PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

MARCELA DE ANDRADE BERNAL FAGIANI

BENEFÍCIOS DA SUPLEMENTAÇÃO COM L-ARGININA EM RATAS *WISTAR* SUBMETIDAS À QUIMIOTERAPIA COM 5-FLUOROURACIL

Presidente Prudente - SP
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Dissertação apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal – Área de concentração: Fisiopatologia Animal.

Orientador:
Prof. Dr. Luis Souza Lima de Souza Reis

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Presidente Prudente, 22 de Janeiro de 2019

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"Resiliência é a capacidade de sair de dentro de si, mesmo naqueles momentos em que tudo parece estar muito ruim e encarar o mundo do lado de fora de cabeça erguida, olho no olho e com o maior sorriso que tiver em seu arsenal de sorrisos".

(Autor Desconhecido)

RESUMO

Benefícios da suplementação com L-Arginina em ratas *Wistar* submetidas à quimioterapia com 5-Fluorouracil

O câncer é considerado a segunda principal causa de morte no mundo, com custos relacionados ao tratamento estimados em 150 bilhões de dólares para 2020. O 5-Fluorouracil é um quimioterápico utilizado no tratamento do câncer há mais de 50 anos, desde 1957, principalmente em neoplasias de cólon, estômago, esôfago, cabeça e pescoço e próstata, que pode causar efeitos colaterais, como a mielossupressão, imunossupressão, mucosite, perda de peso e apetite, bem como danos ao DNA. Na tentativa de minimizar estes efeitos indesejáveis, vêm se estudando alguns nutrientes que estimulam a resposta imune, como a L-arginina, porém as doses encontradas na literatura foram utilizadas em seres humanos e em estudos com ratos machos, apresentando diferenças nas recomendações. O objetivo foi avaliar o efeito de doses baixas de suplementação com L-arginina sobre o hemograma, a integridade do DNA e do baço, no infiltrado inflamatório no jejuno e no coagulograma de ratas *Wistar* submetidas à quimioterapia com 5-FU. Foram utilizadas 32 ratas da linhagem *Wistar*, alimentadas com ração comercial balanceada e água filtrada *ad libitum*, distribuídas em 4 grupos experimentais (n=8 ratas/grupo): no grupo controle normal aplicou-se 1 mL de solução fisiológica 0,9%, por via intraperitoneal, para simular a aplicação do 5-FU nos demais grupos; no $G_{5\text{-}FU}$ aplicou-se uma dose de 200 mg 5-FU/Kg; nos grupos G_{Arg50} e G_{Arg100} aplicou-se uma dose de 200 mg 5-FU/Kg e as ratas foram suplementadas com 50 e 100 mg L-arginina/dia, respectivamente, adicionada na água do bebedouro *ad libitum*. Após a quimioterapia, as ratas dos grupos $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} reduziram o consumo de ração ($P>0,05$) e o $G_{5\text{-}FU}$ apresentou redução no consumo médio de água. O grupo G_{Arg50} não perdeu peso após a quimioterapia, mas nos grupos $G_{5\text{-}FU}$ e G_{Arg100} as ratas perderam peso. O G_{Arg50} apresentou policitemia devido à desidratação causada por diarreia gerada pelo 5-FU. As ratas do G_{Arg100} tiveram aumento na contagem de leucócitos totais, eosinófilos, linfócitos e no índice total de danos ao DNA, apresentaram ainda redução no tempo de protrombina e no índice de depleção no baço. As ratas dos grupos $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} tiveram infiltrado inflamatório moderado semelhante no jejuno. Conclui-se que a suplementação com 100 mg de L-arginina/dia minimizou a imunossupressão, a depleção no baço e o tempo de protrombina e contribuiu para a quebra do DNA gerada pela 5-FU em ratas Wistar. A suplementação com 50 mg de L-arginina/dia diminuiu a perda de peso gerada pelo 5-FU em ratas Wistar. As suplementações com 50 ou 100 mg de L-arginina não interferiram no infiltrado inflamatório no jejuno gerado pelo 5-FU.

Palavras-chave: Arginina. Neoplasias. Tratamento Farmacológico. Citotoxicidade Imunológica. Fluorouracila.

ABSTRACT

Benefits of L-Arginine supplementation in *Wistar* rats submitted to 5-Fluorouracil chemotherapy

Cancer is considered the second leading cause of death in the world with treatment costs estimated at $150 billion by 2020. 5-Fluorouracil has been used in the treatment of cancer for more than 50 years since 1957, mainly in neoplasias of colon, stomach, esophagus, head and neck and prostate, which can cause side effects such as myelosuppression, immunosuppression, mucositis, weight loss and appetite as well as DNA damage. In order to minimize these undesirable effects, some nutrients that stimulate the immune response, such as L-arginine, have been studied, but the doses found in the literature have been used in humans and in studies with male rats, with differences in recommendations. The objective was to evaluate the effect of low doses of L-arginine supplementation on blood count, coagulation cascade, DNA integrity, spleen and jejunal inflammatory infiltrate in rats submitted to 5-fluorouracil chemotherapy. Thirty-two *Wistar* rats were fed ad libitum filtered water and fed commercially balanced feed, divided into 4 experimental groups containing 8 rats per group, which were distributed in two rats per box: normal control group was applied 1 mL of 0.9% physiological solution intraperitoneally to simulate the application of 5-FU in the other groups; G5-FU was applied a dose of 200 mg 5-FU/Kg; G_{Arg50} and G_{Arg100} a dose of 200 mg 5-FU/Kg was added and supplemented with 50 and 100 mg L-arginine/day, respectively, added into the drinking water ad libitum. After the chemotherapy in the rats, it was observed that: The $G_{5\text{-}FU}$, G_{Arg50} and G_{Arg100} groups reduced the feed consumption, presenting similar consumption of the same, and the G5-FU presented reduction in the average water consumption. G_{Arg50} group showed no weight loss, and the $G_{5\text{-}FU}$ and G_{Arg100} groups lost weight. G_{Arg50} presented polycythemia due to dehydration caused by diarrhea generated by 5-FU and in the hemogram, coagulation cascade and depletion in the spleen there was no difference of the G5-FU group. G_{Arg100}, reduced prothrombin time and depletion index in the spleen, increased the total leukocyte, eosinophil and lymphocyte counts. G_{Arg100} still showed the highest total DNA damage index, and did not alleviate the inflammatory infiltrate in the gut, thus preventing the occurrence of mucositis, with the rats showing moderate inflammation in the jejunum. Supplementation with 100 mg L-arginine/day has the potential to minimize immunosuppression and depletion in the spleen caused by 5-FU chemotherapy. In addition, it can still act on the coagulation cascade, reducing blood coagulation time and contributing to DNA breakage generated by 5-FU. The dose of 50 and 100 mg of L-arginine/day, do not contribute to the reduction of mucositis in the intestine of rats.

Keywords: Arginine. Neoplasms. Drug Therapy. Cytotoxicity, Immunologic. Fluorouracil.

SUMÁRIO

1 ARTIGO A SER SUBMETIDO À REVISTA *NUTRITON*

Highlights

- Suplementação L-arginina minimizou a imunossupressão causada pelo 5-FU.
- Suplementação com L-arginina potencializou o dano no DNA gerado pelo 5-FU.
- Suplementação com L-arginina reduziu o tempo de protrombina.

Benefícios da suplementação com L-arginina em ratas *Wistar* submetidas à quimioterapia com 5-fluorouracil

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Resumo

Objetivo: Avaliar o efeito de doses baixas de suplementação com L-arginina sobre o hemograma, a integridade do DNA e do baço, no infiltrado inflamatório no jejuno e no coagulograma de ratas *Wistar* submetidas à quimioterapia com 5-FU. **Métodos:** Foram utilizadas 32 ratas, da linhagem *Wistar*, alimentadas com ração comercial e água filtrada *ad libitum*, distribuídas em 4 grupos (8 ratas/grupo): grupo controle recebeu solução fisiológica 0,9% para simular a aplicação do 5-FU nos demais grupos; $G_{5\text{-}FU}$ recebeu uma dose de 5-FU; G_{Arg50} e G_{Arg100} receberam uma dose de 5-FU e foram suplementadas respectivamente, com 50 e 100 mg L-arginina/dia adicionados na água do bebedouro. **Resultados:** As ratas do grupo G_{Arg50} não perderam peso após a quimioterapia. O G_{Arg50} apresentou policitemia devido à desidratação causada pela diarreia gerada pelo 5-FU. As ratas do G_{Arg100} tiveram aumento na contagem de leucócitos totais, eosinófilos, linfócitos e no índice total de danos ao DNA, apresentaram redução no tempo de protrombina e no índice de depleção no baço. As ratas dos grupos $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} apresentaram infiltrado inflamatório semelhante no jejuno, de grau moderado. **Conclusão:** A suplementação com 100 mg de L-arginina/dia minimizou a imunossupressão, a depleção no baço, interferiu na coagulação sanguínea e contribuiu para a quebra do DNA gerada pela 5-FU em ratas Wistar. A suplementação com 50 mg de L-arginina/dia diminuiu a perda de peso das ratas, gerada pelo 5-FU. As suplementações com 50 ou 100 mg de L-arginina não interferiram no infiltrado inflamatório gerado pelo 5-FU no jejuno.

Palavras-chave: Amino acid; Immunomodulator; Neoplasm; 5-Fluorouracil; Genotoxicity.

Introdução

Segundo a *World Health Organization* (WHO) [1], o câncer é a segunda maior causa de morte no mundo. No Brasil, o Instituto Nacional do Câncer (INCA) [2] estimou que ocorrerão 600 mil novos casos de câncer entre 2018 e 2019, com os custos de tratamento avaliados em US$150 bilhões para 2020 [3,4].

Dentre as alternativas de tratamento, tem sido empregada a quimioterapia com o 5-Fluorouracil (5-FU) que, apesar de seu prolongado tempo de utilização na terapêutica, ainda é eficaz no tratamento do câncer do trato digestório [5,6].

O efeito citotóxico causado pelo 5-FU nas células se dá pela inibição da síntese de DNA [7]. No entanto, como o 5-FU possui baixa especificidade pelas células neoplásicas, este quimioterápico também causa genotoxicidade sobre as células normais dos tecidos, gerando efeitos colaterais, como a mucosite, imunossupressão e mielossupressão, afetando órgãos imunitários, como baço [8] e medula óssea [9,10].

Visando minimizar os efeitos colaterais do 5-FU, e proporcionar melhor qualidade de vida aos pacientes, têm sido utilizados alguns nutrientes imunomoduladores, como a L-arginina [11,12], que é um aminoácido semi-essencial, com potencial para estimular a resposta imunológica, propriedades anti-inflamatórias, que atua na manutenção da integridade da mucosa do trato gastrointestinal por estar envolvida no processo de renovação celular [13,14].

A L-arginina tem potencial para minimizar a mucosite [11], possui ação no processo de proliferação e memorização das células T, melhorando a atividade antitumoral [15] e ainda, pode estimular a produção de linfócitos B, aumentando a síntese do hormônio do crescimento e cicatrização [16].

Apesar dos benefícios da suplementação de L-arginina, as doses indicadas para os seres humanos parecem não estar padronizadas devido às discrepâncias encontradas na bibliografia, pois o *Dietary References Intake* (DRI) [17] indica a dose de 4,2 g/dia, enquanto Olszewer [16] e Mahan, Escott-Stump e Raymond [18] recomendam 1,5 e 10 g de arginina/dia, respectivamente.

No entanto, a suplementação com dose elevada de L-arginina por longos períodos exige cautela, pois poderá acarretar uma produção excessiva do fator vasodilatador óxido nítrico, podendo causar doenças cardiovasculares [19, 20].

O objetivo foi avaliar o efeito de doses baixas de suplementação com L-arginina sobre o hemograma, a integridade do DNA e do baço, no infiltrado inflamatório no jejuno e no coagulograma de ratas *Wistar* submetidas à quimioterapia com 5-FU.

Material e Métodos

O protocolo experimental foi aprovado pela Comissão de Ética no Uso de Animais da Universidade do Oeste Paulista (Unoeste), Presidente Prudente, SP, Brasil, protocolo de número 2955, seguindo os critérios de recomendações do Conselho Nacional de Controle de Experimentação Animal (CONCEA) [21] e *Guide for the Care and Use of Laboratory Animals of the Committee for the Update of the Guide for the Care and Use of Laboratory Animals* [22].

Procedimentos e Delineamento experimental

Utilizou-se 32 ratas, *Rattus novergicus* da linhagem *Wistar*, com peso corporal médio de 232,0 ± 22,8 g que, no início do experimento, estavam na fase de metaestro do ciclo estral e, no final, no estro.

As ratas foram mantidas em ambiente controlado, em fotoperíodo de 12/12 horas e temperatura ambiente de aproximadamente 23 ± 2°C [21,22].

Foi realizada a distribuição das ratas randomicamente em 4 grupos experimentais (8 ratas/grupo), contendo duas ratas por caixa, que receberam água filtrada e ração comercial balanceada (Supralab®, Alisul, Brasil) *ad libitum*, atendendo às exigências nutricionais segundo as recomendações do National Research Council (NRC) [23]: o grupo controle (G_c) foi incluído para obter os valores de referência dos parâmetros analisados, e cada rata recebeu 1 mL de cloreto de sódio 0,9%, por via intraperitoneal, para representar a aplicação do 5-FU realizada nos demais grupos. O grupo 5-FU ($G_{5\text{-}FU}$) recebeu uma dose de 5-FU [24]. Os grupos Arginina 50+5-FU (G_{Arg50}) e Arginina 100+5-FU (G_{Arg100}) receberam também uma dose de 5-FU e foram adicionados 50 e 100 mg L-arginina/dia respectivamente, na água *ad libitum* [24].

A dose de 5-FU que as ratas do $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} receberam foi de 200 mg de 5-FU/Kg de peso corporal, aplicada por via intraperitoneal [24].

A suplementação com L-arginina foi realizada, utilizando-se comprimidos efervescentes de aspartato de arginina (Tagifor®, Sanofi-Aventis, Brasil), dissolvidos na água do bebedouro.

As doses de L-arginina/dia para suplementar as ratas do G_{Arg100} e G_{Arg50}, foram extrapoladas alometricamente segundo Cubas e Joppert [25], de acordo com a dose recomendada para o ser humano segundo Olszewer [16], que é de 1,5g de L-arginina/dia

via oral, e a suplementação das ratas do G_{Arg50}, correspondeu à metade da dose recomendada.

Para o controle do consumo diário de água e L-arginina, o volume da água era medido antes de colocá-la no bebedouro. A cada 24 horas, subtraía-se o valor da sobra de água do valor inicial que foi adicionado no bebedouro. O volume que sobrou de água foi dividido pelo número de ratas por caixa, determinando o consumo médio de água e L-arginina por rata [11,24].

O consumo da ração comercial foi controlado, pesando a ração antes de colocá-la no comedouro e a cada 24 horas, subtraiu-se o valor da sobra da ração do valor inicial que foi adicionado no comedouro, o peso restante foi dividido pelo número de ratas por caixa, determinando o consumo médio de ração. Posteriormente, foram realizadas as análises da oferta calórica e protéica da ração.

Os primeiros sete dias do experimento foram considerados o período de adaptação das ratas às condições experimentais, e da padronização do consumo de L-arginina, água e ração. Considerou-se como o dia zero do experimento aquele em que foi aplicado o 5-FU nas ratas.

Colheita de sangue, intestino delgado e baço

As amostras de sangue, intestino delgado e baço foram colhidas 72 horas após a administração do 5-FU [24]. No dia da colheita das amostras de sangue, as ratas foram anestesiadas com 40 mg de Tiopental/Kg de peso corporal (Thiopentax®, Cristália, Brasil) [26] e colheram-se duas amostras de sangue por meio da punção cardíaca: a primeira amostra de sangue foi colhida em tubos a vácuo contendo EDTA para realização do teste do cometa, hemograma completo, contagem de plaquetas, concentração de fibrinogênio e Proteína Plasmática Total (PPT); a segunda amostra foi colhida em tubos a vácuo contendo citrato de sódio a 3,8%, para determinação dos tempos de protrombina (TP) e tromboplastina parcial ativada (TTPA).

Após as colheitas de sangue, as ratas morreram por “overdose” de barbitúrico, utilizando 100 mg de tiopental/Kg de peso corporal (Thiopentax®, Cristália, Brasil) [21].

Análises laboratoriais

Análise do sangue

Eritrócitos, Hemoglobina (Hb), Volume Globular (VG), Volume Corpuscular Médio (VCM), Hemoglobina Corpuscular Média (HCM), Concentração de Hemoglobina Corpuscular Média (CHCM), amplitude de distribuição do diâmetro das hemácias (RDW) e contagem total de leucócitos, foram determinados utilizando o analisador hematológico POCH-100$_i$V DIFF (Sysmex®, Brasil).

Para a contagem diferencial dos leucócitos, confeccionaram-se esfregaços sanguíneos corados por coloração rápida (Panótico, Laborclin®, Brasil), para posterior análise em microscópio óptico (E-200, Nikon®, Japão).

A concentração de fibrinogênio foi determinada pela técnica de refratometria calorimétrica de precipitação (56 ºC) pelo refratômetro ATC-ITREF-200 (Instrutemp®, Brasil) e os resultados estão expressos em mg/dL.

A concentração da PPT foi quantificada pelo refratômetro ATC-ITREF-200 (Instrutemp®, Brasil) e os resultados estão expresso em g/dL.

O TP foi determinado por meio da técnica de formação do coágulo utilizando-se kit comercial (TP clot®, BIOS Diagnótica Indústria e Comércio de Produtos Biológicos Ltda, Brasil) e os resultados estão expressos em segundos.

O TTPA foi determinado por meio da técnica de formação do coágulo, utilizando-se kit comercial (TTPA clot®, BIOS Diagnóstica Indústria e Comércio de Produtos Biológicos Ltda, Brasil).

Ensaio do cometa

A técnica do ensaio cometa foi adaptada de Silva [27] para coloração com Giemsa, conforme descrita por Fagiani et al. [28].

Para a realização da técnica do ensaio cometa, foram preparadas as soluções PBS (Phosphate Buffer Solution pH=7,4) (10X), solução de lise (1% Triton X-100, 10% DMSO, N_aCl 2,5M, EDTA 100mM, Tris 10, pH = 10), tampão para eletroforese (300mM N_aOH/1mM EDTA, contendo Solução A = N_aOH + água destilada e Solução B = EDTA + água destilada), tampão neutralizador [$NH_2.C\ (CH_2OH)^3$ pH = 7,5], agarose 1,5% (Sigma-Aldrich®), agarose 0,75% (Sigma-Aldrich® A/S, Brondby, Denmark) e Giemsa (Bioclin®, Brasil).

A amostra sanguínea das ratas foram distribuídas sobre as lâminas previamente preparadas com gel de agarose, as quais foram submetidas à corrida eletroforética.

As leituras das lâminas foram realizadas em microscópio óptico contando-se 200 cometas por rata. A especificação do dano ao DNA correspondeu a análise do diâmetro da cabeça do cometa em relação ao comprimento da cauda, classificados em classes de danos ao DNA de 0 a 4, conforme a descrição de Vilela et al. [29].

O índice total de danos (ITD) ao DNA foi calculado, aplicando-se a fórmula: ITD = (0 x n classe 0) + (1 x n classe 1) + (2 x n classe 2) + (3 x n classe 3) + (4 x n classe 4), onde n corresponde ao número de cometas de cada classe. Assim, o resultado do índice total de danos pode variar de 0 (0 x 200), onde não há danos, até 800 (4 x 200), com o DNA totalmente danificado [29].

Análises dos Cortes Histológicos do jejuno e do baço

Os tecidos do jejuno e baço foram coletados e fixados em formalina a 10%, durante 48h. Depois de cortados e acondicionados em cassetes histológicos, foram embebidos em blocos de parafina. Os cortes histológicos foram realizados mediante o corte do bloco de parafina por meio de um micrótromo, sendo que os cortes possuíram 3 µm de espessura, e foram dispostos em lâminas para microscopia, corados com hematoxilina-eosina e analisados em microscópio óptico (E200, Nikon®, Japão) [11].

As avaliações das lâminas foram realizadas aplicando-se os escores propostos por Gerez et al. [30]. Examinou-se uma área de 2,0-2,5 mm^2 para identificação das áreas íntegras e das lesões como depleção, centro germinativo, apoptose e necrose.

O escore lesional do baço pode variar de 0 (ausência de lesão) até 30 (altamente lesionado), que foi calculado aplicando-se a fórmula [30]:

Escore lesional = [(área da depleção x 1) + (área do centro germinativo x 1) + (área de apoptose x 2) + (área de necrose x 2)]

Para a avaliação do processo inflamatório do jejuno, aplicou-se o escore proposto por Gerez et al. [30] onde 0 ausência de inflamação; 1 corresponde a inflamação leve; 2 corresponde a inflamação média e 3 corresponde a inflamação grave.

Análise estatística

Os dados do consumo de água e ração, hemoglobina, HCM, linfócitos, as concentrações de fibrinogênio, classes de danos ao DNA, o ITD ao DNA, escore lesional

do baço e o infiltrado inflamatório do jejuno seguiram uma distribuição não paramétrica pelo teste de Shapiro-Wilk, foram analisados pelo teste de Kruskall-Wallis e as medianas foram comparadas pelo teste de Dunn com significância de 5%. Os resultados foram expressos em mediana ± desvio interquartílico [31].

Para a comparação dos dados do consumo de água e ração entre os períodos anterior e posterior à aplicação do 5-FU, dentro de cada grupo experimental, foi utilizado o teste de Wilcoxon com significância de 5%. Os resultados foram expressos em mediana ± desvio interquartílico [31].

Os dados do VG, VCM, MCV, CMHC, RDW-SD, plaquetas, leucócitos totais, neutrófilos, eosinófilos, monócitos, PPT e o processo inflamatório do intestino mostraram distribuição normal pelo teste de Shapiro-Wilk e foram analisados pela ANOVA *one-way*, e as médias, comparadas pelo teste de Tukey com significância de 5%. Os resultados estão expressos em média ± desvio padrão [31].

Resultados

Antes da quimioterapia, as ratas do G_c, $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} apresentaram peso corporal e consumo de ração semelhantes ($P>0,05$) (Tabela 1).

Após a quimioterapia, as ratas do $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} apresentaram redução ($P < 0,01$) no consumo de ração (Tabela 1) e somente as do G_{Arg50} não perderam peso corporal. As ratas do $G_{5\text{-}FU}$ e G_{Arg100} perderam respectivamente 9,96% e 9,66% de peso corporal ($P < 0,01$) (Tabela 1).

As ratas do $G_{5\text{-}FU}$, antes e após a quimioterapia, apresentaram o menor consumo de água ($P < 0,01$) (Tabela 1); o G_{Arg50} manteve o consumo de água, enquanto que o $G_{5\text{-}FU}$ e G_{Arg100} apresentaram redução no consumo de água ($P < 0,01$) (Tabela 1).

Tabela 1. Peso corporal, ingestão de ração e água de ratas *Wistar* suplementadas com L-arginina após aplicação do 5-Fluorouracil (média ± dp).

Grupos experimentais	Peso corporal (g)		Ingestão de ração (g/dia)		Ingestão de água (mL/dia)	
	Período de Adaptação	Depois da aplicação do 5-FU	Período de Adaptação	Depois da aplicação do 5-FU	Período de Adaptação	Depois da aplicação do 5-FU
Controle	233.4 ± 21.9^{Aa}	231.4 ± 21.6^{Aa}	22.5 ± 3.4^{Aa}	20.5 ± 1.3^{Ab}	45.5 ± 3.0^{ABa}	39.5 ± 4.3^{ABb}
$G_{5\text{-}FU}$	222.9 ± 22.8^{Aa}	200.7 ± 19.2^{Bb}	22.5 ± 0.7^{Aa}	12.3 ± 3.0^{Bb}	32.3 ± 1.7^{Ba}	28.4 ± 12.0^{Bb}
G_{Arg50}	228.4 ± 21.9^{Aa}	227.1 ± 17.7^{ABa}	22.9 ± 2.5^{Aa}	21.2 ± 1.6^{Ab}	50.8 ± 11.5^{Aa}	58.3 ± 2.5^{Aa}
G_{Arg100}	227.7 ± 23.7^{Aa}	205.7 ± 19.9^{ABb}	22.9 ± 2.6^{Aa}	19.0 ± 8.2^{ABb}	48.8 ± 9.8^{Aa}	37.9 ± 13.6^{Bb}

$^{A,B,C-}$ Letras maiúsculas diferentes nas colunas indicam diferença estatística entre os grupos ($P < 0.01$).
$^{a,b-}$ Letras minúsculas nas linhas indicam diferenças estatísticas antes e depois da aplicação do 5-FU dentro de cada grupo ($P < 0.01$).

As concentrações dos eritrócitos e da hemoglobina das ratas do G_{Arg50} foram maiores do que as do G_c (Tabela 2), mas não houve diferença entre o $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} (Tabela 2).

O RDW-SD das ratas do $G_{5\text{-}FU}$ e G_{Arg50} foram menores em comparação com o G_c (Tabela 2).

O VCM, HCM e CHCM não diferiram entre os grupos experimentais (Tabela 2).

Tabela 2. Eritrograma de ratas *Wistar* suplementadas com L-arginina após aplicação do 5-Fluorouracil.

Parâmetros analisados	Grupos experimentais				Valor de P
	Controle	$G_{5\text{-}FU}$	G_{Arg50}	G_{Arg100}	
Eritrócitos (x10^3/µL)	7,4±0.5^{B}	9,1±0.6^{AB}	9,9±0.9^{A}	8,0±0.4^{AB}	0.05
Hemoglobina (g/dL)	14.6±1.17^{B}	17.9±0.8^{AB}	19.3±0.9^{A}	15.6±5.4^{AB}	0.05
Volume Globular (%)	42.0±4.3^{B}	48.5±4.3^{AB}	53.6±3.2^{A}	45.4±7.3^{B}	0.05
VCM (fL)	54.4±0.8^{A}	53.5±1.6^{A}	54.6±1.7^{A}	53.9±1.7^{A}	0.51
HCM (pg)	19.4±0.6^{A}	19.4±0.7^{A}	19.8±0.5^{A}	19.7±0.7^{A}	0.57
CHCM (g/dL)	35.8±0.8^{A}	36.5±0.9^{A}	36.3±0.8^{A}	36.8±0.7^{A}	0.18
RDW-SD (fL)	26.8±0.5^{A}	24.8±1.0^{B}	24.7±1.2^{B}	25.5±2.2^{AB}	0.02

$^{A, B}$ – Letras diferentes nas linhas indicam diferença estatística entre os grupos.
VCM = volume corpuscular médio; HCM = hemoglobina corpuscular média; CHCM = concentração média de hemoglobina corpuscular; RDW-SD = Amplitude de Distribuição dos Eritrócitos medida como Coeficiente de Variação.

As ratas do G_{Arg100} apresentaram aumento na contagem de leucócitos totais, eosinófilos e linfócitos em relação ao $G_{5\text{-}FU}$ e G_{Arg50} (Tabela 3). A contagem de neutrófilos não apresentou diferença entre os grupos experimentais (Tabela 3).

As contagens de leucócitos totais, eosinófilos e linfócitos das ratas do $G_{5\text{-}FU}$ e G_{Arg50} foram semelhantes (Tabela 3).

As concentrações da PPT das ratas foram semelhantes entre os grupos experimentais (Tabela 3).

Tabela 3. Leucograma e proteína plasmática total de ratas *Wistar* suplementadas com L-arginina após aplicação do 5-Fluorouracil.

Parâmetros	Grupos experimentais				Valor de P
	Controle	$G_{5\text{-}FU}$	G_{Arg50}	G_{Arg100}	
Leucócitos totais (10^3/µL)	2,785±1,055^{AB}	1,844±505.5^{B}	1,975±774.1^{B}	3,387±565.6^{A}	0.02
Neutrófilos (10^3/µL)	1,110±926^{A}	1,376±46^{A}	1,420±269^{A}	1,231±1,046^{A}	0.85
Eosinófilos (10^3/µL)	75.2±26.8^{A}	20.8±11.2^{B}	29.8±15.3^{B}	79.0±52.8^{A}	0.01
Linfócitos (10^3/µL)	1,568±608.8^{A}	435.5±233.0^{B}	376.0±181.0^{B}	1,474±782^{A}	0.01
Monócitos (10^3/µL)	186.3±96.5^{A}	65.1±49.3^{B}	33.5±28.8^{B}	106.6±34.4^{AB}	0.01
Proteína plasmática total (g/dL)	6.9±0.7^{A}	7.2±0.9^{A}	7.5±0.7^{A}	7.1±1.1^{A}	0.67

$^{A, B}$ – Letras diferentes nas linhas indicam diferença estatística entre os grupos da mesma coluna.

As ratas do G_{Arg100} apresentaram menor escore lesional ($p<0,045$) na depleção do baço (1,00 ± 1,00), em comparação com o $G_{5\text{-}FU}$ (2,00 ± 0,0) e G_{Arg50} (3,00 ± 0,0) (Figura 1B, 1C e 1 D). O escore lesional na depleção do baço das ratas do G_{Arg100} eram semelhantes ($P>0,05$) ao G_c (1,00 ± 1,00) (Figura 1A e 1 B). O escore lesional na depleção do baço não diferenciou ($P>0,05$) entre o $G_{5\text{-}FU}$ e G_{Arg50} (Figura 1C e 1 D).

Os escores lesionais do baço como o centro germinativo (Controle = 1,00 ± 0,0; G5-FU = 2,00 ± 0,0; GArg50 = 3,00 ± 1,00 e GArg100 = 1,00 ± 1,00) e apoptose (Controle = 13,00 ± 1,00; G5-FU = 2,00 ± 0,0; GArg50 = 2,00 ± 0,0 e GArg100 = 2,00 ± 0,0) não apresentaram diferenças ($P>0,05$) entre os grupos experimentais (Figura 1A, 1B, 1C e 1 D).

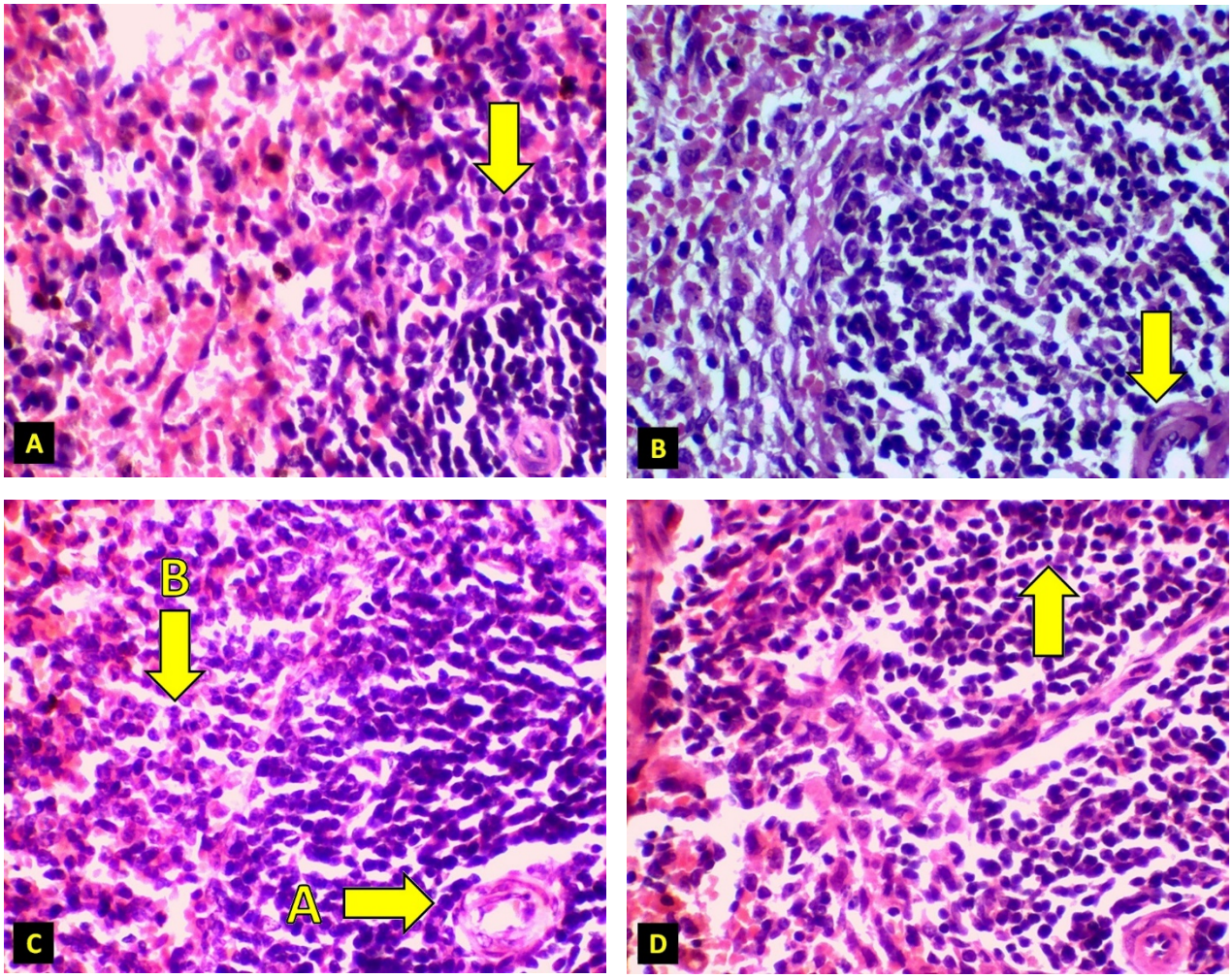

Figura 1. Fotomicrografia do baço de ratas *Wistar* suplementadas com L-arginina e submetidas à quimioterapia com 5-Fluorouracil (H.E. 400x).

A: G_c, seta apontando linfócito. B: $G_{5\text{-}FU}$, seta apontando o centro germinativo. C: G_{Arg50}, seta A: célula apoptótica e B: centro germinativo. D: G_{Arg100}, seta apontando linfócito.

As ratas do G_{Arg100} apresentaram o menor TP, em comparação com o $G_{5\text{-}FU}$ e G_{Arg50} (Tabela 4).

A contagem de plaquetas, as concentrações de fibrinogênio e TTPA das ratas não apresentaram diferenças estatísticas entre os grupos experimentais (Tabela 4).

Tabela 4. Concentração de plaquetas, tempo de protrombina, tromboplastina parcial ativada e fibrinogênio de ratas *Wistar* suplementadas com L-arginina após aplicação do 5-Fluorouracil.

Parâmetros	Grupos experimentais				Valor de P
	Controle	$G_{5\text{-}FU}$	G_{Arg50}	G_{Arg100}	
Plaquetas ($x10^3/\mu L$)	$1,195\pm201.2^{A}$	$1,002\pm304.2^{A}$	$1,033\pm160.0^{A}$	$1,094\pm251.0^{A}$	0.39
Tempo de protrombina (s)	17.6 ± 0.8^{AB}	18.0 ± 1.2^{A}	18.8 ± 2.0^{A}	15.5 ± 1.2^{B}	0.02
Tempo de tromboplastina parcial ativada (s)	32.2 ± 2.7^{A}	34.4 ± 3.8^{A}	34.5 ± 6.9^{A}	32.1 ± 8.1^{A}	0.92
Fibrinogênio (mg/dL)	275.0 ± 103.5^{A}	325.0 ± 103.5^{A}	325.0 ± 103.5^{A}	300.0 ± 106.9^{A}	0.74

$^{A, B}$ – Letras diferentes nas linhas indicam diferença estatística entre os grupos experimentais.

O escore do infiltrado inflamatório no jejuno das ratas do $G_{5\text{-}FU}$ (2,54±0,84); G_{Arg50} (3,20±0,63) e G_{Arg100} (2,75±0,57) não apresentaram diferença (P>0,05) (Figura 2).

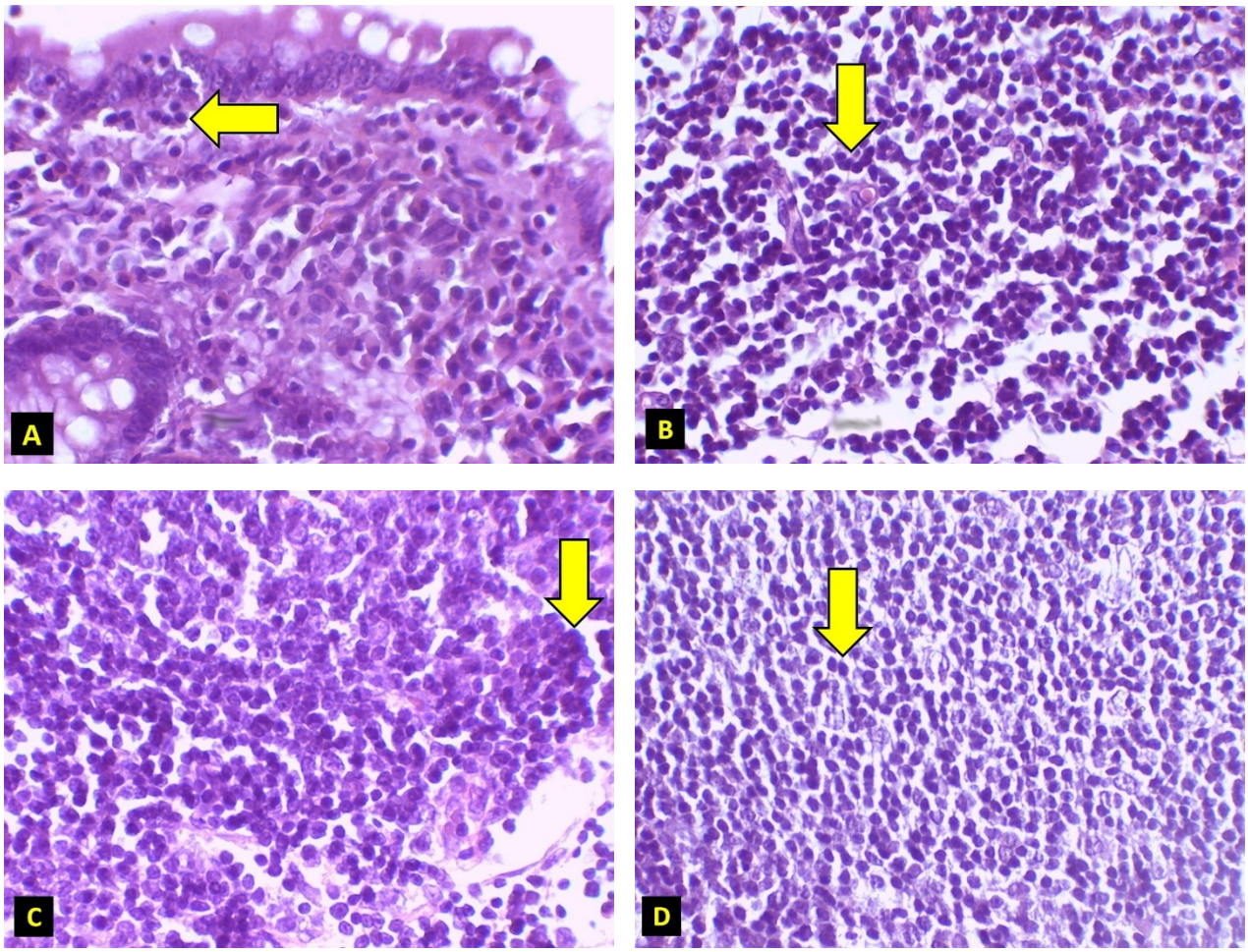

Figura 2. Fotomicrografia do infiltrado inflamatório no jejuno de ratas *Wistar* suplementadas com L-arginina e submetidas à quimioterapia com 5-Fluorouracil (H.E. 400x).

A: G_c. B: $G_{5\text{-}FU}$. C: G_{Arg50}. D: G_{Arg100}. Setas apontando linfócitos.

As ratas do $G_{5\text{-}FU}$ e G_{Arg100} apresentaram redução (P=0,03) nas quantidades de DNA íntegro em relação ao G_c (Figura 3a). No entanto, as quantidades de DNA íntegro e classe 3 de danos ao DNA das ratas do $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} foram semelhantes (P>0,05; Figuras 3a e 3d, respectivamente).

As quantidades de danos ao DNA das classes 1 e 4 não apresentaram diferenças (P>0,05) entre os grupos experimentais (Figuras 3b e 3e, respectivamente).

As ratas do G_{Arg100} apresentaram valores semelhantes (P>0,05) de danos ao DNA da classe 2 em relação às ratas do $G_{5\text{-}FU\ e}$ G_{Arg50} (Figura 3c). O G_{Arg50} apresentou redução (P<0,01) de danos ao DNA da classe 2 em relação ao $G_{5\text{-}FU}$ (Figura 3c).

As ratas do G_{Arg100} apresentaram o maior (P<0,05) ITD ao DNA (Figura 3f). O $G_{5\text{-}FU}$ foi semelhante (P>0,05) ao G_{Arg50} e maior do que o G_c (P>0,05) (Figura 3f). O ITD ao DNA das ratas G_{Arg50} não diferenciou do G_c e $G_{5\text{-}FU}$ (Figura 3f).

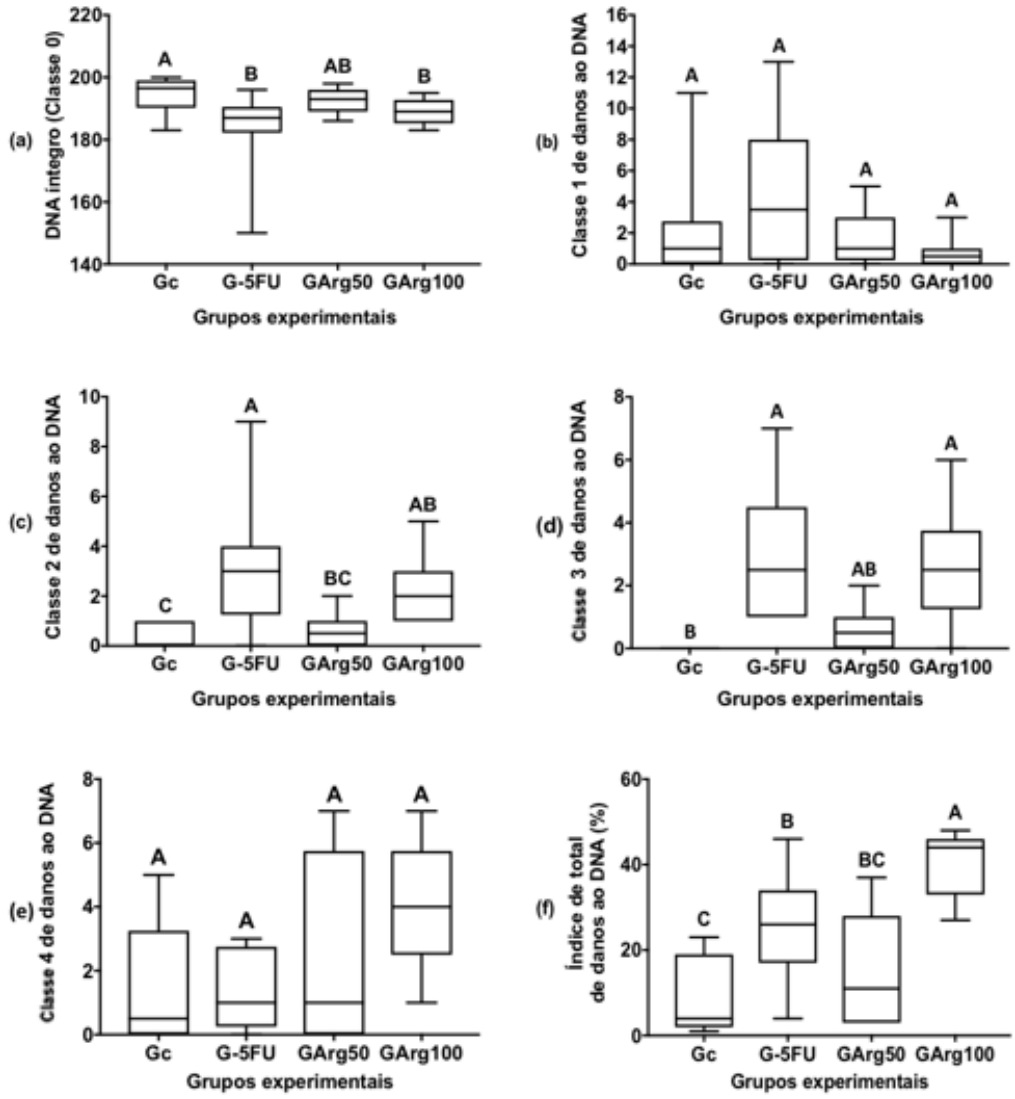

Figura 3. Contagem de DNA íntegro e danificado (classes 0 a 4) e índice total de danos ao DNA de ratas *Wistar* suplementadas com L-arginina após aplicação do 5-Fluorouracil. (a) DNA íntegro. (b) Classe 1 de danos ao DNA. (c) Classe 2 de danos ao DNA. (d) Classe 3 de danos ao DNA. (e) Classe 4 de danos ao DNA. (f) Índice total de danos ao DNA.

[A,B]- Medianas seguidas de pelo menos uma letra minúscula distinta indicam diferença estatística (P < 0,05) entre os grupos experimentais dentro das classes de dano ao DNA.

Discussão

A determinação do período do ciclo estral das ratas demonstrou que não houve interferência hormonal do ciclo sobre o hemograma, consumo de água, ração e perda de peso das ratas [32,33].

A redução no consumo de ração nos grupos $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} após a aplicação do 5-FU foi provavelmente devido aos efeitos adversos da quimioterapia, como a mucosite [34, 35] que causou dificuldades para a mastigação e deglutição da ração [36]. Porém, as ratas do G_{Arg50}, mesmo com a redução do consumo de ração, mantiveram seu peso corporal após a quimioterapia, fato que é benéfico para a saúde dos pacientes, pois segundo Aoyama et al., [37] a perda de peso, é um fator de risco para a continuidade do tratamento oncológico, mas não foi possível esclarecer os efeitos fisiológicos que estão envolvidos neste processo.

Segundo a análise dos componentes da ração consumida pelas ratas, ela foi classificada como fermentativa (64,86% de carboidratos), anabólica, devido à sua relação de nitrogênio:calorias não protéicas de 98:1, e não inflamatória, pois a porcentagem da distribuição de lipídios é inferior à soma da porcentagem de carboidratos e proteínas [38].

A policitemia apresentada pelo $G_{5\text{-}FU}$ e G_{Arg50} pode ter ocorrido devido ao quadro de diarreia ocorrido após a quimioterapia, levando à desidratação que segundo Silveira [39], são alterações hematológicas indicativas desta condição. Entretanto, a diarreia que ocorreu no G_{Arg100} foi de menor intensidade, não causando a desidratação, pelo fato da concentração dos eritrócitos, VG e PPT permanecerem dentro da normalidade com valores semelhantes aos do G_c, sendo esta a dose mais indicada neste caso.

Os índices hematimétricos das ratas do G_c, $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} permaneceram dentro da normalidade, com hemácias normocíticas e normocrômicas [40,41], corroborando com Balmant et al. [11], demonstrando que as suplementações com até 458 mg de L-arginina/dia não interferem no tamanho e na coloração das hemácias.

A suplementação com 100 mg de L-arginina/dia minimizou a imunossupressão causada pelo 5-FU, uma vez que a contagem de leucócitos totais e linfócitos das ratas do G_{Arg100} era semelhante ao G_c e permaneceram dentro da normalidade, corroborando com o estudo realizado por Balmant et al. [11], que também demonstraram que as suplementações com 295 e 458 mg de L-arginina/dia minimizaram a imunossupressão gerada pelo 5-FU. Isto é benéfico para pacientes em quimioterapia, pois influencia na tolerância do indivíduo ao quimioterápico e tornando-o mais resistente a infecções [42,43], e as doses baixas de suplementação garantem menor custo com a suplementação.

As ratas do G_{Arg100} apresentaram 200% e 300% menos depleção no baço do que o $G_{5\text{-}FU}$ e G_{Arg50} respectivamente, ocorrendo maior concentração de linfócitos no centro germinativo, demonstrando que esta dose amenizou a imunossupressão gerada pelo 5-FU, pois segundo Murphy [44], o centro germinativo é composto por linfócitos B e T que podem conferir a essas ratas, melhor resistência imunológica, por meio da resposta imunomediada pelos linfócitos.

O G_{Arg50} não apresentou redução da imunossupressão no baço, devido à possível mielossupressão causada pela quimioterapia, expondo o organismo ao risco de desenvolver infecções [34, 45], e a necessidade de reduzir a intensidade das doses de quimioterapia.

As ratas do $G_{5\text{-}FU}$, G_{Arg50} e G_{Arg100} apresentaram inflamação semelhante no jejuno, de intensidade moderada gerada pelo 5-FU, denominada como mucosite, que não foi minimizada pelas suplementações. Entretanto, Balmant et al. [11], utilizando doses maiores de L-arginina (295 e 458 mg/dia) e Iwase et al. [46], observaram redução no processo inflamatório gerado pela quimioterapia.

O TP do $G_{5\text{-}FU}$ foi elevado pela quimioterapia, indicando maior tempo para o sangue coagular. Isto é indesejável para o tratamento antineoplásico cirúrgico, e poderá inviabilizar o procedimento devido ao risco de hemorragia. Entretanto, a suplementação do G_{Arg100} demonstrou minimizar o efeito colateral do 5-FU, reduzindo o TP, favorecendo a ativação da cascata da coagulação [40].

A contagem de plaquetas e o TTPA dos grupos foram semelhantes ao G_c, indicando o 5-FU e as suplementações com L-arginina não interferiram nestes parâmetros sanguíneos. Porém, Balmant et al. [12], utilizando doses maiores de suplementação com L-arginina (295 e 458 mg/dia) e Iwase et al. [46], observaram aumento na contagem de plaquetas.

O G_{Arg100} apresentou aumento no ITD ao DNA, que pode potencializar o mecanismo de ação do 5-FU e, assim, contribuir para a inibição do crescimento tumoral [47].

Em contrapartida, o G_{Arg50} apresentou porcentagem de DNA íntegro semelhante ao G_c e redução no ITD ao DNA, indicando a preservação da do DNA, fato que vai contra o mecanismo de ação do 5-FU, o que se torna indesejável, por talvez prejudicar a involução tumoral.

Conclusão

A suplementação com 100 mg de L-arginina/dia foi benéfica para minimizar a imunossupressão e a depleção no baço, causada pela quimioterapia com 5-FU. Além disso, ainda pode reduzir o tempo de coagulação sanguínea e contribuir para a quebra do DNA gerada pelo 5-FU.

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ANEXO A: PARECER FINAL DO COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) E DA COMISSÃO DE ÉTICA EM USO DE ANIMAIS (CEUA)

UNOESTE - Universidade do Oeste Paulista

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

PPG - Programa de Pesquisa de Pós-Graduação
PEIC - Programa Especial de Iniciação Científica

Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"EFEITO DA SUPLEMENTAÇÃO COM L-ARGININA NA INTEGRIDADE DO BAÇO, FIGADO E INTESTINO DELGADO DE RATOS WISTAR SUBMETIDOS À QUIMIOTERAPIA COM 5-FLUOROURACIL"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **4900** e tendo como participante(s) **MARCELA DE ANDRADE BERNAL FAGIANI (discente), MARINA GONCALVES AVANTE (discente), KESIA ARAUJO DA SILVA (discente), SARA BERTOZO BEZERRA DA SILVA (discente), RENAN SANTANA CELESTINO (discente), PAULO FELIPE IZIQUE GODOZO (docente), LUIS SOUZA LIMA DE SOUZA REIS (orientador responsável)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 12/09/2018.

MATERIAL ARMAZENADO/DOADO

Protocolo(s)	Data Aprovação	Armazenado (local)	É doação	Detalhes armazenamento
2955	16/03/2016	UNOESTE	NÃO	Laboratório de Patologia do Hospital Veterinário da Unoeste

Presidente Prudente, 23 de Setembro de 2018.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CPDI

Profª Ms. Adriana Falcão de Reis
Coordenadora da CEUA - UNOESTE

ANEXO B: CERTIFICADO PREMIAÇÃO NO CONGRESSO GANEPÃO (2018)

GANEPÃO 2018
40 anos

CERTIFICADO

CERTIFICAMOS QUE

Marcela de Andrade Bernal Fagiani, Antônio Fluminhan, Fabiola de Azevedo Mello, Sandra Cristina Genaro, Luís Souza Lima de Souza Reis

Receberam a Menção Honrosa com o trabalho intitulado na **EFEITO DA SUPLEMENTAÇÃO COM L-ARGININA NA INTEGRIDADE DO ÁCIDO DEXORRIBONUCLEICO EM RATOS SUBMETIDOS À QUIMIOTERAPIA COM 5-FLUOROURACIL** na modalidade de Temas Livres – Experimental no **20º Fórum Paulista de Pesquisa em Nutrição Clínica e Experimental** por ocasião do Ganepão 2018, 8º ICNO/CBNC - International Conference of Nutritional Oncology Congresso Brasileiro de Nutrição em Câncer e 2º NEXSA - Congresso Internacional de Nutrição, Exercício e Saúde.

São Paulo, 15 de junho de 2018

Dan L. Waitzberg
Presidente

Maria de Lourdes Teixeira da Silva
Secretária Geral

Angela F. L. Waitzberg
Secretária Geral

Giliane Belarmino
Coordenador Científico

8º ICNO CBNC

2º NEXSA
Congresso Internacional de Nutrição, Exercício e Saúde

ANEXO C: INSTRUÇÕES PARA AUTORES DA REVISTA *NUTRITION*

GUIDE FOR AUTHORS

Your Paper Your Way

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To find out more, please visit the Preparation section below. INTRODUCTION

Nutrition provides an international forum for professionals interested in the applied and basic biomedical nutritional sciences, and publishes papers both of clinical interest and of scientific import. Investigators are encouraged to submit papers in the disciplines of nutritionally related biochemistry, genetics, immunology, metabolism, molecular and cell biology, neurobiology, physiology, and pharmacology. Papers on nutrition-related plant or animal sciences which are not of direct relevance to man, whereas occasionally of interest are not the main focus of the Journal.

Nutrition publishes a wide range of articles, which includes original investigations, review articles, rapid communications, research letters, case reports and special category manuscripts. Manuscripts must be prepared in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" developed by the International Committee of Medical Journal Editors (N Engl J Med 1991;324:424-428). All submissions are peer reviewed.

Original Investigation (3000-5000 words including tables, figures and references)

Original investigations are considered full-length applied (human) or basic (bench work) research reports. They cover topics relevant to clinical and basic studies relevant to man in the following areas nutritionally related biochemistry, genetics, immunology, metabolism, molecular and cell biology, neurobiology, physiology, and pharmacology. Studies in adult and pediatric populations are welcome. The work presented in the manuscript must be original; studies confirming previous observations will be considered. Other considerations of a paper's publishability are its importance to the science, the soundness of the experimental design, the validity of methods, the appropriateness of the conclusions and the quality of presentation.

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Papers representing concise and original studies of scientific importance are considered. In the cover letter the author should justify the request for Rapid Communication. The review process is 10 days, authors are allowed one revision if accepted, and the final version of the paper appears in the next available issue of the journal.

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A Research Letter contains new data or a clinical observation, in a format that allows for rapid publication.

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In-depth, comprehensive state of the art reviews on a nutritional topic are welcomed. Reviews may be invited by the Editor or may be unsolicited viewpoints.

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Editorials express opinions on current topics of interest, or provide comments on papers published in Nutrition or other journals. Editorials are generally solicited by one of the Editors.

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Collections of abstracts representing the proceedings of organizational meetings are not subjected to customary peer review. It is the view of the Editorial Board that it is of service to the nutrition community to present such material as promptly as possible.

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Novel insights into a significant questions or clinical issues are welcome, and will be peer reviewed. As the definition of âhypothesisâ suggests, articles of this type should be, although they lack direct experimental evidence, closely tied to empirical data and lead to testable predictions.

Special Article (up to 5000 words including tables, figures and references)

Associated with a particular special event, invitation or announcement; for example, the annual John M. Kinney Awards papers.

SUBMISSION_CHECKLIST

Ensure that the following information and files have been included. One author has been designated as the corresponding author with contact details: E-mail address Full postal address

Manuscript: Word doc or similar required. PDF is not suitable for review and production. Include keywords. Has been spell-checked and grammar checked. Has been edited by professional, preferably native-English-speaking editor.

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If applicable include as separate files: Graphical abstracts Highlights (3-5, document file) Supplemental files

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BEFORE YOU BEGIN

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