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ORIGINAL ARTICLE

Nutritional supplementation with L-arginine prevents pelvic radiation-induced changes in morphology, density, and regulating factors of blood vessels in the wall of rat bladder

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Abstract
Purpose To determine whether L-arginine has protective effects against radiation-induced alterations in the morphology and regulatory factors of vesical blood vessels in rats.
Methods Male rats aged 3–4 months were divided into groups of 10 animals each: (a) controls, consisting of non-treated animals; (b) radiated-only rats; and (c) radiated rats receiving L-arginine supplementation. Radiation was in one session of 10 Gy and was aimed at the pelvic-abdominal region. L-arginine was administered once a day (0.65 g/kg body weight), starting 7 days before radiation and continuing until killing on the 16th day after radiation. The density, relative area, and wall thickness of blood vessels were measured in the vesical lamina propria using histological methods, and the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) in the bladder wall was assessed by RT-PCR.
Results Compared with controls, radiation alone decreased the density and relative area of blood vessels by 32 % ($p < 0.01$) and 25 % ($p < 0.05$), respectively, and reduced the arterial wall thickness by 42 % ($p < 0.004$). VEGF and FGF mRNA levels after radiation were diminished by 67 % ($p < 0.002$) and 56 % ($p < 0.04$), respectively. The radiated animals supplemented with L-arginine were not significantly different from controls.
Conclusions Pelvic radiation leads to significant vesical modifications, as in the morphology of blood vessels and in VEGF and FGF expression. All these changes, however, were prevented by L-arginine treatment. These results emphasize, therefore, the potential use of this amino acid as a radioprotective drug.

Keywords Bladder · Radiotherapy · Blood vessels · L-arginine

Introduction

Radiotherapy plays an important role in the treatment for pelvic malignancies and significantly improves patient prognosis [1]. However, radiation-induced early or late injuries in normal tissues are still a limiting factor for the treatment. The incidence of acute symptoms varies from 23 to 80 % [2], although gross hematuria only occurs in about 8 % of patients [3].

In spite of recent advances that improve range and direction of radiation, external beam radiotherapy targeted at pelvic organs malignancies may still affect adjacent structures. Indeed, the urinary bladder is often injured during radiotherapy targeting the pelvic region [4, 5]. Progression of radiation-induced bladder injury in humans, as well as in mouse models, is commonly described as having three phases. Initial damage is observed while the patient is still under treatment, but it gradually disappears once radiotherapy is interrupted. There is then a clinically quiescent period that may last many years, after which conditions may worsen and lead to irreversible lesions [6].

Fibrosis is a common morphological alteration after radiation, especially around blood vessels [5, 6]. In large vessels, radiation-induced damage consists mainly of

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atherosclerosis, which results in thromboembolism and stenosis [5]. In smaller vessels, injury is marked by telangiectasia and bleeding, which sometimes requires surgical intervention [7]. Additionally, modifications have been observed in capillaries, which are responsible for ischemia and neovasculogenesis [8].

Preliminary data from animals have shown that deleterious effects of radiation can be minimized by dietary supplements such as L-arginine, which are taken before and during radiotherapy sessions [9, 10]. L-arginine is an essential amino acid that is the substrate for the production of nitric oxide (NO) [11], which acts in intracellular signaling and as a toxin in various pathologic processes [12]. L-arginine stimulates the production of anabolic hormones, such as growth hormone and insulin, which can have immunomodulatory effects, and promotes wound healing, while NO causes vasodilatation, stimulates microcirculation, and modulates the secretion of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which themselves also stimulate angiogenesis [11, 13]. Studies in humans have shown that the bioavailability of L-arginine is about 70 % and that when administered orally, it is relatively free from side effects [14]. Thus, L-arginine is potentially a radioprotective drug, especially against injuries due to vascular modifications [10].

In spite of this, little is known about the effects of L-arginine on VEGF and FGF in the bladder wall. Hence, we used a rat model of pelvic radiation and analyzed the morphology of blood vessels in the lamina propria and the expression of VEGF and FGF in the bladder wall. We focused the analyses on the lamina propria because it is rich in blood vessels [15] and it is the region that is more severely affected by radiotherapy in animal models [16].

The aim of the present investigation was therefore to determine whether L-arginine has protective effects against radiation-induced vascular alterations in the bladder wall.

Materials and methods

The Ethical Committee for the Care and Use of Laboratory Animals of the State University of Rio de Janeiro reviewed and approved this study.

Animals and treatments

Male Wistar rats aged 3–4 months and weighing 270–310 g were randomly assigned to the following groups of 10 animals each: (a) controls; (b) radiated-only rats; and (c) irradiated rats receiving L-arginine supplementation.

Previous investigations have characterized a rat model of single-dose, pelvic-abdominal radiation that causes extensive short-term morphological and functional damage to the pelvic organs [9, 10]. Immobilized animals were exposed in one session to a total dose of 10 Gy using a 10 MeV photon beam generated by a linear accelerator aimed at the pelvic-abdominal region. L-arginine was administered daily by gavage at a dose of 0.65 g/kg^{-1}, starting 7 days before radiation and continuing until killing [10]. All animals were killed on the 16th day after radiation by an overdose of thiopental.

Tissue preparation

We divided the bladder into a superior and an inferior portion, excluding the trigone and the bladder dome extremity. The upper portion was frozen at −80 °C and used for RT-PCR, while the lower portion was fixed in 10 % formalin and processed for paraffin embedding. Five-micrometer sections were stained with hematoxylin–eosin (HE) for identification of blood vessels in general, and with the Weigert's resorcin-fuchsin for elastic system fibers [17].

Density and relative area of blood vessels

These were assessed only in the lamina propria of the vesical wall. Using the ImageJ software (NIH, Bethesda, Maryland, USA) and HE-stained sections captured at a magnification of 200×, a continuous segment of lamina propria was outlined and its surface area was measured. The relative area occupied by blood vessels, including their luminal spaces, was estimated by point counting and expressed as percent of the reference space [18, 19].

Vascular density was estimated using the "Cell Counter" plugin of ImageJ. Thus, the number of blood vessels in the outlined lamina propria was counted and divided by its previously measured area. For each animal, 25 evenly spaced segments of lamina propria were used for these quantifications. Results were expressed as number of blood vessels per μm^2.

Thickness of the arterial wall

These measurements were carried out only in arteries located in the lamina propria, as above. Weigert-stained sections were captured at a magnification of 1,000×, and the linear distance (μm) between the internal and external elastic membranes was then measured using the ImageJ. This region of the arterial wall roughly corresponds to the tunica media. Ten measurements were obtained from each artery, and from these values, a mean thickness was calculated. About 30 arteries were measured per animal.

RT-PCR

Total RNA from bladder tissue was extracted using TRIZOL reagent (Invitrogen, California, USA), and all RNA samples were rid of contaminating DNA by using DNA-free reagents

(Invitrogen) according to the manufacturer's protocol. Then, 1 mg of RNA sample was used in a 20 µl cDNA reaction using Oligo dT and the Superscript cDNA synthesis system (Invitrogen) according to the manufacturer's protocol. All amplified cDNA fragments of VEGF and FGF were run on a 1.5 % agarose gel stained with ethidium bromide, visualized under UV transillumination, and analyzed with ImageJ.

Statistical analysis

For each variable under study, we used ANOVA. When significance was detected, the Bonferroni method was applied. $p < 0.05$ was considered statistically significant.

Results

The different combinations of treatments significantly affected the density (one-way ANOVA, $p < 0.005$; Fig. 1a) and relative area (one-way ANOVA, $p < 0.025$; Fig. 1b) of blood vessels in the lamina propria of the rat bladder, 15 days after radiation. Radiation alone decreased the density of blood vessels by 32 % when compared with non-radiated animals (7.35 ± 1.12 vessels/µm^2 vs. 10.87 ± 2.42 vessels/µm^2, respectively; $p < 0.01$). Radiation also reduced the relative vascular area by the similar amount of 25 % in relation to non-radiated controls (11.97 ± 2.06 % vs. 16.02 ± 4.10 %, respectively; $p < 0.05$).

Results from the group that received L-arginine supplementation showed that it prevented some adverse effects of radiation. Accordingly, both the density (11.04 ± 2.37 vessels/µm^2; Fig. 1a) and relative area (17.79 ± 4.76 %; Fig. 1b) of blood vessels in supplemented animals were not significantly different from those in controls. These changes in blood vessel morphology can also be seen in tissue sections of the bladder wall (Fig. 2).

The arterial wall thickness was significantly affected by the treatments (one-way ANOVA, $p < 0.001$; Fig. 1c). Radiation alone reduced arterial wall thickness by 42 % compared with non-radiated controls (6.06 ± 2.55 µm vs. 10.42 ± 1.71 µm, respectively; $p < 0.004$), but in radiated and supplemented animals (10.10 ± 1.77 µm), this parameter was not significantly different from that of non-radiated controls. Thus, radiation-induced decrease in arterial wall thickness was prevented by L-arginine supplementation.

Levels of mRNA for the soluble factors VEGF (Fig. 3a) and FGF (Fig. 3b) were similarly changed by the treatments (one-way ANOVA, $p < 0.005$ and $p < 0.025$, respectively). Thus, mRNA levels of VEGF (0.177 ± 0.092) and FGF (0.557 ± 0.227) after radiation were significantly reduced by 67 % ($p < 0.002$) and 56 % ($p < 0.04$), respectively, when compared to control animals (0.543 ± 0.175 and 1.253 ± 0.562). However, in supplemented animals VEGF (0.565 ± 0.243) and FGF (0.874 ± 0.302) did not significantly differ from those of non-radiated controls. Therefore, L-arginine prevented the reductions in mRNA levels of VEGF and FGF caused by radiation.

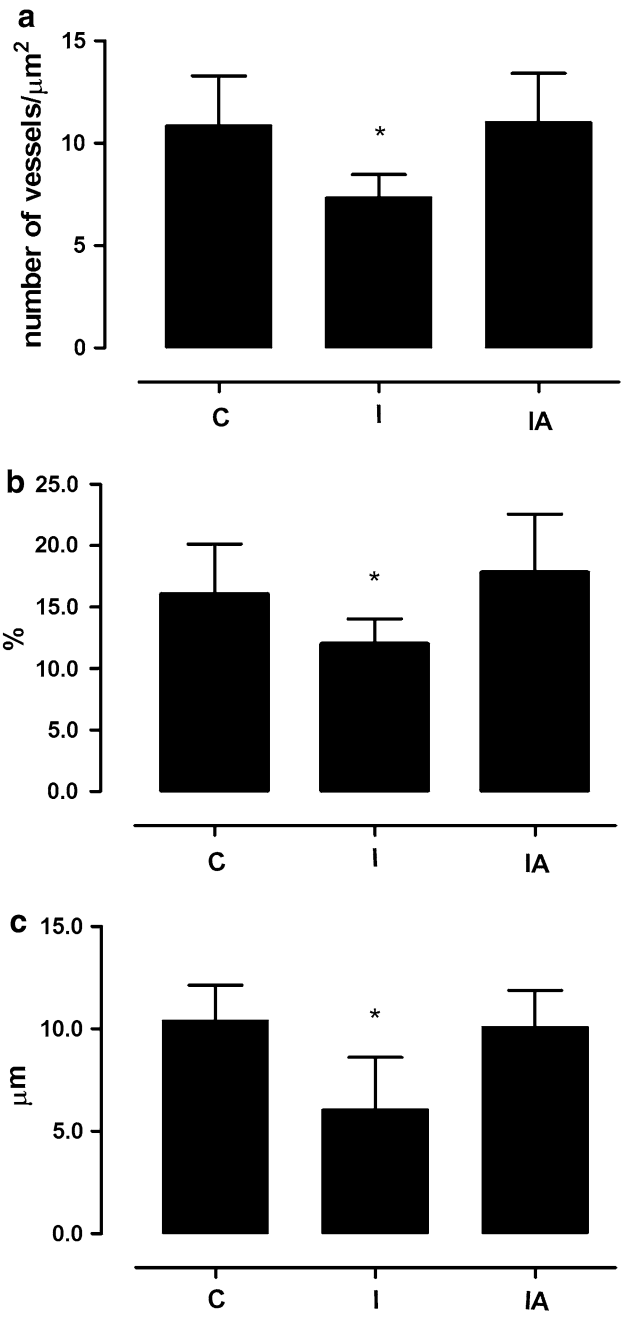

Fig. 1 **a** Density of blood vessels in the lamina propria of the bladder wall. Results are given as number of vessels per µm^2. **b** Relative area of blood vessels in the lamina propria of the bladder wall. Results are given as percent of lamina propria area. **c** Wall thickness of arteries in the lamina propria of the bladder wall. Results are given in µm. *Bars* represent the mean and standard deviation. (*C*) Control group; (*I*) irradiated-only group; (*IA*) irradiated and L-arginine-treated group. In the three images means are significantly different (one-way ANOVA), and significant differences between each group and group C are indicated by an *asterisk* (Bonferroni posttest)

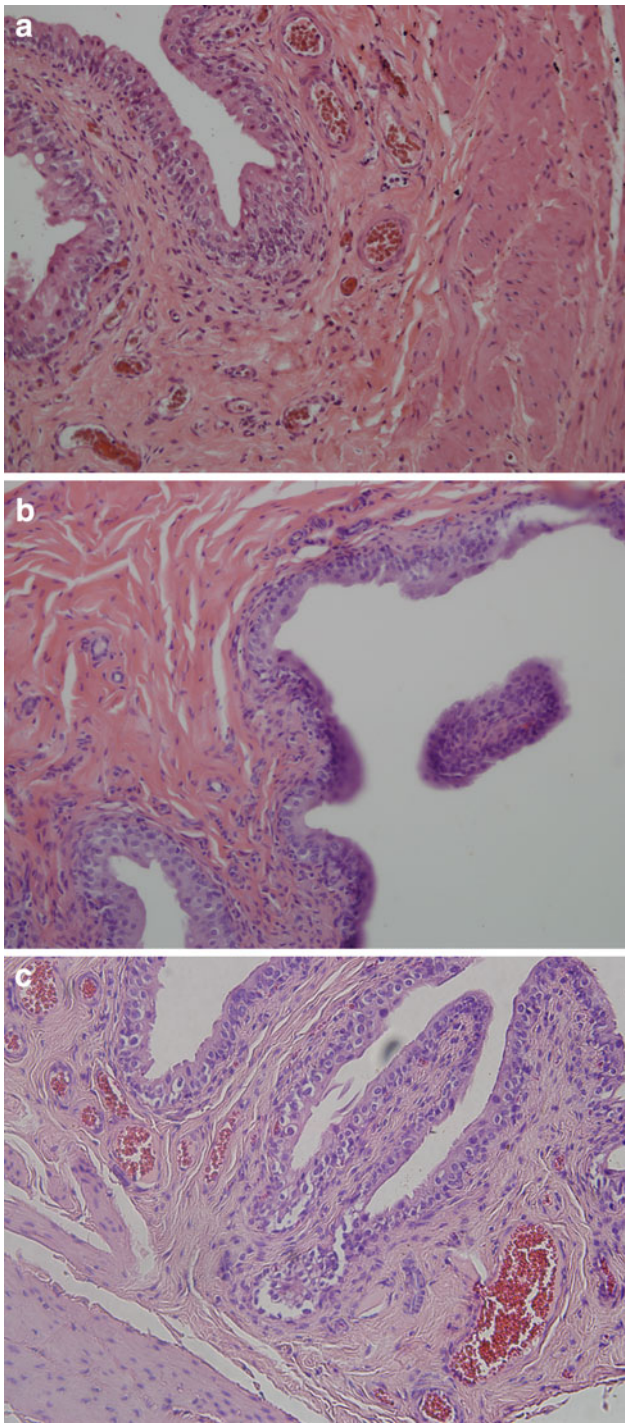

Fig. 2 Photomicrograph of the bladder wall showing the blood vessels in the lamina propria, HE ×200. **a** control group. **b** Irradiated-only group. Note decrease in blood vessel density; **c** irradiated and L-arginine-treated group, showing recovering of blood vessels density

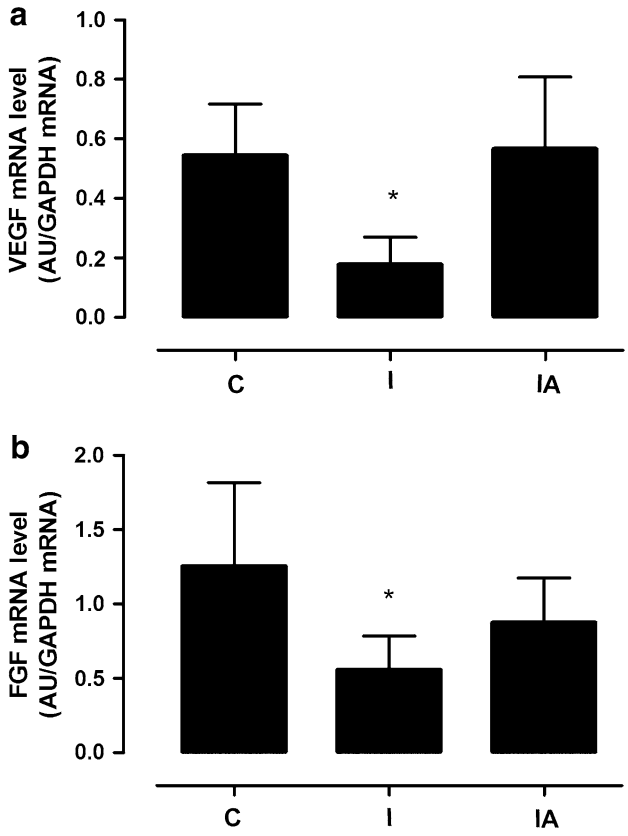

Fig. 3 **a** VEGF mRNA expression in the bladder wall **b** FGF mRNA expression in the bladder wall. *Bars* represent the mean and standard deviation. (*C*) Control group; (*I*) irradiated-only group; (*IA*) irradiated and L-arginine-treated group. In the two images means are significantly different (one-way ANOVA), and significant differences between each group and group C are indicated by an *asterisk* (Bonferroni posttest)

Discussion

Experimental animal models have shown that the adverse effects of pelvic radiation on the bladder are time and dose dependent. Although many studies on the side effects of radiotherapy have focused on collagen changes, vascular injury is one of the earliest alterations of tissues that are exposed to radiation. Damage to blood vessels, and the ensuing hypoxia and ischemia contribute to the more severe alterations, such as fibrosis and/or necrosis [20].

Fibrosis-inducing cytokines and growth factors are involved in the mechanisms of vascular lesions and fibrosis, in the migration and proliferation of smooth muscle cells, in enhanced collagen expression, and in the remodeling of the extracellular matrix [20]. VEGF is a cytokine that increases vascular permeability and is an endothelial mitogen. In addition to its angiogenic effects, VEGF also inhibits endothelial and smooth muscle cell apoptosis [21]. Additionally, it has been shown that VEGF protects endothelial cells in vitro against radiation [20]. These findings are consistent with our results, which showed that the decrease in the density and relative area of blood vessels in the lamina propria was accompanied by a decrease in the expression of VEGF. On the other hand, in L-arginine-treated animals, the greater availability of NO, which is a

mediator of the angiogenic stimuli from different growth factors, including VEGF [11], might explain the protective effects of this amino acid against these changes. NO in addition promotes the secretion of VEGF [11]. It is worth noting that therapies that stimulate angiogenesis through the administration of growth factors have been used in different situations [22], and experiments have shown that this effect reduces ischemia [23]. It has been shown that VEGF expression might be correlated with bladder cancer aggressiveness. However, this effect depends on the overexpression of VEGF [24], while in our experiment, L-arginine simply brought the expression back to normal levels. Thus, it can be speculated that L-arginine supplementation for protecting the bladder from irradiation side effects should not increase VEGF-related tumor aggressiveness.

Although one investigation has shown that arginine per se may induce acute-phase damage in the intestinal epithelium [25], most experimental data, including the results of the present study, demonstrate that this amino acid, taken before and after radiotherapy sessions, minimizes the deleterious effects of radiation [9, 10].

Radiation decreased the density of blood vessels and the relative vascular area by similar amounts. Thus, it can be inferred that the decrease in blood vessel density was not accompanied by appreciable changes in vascular lumen. Blood supply to the bladder wall is likely, therefore, to be diminished. By protecting the bladder against these alterations, arginine should thus maintain normal blood supply. This would in turn prevent an ischemic condition, which should certainly prevent or diminish epithelium and nerve damage, as well an ensuing inflammatory response.

The results obtained in the present study with regard to density and area of blood vessels, together with published data, indicate that radiation leads to a reduction in angiogenesis, either through an increase in the apoptosis of endothelial cells, or through a reduction in angiogenesis-stimulating factors [7, 8]. Several members of the FGF family have the capacity to protect tissues against the harmful effects of radiation [26]. FGF enhances migration and replication of endothelial cells, epithelial cells, and fibroblasts [27] and induces branching of small vessels, as occurs in vascular tissue recovering from lesions [28]. Thus, and much like VEGF, FGF also acts upon angiogenic pathways. Further, the continuous signaling of FGF is thought to play a critical role in maintaining vascular integrity and in regulating permeability of the underlying basement membrane [29].

In the present study, we showed that FGF expression was reduced in the radiated-only group, but in animals treated with radiation and L-arginine, this expression was similar to that of controls. It is likely that the increased availability of NO provided by the L-arginine supplementation reduced the effects of radiation on VEGF synthesis, so that expression of this angiogenic factor did not suffer significant alterations even after exposure to radiation. It should be noted that, while FGF signaling also involves NO, its angiogenic effects are less clearly tied to NO compared with VEGF [30].

Our results also showed that radiation decreased the thickness of the arterial wall in the bladder lamina propria. Because the part of the arterial wall that was included in our measurements was basically the tunica media, it can be inferred that this reduction in thickness was due mainly to atrophy or loss of smooth muscle cells, either as a direct effect of ionizing radiation, or through activation of apoptosis cascades [7, 8]. As L-arginine restored normal expression levels of VEGF and FGF in radiated animals, this protection against decrease in arterial wall thickness could also be attributed to the angiogenic effects of these growth factors, and to inhibition of smooth muscle cell apoptosis by VEGF, as discussed above.

Our results showed that pelvic radiation leads to significant changes in the morphology and density of blood vessels of the vesical lamina propria, and to marked reductions in the expression of VEGF and FGF in the bladder wall. These changes may compromise normal bladder function, but they could all be prevented by supplementing animals with L-arginine. These results emphasize, therefore, the potential use of this amino acid as a radioprotective drug.

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Conflict of interest None.

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