The evaluation of cell proliferation and viability was performed by Trypan Blue (0.5%) staining and counting in a Neubauer chamber. Data are expressed as means ± SEM and analyzed by Student *t* test. ***p<0,0001 *versus* control.

FIGURE 2:

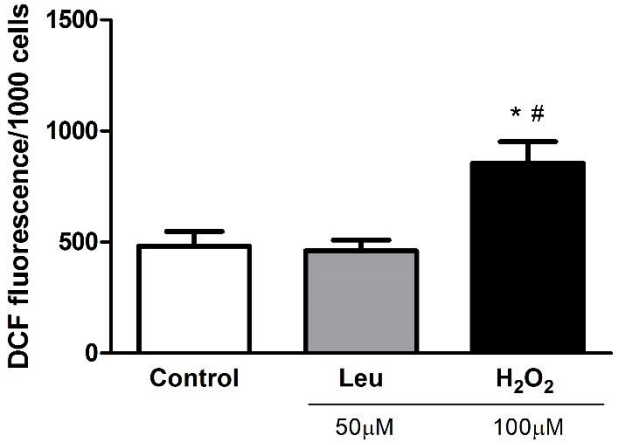

Figure 2. Effects of Leucine supplementation on MC3T3-E1 ROS release. Cells were treated for 48 h with Leu (50μM) and H_2O_2 (100 μM) as a positive control. Data represent the means ± SEM. Results were expressed as DCF Fluorescence/1000 cells. *p<0,05 *versus* control; #p<0,05 *versus* Leu (ANOVA, followed by the Tukey post-hoc test)

FIGURE 3:

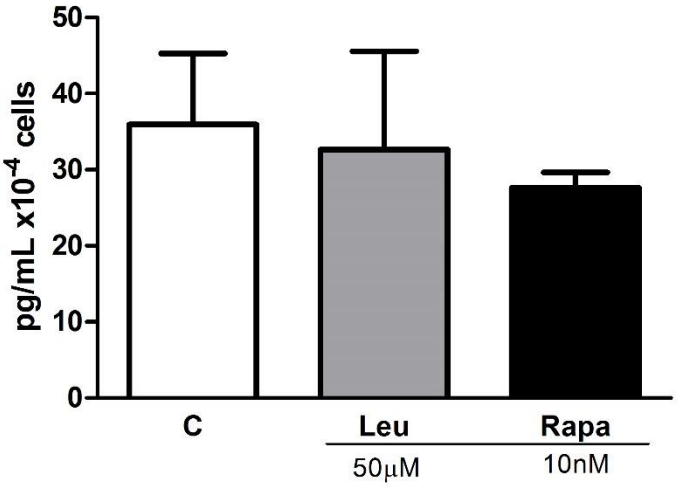

Figure 3. MCP-1 in cell supernatant after 48 hours of treatment with Leu (50μM) and Rapa (10nM), measured by flow cytometry using the Cytometric Bead Array (CBA). Data are expressed as means ± SEM and analyzed by ANOVA, followed by the Tukey post-hoc test. It was no difference between groups.

FIGURE 4:

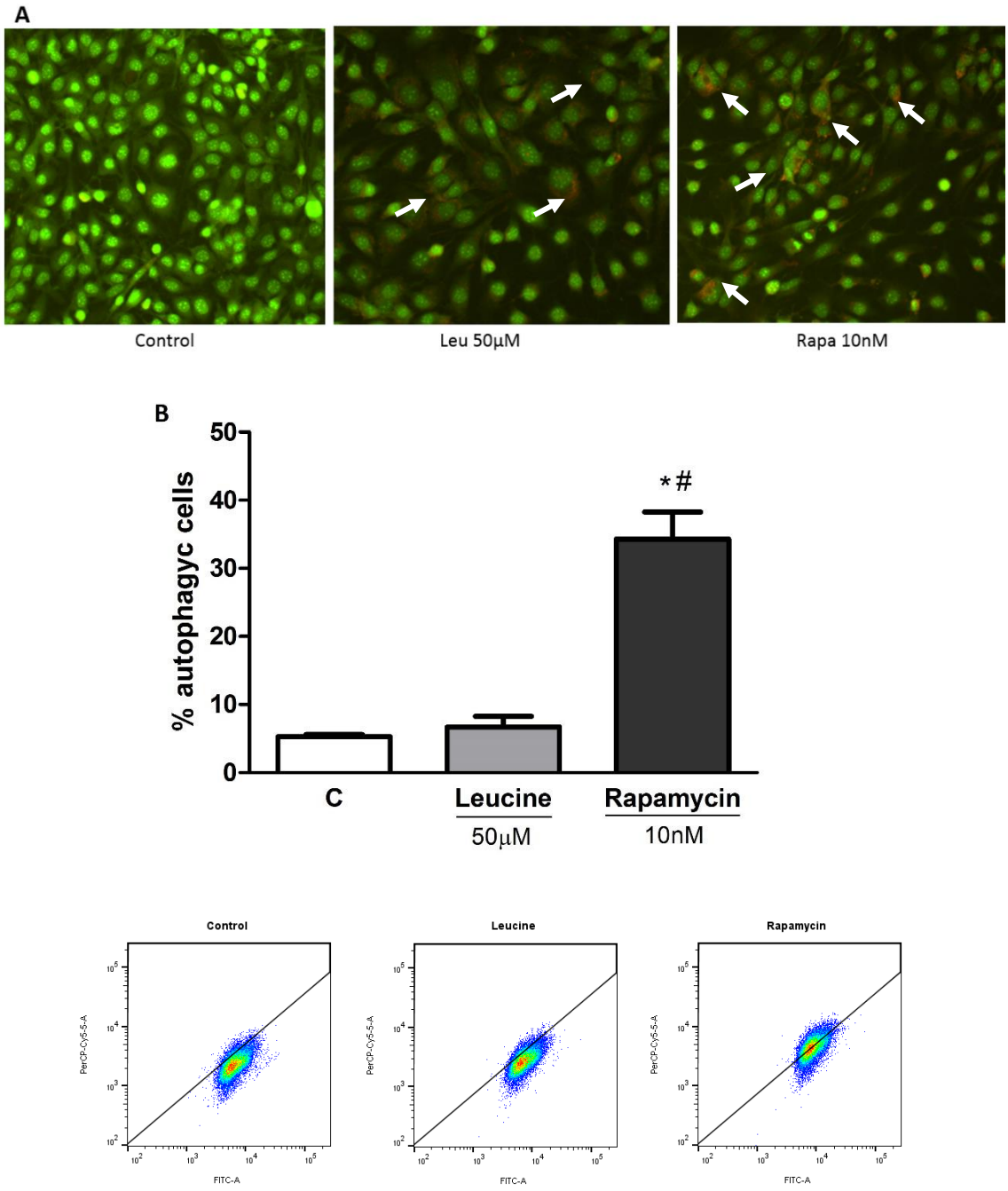

Figure 4. A: Images of cells submitted to treatment with leucine and rapamycin for 48 hours and stained using the Acridine Orange method. Red areas show acidic organelles as lysosomes and autolisossomes. Magnification 40x. **B:** Flow citometry of cells submitted to treatment with leucine and rapamycin for 48 hours and stained using the Acridine Orange method. Data are expressed as means ± SEM and analyzed by ANOVA, followed by the Tukey post-hoc test. p<0,05 **versus* control, #*versus* leucine.

FIGURE 5:

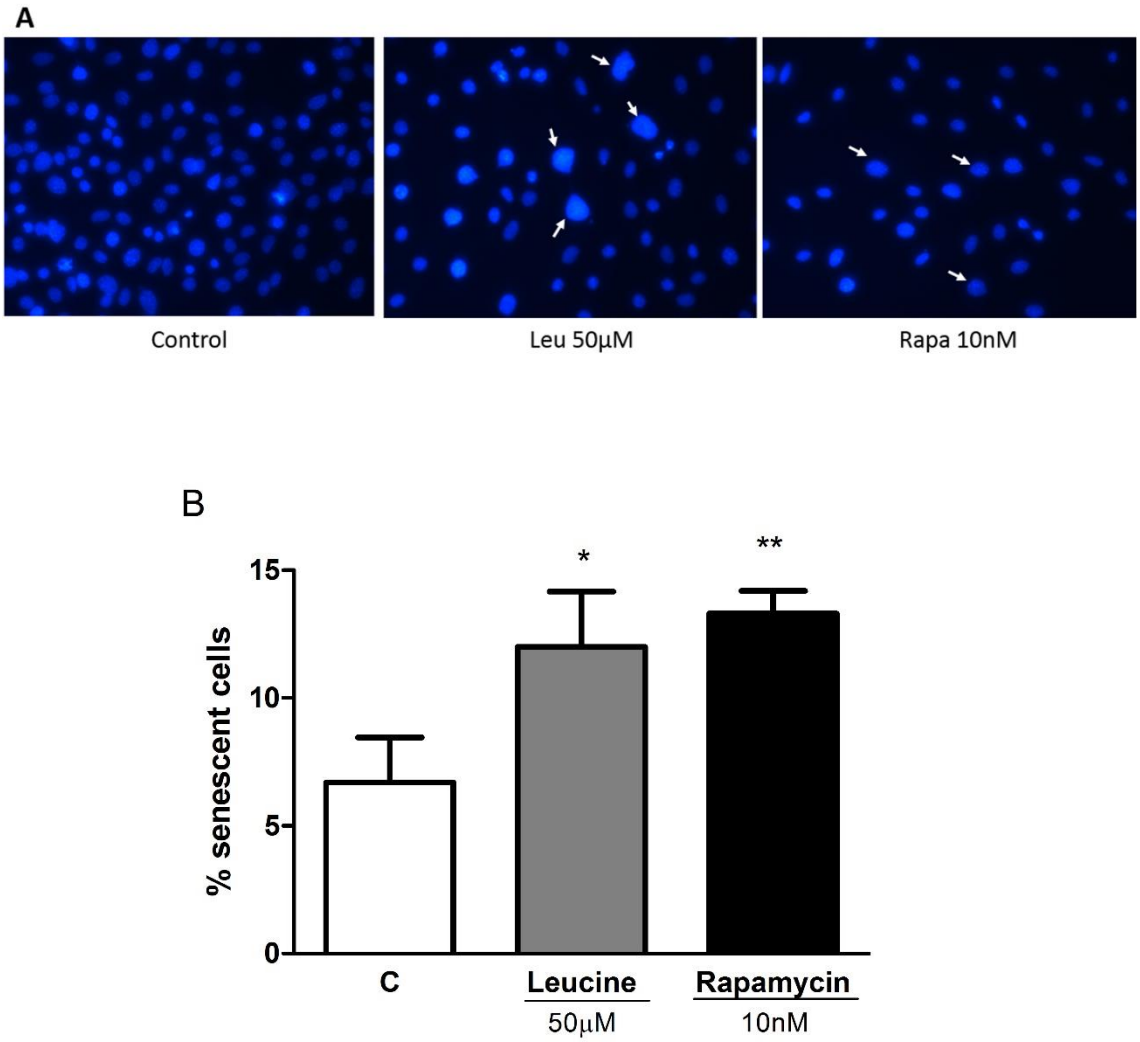

Figure 5. A: Images of cells submitted to treatment with leucine and rapamycin for 48 hours and stained using the 4′,6-diamidino-2-phenylindole (DAPI) method. Senescent cells, indicate by white arrows, show increased nucleus. Magnification 40x. **B:** Image analisys of cells submitted to treatment with leucine and rapamycin, for 48 hours and stained using the 4′,6-diamidino-2-phenylindole (DAPI) method. Data are expressed as means ± SEM and analyzed by ANOVA, followed by the Tukey post-hoc test. *p<0,05 *versus* control, ** p<0,001 *versus* control.

FIGURE 6:

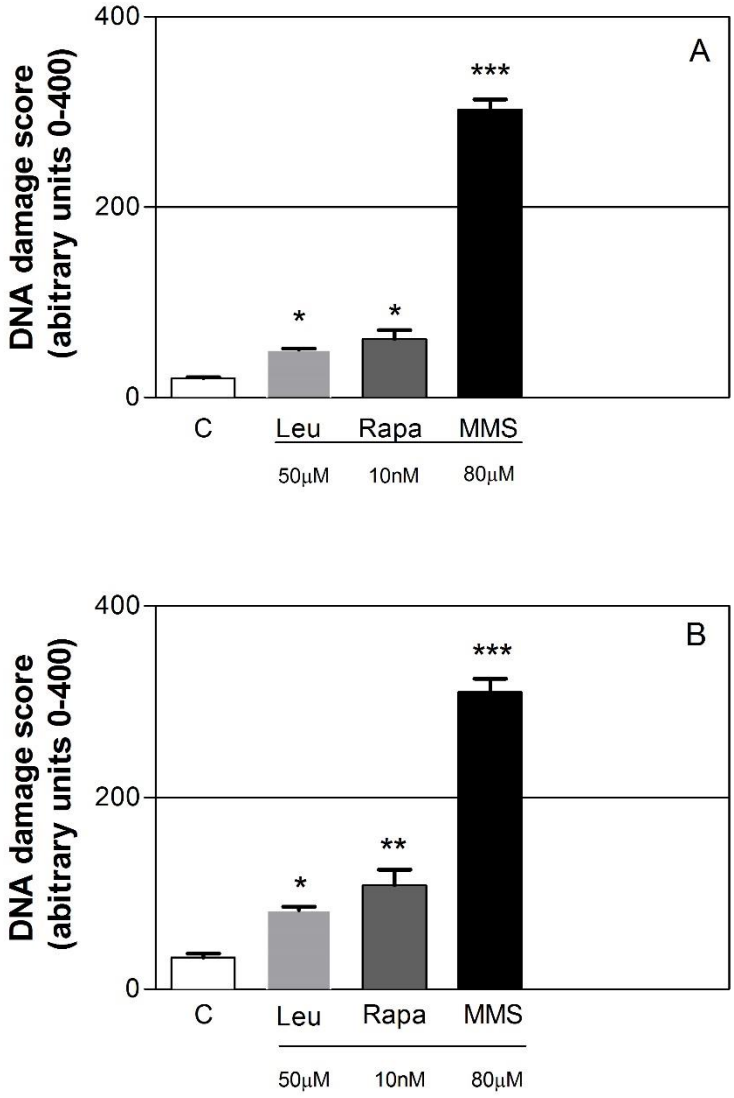

Figure 6. Effect of Leucine and Rapamycin after 6 h (**A**) and 12 h (**B**) of treatment on DNA damage index using MC3T3-E1 pre-osteoblast lineage cells, determined by alkaline comet assay. Control (untreated) or treated cells with Leucine at 50 μM and Rapamycin at 10 nM concentrations were used. Data are expressed as mean ± SD of three independent experiments. *p< 0.05, **p < 0.01 and ***p < 0.001 compared to the control group.

CAPÍTULO IV

CONSIDERAÇÕES FINAIS

1 CONSIDERAÇÕES FINAIS

A busca pela oferta adequada de nutrientes e sua relação com a saúde humana tem sido alvo de muitas pesquisas nas últimas décadas. Apesar do avanço da ciência da nutrição, pouco ainda se sabe sobre os efeitos que o excesso de nutrientes pode causar nos diferentes órgãos e tecidos do organismo humano. Neste sentido, este trabalho apresenta algumas evidências que podem contribuir para a melhor compreensão destes efeitos sobre o tecido ósseo.

Em conjunto, esta pesquisa verificou os efeitos da suplementação de Leu sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1. Em se tratando de um estudo inédito, o primeiro objetivo deste ensaio *in vitro* foi o de identificar as ações do AA sobre a proliferação celular. Os resultados encontrados demonstraram um potencial efeito nocivo da Leu, já que a suplementação levou a uma diminuição da proliferação celular. Este efeito antiproliferativo foi alcançado com um acréscimo de pouco mais de 12% (50 μM) acima dos níveis considerados ideais para o meio de cultura, num período de 48h. Além disso, quando comparamos os efeitos da Leu com outros AA, identificamos que a ação antiproliferativa é atribuída somente a este nutriente, o que demonstra que a Leu provavelmente possui um mecanismo específico para esta ação.

A partir deste resultado, iniciamos a busca de possíveis mecanismos envolvidos na diminuição da proliferação destas células. Na primeira parte do trabalho, realizamos ensaios que nos permitiram avaliar a citotoxicidade da substância, bem como os efeitos da suplementação sobre mecanismos de morte celular programada e dano à membrana celular por estresse oxidativo. Os resultados desta etapa demonstraram que a diminuição da proliferação das células MC3T3-E1 suplementadas com Leu não foi relacionada a nenhum dos mecanismos supracitados, já que não obtivemos diferenças estatisticamente significativas nos níveis de LDH, de células marcadas com Anexina V e de TBARS.

Tendo o conhecimento de que a Leu tem um papel essencial em rotas de proliferação celular, como a mTOR, ainda no primeiro estudo, investigamos quais os efeitos deste AA em comparação a substâncias que,

conhecidamente, atuam inibindo ou ativando esta via. Desta forma, a Rapa, um inibidor do mTOR, e a insulina, um ativador do mTOR, demonstraram resultados previsíveis, já que foram capazes de diminuir e aumentar a proliferação celular, respectivamente. Porém, quando a Leu foi associada a estas substâncias, identificamos uma incapacidade do AA em potencializar os efeitos antiproliferativos da Rapa e de reverter os efeitos proliferativos da insulina, sugerindo que a diminuição da proliferação causada por este nutriente acontece por uma via independente do mTOR. Na tentativa de compreender melhor este resultado, avaliamos os níveis de TGF-B1, uma substância anti-inflamatória que encontra-se elevada, quando o mTOR está inibido. O resultado deste ensaio corroborou com a ideia de que a Leu não depende do mTOR para diminuir a proliferação, já que não foi capaz de aumentar os níveis de TGF-B1, como fez a Rapa, que atua inibindo esta rota.

Outra hipótese descartada na primeira fase deste estudo, foi a de que a suplementação de Leu promoveria uma diferenciação precoce, e com isso, uma diminuição da proliferação celular. Não foi possível identificar mudanças nos níveis de colágeno e de fosfatase alcalina, importante marcadores de diferenciação das células MC3T3-E1.

Embora tenhamos tido várias respostas importantes com os ensaios realizados na primeira fase do estudo, ainda não estava claro qual o mecanismo envolvido da diminuição da proliferação das células MC3T3-E1 suplementadas com Leu. Assim, o segundo estudo estendeu as investigações, no sentido de ampliar os conhecimentos sobre o estresse oxidativo, o perfil inflamatório e os mecanismos de morte celular programada.

Desta forma, foi possível confirmar, a partir de técnicas complementares, vários dos achados do primeiro estudo. Demonstramos que a Leu não promove estresse oxidativo, já que não aumenta a produção de ROS, que foi avaliado pelos níveis de DCFH. Este resultado é coerente com o resultado encontrado no primeiro estudo, que não identificou aumento da peroxidação lipídica, indicando que não houve dano à membrana celular, associado ao estresse oxidativo. Outro resultado que corrobora com os achados anteriores, foi o de que a Leu não altera parâmetros inflamatórios, ao contrário do que fez a Rapa, que conhecidamente é uma substância anti-inflamatória.

Tendo em vista que o excesso de nutrientes tem sido associado ao aumento da autofagia e da senescência celular, investigamos, de forma complementar a apoptose (analisada no primeiro estudo), se estes mecanismos estariam envolvidos na diminuição da proliferação celular. A autofagia, assim como a apoptose, não confirmou-se como o mecanismo que explicaria a diminuição da proliferação celular causada pela suplementação de Leu. Por outro lado, o AA foi capaz de promover a senescência celular, demonstrada através da modificação da morfologia dos núcleos celulares, que tornaram-se grandes e achatados.

Muitos são os motivos pelos quais a célula pode entrar em senescência. Entretanto, baseados em estudos prévios que avaliaram os efeitos do excesso de Leu, demonstramos que a Leu provoca dano ao DNA. Este resultado sugere que esta é a causa da senescência, que por sua vez provoca a diminuição da proliferação celular dos pré-osteoblastos.

Os resultados obtidos nesta tese nos conduziram a um racional que pode explicar os efeitos deletérios da suplementação de Leu sobre a proliferação das células MC3T3-E1, bem os possíveis mecanismos envolvidos nesta ação. Os efeitos da suplementação deste AA sobre pré-osteoblastos está sumarizado na Figura 1.

Estes achados apontam para novas perspectivas de estudo e ampliam o conhecimento atual sobre a suplementação de nutrientes. A evidência de que a suplementação de Leu provoca dano ao DNA, com consequente aumento da senescência e diminuição da proliferação celular de pré-osteoblastos, é um alerta sobre os potenciais efeitos adversos da suplementação dietética deste aminoácido sobre a saúde óssea e reforça a ideia de que uma avaliação criteriosa sempre é necessária. É importante ainda considerar as limitações deste estudo e, apesar de ensaios *in vitro* serem importantes para a identificação de mecanismos celulares, os resultados não podem ser extrapolados para seres humanos. Por este motivo, mais estudos não necessários para a melhor compreensão da ação deste AA sobre a saúde humana.

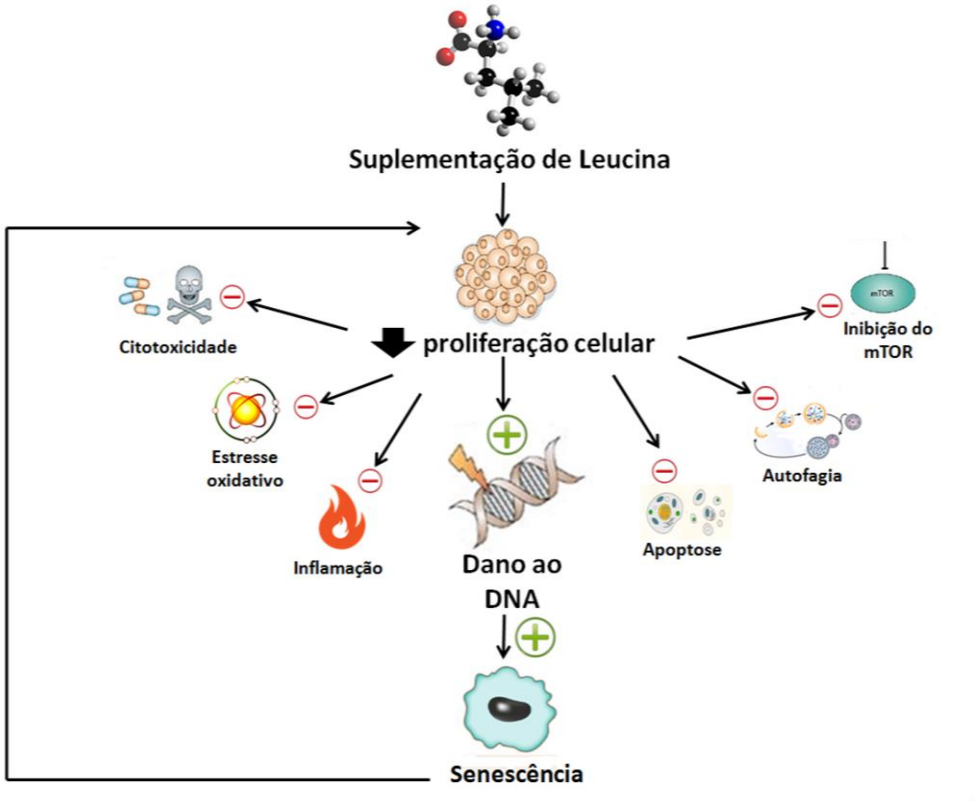

Figura 1. Efeito da suplementação de leucina sobre a proliferação de pré-osteoblastos da linhagem MC3T3-E1: a leucina causa diminuição da proliferação celular por causas não relacionadas a citotoxicidade, estresse oxidativo, inflamação, apoptose, autofagia ou inibição do mTOR. O mecanismo que explica a diminuição da capacidade proliferativa é o dano ao DNA, que promove a senescência das células com consequente diminuição da proliferação.