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L-arginine minimizes immunosuppression and prothrombin time and enhances the genotoxicity of 5-fluorouracil in rats

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of low doses of L-arginine supplementation on hemogram, integrity of DNA and spleen, inflammatory infiltrate in the jejunum, and in the coagulogram of rats submitted to 5-fluorouracil (5-FU) chemotherapy.
Methods: Thirty-two Wistar rats were fed commercial feed and water ad libitum and grouped into four (eight rats per group): The control group was given a 0.9% physiologic solution to simulate the application of 5-FU in the other groups; the $G_{5\text{-FU}}$ group was given a dose of 5-FU; the G_{Arg50} and G_{Arg100} groups were given a dose of 5-FU and supplemented with 50 and 100 mg L-arginine/d added in the drinking water ad libitum.
Results: The rats in the G_{Arg50} group did not lose weight after chemotherapy. G_{Arg50} rats presented polycythemia owing to dehydration caused by diarrhea generated by 5-FU. G_{Arg100} rats had increased total leukocyte count, eosinophils, lymphocytes, and index in the total index of DNA damage, yet showed a reduction in prothrombin time and in the spleen depletion index. Rats in the $G_{5\text{-FU}}$, G_{Arg50}, and G_{Arg100} groups had similar moderate inflammatory infiltrate in the jejunum.
Conclusion: Supplementation with 100 mg/d of L-arginine minimized immunosuppression, spleen depletion, and prothrombin time and contributed to the breakdown of 5-FU–generated DNA in Wistar rats. Supplementation with 50 mg/d of L-arginine decreased the weight loss generated by 5-FU in Wistar rats. Supplements with 50 or 100 mg of L-arginine did not interfere with 5-FU–generated jejunal inflammatory infiltrate.

Introduction

Among the alternatives for treatment of neoplasias, chemotherapy has been used with 5-fluorouracil (5-FU). This chemotherapy is effective in the treatment of the colon, rectum, anus, esophagus, stomach, liver, bile duct, pancreas, and salivary gland cancers [1,2].

According to the World Health Organization [3], cancer is the second leading cause of death in the world, resulting in the deaths of 8.8 million individuals in 2015, and in Brazil, the National Cancer Institute [4] estimated that 600 000 new cancer cases occurred in 2018 and 2019, with treatment costs estimated to be $150 billion by 2020, involving surgeries, health covenants, and improved technologies [5,6].

The cytotoxic effect caused by 5-FU in cells is related to the inhibition of DNA synthesis [7] in which 5-FU is converted by the pyrimidine monophosphate kinase and pyrimidine diastasis kinase enzymes [8] into 5-fluoro-2′-deoxyuridine-5′-triphosphae (5-FdUTP), which integrates into the DNA molecule causing the

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breakage of its ribbons and consequently leads to cellular apoptosis [9]. However, because 5-FU has low specificity for neoplastic cells, this chemotherapeutic drug also causes genotoxicity in normal tissue cells, generating side effects such as mucositis, nausea, vomiting, diarrhea, anorexia, stomatitis, immunosuppression, and myelosuppression and affecting the spleen [10]. These symptoms can affect 60% to 100% of patients undergoing treatment, further compromising their health [11,12].

To minimize the side effects of 5-FU and to provide better quality of life to patients during chemotherapy, some nutrients, such as arginine, have been used [13,14]. L-arginine is a semi-essential amino acid that has the potential to stimulate the immune response, has anti-inflammatory properties, acts to maintain the mucosal integrity of the gastrointestinal tract by being involved in the cell renewal process, and also can minimize the occurrence of bacterial translocation in the intestine [15,16].

L-arginine may impair the neoplastic proliferation and the metastasis process because it is a substrate of the enzymes arginase and nitric oxide (NO) synthase, which promotes the production of NO and acts on the modulation of neoplastic cell growth [17]. In addition, this amino acid has the potential to minimize mucositis [13]; plays a role in the proliferation and storage of T cells derived from T lymphocytes, improving antitumor activity [18]; and may stimulate the production of B lymphocytes, increasing the synthesis of growth hormone and healing [19].

Despite the benefits of L-arginine supplementation, the doses indicated for humans do not appear to be standardized owing to discrepancies found in the literature. The Dietary Reference Intake [20] indicates a dose of 4.2 g/d, whereas Olszewer [19] and Mahan et al. [21] recommend 1.5 and 10 g/d of arginine, respectively.

Some studies evaluating the effects of L-arginine associated with mucositis amelioration and immunosuppression generated by 5-FU chemotherapy have used the highest doses of L-arginine that are indicated for humans [13,22]. However, high-dose supplementation of L-arginine for long periods may lead to excessive production of the vasodilator NO, associated with the vascular endothelium, and may cause cardiovascular diseases [23,24].

The aim of this study was to evaluate the effect of low doses of L-arginine supplementation on hemogram, integrity of DNA, and spleen in the inflammatory infiltrate in the jejunum and in the coagulogram of Wistar rats submitted to 5-FU chemotherapy.

Material and methods

The experimental protocol was approved by the Ethical Committee on Animal Use of the Universidade do Oeste Paulista, Presidente Prudente, SP, Brazil.

Procedures and experimental design

The study included 32 female Wistar rats, with a mean body weight (BW) of 232 ± 22.8 g. At the beginning of the experiment, the rats were in the metaestrus phase of the estrous cycle and in the estrus phase at the end of the experiment. The rats were kept in a controlled environment, with a photoperiod of 12/12 h at an ambient temperature of ~23°C ± 2°C [25,26].

Rats were randomly distributed into four experimental groups (eight rats per group), containing two rats per box that received filtered water and balanced commercial feed (Supralab, Alisul, Brazil) ad libitum: The control group (Gc) was included to obtain the reference values of the parameters analyzed and 1 mL of 0.9% sodium chloride was applied intraperitoneally to simulate the application of 5-FU assayed in group 5-FU ($G_{5\text{-}FU}$) [22] and the groups supplemented with 50 (G_{Arg50}) and 100 mg/d (G_{Arg100}) L-arginine, respectively, in water ad libitum [22].

The dose of 5-FU that the rats in the three 5-FU groups received was 200 mg of 5-FU/kg BW, applied intraperitoneally [22].

L-arginine supplementation of the rats was performed using effervescent arginine aspartate tablets, without addition of vitamin C (Tagifor, Sanofi-Aventis, Brazil), dissolved in the drinking water.

The concentrations of L-arginine used in the experiment were extrapolated allometrically [27] to the rats from the dose of 1.5 g/d of L-arginine recommended by Olszewer [19] for humans.

The daily consumption of L-arginine was controlled so that the volume of water was measured before being placed in the rat drinker, and after 24 h, the volume of leftover water was measured in the drinking fountain. By subtracting the second volume of water from the first volume, the average daily water consumption of the animals in each box, containing two rats, was determined and divided by the number of rats. Thus, mean water and L-arginine consumption per rat was determined [13,22].

The commercial ration used was Supralab (Alisul, Brazil), and its consumption was measured daily by the difference of weights between the diet offered and the rest of the diet consumed in the boxes during 24 h. After 24 h, the remainder of the feed was weighed on a scale, and the first weight of the second weight, averaged in grams, was subtracted from the feed consumption of each treatment group. The average dietary intake of the group was divided by the number of rats in the box to determine the individual dietary intake.

During the first 7 d of the experiment, the period of adaptation of the rats to the experimental conditions was considered, such as the environment, the standardization of L-arginine, and water and feed intake. After this period, day 0 of the experiment was considered the day that was applied to 5-FU in the rats.

Harvest of blood, small intestine, and spleen

Samples of blood, small intestine, and spleen were collected 72 h after administration of 5-FU, which is the time required for induction of mucositis [22]. On the day of collection of the blood samples, rats were anesthetized with 40 mg/kg BW of thiopental (Thiopentax, Cristália, Brazil) [28], and two blood samples were collected by cardiac puncture: The first blood sample was collected in vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) to perform the comet test, blood count, platelet count, fibrinogen concentration, and total plasma protein (PPT); the second blood sample was collected in vacuum tubes containing 3.8% sodium citrate for determination of prothrombin times (PT) and activated partial thromboplastin (aPTT).

After blood harvest, the rats died of barbiturate overdose, using a dose of 100 mg/kg BW thiopental [25], and samples of the small intestine (jejunum) and spleen were collected.

Laboratory analysis

Blood analysis

Hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW-SD), and total leukocyte count were determined using the POCH-100 iV DIFF hematology analyzer (Sysmex, Brazil).

For differential counting of leukocytes, stained blood smears were prepared by rapid staining (Panótico, Laborclin, Brazil) for later analysis under an optical microscope (E-200, Nikon, Japan) with 100 × magnification.

The concentration of fibrinogen was determined by the precipitometric calorimetric refractometry technique (56°C) by the refractometer ATC-ITREF-200 (Instrutemp, Brazil), and the results are expressed as mg/dL.

The PPT concentration was quantified by the ATC-ITREF-200 refractometer (Instrutemp), and the results are expressed as g/dL.

Prothrombin time (PT) was determined by the clot formation technique using a commercial kit (TP Clot, BIOS Diagnótica Indústria e Comércio de Produtos Biológicos Ltda, Brazil), and the results are expressed in seconds.

The aPTT was determined using the clot formation technique using commercial kit (TTPA Clot, BIOS Diagnostica Indústria e Comércio de Produtos Biológicos Ltda).

Comet test

The comet assay technique was adapted from Silva [29] for staining with Giemsa as described by Fagiani et al. [30].

To carry out the comet assay technique, PBS solutions (10 X), lysis solution (1% Triton X-100, 10% DMSO, 2.5 M NaCl, 100 mM EDTA pH = 10), electrophoresis buffer (300 mM NaOH/1 mM EDTA, containing Solution A = NaOH + distilled water and Solution B = EDTA + distilled water), neutralizing buffer (NH2 C[CH2 OH]3; pH = 7.5), 1.5% agarose (Sigma-Aldrich, St. Louis, MO, USA) for precoating the slides, and 0.75% agarose (Sigma-Aldrich).

The technique was performed by the blood sample distribution of the rats on the slides previously prepared with agarose gel, which were submitted to the electrophoretic run, applying 23 to 25 V and 310 and 360 mA of electrical current (Model 250 Gibco BRL Electrophoresis Power supply, Life Technologies, Carlsbad, CA, USA) for 20 min.

The slides were stained with Giemsa (Bioclin, Brazil) for 5 min, washed with distilled water, and read under an optical microscope (Nikon, Japan), counting 200 comets and classifying the damage observed to the DNA in relation to the head diameter in relation to the tail length, classifying in classes of damages to the DNA from 0 to 4 as described by Vilela et al. [31]: Class 1 damage was when the head-to-tail ratio ≤1; class 2 damage was when the head-to-tail ratio ranged from 1 to

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

Experimental groups	Body weight (g)		Feed intake (g/d)		Water intake (mL/d)		Consumed L-arginine (mg/d)
	Before 5-FU treatment	After 5-FU treatment	Before 5-FU treatment	After 5-FU treatment	Before 5-FU treatment	After 5-FU treatment	
Control	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^{Bb}	32.3 ± 1.7^{Ba}	28.4 ± 12^{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}	50
G_{Arg100}	227.7 ± 23.7^{Aa}	205.7 ± 19.9^{ABb}	22.9 ± 2.6^{Aa}	19 ± 8.2^{ABb}	48.8 ± 9.8^{Aa}	37.9 ± 13.6^{Bb}	100

5-FU, 5-fluorouracil; $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L-arginine.
Different uppercase letters (A, B, C) in a column indicate statistical difference between groups.
Different lowercase letters (a, b) in a row indicate difference between before and after administration of 5-FU treatment for the same group ($P < 0.01$).

2; class 3 damage was considered when the head-to-tail ratio was ≥2; and in class 4, it was not possible to differentiate the head-to-tail ratio from the comet.

The total damage index (TDI) to DNA was calculated by multiplying the number of comets of each class (n class) by the denominator digit of the damage class (0, 1, 2, 3, and 4), and the following formula was applied:

$$\text{TDI} = (0 \times \text{n class } 0) + (1 \times \text{n class } 1) + (2 \times \text{n class } 2) + (3 \times \text{n class } 3) + (4 \times \text{n class } 4).$$

Thus, the result of the total DNA damage index can vary from 0 (0 × 200), where there is no DNA damage, up to 800 (4 × 200), with DNA totally damaged [31].

Histologic sections analysis of the small intestine and spleen

The tissues of the small intestine (jejunum) and spleen were collected and fixed in 10% formalin for 48 h, embedded in paraffin blocks, and 3-μm thick slices were arranged on microscopic slides stained with hematoxylin and eosin and analyzed under a light microscope [13].

The evaluation of the spleen slides was carried out by applying the scores proposed by Gerez et al. [32]. A 2 to 2.5 mm^2 area under optical microscopy (E200, Nikon, Japan) was examined to apply lesion score, identification of intact areas, and lesions, such as depletion, germinal center, apoptosis, and necrosis.

For the evaluation of the inflammatory process of the small intestine (jejunum), the score proposed by Gerez et al. [32] was used: 1 corresponds to mild inflammation (25% of the cut of the affected jejunum); 2 corresponds to the mean inflammation (50% of the cut of the affected jejunum); and 3 corresponds to severe inflammation (75% of the cut of the affected jejunum).

Statistical analysis

Data on water and feed intake, Hb, MCH, lymphocytes, fibrinogen concentrations, DNA damage classes (between groups within each class of damage and between classes of damage within each group), total DNA damage, spleen lesion score, and small intestinal inflammatory infiltrate (jejunum) following a nonparametric distribution using the Shapiro–Wilk test, were analyzed by the Kruskall–Wallis test and the medians were compared by the Dunn test with significance of 5%. The results are expressed as median ± interquartile range [33].

The Wilcoxon test with significance of 5% was used to compare water and feed intake data between the periods before and after application of 5-FU. The results are expressed as median ± interquartile deviation [33].

Data from the HHt, MCV, MCH, MCHC, RDW-SD, platelets, total leukocytes, segmented, eosinophils, monocytes, total plasma protein, and inflammatory bowel process showed normal distribution by the Shapiro–Wilk test and were analyzed by one-way analysis of variance, and the means were compared by the Tukey test with significance of 5%. The results are expressed as mean ± SD [33].

Results

Before chemotherapy, rats from the control, $G_{5\text{-FU}}$, G_{Arg50}, and G_{Arg100} groups had similar BW and feed intake ($P > 0.05$; Table 1).

After chemotherapy, rats in the $G_{5\text{-FU}}$, G_{Arg50}, and G_{Arg100} groups showed a reduction ($P < 0.01$) in feed intake (Table 1), and rats in the G_{Arg50} group did not lose BW, whereas rats of the $G_{5\text{-FU}}$ groups and G_{Arg100} lost BW ($P < 0.01$) on the order of 9.96% and 9.66%, respectively (Table 1).

The $G_{5\text{-FU}}$ rats, before and after chemotherapy, had the lowest water consumption ($P < 0.01$; Table 1), and the G_{Arg50} group maintained water consumption, whereas the $G_{5\text{-FU}}$ and G_{Arg100} groups presented a reduction in water consumption ($P < 0.01$; Table 1).

The erythrocyte and Hb concentrations of the G_{Arg50} rats were higher than those in the control group (Table 2), but there was no difference among the $G_{5\text{-FU}}$, G_{Arg50}, and G_{Arg100} groups (Table 2).

The RDW-SD of the $G_{5\text{-FU}}$ and G_{Arg50} groups were smaller than the control group (Table 2).

The MCV, MCH, and CHCM did not differ between the experimental groups (Table 2).

Rats in the G_{Arg100} group showed increased counts of total leukocytes, eosinophils, and lymphocytes in relation to the $G_{5\text{-FU}}$ and G_{Arg50} groups (Table 3). Neutrophil count did not differ between the experimental groups (Table 3).

The total leukocyte, eosinophil, and lymphocyte counts of the $G_{5\text{-FU}}$ and G_{Arg50} rats were similar (Table 3).

The PPT concentrations of the rats were similar between the experimental groups (Table 3).

Rats in the G_{Arg100} group had the lowest ($P = 0.045$) lesion score in spleen depletion (1.00 ± 1.00) compared with the $G_{5\text{-FU}}$ (2.00 ± 0.0) and G_{Arg50} (3.00 ± 0.0) groups (Fig. 1B–D). Still, the lesion scores in the spleen depletion of the G_{Arg100} rats were similar ($P > 0.05$) to that of the control group (1.00 ± 1.00; Fig. 1A and B). The lesion score in spleen depletion was not different ($P > 0.05$) between the $G_{5\text{-FU}}$ and G_{Arg50} groups (Fig. 1C and D).

Table 2
Erythrogram in Wistar rats supplemented with L-arginine after 5-FU treatment

Parameters	Experimental lots				*P-value*
	Control	$G_{5\text{-FU}}$	G_{Arg50}	G_{Arg100}	
Blood cells (× $10^3/\mu L$)	7.4 ± 0.5^{B}	9.1 ± 0.6^{AB}	9.9 ± 0.9^{A}	8.0 ± 0.4^{AB}	0.05
Hemoglobin (g/dL)	14.6 ± 1.17^{B}	17.9 ± 0.8^{AB}	19.3 ± 0.9^{A}	15.6 ± 5.4^{AB}	0.05
Hematocrit (%)	42.0 ± 4.3^{B}	48.5 ± 4.3^{AB}	53.6 ± 3.2^{A}	45.4 ± 7.3^{B}	0.05
MCV (fL)	54.4 ± 0.8^{A}	53.5 ± 1.6^{A}	54.6 ± 1.7^{A}	53.9 ± 1.7^{A}	0.51
MCH (pg)	19.4 ± 0.6^{A}	19.4 ± 0.7^{A}	19.8 ± 0.5^{A}	19.7 ± 0.7^{A}	0.57
MChR (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^{B}	24.7 ± 1.2^{B}	25.5 ± 2.2^{AB}	0.02

5-FU, 5-fluorouracil; $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L-arginine; MCH, mean corpuscular hemoglobin; MChR, mean corpuscular hemoglobin concentration MCV, mean corpuscular volume; RDW-SD, red blood cell distribution width.
Different letters (A, B) in a row indicate statistical difference between groups.

Table 3
Leukogram and total plasma protein in Wistar rats supplemented with L-arginine after 5-FU treatment

Parameters	Experimental lots				*P*-value
	Control	$G_{5\text{-FU}}$	G_{Arg50}	G_{Arg100}	
Total leukocytes (/μL)	2,785 ± 1,055 ^{AB}	1,844 ± 505.5 ^{B}	1,975 ± 774.1 ^{B}	3,387 ± 565.6 ^{A}	0.02
Neutrophils (/μL)	1,110 ± 926 ^{A}	1,376 ± 46 ^{A}	1,420 ± 269 ^{A}	1,231 ± 1,046 ^{A}	0.85
Eosinophils (/μL)	75.2 ± 26.8 ^{A}	20.8 ± 11.2 ^{B}	29.8 ± 15.3 ^{B}	79.0 ± 52.8 ^{A}	0.01
Lymphocytes (/μL)	1,568 ± 608.8 ^{A}	435.5 ± 233.0 ^{B}	376.0 ± 181.0 ^{B}	1,474 ± 782 ^{A}	0.0003
Monocytes (/μL)	186.3 ± 96.5 ^{A}	65.1 ± 49.3 ^{B}	33.5 ± 28.8 ^{B}	106.6 ± 34.4 ^{AB}	0.0004
Total plasma proteins (g/dL)	6.9 0.7 ^{A}	7.2 ± 0.9 ^{A}	7.5 ± 0.7 ^{A}	7.1 ± 1.1 ^{A}	0.67

5-FU, 5-fluorouracil; $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L-arginine.
Different letters (A, B) in a row indicate statistical difference between groups.

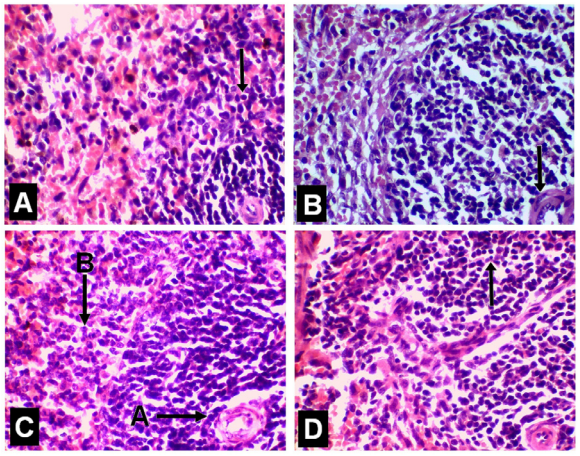

Fig. 1. Photomicrograph of spleen of Wistar rats supplemented with L-arginine and submitted to chemotherapy with 5-fluorouracil (H&E 400 ×). (**A**) Control group, chief lymphocyte arrow. (**B**) Group $G_{5\text{-FU}}$, arrow direction the germinal center. (**C**) Group G_{Arg50}, arrow (**A**) apoptotic cell and (**B**) germinal center. (**D**) Group G_{Arg100}, chief lymphocyte arrow. $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L- arginine.

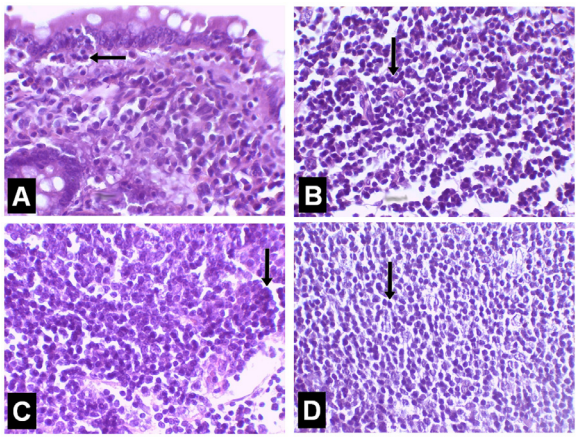

Fig. 2. Photomicrography of the inflammatory infiltrate in the jejunum of Wistar rats supplemented with L-arginine and submitted to chemotherapy with 5-fluorouracil (H&E 400 ×). (**A**) Control group. (**B**) Group $G_{5\text{-FU}}$. (**C**) Group G_{Arg50}. (**D**) Group G_{Arg100}. Arrows pointing to lymphocytes. $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L-arginine.

The lesion scores of the spleen as the germinal center (control = 1.00 ± 0.0, $G_{5\text{-FU}}$ = 2.00 ± 0.0; G_{Arg50} = 3.00 ± 1.00, and G_{Arg100} = 1.00 ± 1.00) and apoptosis (control = 13.00 ± 1.00; $G_{5\text{-FU}}$ = 2.00 ± 0.0; G_{Arg50} = 2.00 ± 0.0, and G_{Arg100} = 2.00 ± 0.0) showed no difference ($P > 0.05$) between the experimental groups (Fig. 1A–D).

Rats in the G_{Arg100} group had the shortest PT compared with the $G_{5\text{-FU}}$ and G_{Arg50} groups (Table 4).

The platelet count, fibrinogen concentrations, and aPTT of the rats did not present statistical differences between the experimental groups (Table 4).

Figure 2 shows that the inflammatory infiltrate score in the jejunum of the rats of the $G_{5\text{-FU}}$ groups (2.54 ± 0.84), G_{Arg50} (3.20 ± 0.63), and G_{Arg100} (2.75 ± 0.57) did not differentiate between them ($P > 0.05$).

Rats in the $G_{5\text{-FU}}$ and G_{Arg100} groups showed a reduction ($P = 0.03$) in the amounts of intact DNA in relation to the control group (Fig. 3A). However, the amounts of DNA integrity and class 3-DNA damage of the $G_{5\text{-FU}}$, G_{Arg50}, and G_{Arg100} groups were similar ($P > 0.05$; Fig. 3A and D, respectively).

The amounts of DNA damage of classes 1 and 4 did not show differences ($P > 0.05$) between experimental groups (Figs. 3B and E, respectively).

Rats in the G_{Arg100} group showed similar amounts ($P > 0.05$) of damage to class 2-DNA relative to the rats of the $G_{5\text{-FU}}$ and G_{Arg50} groups (Fig. 3C). However, the ratios of the G_{Arg50} group had lower ($P < 0.01$) amounts of damage to the DNA of class 2 in relation to the rats of the $G_{5\text{-FU}}$ groups (Fig. 3C).

Rats in the G_{Arg100} group had the highest ($P < 0.05$) TDI in the DNA (Fig. 3F). The $G_{5\text{-FU}}$ group was similar ($P > 0.05$) to the G_{Arg50} group and higher than the control group ($P > 0.05$; Fig. 3F). The TDI

Table 4
Platelets, prothrombin time, activated partial thromboplastin time, and fibrinogen in Wistar rats supplemented with L-arginine after 5-FU treatment

Parameters	Experimental lots				*P*-value
	Control	$G_{5\text{-FU}}$	G_{Arg50}	G_{Arg100}	
Platelets ($\times 10^3$/μL)	1195 ± 201.2 ^{A}	1002 ± 304.2 ^{A}	1033 ± 160^{A}	1094 ± 251^{A}	0.39
Prothrombin time (s)	17.6 ± 0.8 ^{AB}	18 ± 1.2 ^{A}	18.8 ± 2 ^{A}	15.5 ± 1.2 ^{B}	0.02
Activated partial thromboplastin time (s)	32.2 ± 2.7 ^{A}	34.4 ± 3.8 ^{A}	34.5 ± 6.9 ^{A}	32.1 ± 8.1 ^{A}	0.92
Fibrinogen (mg/dL)	275 ± 103.5 ^{A}	325 ± 103.5 ^{A}	325 ± 103.5 ^{A}	300 ± 106.9 ^{A}	0.74

5-FU, 5-fluorouracil; $G_{5\text{-FU}}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L-arginine.
Different letters (A, B) in a row indicate statistical difference between groups.

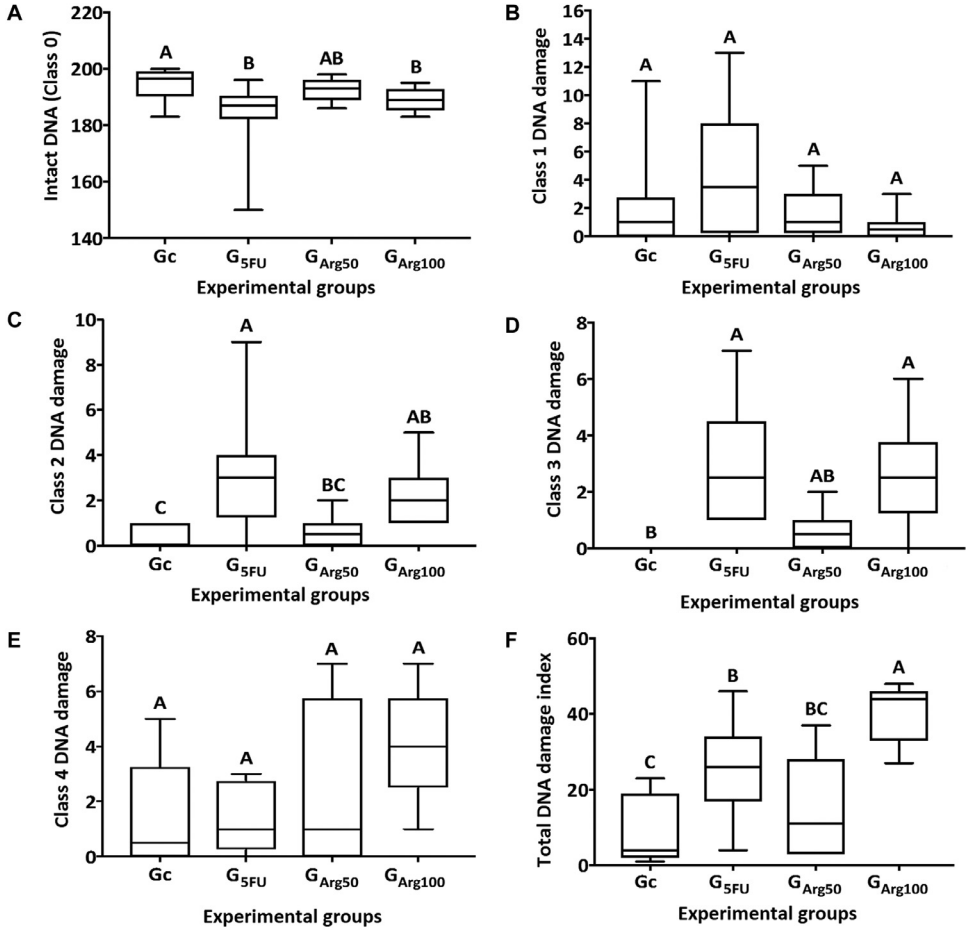

Fig. 3. Damaged and intact DNA count (classes 0–4) and total DNA damage index of Wistar rats supplemented with L-arginine after application of 5-fluorouracil. (**A**) Whole DNA. (**B**) Class 1 DNA damage. (**C**) Class 2-DNA damage. (**D**) Class 3-DNA damage. (**E**) Class 4 DNA damage. (**F**) Total DNA damage index. Medians followed by at least one distinct lowercase letter indicate statistical difference ($P < 0.05$) between the experimental groups within the DNA damage classes. $G_{5\text{-}FU}$, rats receiving 5-FU; G_{Arg50}, rats receiving 5-FU and 50 mg/d L-arginine; G_{Arg100}, rats receiving 5-FU and 100 mg/d L- arginine; GC, control group.

to the DNA of the G_{Arg50} rats did not differ from the control and $G_{5\text{-}FU}$ groups (Fig. 3F).

Discussion

Treatments that attenuate the side effects of 5-FU administration for patients with cancer are urgent given the widespread use of this drug. In this sense, the findings of the present study are promising because they indicate that arginine supplementation at low doses can counteract 5-FU cytotoxicity in rats. That is a feasible alternative because arginine is an affordable and available supplement.

Determination of the estrous cycle period of the rats was performed to homogenize the experimental groups and, because all the rats at the beginning of the experiment were in the metaestrus phase and at the end in the estrus phase, there was no hormonal interference in the cycle in the results found in the hemogram, water consumption, and ratio and weight loss of rats. Because rats during estrus can alter their eating behavior, reduction in feed intake at this stage [34] and during the proestrus phase interfere with the production of antibodies and proteins that act on the immune system and may lead to immunosuppression [35].

The reduction in feed intake of the rats in the $G_{5\text{-}FU}$, G_{Arg50}, and G_{Arg100} groups after application of 5-FU was probably owing to the adverse effects that occurred in rats, such as mucositis [8,36], which caused difficulties in chewing and swallowing the ration [37]. However, rats in the G_{Arg50} group, even with reduced feed intake, maintained BW after chemotherapy. This fact is beneficial for the health of the patients because weight loss, according to Aoyama et al. [38], mainly the loss of lean mass, is a risk factor for the continuity of treatment of neoplasia. However, it was not possible to clarify the physiologic effects that are involved in this process.

The rats in the $G_{5\text{-}FU}$ and G_{Arg50} groups presented polycythemia because the concentration of Hb, Hct, and PPT were above the values of the control group. This may have occurred owing to the diarrhea that these rats presented after chemotherapy, leading to mild dehydration. According to Silveira [39], these hematologic alterations are indicative of dehydration. Loss of body fluids, plasma volume reduction, and dehydration may occur as side effects of chemotherapy [40,41], which is undesirable for the health of patients. However, the diarrhea that occurred in the G_{Arg100} group was of lower intensity and did not cause dehydration, because hematologic parameters indicative of this condition, such as the concentration of erythrocytes, Hct, and PPT, remained within the normal range, with values similar to the control group. This is the most frequently indicated dose in this case.

The hematimetric indexes of the rats in the control groups, $G_{5\text{-}FU}$, G_{Arg50}, and G_{Arg100}, remained within the normal range, indicating that the erythrocytes were normocytic and normochromic

[42,43]. A similar fact was reported by Balmant et al. [12], who supplemented rats with 295 and 458 mg/d L-arginine, demonstrating that supplementation with up to 458 mg/d of L-arginine did not interfere with the size and staining of the red blood cells.

Supplementation with 100 mg/d of L-arginine ameliorated leukopenia, lymphopenia, and eosinopenia caused by 5-FU chemotherapy because the total leukocyte and lymphocyte counts of the G_{Arg100} group were similar to the control group. This is beneficial for patients with cancer because leukocyte and lymphocyte counts are relevant clinical indicators in chemotherapy, directly influencing the individual's tolerance to chemotherapy and making them more resistant to infections [44,45]. This corroborates the study by Balmant et al. [12], who also demonstrated that supplementation with 295 and 458 mg/d of L-arginine minimized the 5-FU–induced immunosuppression, similar to supplementation with 100 mg/d of L-arginine, resulting in lower costs with supplementation and the vasodilator effect [22].

In addition, rats in the G_{Arg100} group showed a higher concentration of lymphocytes at the germinal center of the spleens, demonstrating that supplementation with 100 mg/d of L-arginine alleviated the immunosuppression generated by 5-FU. Murphy [46] reported that the germinal center consists of B and T lymphocytes that can confer better resistance and immune response mediated by lymphocytes to these rats. However, new studies are needed to elucidate the physiologic mechanisms by which L-arginine ameliorated immunosuppression.

Supplementation with 50 mg/d of L-arginine (G_{Arg50} group) was not able to minimize the leukopenia, lymphopenia, eosinopenia, and high lymphocyte depletion that occurred in the spleen due to the possible myelosuppression caused by chemotherapy.

$G_{5\text{-}FU}$, G_{Arg50}, and G_{Arg100} rats presented a similar inflammatory process in the jejunum of moderate intensity generated by 5-FU that was due to the side effect of mucositis, and supplementation with L-arginine did not minimize this process. However, Balmant et al. [12], using higher doses of L-arginine supplementation (295 and 458 mg/d), and Iwase et al. [47] observed a reduction in the inflammatory process generated by chemotherapy.

The PT of rats in the $G_{5\text{-}FU}$ group was increased by chemotherapy, indicating that there would be longer time for the blood to coagulate, which is undesirable because the antineoplastic treatment may be associated with surgically extirpating the tumor. This may make the surgical procedure unfeasible due to the risk for hemorrhage. However, supplementation with 100 mg/d of L-arginine reduced this side effect of 5-FU because this dose reduced the PT and could favor the activation of the coagulation cascade, resulting in the conversion of fibrinogen to fibrin, forming the clot [39].

The platelet count and activated thromboplastin time of the experimental groups were similar to the control group, indicating that 5-FU chemotherapy and L-arginine supplementation did not interfere with these blood parameters. However, Balmant et al. [12], using higher doses of L-arginine supplementation (295 and 458 mg/d), observed an increase in platelet count in rats submitted to 5-FU chemotherapy.

Another interesting fact was that the rats supplemented with 100 mg/d of L-arginine had an increase in the total DNA damage index, and this increase in DNA breakdown may benefit by potentiating the mechanism of action of 5-FU chemotherapy and thus contribute to inhibition of tumor growth [48].

After being metabolized, L-arginine can be integrated into the DNA structure through methylation, cause flaws in this structure [49], and decrease the DNA repair capacity [50]. Reynolds et al. [51] observed that inhibition of tumor growth occurred with some types of high immunogenicity tumors; however, tumor growth was stimulated in the presence of low immunogenicity tumors, indicating that inhibition of tumor growth by L-arginine supplementation is only possible when the tumors were recognized and eliminated by the immune system.

In contrast, rats receiving daily supplementation of 50 mg/d of L-arginine had a percentage of intact DNA similar to that of the control group and a reduction in the DNA TDI, indicating that this supplementation may preserve DNA integrity. This goes against the mechanism of action of 5-FU, which is to incorporate its cyclic ring in the DNA and RNA to cause apoptosis of the cells, which becomes undesirable owing to its potential to harm the tumor involution.

It is possible that arginine supplementation at 1.5 g/d throughout the chemotherapy cycle may attenuate the side effects of 5-FU in humans, helping those with cancer. However, before it is released for human use, further studies are needed to assess the effects of arginine consumption on tumor growth and involution and other possible actions of this amino acid in humans.

Conclusion

The results of the present study demonstrated that supplementation with 100 mg/d of L-arginine has the potential to minimize immunosuppression and depletion in the spleen caused by 5-FU chemotherapy. In addition, it likely reduces blood coagulation time and contributes to DNA breakage generated by 5-FU.

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