



Protective effects of L-glutamine on the bladder wall of rats submitted to pelvic radiation

Leilane M. Barcellos, Waldemar S. Costa, Jorge L. Medeiros Jr., Beatriz R. Rocha, Francisco J.B. Sampaio, Luiz E.M. Cardoso*

Urogenital Research Unit, State University of Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

Radiotherapy is often used to treat prostate tumors, but the normal bladder is usually adversely affected. Using an animal model of pelvic radiation, we investigated whether glutamine nutritional supplementation can prevent radiation-induced damage to the bladder, especially in its more superficial layers. Male rats aged 3–4 months were divided into groups of 8 animals each: controls, which consisted intact animals; radiated-only rats, which were sacrificed 7 (R7) or 15 (R15) days after a radiation session (10 Gy aimed at the pelvico-abdominal region); and radiated rats receiving L-glutamine supplementation (0.65 g/kg body weight/day), which were sacrificed 7 (RG7) or 15 (RG15) days after the radiation session. Cells and blood vessels in the vesical lamina propria, as well as the urothelium, were then measured using histological methods. The effects of radiation were evaluated by comparing controls vs. either R7 or R15, while a protective effect of glutamine was assessed by comparing R7 vs. RG7 and R15 vs. RG15. The results showed that, in R7, epithelial thickness, epithelial cell density, and cell density in the lamina propria were not significantly affected. However, density of blood vessels in R7 was reduced by 48% ($p < 0.05$) and this alteration was mostly prevented by glutamine ($p < 0.02$). In R15, density of blood vessels in the lamina propria was not significantly modified. However, epithelial thickness was reduced by 25% ($p < 0.05$) in R15, and this effect was prevented by glutamine ($p < 0.01$). In R15, epithelial cell density was increased by 35% ($p < 0.02$), but glutamine did not protect against this radiation-induced increase. Cell density in the lamina propria was likewise unaffected in R15. Density of mast cells in the lamina propria was markedly reduced in R7 and R15. The density was still reduced in RG7, but a higher density in RG15 suggested a glutamine-mediated recovery. Alpha-actin positive cells in the lamina propria formed a suburothelial layer and were identified as myofibroblasts. Thickness of this layer was increased in R7, but was similar to controls in RG7, while changes in R15 and RG15 were less evident. In conclusion, pelvic radiation leads to significant acute and post-acute alterations in the composition and structural features of the vesical lamina propria and epithelium. Most of these changes, however, can be prevented by glutamine nutritional supplementation. These results emphasize, therefore, the potential use of this amino acid as a radioprotective drug.

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1. Introduction

Radiotherapy is often used in the treatment of different pelvic tumors, including those of the prostate, rectum, cervix, and endometrium. However, radiation-induced adverse effects may occur in normal tissues that are close to the target organ. For example, the urinary bladder usually undergoes injuries when radiotherapy is aimed at the pelvic region to treat tumors in other

organs (Jaal and Dorr, 2010). Indeed, patients receiving radiotherapy for prostate cancer are at a higher risk of bladder cancer when compared to those who underwent radical prostatectomy (Nieder et al., 2008).

In experimental models of radiotherapy, mice receiving a radiation dose of 20Gy show a decrease in the number of cells of the urothelium in the early (0–31 days) and late stages (90–120 days) (Jaal and Dorr, 2006a). Submitting mice to this same dose of radiation also leads to inflammatory changes in the bladder, in which other cells are involved (Jaal and Dorr, 2006b). The bladder lamina propria has several cell types such as fibroblasts, myofibroblasts, and fibrocytes. These cells are implicated in the synthesis of extracellular matrix and stromal remodeling associated with inflammation and tumors (Nimphius et al., 2007). Myofibroblasts

* Corresponding author at: Urogenital Research Unit – UERJ, Av. 28 de Setembro, 87, fundos-FCM-terreo, 20551-030 Rio de Janeiro, RJ, Brazil. Tel.: +55 21 2587 6499; fax: +55 21 2587 6121.

E-mail address: luizemcardoso@gmail.com (L.E.M. Cardoso).

exhibit a hybrid phenotype between fibroblasts and smooth muscle cells. They are characterized by the expression of α -actin and other intracellular filaments and have a central role in the formation of fibrotic tissue (Gan et al., 2007). In the human bladder, they are located in the suburothelial layer near the capillary network (Paner et al., 2009). Myofibroblasts play an important role in the initial reflex of voiding due to its position between the urothelium and nerve endings (Fry et al., 2007), and their increase in number is associated with enhanced spontaneous contraction of the bladder (Sui et al., 2008). Experimental data have shown that myofibroblasts are activated immediately after a radiation session (Devalia and Mansfield, 2008). Mast cells are also present in the vesical lamina propria, and they release a series of chemical mediators related to inflammation and angiogenesis (Ranieri et al., 2009; Rickard et al., 2005).

Nutritional supplementation with L-glutamine has been shown to reduce the morbidity and mortality of severe diseases, and it has important effects against radiation-induced tissue lesions (Savarese et al., 2003). For example, several experimental evidences indicate that glutamine is effective in the protection and repair of the intestinal wall of rats submitted to pelvic radiation (Chun et al., 1997; Ersin et al., 2000; Diestel et al., 2007) or to chemotherapy (Tazuke et al., 2011; Owari et al., 2012). In humans, it has been shown that glutamine prevents post-radiotherapy esophagitis (Algara et al., 2007). Additionally, glutamine has antitumorigenic effects, which is thought to be mediated by glutathione, of which it is a precursor (Kaufmann et al., 2008).

In spite of what has been discussed above, there is little data about the effects of radiation on the bladder when it is not the target organ in pelvic radiotherapy. As already mentioned, this is a common outcome during treatment for prostate cancer (Nieder et al., 2008). Likewise, little is known about the use of oral supplements to protect the bladder against the adverse effects of radiation. A previous work by our group (Rocha et al., 2011) used a rat model of pelvic radiation and concentrated the analysis on the gene expression of collagen in the whole bladder tissue. The results showed that there were changes following radiation, and that they could be prevented by treating the animals with glutamine. However, there is no data yet about the possible radioprotective effects of glutamine on the cellular and vascular components of the bladder wall.

The aim of the present study was thus to investigate whether glutamine supplementation protects these structural components of the rat bladder wall against radiation-induced damage. We focused the analysis on the more superficial layers of the bladder wall, such as the urothelium and the lamina propria, and used quantitative histochemical and immunohistochemical methods.

2. Materials and methods

The present experimental protocol was reviewed and approved (approval number CEA/224/2009) by the Animal Care Committee of the State University of Rio de Janeiro, Brazil.

2.1. Animals and treatments

Male Wistar rats aged from three to four months and weighing from 270 to 310 g were randomly assigned to one of the following five groups of eight animals each: controls, which consisted of non-irradiated, non-treated animals; irradiated-only rats, which were sacrificed seven (R7) or 15 (R15) days after the radiation session; and irradiated rats receiving L-glutamine supplementation, which were also sacrificed seven (RG7) or 15 (RG15) days after the radiation session. Animal housing and maintenance conditions were as described elsewhere (Diestel et al., 2007).

Previous investigations have characterized a rat model of single-dose, pelvico-abdominal radiation that causes extensive short-term morphological and functional damage to the intestinal tissue (Ersin et al., 2000; Diestel et al., 2007). These established experimental protocols of radiation and treatment were thus used herein to ascertain whether the urinary bladder is also affected. Briefly, 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 (Clinac 2100C, Varian, Palo Alto, USA). The dose was delivered at a source-to-skin distance of 100 cm, at a rate of 2.4 Gy/min for 4.16 min, and was aimed at the pelvico-abdominal region, while other fields were shielded off. L-Glutamine (Resource Glutamine, Novartis, Rio de Janeiro, Brazil) was prepared as a 4% aqueous solution and was administered once a day by gavage at a dose of 0.65 g per kg of body weight, starting seven days before radiation and continuing until sacrifice. A dose similar to this one has been shown to have protective effects against changes in the colon using the same rat radiation model (Diestel et al., 2007). The groups that were not under L-glutamine supplementation received, also by gavage, a corresponding volume of water for the same period. All animals were sacrificed by an overdose of thiopental.

2.2. Histological methods

Immediately after sacrifice, the lower half of the urinary bladder was removed, fixed in 10% formalin prepared in PBS, and routinely processed for paraffin embedding. Five- μ m thick sections were obtained and then stained with hematoxylin–eosin, 1% toluidine blue, and Masson's trichrome.

Indirect immunofluorescence was used for labeling smooth muscle and related cells in the lamina propria using as primary antibody an anti-smooth muscle α -actin (product code 454863A, monoclonal, predilute, Zymed, Carlsbad, CA, USA). Endothelial cells were also detected by immunofluorescence, using as primary antibody an anti-CD31 (product code ab28364, polyclonal, Abcam, Cambridge, MA, USA) at a dilution of 1:30. The secondary antibody for these two immunofluorescence reactions was an Alexa Fluor 488 Goat anti-rabbit IgG (antibody no. 2, product code A11001, Invitrogen, Carlsbad, CA, USA) at a dilution of 1:50, which was used according to the manufacturer's instructions.

Bright-field histological images were captured on a digital camera (DP71, Olympus, Tokyo, Japan) coupled to a light microscope (BX51, Olympus). Immunofluorescence labelings were observed and photographed on a Zeiss LSM 510 confocal laser microscope (Zeiss, Jena, Germany).

2.3. Morphometric methods

Epithelial thickness, epithelial cell density, and cell density in the lamina propria were evaluated on Masson's trichrome-stained sections captured at a magnification of 1000 \times . For epithelial thickness, ten linear measurements (μ m), taken from the basement membrane to the luminal surface of the epithelium, were carried out per field for each tissue section using the software ImageJ version 1.45 (National Institute of Health, Bethesda, MD, USA) and a total of five sections per animal. From these 50 measurements, a mean epithelial thickness was calculated for each animal.

Epithelial cell density was determined by counting the number of nuclei in an outlined area of epithelium using the "Cell Counter" plugin of ImageJ. This number was then divided by the surface area of the outlined region, and the results were expressed as number of nuclei per mm². Five outlined regions were counted per section, and five sections were analyzed per animal. Cell density in the lamina propria was also assessed with "Cell Counter" plugin. In an outlined region of lamina propria, all nuclei were counted, with the exception of those of mast cells and of blood vessels, and the

Table 1
Morphometric data obtained from bladders of the control (CONTR), radiated-only (R7), and radiated plus glutamine supplementation (RG7) groups. All animals were sacrificed seven days after the radiation session.

Variables	Animal groups			ANOVA
	CONTR	R7	RG7	
Epithelial thickness (μm)	42.82 \pm 10.13	38.66 \pm 4.14	38.74 \pm 3.10	$p > 0.05$
Epithelial cell density (nuclei/ mm^2)	5.91 \pm 1.51	6.15 \pm 0.77	6.32 \pm 0.44	$p > 0.05$
Cell density in lamina propria (nuclei/ mm^2)	52.13 \pm 11.32	55.48 \pm 9.29	53.04 \pm 15.39	$p > 0.05$
Density of blood vessels in lamina propria (number of vessels/ mm^2)	55.20 \pm 20.09	29.00 \pm 8.28 $p < 0.05$	50.00 \pm 9.43 $p < 0.02$	$p < 0.025$

For each variable, means of the three groups were first analyzed by one-way ANOVA (p values under the ANOVA heading), and when significance was found, pairwise comparisons were carried out using the Bonferroni method. Pairs consisted of R7 vs. CONTR, and RG7 vs. R7, and statistical significance is indicated by a p -value under R7 or RG7, respectively.

count was then divided by the surface area of the outlined region. A total of 25 determinations were carried out per animal, and cell density in the lamina propria was expressed as number of nuclei per mm^2 . Using these same procedures that were employed for cell density determination, the density of mast cells in the lamina propria was evaluated by counting metachromatic cells on toluidine blue-stained sections, using a total of 25 areas per animal. Similarly, CD31-positive blood vessels were counted in images of the lamina propria captured at a magnification of 1000 \times and using 25 areas per animal. Blood vessel density was then expressed as number of vessels per mm^2 .

2.4. Statistical analysis

Statistical procedures were carried out according to Sokal and Rohlf (1995). Experiments terminated at 7 and 15 days after the radiation session were analyzed separately, and for each morphometric variable, means from the three groups were first compared by one-way ANOVA. When significance was found, pairwise comparisons using the Bonferroni multiple comparisons test were performed to determine: (a) if the variable was affected by radiation; and (b) if glutamine supplementation had a protective effect against eventual changes as detected in the previous comparison. All results are reported as mean \pm standard deviation, and statistical significance was considered when $p < 0.05$.

3. Results

In the present study, the basic metabolic responses of animals were quite similar to those that have been described in detail in a previous investigation (Diestel et al., 2007), as in both cases animal strains and experimental conditions were the same. Briefly, after the radiation session aimed at the pelvico-abdominal region, all animals had diarrhea, which started around the third day post-radiation and lasted about two days. Compared with the control group, food intake decreased by about 33% during this short period, after which it gradually returned to near normal values. Body

weight loss at the end of the experiments was of about 15% on average in the animal groups submitted to radiation. However, this reduction in body weight did not differ significantly among these groups (data not shown).

In the groups that were sacrificed seven days after the single-dose radiotherapy session, radiation did not affect, in the bladder wall, epithelial thickness, epithelial cell density, and cell density in the lamina propria (Table 1). However, density of blood vessels in the lamina propria was altered by the treatments ($p < 0.025$), and in radiated-only animals this density was reduced by 48% ($p < 0.05$) compared with the control group. Notably, this alteration was mostly prevented by glutamine, because in radiated animals receiving this supplement, blood vessel density was significantly ($p < 0.02$) increased with regard to radiated-only animals. Thus, and as shown in Table 1, in the radiated group treated with glutamine, density of blood vessels in the lamina propria was close to that of control animals.

In contrast, in animals sacrificed at the 15-day time point, density of blood vessels in the lamina propria was not modified by the different experimental conditions (Table 2), while epithelial thickness and epithelial cell density were all significantly affected ($p < 0.01$ and $p < 0.025$, respectively) (Table 2 and Fig. 1A–C). Compared with controls, epithelial thickness was reduced by 25% ($p < 0.05$) after radiation, and this effect was also prevented by glutamine supplementation ($p < 0.01$). In the radiated-only group, epithelial cell density was increased by 35% ($p < 0.02$). However, the value for this variable in the supplemented group was not significantly different from that of radiated-only animals, which implies that glutamine did not protect epithelial cell density against the aforementioned radiation-induced increase. As in the previous time point, cell density in the lamina propria was likewise unaffected in animals sacrificed 15 days after radiation.

Because the density of mast cells in the lamina propria was rather variable, data analysis was restricted to calculating means and the full range. Nevertheless, the results showed a noticeable and consistent trend. Thus, the density was markedly reduced seven (mean 1.4; full range 0–3) and 15 (1.0; 0–2) days after

Table 2
Morphometric data obtained from bladders of the control (CONTR), radiated-only (R15), and radiated plus glutamine supplementation (RG15) groups. All animals were sacrificed 15 days after the radiation session.

Variables	Animal groups			ANOVA
	CONTR	R15	RG15	
Epithelial thickness (μm)	42.82 \pm 10.13	32.03 \pm 7.85 $p < 0.05$	51.31 \pm 9.74 $p < 0.01$	$p < 0.01$
Epithelial cell density (nuclei/ mm^2)	5.91 \pm 1.51	7.99 \pm 1.11 $p < 0.02$	6.84 \pm 1.45	$p < 0.025$
Cell density in lamina propria (nuclei/ mm^2)	52.13 \pm 11.32	50.60 \pm 8.61	50.62 \pm 14.26	$p > 0.05$
Density of blood vessels in lamina propria (number of vessels/ mm^2)	55.20 \pm 20.09	59.20 \pm 13.61	64.60 \pm 6.91	$p > 0.05$

For each variable, means of the three groups were first analyzed by one-way ANOVA (p values under the ANOVA heading), and when significance was found, pairwise comparisons were carried out using the Bonferroni method. Pairs consisted of R15 vs. CONTR, and RG15 vs. R15, and statistical significance is indicated by a p -value under R15 or RG15, respectively.

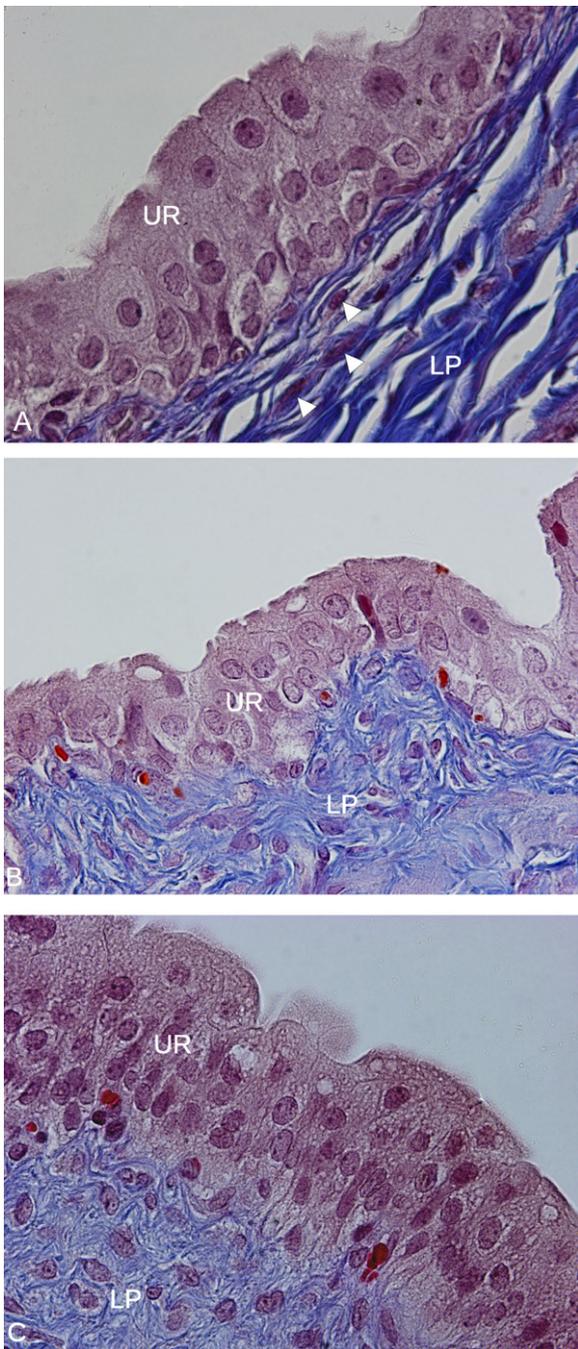


Fig. 1. Superficial layers of the bladder wall from animals sacrificed 15 days after radiation. In radiated-only animals (B) there is a decrease in the thickness of the urothelium (UR), and an increase in its cell density, as compared to the control group (A). In the radiated plus glutamine supplementation group (C), the urothelium is more similar to that of controls (A). In A, note a layer of longitudinally sectioned, elongated cells (arrowheads) in the lamina propria (LP), just under the urothelium. In B and C, these cells are more transversely sectioned. Representative images of each group stained with Masson's trichrome and captured at a final magnification of 1000 \times .

radiation, compared with controls (5.2; 0–9). In the supplemented groups, the density was still reduced seven days after radiation (1.4; 0–4), but at the 15-day time point, the higher density (3.8; 1–7) suggests a glutamine-mediated recovery.

A semi-quantitative analysis of smooth muscle and related cells was also carried out in the lamina propria by labeling bladder samples with an anti-smooth muscle alpha-actin antibody. The results showed that, in all groups, antibody positivity was limited to

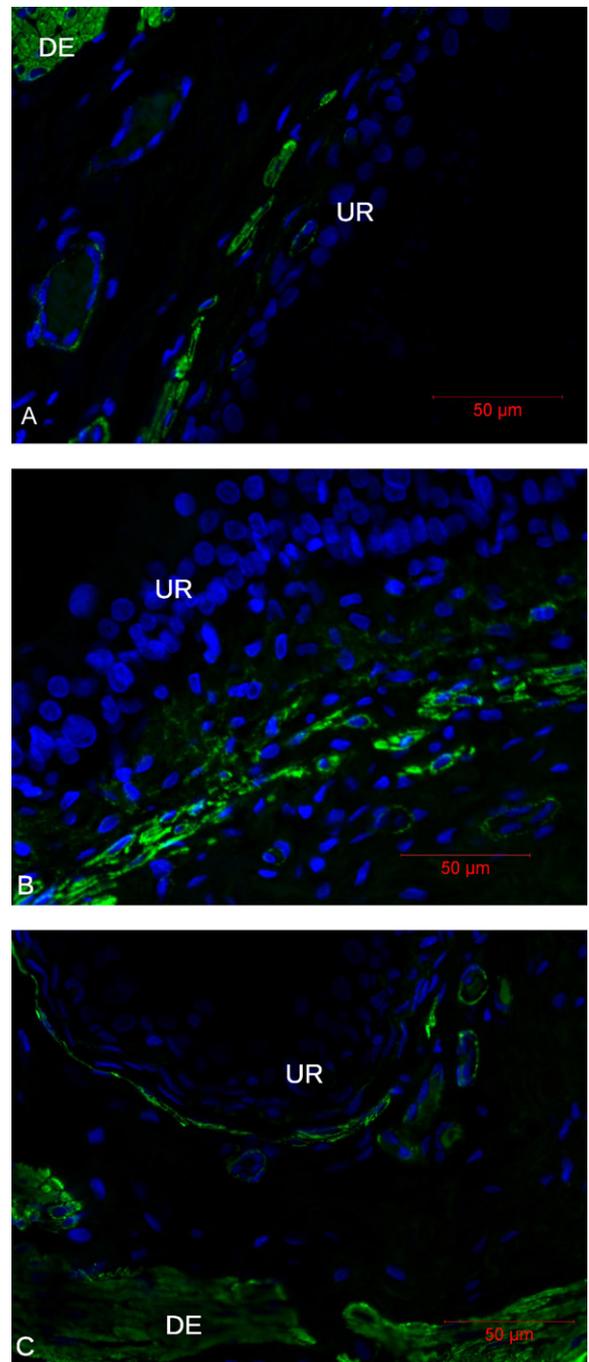


Fig. 2. Immunohistochemical analysis of the bladder lamina propria from animals sacrificed seven days after radiation using an anti-smooth muscle alpha-actin antibody. In this preparation, alpha-actin labeling is shown as a green fluorescence, whereas all nuclei stain blue. Smooth muscle alpha-actin-positive cells include myofibroblasts, which are elongated cells just under the urothelium (UR) (see also Fig. 1A), and the smooth muscle cell bundles of the detrusor layer (DE), which is located deeper in the bladder wall. Myofibroblasts are seen in variable amounts in the control (A), radiated-only (B), and radiated plus glutamine supplementation (C) groups. All images were captured at a 400 \times magnification, and are representative of each group.

elongated cells, which formed a single layer of varying thickness located just under the urothelium (Fig. 2A–C). This layer should therefore correspond to a similar structure as seen in bright-field images (Fig. 1A). In controls, this layer was mostly one cell deep (Fig. 2A). In the groups sacrificed at the seven-day time point, however, this thickness was noticeably increased in the radiated-only group, and the layer was two to four cells deep (Fig. 2B). On

the other hand, in the glutamine-supplemented group (Fig. 2C), the layer of labeled cells had a thickness similar to that of controls. Changes in animals sacrificed at the 15-day time point were less evident (not shown).

4. Discussion

Although radiotherapy is widely used in the treatment of cancer, the response of tissues to radiation is variable (Ross, 1999). For example, in a study using rats (Antonakopoulos et al., 1982), the bladder urothelium was mostly normal one month after the animals had received 20 Gy of radiation. In other studies, however, mice were submitted to this same dose of radiation, and 13 days later it was observed a decrease in the total number of urothelial cells (Jaal and Dorr, 2006a, 2010). Thus, the short- and longer-term changes the urothelium undergo after radiation are different. Our results are consistent with these findings, as they showed that radiation-induced modifications in the bladder wall are time dependent. Accordingly, epithelial thickness and density were unchanged in R7, while in R15 they were significantly decreased and increased, respectively. The fact that these two particular changes were in different directions, but had similar magnitudes, implies that cell volume decreased in the urothelium. It should be noted also that this response to radiation occurred in the post-acute phase. A radiation dose of 10 Gy, as used in previous reports (Diestel et al., 2007) and in the present one, can be considered to be intermediate between low doses ranging from 0.015 to 2 Gy (Kim et al., 2007) and high doses from 30 to 45 Gy (Lerouxel et al., 2009). Because in RG15 epithelial thickness was similar to that of the control group, while epithelial cell density remained altered, this protective effect of glutamine should be due to enhanced cell proliferation, rather than to changes in cell volume. Indeed, in rats that were submitted to chemotherapy, the intestinal epithelium was injured, but in animals that were treated with glutamine, there was an increase in the mRNA expression of both the glutamine transporter and of the proliferating cell nuclear antigen (PCNA) in cells of the intestinal epithelium (Tazuke et al., 2011). In a similar experiment using chemotherapy-treated rats, glutamine increased the concentration of the antioxidant glutathione, and diminished apoptosis (Owari et al., 2012). In these two studies, epithelial cell proliferation was much enhanced. These mechanisms might underlie, therefore, the protective effect of glutamine on the bladder urothelium, as shown by our results.

In contrast, the total cell density in the lamina propria remained unchanged in R7 and R15. However, these results exclude mast cells and blood vessels, which were affected by radiation.

Ionizing radiation promotes, in mast cells, the release of tryptase, histamine granules, and pro-angiogenic factors (Müller and Meineke, 2011), as well as proliferation factors of fibroblasts and endothelial cells (Fajardo and Pejler, 2003; Ribatti et al., 2010; Sant et al., 2007; Stein et al., 2007). The evaluation of mast cells showed a pronounced reduction in the radiated groups as compared with control animals. A mild recovery was observed in RG15. These changes can be explained by the high sensitivity of these cells to radiation (Forsgren et al., 2001; Norrby et al., 1984). The recovery of mast cells in RG15 might be related to the protection of these cells by the action of L-glutamine, as already described in the gastric mucosa of rats (Mittra and Pal, 1977).

The bladder lamina propria has a relatively high density of small blood vessels such as arterioles, capillaries and venules. Changes in blood vessels are one of the side effects of radiation, and may be related to the evolution of symptoms developed over time (Lagrange, 1997). In CD31-labeled sections, we observed a marked decline in the fluorescence of endothelial cells in R7 compared with controls. In RG7, however, the fluorescence was similar to that of

the control group, thereby indicating a protective effect of glutamine on the acute phase of the tissue response. Glutamine has angiogenic effects, and decreasing its concentration limits tumor neovascularization and growth (Maity et al., 2003). In R15, the density of blood vessels was similar to that of controls, which suggests a spontaneous recovery due to neovascularization, as normally occurs after injury.

Our results showed a distinct layer of smooth muscle alpha-actin-positive cells just under the urothelium. This layer also exists in humans, and has been designated as suburothelial band of myofibroblasts (Paner et al., 2009). However, and unlike humans, there was no other actin-positive layer of cells between this suburothelial band of myofibroblasts and the detrusor layers. Thus, apparently the muscularis mucosae, as described in humans (Paner et al., 2009), is lacking in the rat. This difference should be relevant for understanding the physiology and cell responses of the rat bladder, especially when it is used as an experimental model for human diseases. In fact, and compared with humans, the genitourinary tract of the rat has other differences regarding smooth muscle-related cells, as it has been previously shown (Pinheiro et al., 2000).

The increase in myofibroblasts is associated with spontaneous contraction activity of the bladder and overactive bladder in humans (Sui et al., 2008; Haab et al., 2010; Ikeda et al., 2007; Roosen et al., 2009). Only in radiated animals sacrificed at the seven-day time point it was observed an increase in the number of myofibroblasts. Radiation causes cell damage, and the ensuing release of cytokines has a role in the activation and regulation of myofibroblasts in the healing process (Devalia and Mansfield, 2008). This could explain the increased thickness of the suburothelial band of myofibroblasts in R7. Glutamine prevented this change, yet there is no direct evidence relating glutamine and myofibroblast growth and/or modulation. In the liver, apparently glutamine has an opposite effect, as greater availability of this aminoacid through enhanced expression of glutamine synthetase parallels modulation of precursor cells into myofibroblast-like cells (Bode et al., 1998). However, the effects on the rat intestinal smooth muscle cells are more similar to our results. Thus, glutamine prevents post-injury growth of smooth muscle cells via suppression of inflammatory mediators (San-Miguel et al., 2010). It should be noted that, as cell density in the lamina propria was unaffected in all animal groups, these changes in myofibroblasts imply that these cells, and precursors thereof, are undergoing phenotypic modulation and apoptosis.

5. Conclusion

Our findings show that pelvic radiation leads to significant acute and post-acute alterations in the composition and structural features of the vesical lamina propria and epithelium. These include changes in the density of blood vessels, mast cells, myofibroblasts, and urothelial cells. Most of these changes, however, could be prevented by glutamine nutritional supplementation. These results emphasize, therefore, the potential use of this aminoacid as a protective drug against the adverse effects of pelvic radiotherapy.

Conflicts of interest

The authors declare that they have no financial and non-financial conflicts of interest.

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