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

TALITA RIZO PEREIRA

INFLUÊNCIA DA SUPLEMENTAÇÃO COM L-ARGININA NA REMODELAÇÃO CARDÍACA DE RATOS SUBMETIDOS AO 5-FLUOROURACIL

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

Orientadora: Prof. Dra. Francis Lopes Pacagnelli

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Presidente Prudente, 26 de Março de 2021.

BANCA EXAMINADORA

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DEDICATÓRIA

Poderia dedicar aos mortos, já que tenho dois deles expressos e grafados em minha memória e coração exatamente todos os dias ao levantar e ao me deitar como se ainda estivessem nesta dimensão e a qualquer momento pudessem me chamar ou ligar...

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“Não tentes ser bem sucedido, tenta antes ser um homem de valor.”
Albert Einsten

RESUMO

Influência da suplementação com L-arginina na remodelação cardíaca de ratos submetidos ao 5-fluorouracil

A remodelação cardíaca (REM) é uma condição que afeta os cardiomiócitos, vasos sanguíneos e a matriz extracelular (ECM), podendo ser resultante de inúmeros fatores como, cardiomiopatias e drogas antioneoplásicas. O 5-fluorouracil (5-FU) é uma destas drogas, utilizado no tratamento de canceres de mama, cabeça, pescoço e esôfago, é um quimioterápico antimetabólito que está relacionado a cardiotoxidade por promover isquemia. A L-arginina (ARG), um aminoácido semi-essencial sintetizado no intestino delgado e nos rins, tem sido empregado como suplemento e cooperativo na presença de doenças que afetam o sistema cardiovascular e poderia ser um recurso terapêutico cardioprotetor aplicado nesta condição. Portanto, o objetivo deste trabalho foi avaliar o efeito de baixas doses de suplemento de L-arginina na REM de ratos *Wistar* submetidos à quimioterapia com 5-FU. Foram utilizados 48 ratos machos com 40 dias de idade, peso corporal médio de 140 ± 10g, divididos em 4 grupos, n= 12 cada grupo: controle (CT): tratados com soro fisiológico, grupo 5-FU (1 dose de 200 mg), grupo ARG: tratados preventivamente com ARG (100mg) em água filtrada e 5-fluorouracil + L-arginina (5-FU+ARG). Após a eutanásia, 72 horas da introdução do quimioterápico, o coração foi pesado, dissecado e processado para análise histológica e de expressão gênica. Os ratos tratados com 5-FU, ARG e ARG+FU apresentaram redução do peso corporal e nenhum grupo apresentou hipertrofia. Houve uma redução da fractabilidade e melhora da organização celular no grupo ARG+5-FU e o grupo tratado apenas com ARG apresentou fibrose. A expressão gênica dos colágenos (*Col) 1a1, 1a2* e *3*, do marcador de vasodilatação *(Nos 3)* e do fator induzível de hipóxia *(Hif1-α)* apresentaram redução nos ratos submetidos ao quimioterápico. A associação 5-FU+ARG não alterou essa diminuição. Concluímos que apenas uma sessão de 5-fluorouracil promoveu uma expressão gênica cardíaca alterada e que sua associação com L-arginina não reverteu a diminuição dos colágenos, do óxido nítrico e do fator induzível de hipóxia.

Palavras-chave: agente antineoplásico; suplementação; miócitos; colágenos fibrilares; remodelação ventricular.

ABSTRACT

Influence of L-arginine supplementation on cardiac remodeling in rats discovered with 5-Fluorouracil

Cardiac remodeling (REM) is a condition that affects cardiomyocytes, blood vessels and the extracellular matrix (ECM), and may be the result of numerous factors such as cardiomyopathies and antioneoplastic drugs. 5-fluorouracil (5-FU) is one of these drugs, used to treat cancers of the breast, head, neck and esophagus, it is an antimetabolite chemotherapy that is related to cardiotoxicity by promoting ischemia. L-arginine (ARG), a semi-essential amino acid synthesized in the small intestine and kidneys, has been used as a supplement and cooperative in the presence of diseases that affect the cardiovascular system and could be a cardioprotective therapeutic resource applied in this condition. Therefore, the objective of this work was to evaluate the effect of low doses of L-arginine supplement on REM in *Wistar* rats submitted to chemotherapy with 5-FU. 48 male rats with 40 days of age, average body weight of 140 ± 10g were used, divided into 4 groups, n = 12 each group: control (CT): treated with saline, group 5-FU (1 dose of 200 mg), ARG group: preventively treated with ARG (100mg) in filtered water and 5-fluorouracil + L-arginine (5-FU + ARG). After euthanasia, 72 hours after the introduction of chemotherapy, the heart was weighed, dissected and processed for histological analysis and gene expression. The rats treated with 5-FU, ARG and ARG + FU showed a reduction in body weight and none of the groups presented hypertrophy. There was a reduction in fractability and improvement in cell organization in the ARG + 5-FU group and the group treated only with ARG showed fibrosis. The gene expression of collagens *(Col) 1a1, 1a2* and *3*, of the vasodilation marker *(Nos 3)* and of the inducible hypoxia factor *(Hif1-α)* showed a reduction in rats submitted to chemotherapy. The association 5- FU + ARG did not alter this decrease. We concluded that only one session of 5-fluorouracil promoted altered cardiac gene expression and that its association with L-arginine did not reverse the decrease in collagen, nitric oxide and the inducible hypoxia factor.

Keywords: antineoplastic agent; dietary supplements; myocytes; fibrillar collagens; ventricular remodeling.

LISTA DE SIGLAS

ANOVA	–	Analysis of Variance
ARG	–	L-arginine
AT	–	Atria
BH4	–	Tetrahydrobiopterin cofactor
BW	–	Body weight
BWF	–	Final body weight
CT	–	Control
ECM	–	Extracellular Matrix
FD	–	Fractal dimension
FBW	–	Final body weight
Gapdh	–	Glyceraldehyde-3-phosphate dehydrogenase
HE	–	Hematoxylin-Eosin
Hif-1α	–	Hypoxia-inducible factor 1 alpha
LV	–	Left ventricles
NO	–	Nitric oxide
Nos2	–	Inducible nitric oxide synthase
Nos3	–	Endothelial nitric oxide synthase
PSR	–	Picrosirius red
REM	–	Heart Remodeling
ROS	–	Reactive oxygen species
RV	–	Right ventricles
TBP	–	TATA-Box Binding Protein
5-FU	–	5- Fluorouracil

SUMÁRIO

1 ARTIGO- CAN L-ARGININE ATTENUATE 5-FLUOROURACIL-INDUCED CARDIOTOXICITY IN RATS?

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Abstract

Objective: The aim of this study was to evaluate the effects of low dose L-arginine (ARG) supplements on myocardial structures and the gene expression of vasodilator and hypoxia markers of *Wistar* rats submitted to 5-fluorouracil (5-FU) chemotherapy.

Methods: Forty-eight male *Wistar* rats (40 days old, weights of 140g ± 10g) were allocated to four groups (12 rats per group): the control group (CT) received a 0.9% physiological solution to simulate the 5-FU application of the other groups; the 5-FU group received a 200mg dose of 5-FU; the ARG group received 100mg of ARG/day (added to drinking water) for 11 days; and the 5- fluorouracil+L-arginine (5-FU+ARG) group received a dose of 5-FU supplemented with 100 mg of ARG (drinking water).

Results: None of the rats presented cardiac hypertrophy. The ARG group showed fibrosis. The 5-FU+ARG group demonstrated reductions of fractal dimensions and improvements to cellular organization. The 5-FU group exhibited less gene expression of collagen type 1, alpha1 *(Col1a1)*, collagen type 1, alpha2 *(Col1a2)*, collagen type 3 *(Col3)*, nitric oxide synthase 3 *(Nos3)*, and hypoxia-inducible factor 1 alpha *(Hif-1α).*

Conclusion: Acute application of 5-FU promoted alterations in protein-encoding genes related to collagen, hypoxia, and vasodilator response. In the 5-FU+ARG group, the ARG supplement did not reverse decreases in collagen or *Hif-1α.* ARG also did not stimulate a vasodilator response to the 5-FU-provoked reduction in nitric oxide (*Nos3*) gene expression.

Keywords: Antineoplastic Agent; Dietary Supplements; Myocytes; Fibrillar Collagens; Ventricular Remodeling

Introduction

With the surge of neoplasm cases around the world, the use of antineoplastic drugs has increased [1]. In 1957, Duschinsky and collaborators developed 5-FU as an alternative to treat cancers. It is currently the third most commonly used chemotherapeutic agent in the treatment of solid malignancies. It is frequently used in the treatment of several types of cancers, such as colorectal carcinoma, breast and aerodigestive tract tumors, and breast, head, neck, and pancreas cancers [2-4]. 5-FU antimetabolite chemotherapy acts by interfering with the synthesis of DNA and RNA and inhibiting thymine synthesis [5]. However, 5-FU has low specificity for neoplastic cells and causes genotoxic effects on normal cells and side effects that include mucositis, immunosuppression, myelosuppression, and cardiotoxicity [6].

Cardiotoxicity is the second most common effect induced by 5-FU [7]. In the heart, toxicity from 5-FU can provoke coronary artery spasms (vasoconstriction, ischemia and hypoxia), angina during exertion or rest, and acute coronary syndromes (e.g., myocardial infarctions) autoimmune myocardial damage, thrombogenic effects, increased myocardial oxygen consumption, arrhythmias, and sudden death [2,3,8]. 5-Ischemia caused by 5-FU can alter the extracellular matrix (ECM), which disrupts proper cardiac function [9].

ARG, a semi-essential amino acid to the human body, is used to combat ischemia. Among its various functions, ARG acts as a substrate for the enzymatic production of nitric oxide (NO), a cardiac vasodilator [10]. Considering the importance of NO bioavailability in cardiovascular and metabolic disease treatments, the therapeutic potential of ARG supplements to normalize cellular NO levels and promote vasodilation has been discussed [4,11,12].

However, high doses of ARG for long periods can cause excessive production of the nitric oxide vasodilator, leading to cardiotoxicity [6]. In addition, the optimal ARG dose is still unknown and likely varies according to the attributes of the patient and target organ. Several studies have reported 1.5 g/day to 4.2 g/day as appropriate ARG doses for administration in humans [6].

The effects of ARG supplements on structural and molecular mechanisms are still largely unknown. 5-FU chemotherapy can lead to structural changes, cardiac ischemia, and hypoxia from coronary vasospasm; thus, examining the potential of ARG to inhibit these alterations is of great clinical importance for the development of

new treatment strategies. The aim of this study was to evaluate the effects of low doses of ARG supplements on myocardial structures and the gene expression of vasodilator and hypoxia markers in *Wistar* rats submitted to 5-FU chemotherapy.

Methods

Experimental groups

The experimental protocol was approved by the Ethical Committee on Animal Use of the University of Western São Paulo, Presidente Prudente, Sao Paulo, Brazil (protocol number 5832).

We used 48 male *Wistar* rats. They were 40 days old and had mean body weights (BW) of 140 ± 10 g. The rats were kept in a controlled environment (temperatures: 23°C ± 2°C; 12/12 h photoperiods) and had access to commercial rat feed (Supralab, Alisul, Brazil) *ad libitum* [6]. Rats were randomly distributed into four experimental groups (12 rats per group). Experimental groups were divided into three boxes (four rats per box). The control group (CT) received a 0.9% physiological solution to simulate the 5-FU application of the other groups; the 5-FU group received a 200mg dose of 5-FU; the ARG group received 100mg of ARG/day (drinking water) for 11 days; and the 5-FU+ARG group received a dose of 5-FU supplemented with 100 mg of ARG (drinking water) [6,13].

For ARG supplements, we used effervescent arginine aspartate tablets (Tagifor®, Sanofi-Aventis) dissolved in drinking water. The concentrations of ARG used in the experiment were allometrically extrapolated to the rats from the recommended dose of 1.5 g/d of ARG for humans [6]. The allometric scale calculates dose changes based on body surface area comparisons, with appropriate considerations to differences in anatomical, physiological, biochemical, and pharmacokinetic processes between two species [14]. However, no standards of dosing and application exist for ARG in the treatment of cardiovascular conditions [12,15,16].

We controlled ARG consumption by measuring the volume in water before and after placement in the water dispenser. Every 24 hours, we subtracted the final volume (surplus) from the initial volume. To obtain the mean consumption of water/ARG per rat, we divided the resulting volume by the number of rats per box [6].

We considered the first seven days of the experiment as the adaptation period; in this period, the rats adapted to the experimental conditions (e.g., environment, water, food, ARG supplement standardization). Day zero of the experiment occurred when the rats started 5-FU and ARG treatments (after the adaptation period). The rats were euthanized after 72 h of 5-FU application [6].

Evaluation of anatomical parameters

The rats were euthanized (barbiturate overdose: 100 mg/Kg, thiopental) after their final body weights (FBW) were measured [6]. Hearts were removed and dissected to separate the atria (AT), right ventricle (RV), and left ventricle (LV). These parts were then weighed. Anatomical parameters were normalized (AT/FBW, RV/FBW, and LV/FBW) and used as the hypertrophy index [17].

Histology and fractal analysis

Cardiac tissue samples were fixed in 10% buffered formalin solution for a period of 48 h. After fixation, the tissues were stored in paraffin blocks, which enabled coronal histological analysis of the 4-μm sections. The histological sections were stained with hematoxylin-eosin (HE) solution for measurement of cardiomyocyte cross-sectional areas, according to the standard laboratory histological protocol [18]. We selected 15 histological fields from each rat. To make the cardiomyocyte sets from different groups as uniform as possible, we selected 50 rounded and central-core cardiomyocytes in the subendocardial layer of the left ventricular muscle wall (cross-sectional view). We used the average values of sectional areas obtained from each group as cell size indicators [18].

The histological sections were also stained with picrosirius red (PSR), according to standard laboratory histological protocols [18]. For histological slide analysis, images were captured using a LEICA DMLS microscope (DM750, Leica Microsystems, Wetzlar, Germany) with 400 x magnification. We used LV sections stained with PSR to quantify ECM. Analyzes were performed using Image J software, following the software instructions for collagen quantification [18,19].

We used LV sections stained with PSR to assess tissue fractal dimension (FD). For FD analysis, the photographs were binarized for reading, and FD was estimated by the box-counting method using Image J software. The software

considered the box-counting in two dimensions; this method enables pixel distribution quantification even without image texture. With this method, two images with the same pixel distribution (binarized image, gray level image) express the same FD. The analysis of the fractal histological slides was based on the relation between the resolution and the evaluated scale, and the result was quantitatively expressed as the FD of the object, that is FD ¼ (Log Nr/Log r_1; Nr, being the quantity of equal elements needed to fill the original object, was scale applied to the object). FD was calculated using ImageJ software set between 0 and 2, without consideration to different textures [19].

RNA extraction and target gene expression

The mRNA relative abundance of collagen genes collagen type 1- alpha1 (*Col 1a1*), collagen type 1 - alpha2 (*Col1a2*), collagen type 3 (*Col3*), inducible nitric oxide synthase and endothelial nitric oxide syntase (*Nos2*) and (*Nos3*), hypoxia-inducible factor 1 alpha (*HIF-1α*), and reference genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and TATA-Box Binding Protein (*TBP*) were analyzed by RT-PCR according to a previously described method [20]. Total RNA was extracted from LV myocardiums with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and treated with DNase I (Invitrogen Life Technologies). One microgram of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit, according to standard methods (Applied Biosystems, Foster City, CA, USA). Aliquots of cDNA were then submitted to a real-time PCR reaction using a customized assay containing sense and antisense primers and Taqman (Applied Biosystems, Foster City, CA, USA) probes specific to each gene: *Col1a1* (Rn01463848_m1), *Col1a2* (Rn01526720_m1), *Col3* (Rn01437664_g1), *Nos2* (Rn00561646_m1), *Nos3* (Rn07312037_g1), and *Hif-1α* (Rn01472828_m1) [20,21].

Amplification and analysis were performed using Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression data were normalized to reference gene expressions: *Gapdh* (Rn01775763 g1) and *TBP* (Rn01455648_m1). Reactions were performed in duplicate, and expression levels were calculated using the CT comparative method ($2^{-\Delta\Delta CT}$) [20,21].

Statistical analysis

We used the Shapiro–Wilk test for normality analysis. Data are expressed as mean ± standard deviation or median and 25th and 75th percentile. For comparison between groups, the nonparametric Kruskal–Wallis test was used followed by Dunn's post hoc test. For parametric data, we used the ANOVA test (one-way), followed by Tukey's post test. A P <0.05 value was considered significant. GraphPad Prism Version 5.00 (Graph-Pad Software, La Jolla, CA, USA) software was used [18].

Results

Food and water intake

Before and after chemotherapy, rats from the control, 5-FU, ARG, and 5-FU+ARG groups had similar food intake (P > 0.05; Table 1). Before and after chemotherapy, ARG group rats consumed more water than CT group rats (P < 0.05; Table 1).

Table 1. Food and water intake (mean ± SD) in Wistar rats subjected to 5-FU treatment and dietary L-arginine supplementation.

Experimental groups	Food intake (g/day)		Water intake (mL/day)	
	Before 5-FU treatment	After 5-FU treatment	Before 5-FU treatment	After 5-FU treatment
CT	29.20±2.0	27.3±2.1	39.25±4.0	41.7±3.0
5-FU	28.30±6.1	25.7±4.7	36.0±6.6	39.12±3.5
ARG	30.20±5.6	23.3±9.8	51.14±17.5*	49.87±5.4#
5-FU+ARG	37.7±16.1	33.9±5.9	36.81±14.1	34.54±18.1

CT: control, 5-FU: 5-fluorouracil, ARG: arginine, 5-FU+ARG: 5-fluorouracil+ arginine, Values are means ± SD, g/day: grams/day, ml/day: milliliters day, Statistical difference: *ARG before 5-FU treatment vs. CT before 5-FU treatment; # ARG after 5-FU treatment vs. CT after 5-FU treatment, ANOVA and Tukey tests.

Evaluation of anatomical parameters

We observed significant statistical differences between the groups in FBW. The 5-FU group weighed less than the CT; the 5-FU+ARG group weighed less than the ARG group; and the FU+ARG group weighed less than the CT (Table 2).

Table 2. Anatomical data in Wistar rats subjected to 5-FU treatment and dietary L-arginine supplementation.

Parameters	Experimental Groups			
	CT (n= 12)	5- FU (n= 12)	ARG (n= 12)	FU+ARG (n= 12)
BWF (g)	414.4 ± 11.95	368.6 ± 16.47*	404.2 ± 35.05	356.8 ± 17.17*#
AT (g)	0.07 (0.06 -0.09)	0.06 (0.06 – 0.08)	0.07 (0.07 – 0.08)	0.07 (0.06- 0.07)
AT/BWF (mg/g)	0.29 (0.26 – 0.30)	0.23 (0.22 – 0.26)	0.25 (0.23 – 0.28)	0.25 (0.21 – 0.28)
RV (g)	0.96 ± 0.06	0.91 ± 0.09	0.97 ± 0.11	0.87 ± 0.04
RV/BWF (mg/g)	0.17 (0.15- 0.21)	0.17 (0.16 – 0.21)	0.19 (0.18 – 0.21)	0.19 (0.18 – 0.20)
LV (g)	0.69 (0.61 -0.74)	0.65 (0.60 – 0.72)	0.64 (0.58 – 0.69)	0.71 (0.59 – 0.79)
LV/BWF (mg/g)	2.32 ± 0.12	2.48 ± 0.23	2.42 ± 0.16	2.46 ± 0.14

CT: control, ARG: L-arginine, 5-FU: 5-fluorouracil, FU+ARG: 5-fluorouracil+ L-arginine. Data are expressed as mean ± standard deviation or median and 25th and 75th percentile. N: number of animals, BWF: body weight final, AT: atria weight, LV: left ventricle, RV: right ventricle. Statistical difference: *p< 0,05 vs CT; # p < 0.05 vs. ARG. ANOVA and Tukey or Kruskal–Wallis and Dunn.

Cardiomyocyte area analysis

Left ventricular myocyte areas did not differ between groups (Figure 1).

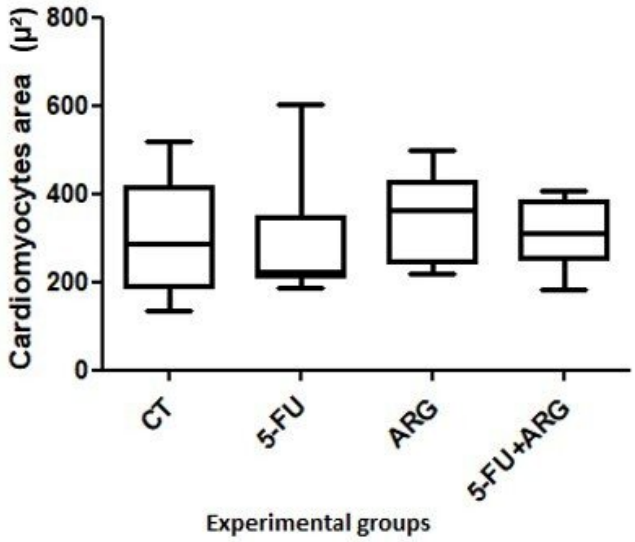
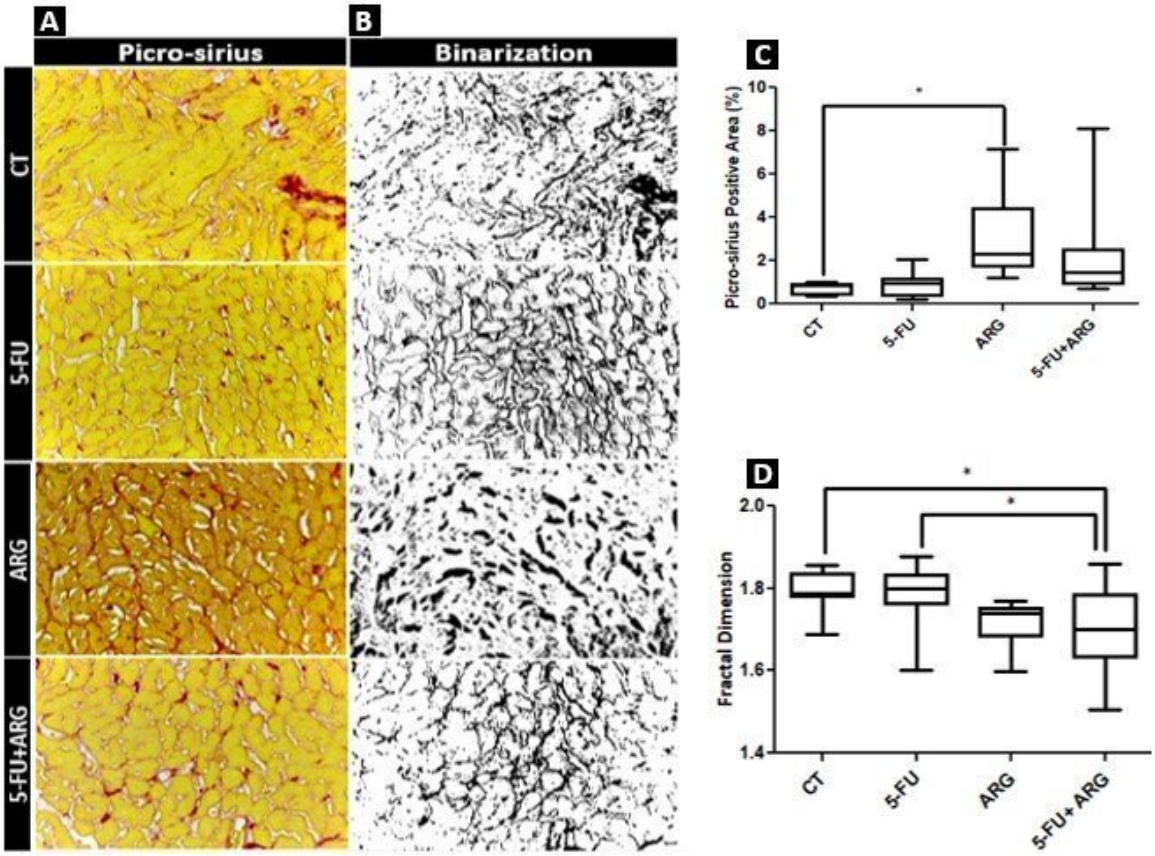


Figura 1. Histomorphometric analysis. CT: control, ARG: L-arginine, 5-FU: 5-fluorouracil, FU+ARG: 5-fluorouracil+ L-arginine. Kruskal-Wallis test followed by Dunn's post-test.

259 **Fibrosis measurement and fractal analysis**

260 Cross-sectional analyzes of left ventricles stained by PSR showed significant
261 increases in the area of collagen content (fibrosis) in the ARG group when compared
262 to the CT group (p= 0.0009) (Figure 4). The FD of collagen fibers stained in PSR was
263 lower in the 5-FU+ARG group than in the 5-FU group (p = 0.012) (Figure 2).

264



265

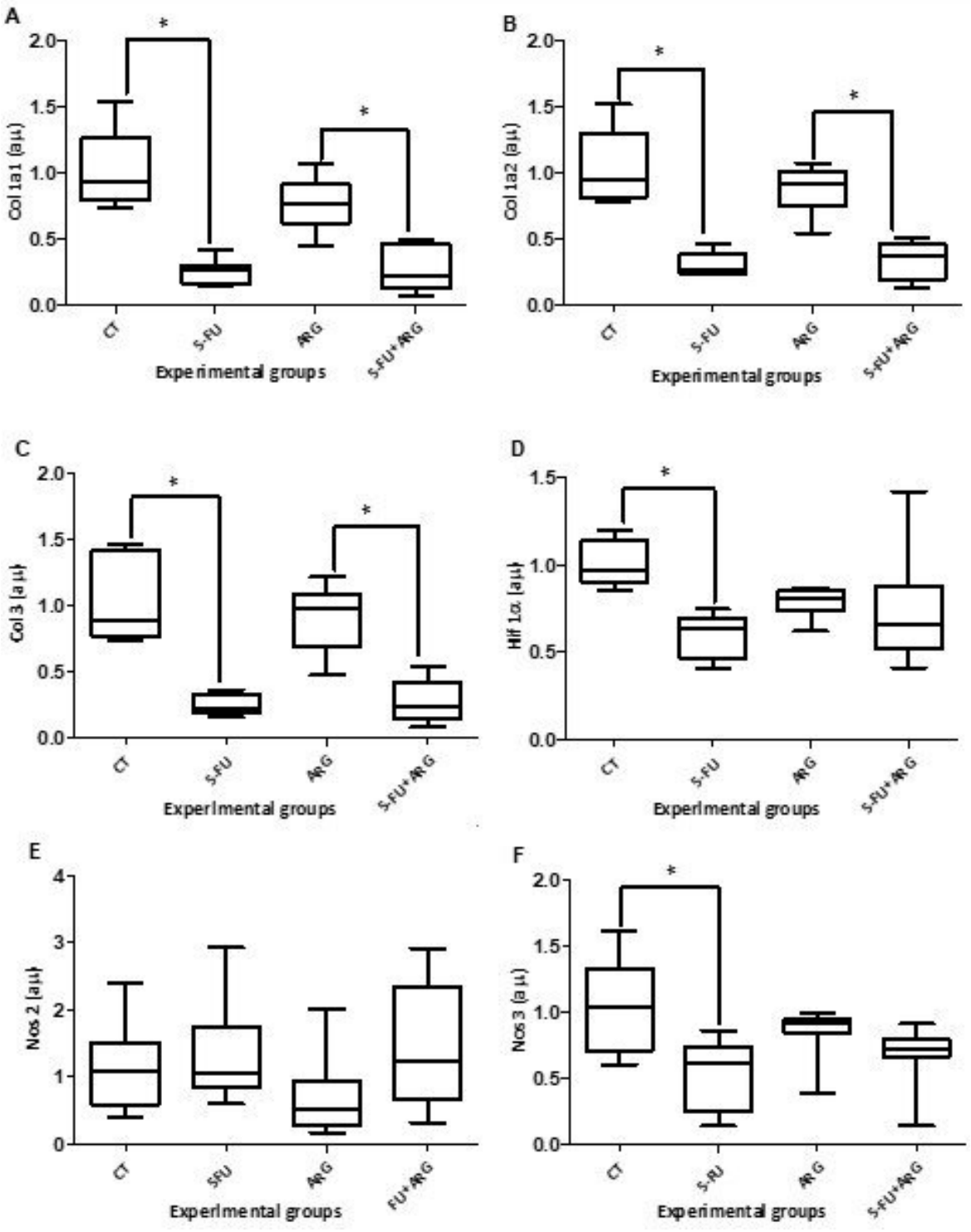
266 **Figure 2.** Histological sections of the left ventricle stained with Picrosírius Red (A). Analysis of the
267 fractal dimension after the binarization process, colagen fibers are black and the rest of the cell is
268 white (B). ×400 magnification. PSR-stained area (C). Collagen fibers organization (D). CT: control,
269 ARG: L-arginine, 5-FU: 5-fluorouracil, FU+ARG: 5-fluorouracil+ L-arginine. Kruskal-Wallis and Dunn's
270 post-test. * p< 0.05.

271

272 **Expression of target genes**

273 When we evaluated the profiles of gene-encoding proteins related to cardiac
274 ECM remodeling, we found a lower abundance of *Col1a1*, *Col1a2*, and *Col3* in the 5-
275 FU group than in the CT group. This was also the case for the 5-FU+ARG group
276 when compared to the ARG group (p<0.001) (Figures 3A, 3B, 3C).

277 The 5-FU group showed a significant reduction in cardiac hypoxia marker
278 *Hif-1α* gene expression when compared to the CT group ($p<0.001$) (Figure 3D). The
279 5-FU group also showed significantly less gene expression of cardiac vasodilation
280 marker *Nos3* when compared to the CT group ($p<0.007$) (Figures 3E and 3F).
281
282



283

Figure 3. Expression of target genes. Col1a1- collagen type 1, alpha1 (A); Col1a2- collagen type 1, alpha2 (B); Col3- alpha 1 collagen type 3 (C); Hlf1α- hypoxia 1-alpha inducible factor (D); Nos2- nitric oxide synthase 2 (E); Nos3- nitric oxide synthase 3 (F). *p<0,001. aμ- arbitrary unity. ANOVA and Tukey or Kruskal- Wallis and Dunn's testes.

Discussion

Considering the widespread use of 5-FU for patients with cancer, treatments that attenuate its cardiotoxic effects are urgent. ARG promotes vasodilation, improving perfusion and the supply of nutrients and oxygen to various organs. It is also cheap and readily available [10]. Our study demonstrated that only one 5-FU treatment session resulted in cardiotoxic effects. However, we could not find evidence that ARG supplements counteract 5-FU cytotoxicity in rats.

Various studies have reported beneficial effects from ARG dietary supplements. However, the effects of ARG on the structure and function of pathologically-altered hearts undergoing treatment with 5-FU have not yet been thoroughly examined.

We observed that 5-FU reduced body weight both with and without ARG supplements. Song et al. 2013 [22] also demonstrated in mice that 5-FU caused a significant reduction in body weights that was accompanied by diarrhea. The authors suggested that 5-FU administration acutely affects animal weight, possibly due to the activation of inflammatory responses followed by gastrointestinal malfunction [22].

In our study, ARG group rats (ingested ARG without 5-FU) consumed more water than rats in other groups, which resulted in better hydration. Araújo [23] showed that this behavior is a result of the palatability of ARG; when ARG is added to water, its taste is pleasant for rats.

We observed an absence of left cardiac hypertrophy in analysis of anatomical parameters and cardiomyocytes; this effect may be due to an acute factor in the chemotherapy. Pahlavani et al. [12] identified right ventricular hypertrophy promoted by chronic ARG supplementation; the supplement (45 days/ARG) seemed to induce physiological hypertrophy, as no signs of myocardial fibrosis were observed [12].

In our study, we observed an increase in fibrosis in the hearts of rats supplemented with ARG. However, we identified no changes in the expression of the genes that encode collagen 1 and 3 related proteins. The fibrosis may have been

provoked by a synthesis of collagen types IV, V, and VI (not investigated in the study) or minimal degradation due to low metalloproteinase activation [24]. To achieve a better understanding of these effects, future studies need to elucidate post translational factors involved in different types of collagen and functional mechanisms [9].

We observed reductions in FD in the 5-FU+ARG group, which suggests organization of the extracellular matrix. Through histological slides, we can use fractal dimensions to identify structural alterations, characterize irregular structures, and quantify existing alterations [18,25]. Fractal dimensions have been used to evaluate left ventricles during human heart transplants, neoplasms, and cardiac variables [18,25]. FD also enables the quantification of differences between reparative fibrosis and reactive fibrosis. Both fibrosis forms manifest in the myocardium extracellular matrix. Reparative fibrosis is characterized by the disorderly filling of collagen in empty spaces (due to myocyte loss). Reactive fibrosis is characterized by increased orderly deposition of collagen and lack of collagen filling (due to minimal myocyte loss) [25].

The expression of collagen-related genes was lower in groups submitted to chemotherapy; ARG did not seem have an effect on expression. The selective and specific reduction of collagen gene expression was one of the most significant cellular effects of 5-FU on fibroblasts and likely caused alterations in the balance of enzymes involved in the extracellular collagen decomposition. After secretion, collagens likely join other ECM components and are degraded by a family of zinc endopeptidases. Low metalloproteinase activation in these scenarios [26] decreases collagen gene expression and may contribute to cardiotoxicity and impaired functional performance of cardiac cells in chemotherapy [24]. However, post-transcriptional and functional evaluations are needed to assess the repercussions of this reduction.

In our study, rats treated with only one dose of 5-FU presented a significant reduction in the gene expression of cardiac vasodilation marker Nos3; this may indicate oxidative stress [7,26]. Reductions to NO production and bioavailability are often combined with an increase in reactive oxygen species (ROS). Inflammation and endothelial pathophysiology frequently occur in such cases [27]. This occurred through reactions with ROS and led to the following: reduction of NO bioavailability;

formation of peroxynitrite; and oxidation of the Nos3 BH4 cofactor, which uncoupled this enzyme and generated further oxidative stress [28].

ARG was expected to cause a vasodilatory response by acting on the increase of *Nos2* and *3*. ARG is the main substrate in the production of nitric oxide synthases, which are expressed as stimuli and smooth muscle vasodilation. ARG has shown significant positive effects when used as a supplement to treat hypertension in humans and animal models. In rat models, ARG supplements seem to regulate hemodynamics and restore renovascular homeostasis [29]. However, factors such as the form of administration, dose, and evaluated tissue can justify the differences in the results.

Although we observed a reduction in *Nos3* expression, we did not record an increase in the hypoxia biomarker. On the contrary, we observed that 5-FU decreased *Hif-1α*; this effect was not altered by ARG in the 5-FU+ARG group [30]. Study reported that high levels of ROS activation leads to degradation and low expression of *Hif-1α* [30].

We used mRNA encoding protein analysis in the heart to gain insights into the molecular events underpinning the effects of ARG supplements on structural and molecular parameters of rats submitted to 5-FU chemotherapy. Gene expression, due to its specificity and sensitivity, has been increasingly used in diagnostics. Although the functionality of individual mRNAs is measurable, this measure is intricately linked to protein expression and activity. Our study does not elucidate the possible effects of ARG supplements on mRNA alterations or modulations of transcription and post-transcription factors that amplify the changes caused by 5-FU.

Conclusion

In our study, just one 5-FU chemotherapy session downregulated the transcription of collagens, vasodilators (Nos2 and Nos3), and Hif-1α target genes. Low dose of ARG supplements combined with 5-FU did not reverse the cardiotoxicity caused by chemotherapy.

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References

[1] Lewandowska AM, Rudzki M, Rudzki S, Lewandowski T, Laskowska B. Environmental risk factors for cancer – review paper. Annals of Agricultural and Environmental Medicine 2019: 26: 1-7. doi: 10.26444/aaem/94299.

[2] Lamberti M, Porto S, Zappavigna S, Addeo E, Marra M, Miraglia N, Sannolo N, Vanacore D, Stiuso P, Caraglia M. A mechanistic study on the cardiotoxicity of 5-fluorouracil in vitro and clinical and occupational perspectives. Toxicology Letters 2014: 227: 151-156. https://doi.org/10.1016/j.toxlet.2014.03.018.

[3] Sara JD, Kaur J, Khodadadi R, Rehman M, Lobo R, Chakrabarti S, Herrmann J, Lerman A, Grothey A. 5-fluorouracil and cardiotoxicity: a review. Therapeutic advances in medical oncology 2018: 10: 1758835918780140.

[4] Francis N. The need for routine monitoring of cardiac function in patients receiving 5- fluorouracil infusion. Clin J Oncol Nurs 2014: 18: 3: 360-362. doi: 10.1188/14.CJON.360-362

[5] Galarza AFA: Avaliação genotípica e fenotípica da enzima diidropirimidina desidrogenase (DPD) e risco de toxicidade com o uso de fluoropirimidinas. Universidade Federal do Rio Grande Do Sul. (2016)

[6] de Andrade Bernal Fagiani M, Fluminhan A, de Azevedo Mello F, Yabuki D, GonçalvesGV, Tsujigushi LK, Pereira LG, da Silva KA, da Silva SBB, Santarem CL, et al. L-arginine minimizes immunosuppression and prothrombin time and enhances the genotoxicity of 5- fluorouracil in rats. Nutrition 2019: 66: 94-100. 10.1016/j.nut.2019.04.012.

[7] Focaccetti C, Bruno A, Magnani E, Bartolini D, Principi E, Dallaglio K, Bucci EO, Finzi G, Sessa F, Noonan DM, Albini A. Effects of 5-fluorouracil on morphology, cell cycle, proliferation, apoptosis, autophagy and ROS production in endothelial cells and cardiomyocytes. Plos one 2015: 11: 10. doi: 10.1371/journal.pone.0115686

[8] Lestuzzi C, Vaccher E, Talamini R, Lleshi A, Meneguzzo N, Viel E, et al. Effort myocardial ischemia during chemotherapy with 5-fluorouracil: an underestimated risk. Ann Oncol 2014: 25:5: 1059-1064. doi: 10.1093/annonc/mdu055

[9] Frangogiannis NG. The extracellular matrix in myocardial injury, repair, and remodeling. The Journal of Clinical Investigation 2017: 127: 1600-1612. doi: 10.1172/JCI87491

[10] Hmaid AAAA, Markelic M, Otasevic V, Masovic S, Jankovic A, Korac B, et al. Structural alterations in rat myocardium induced by chronic l-arginine and l-NAME supplementation. Saudi journal of biological sciences 2018: 25: 537-544. https://doi.org/10.1016/j.sjbs.2016.01.022

[11] Barcelos GT, Rossato DD, Perini JL, Pinheiro LP, Carvalho C, Jaenisch RB, Rhoden CR, Lago PD, Nunes RB. Effects of l-arginine supplementation associated with continuous or interval aerobic training on chronic heart failure rats. Metabolism 2017: 76: 1-10. https://doi.org/10.1016/j.metabol.2017.06.009.

[12] Pahlavani N, Jafari M, Sadeghi O, Rezaei M, Rasad H, Rahdar H, Entezari M. L-arginine supplementation and risk factors of cardiovascular diseases in healthy men: a double-blind randomized clinical trial 2017: 3: doi: 10.12688/f1000research.5877.2.

[13] Leocádio PC, Antunes Mm Fau - Teixeira LG, Teixeira Lg Fau - Leonel AJ, Leonel Aj Fau - Alvarez-Leite JI, Alvarez-Leite Ji Fau - Machado DCC, Machado Dc Fau – Generoso SV, Generoso Sv Fau - Cardoso VN, Cardoso Vn Fau - Correia MITD, Correia MI. L-arginine pretreatment reduces intestinal mucositis as induced by 5-FU in mice.

[14] Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. Journal of basic and clinical pharmacy 2016: 7: 27-31. doi: 10.4103/0976-0105.177703.

[15] Rodrigues-Krause J, Krause M, Rocha IMGd, Umpierre D, Fayh APT. Association of l- Arginine Supplementation with Markers of Endothelial Function in Patients with Cardiovascular or Metabolic Disorders: A Systematic Review and Meta-Analysis. Nutrients 2018: 11: 15. doi: 10.3390/nu11010015.

[16] Ramos L, Labat R, Carvalho FAS, Martin AB, Lopes-Martins RÁB. Efeito da administração oral de arginina sobre a pressão arterial e parâmetros cardíacos em ratos submetidos ao bloqueio crônico da síntese de óxido nítrico. Revista Brasileira de Medicina do Esporte. 2006: 12: 169-174. https://doi.org/10.1590/S1517-86922006000400001

[17] Pacagnelli FL, de Almeida Sabela AK, Okoshi K, Mariano TB, Campos DH, Carvalho RF, et al. Preventive aerobic training exerts a cardioprotective effect on rats treated with monocrotaline. Int J Exp Pathol 2016: 97: 3: 238-247. doi: 10.1111/iep.12166

[18] De Oliveira Mantovani R, Pinheiro DG, De Oliveira GLF, Perrud SN, Teixeira GR, Nai GA, Veras ASC, de Almeida Tavares ME, de Oliveira Mendes L, Pacagnelli FL. Effect of different doses of 2,4-dichlorophenoxyacetic acid (2,4-d) on cardiac parameters in male Wistar rats. Environmental Science and Pollution Research 2021: 28: 3078-3087. doi: 10.1007/s11356-020-10699-y.

[19] Fávero PF, Vieira de Lima VA, Helena Dos Santos P, Marques Andrade AP, Mendes LO, Pacagnelli FL, et al. Differential fractal dimension is associated with extracellular matrix remodeling in developing bovine corpus luteum. Biochem Biophys Res Commun 2019: 27:516: 888-893. doi: 10.1016/j.bbrc.2019.06.002

[20] Reyes DRA, Gomes MJ, Rosa CM, Pagan LU, Damatto FC, Damatto RL, Depra I, Campos DHS, Fernandez AAH, Martinez PF, et al. N-Acetylcysteine Influence on Oxidative Stress and Cardiac Remodeling in Rats During Transition from Compensated Left Ventricular Hypertrophy to Heart Failure. Cellular Physiology and Biochemistry 2017: 44: 2310-2321. doi: 10.1159/000486115.

[21] Brattelid T, Winer LH, Levy FO, Liestøl K, Sejersted OM, Andersson KB. Reference gene alternatives to Gapdh in rodent and human heart failure gene expression studies. BMC molecular biology 2010: 11: 22-22. 10.1186/1471-2199-11-22.

[22] Song M-K, Park M-Y, Sung M-K. 5-Fluorouracil-induced changes of intestinal integrity biomarkers in BALB/c mice. Journal of cancer prevention 2013: 18: 322-329. doi: 10.15430/jcp.2013.18.4.322.

[23] Araújo EON, Junior AF, Silva DAF, Dundi CG, Genaro SC, Nogueira RMNB, Chacur MGM, Reis LSLS: Efeito de diferentes doses de l-arginina em ratos após a quimioterapia com 5-fluorouracil. Mestre, Unoeste. PÓS-GRADUAÇÃO MESTRADO EM CIÊNCIA ANIMAL (2017).

[24] Li L, Zhao Q, Kong W. Extracellular matrix remodeling and cardiac fibrosis. Matrix Biol 2018: 68: 69: 490-506. doi: 10.1016/j.matbio.2018.01.013

[25] Captur G, Karperien AL, Hughes AD, Francis DP, Moon JC. The fractal heart - embracing mathematics in the cardiology clinic. Nat Rev Cardiol 2017: 14: 1: 56-64. doi: 10.1038/nrcardio.2016.161

[26] Bulstrode NW, Mudera V, DA McGrouther, Grobbelaar AO, Cambrey AD. 5-fluorouracil selectively inhibits collagen synthesis. Plast Reconst Sur 2005: 116: 1: 209-221. doi: 10.1097/01.prs.0000169701.16509.d6.

[27] Premer C, Kanelidis AJ, Hare JM, Schulman IH. Rethinking Endothelial Dysfunction as a Crucial Target in Fighting Heart Failure. Mayo Clinic proceedings Innovations, quality outcomes 2019: 3: 1-13. doi: 10.1016/j.mayocpiqo.2018.12.006

[28] Keshet R, Erez A. Arginine and the metabolic regulation of nitric oxide synthesis in cancer. Disease models & mechanisms 2018: 11: 8. doi: 10.1242/dmm.033332

[29] Gokce N. L-Arginine and Hypertension. The Journal of Nutrition 2004: 134: 2807S-2811S. doi: 10.1093/jn/134.10.2807S.

[30] Qutub AA, Popel AS. Reactive oxygen species regulate hypoxia-inducible factor 1alpha differentially in cancer and ischemia. Molecular and cellular biology 2008: 28: 5106-5119. doi: 10.1128/MCB.00060-08.

ANEXO A- PARECER FINAL- COMISSÃO ÉTICA EM USO DE ANIMAIS

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 **"AVALIAÇÃO DO REMODELAMENTO CARDÍACO DE RATOS SUBMETIDOS À QUIMIOTERAPIA COM 5-FLUOROURACIL E TRATADOS COM L-ARGININA"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **5832** e tendo como participante(s) **TALITA RIZO PEREIRA (discente), MARIA VITORIA DA SILVA CARVALHO (discente), ANDRESSA PAOLA PEREGO NUNES (discente), FABIO PIEMONTE LOPES (discente), INES CRISTINA GIOMETTI CEDA (docente), LUIS SOUZA LIMA DE SOUZA REIS (docente), FRANCIS LOPES PACAGNELLI (orientador responsável)**, foi avaliado e **APR. COM RECOMENDAÇÃO** 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 APR. COM RECOMENDAÇÃO em reunião realizada em 13/11/2019.

MATERIAL ARMAZENADO/DOADO

Protocolo(s)	Data Aprovação	Armazenado (local)	É doação	Detalhes armazenamento
4075	19/12/2017	UNOESTE	SIM	no laboratório de Anatomia Patológica

Presidente Prudente, 13 de Dezembro de 2019.

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

Profª Ms. Adriana Falco de Brito
Coordenadora da CEUA - UNOESTE

Coordenadoria de Pesquisa, Desenvolvimento e Inovação – CPDI – 18 3229-2079 – cpdi@unoeste.br
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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.

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Has been spell-checked and grammar checked.
Has been edited by professional, preferably native-English-speaking editor.

Tables:
Include titles, description, footnotes.
Create tables in the document rather than inserting image files, so that changes can be made.

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Ensure all figure and table citations in the text match the files provided.

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

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All references mentioned in the Reference List are cited in the text, and vice versa.
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Relevant declarations of interest have been made.
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