Protective Effect of Leuprorelin on Radiation-induced Intestinal Toxicity

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Abstract. *Background/Aim: Patients with prostate cancer treated with neoadjuvant androgen ablation experience less radiation-induced intestinal toxicity, mostly due to a reduction of the volume of normal tissue exposed to high radiation doses. We aimed to evaluate if the anti-androgenic drug leuprorelin itself exerts a protective effect on irradiated bowel. Materials and Methods: Female, intact and castrated male C57BL/6J mice underwent 12-Gy total body irradiation, with or without a three-month leuprorelin (0.054 mg/kg/month i.p.) pre-treatment. After 24-72 h, mice were sacrificed and intestinal segments collected for histological, immunohistochemical and molecular analyses. Results: Leuprorelin markedly reduced radiation-induced jejunal and colonic histological alterations in mice, increased the number of regenerating crypts vs. irradiation, and reduced radiation-induced nitrotyrosine immunoreactivity. Leuprorelin significantly reduced radiation-induced matrix metalloproteinase-2 (Mmp2) and -13, collagen 1 and -3, transforming growth factor-beta (Tgfb), p53, interleukin 6 (Il6), and B-cell lymphoma 2 (Bcl2)-associated X protein (Bax) gene expressions, and nuclear factor-kappa B (NFκB) and TGFβ protein expression, and hampered radiation-induced BCL2 protein down-regulation. Conclusion: Leuprorelin protects mice from radiation-induced intestinal injury, likely through a reduction of tissue oxidative stress. These findings give a biological interpretation to clinical observations of improved intestinal tolerance in patients undergoing androgen ablation before RT.*

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Androgen deprivation therapy (ADT) is a well-established modality for the treatment of patients with prostate carcinoma. ADT with luteinizing hormone-releasing hormone (LHRH) agonists is administered in combination with radiotherapy (RT) as primary treatment for localized or locally advanced prostate cancer. Randomized phase III studies demonstrated an improved disease-specific and overall survival with combined therapy for high-risk patients (1). ADT exerts a cytoreductive effect, leading to a reduction in tumor bulk and improved tumor control by RT. Moreover, ADT in combination with RT has a potentiating effect through enhancing induction of apoptosis (2).

New techniques have allowed the RT dose to be increased while keeping morbidity acceptable. However, radiation-induced intestinal toxicity remains the major dose-limiting side-effect in abdominal and pelvic RT (3). In the mid-1990s, neoadjuvant ADT was thought to increase radiation-induced toxicity (4). Liu *et al*. observed that short-term (<2 months) neoadjuvant ADT, but not treatment of longer duration, increases the risk of developing toxicity (5). During the course of ADT, the volumes of prostate gland and seminal vesicles shrink; the volume is reduced by 20-50% after 3 months of ADT, after which shrinking occurs at a slower rate (6). Patients who undergo planning computed tomography (CT) shortly after starting ADT can have a significantly larger reduction in prostate volume than patients with longer duration of ADT. This might lead to an unexpected increase of the percentage of rectal wall exposed to intermediate RT doses (7).

The European Organisation for Research and Treatment of Cancer (EORTC) trial 22863, comparing whole-pelvic irradiation with or without a LHRH agonist, did not find any significant differences in all-grade gastrointestinal toxicity between treatment arms (8). However, a lower incidence of grade 3-4 gastrointestinal toxicity in the LHRH agonist-treated arm was reported (6.4% *vs*. 10.9% in patients not treated with LHRH).

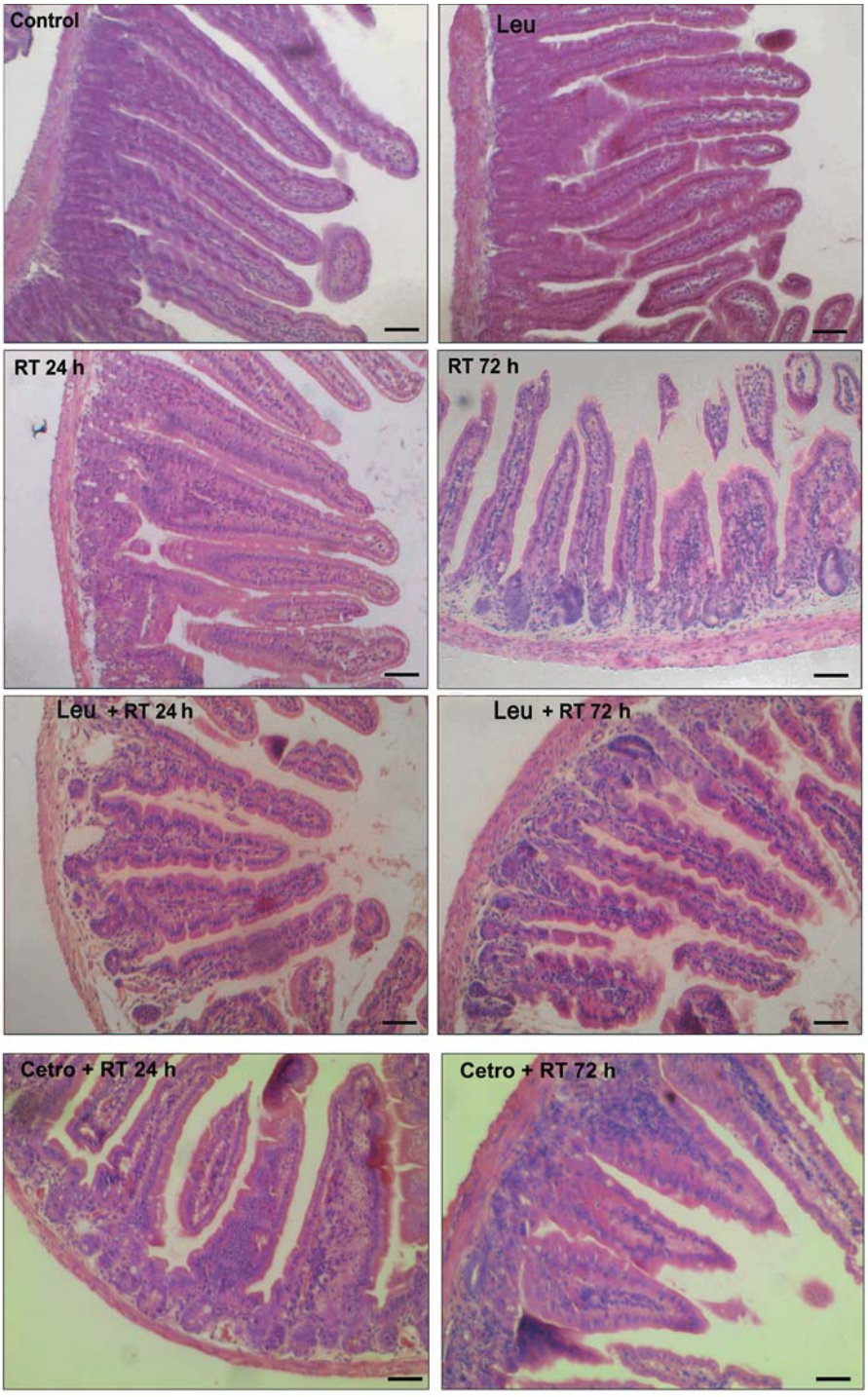

Figure 1. *Leuprorelin (Leu) reduced histological signs of radiotherapy (RT)-induced damage to the jejunum in male mice. Full-thickness images of jejunal wall of intact male mice treated with saline (Control), Leu, 12 Gy RT, Leu plus RT or Cetrorelix (Cetro) plus RT, 24-72 h after irradiation. Bars=100 μm.*

Moreover, a recent study on high-dose RT in combination with anti-androgens starting 2 months before external-beam radiation therapy did not associate ADT with additional RT-related gastrointestinal or genitourinary toxicity (9). Rather, the reduction in tumor bulk due to ADT potentially seems to provide an improvement in treatment planning and normal tissue-sparing (10) by optimizing the geometry of the target volume prior to RT (11).

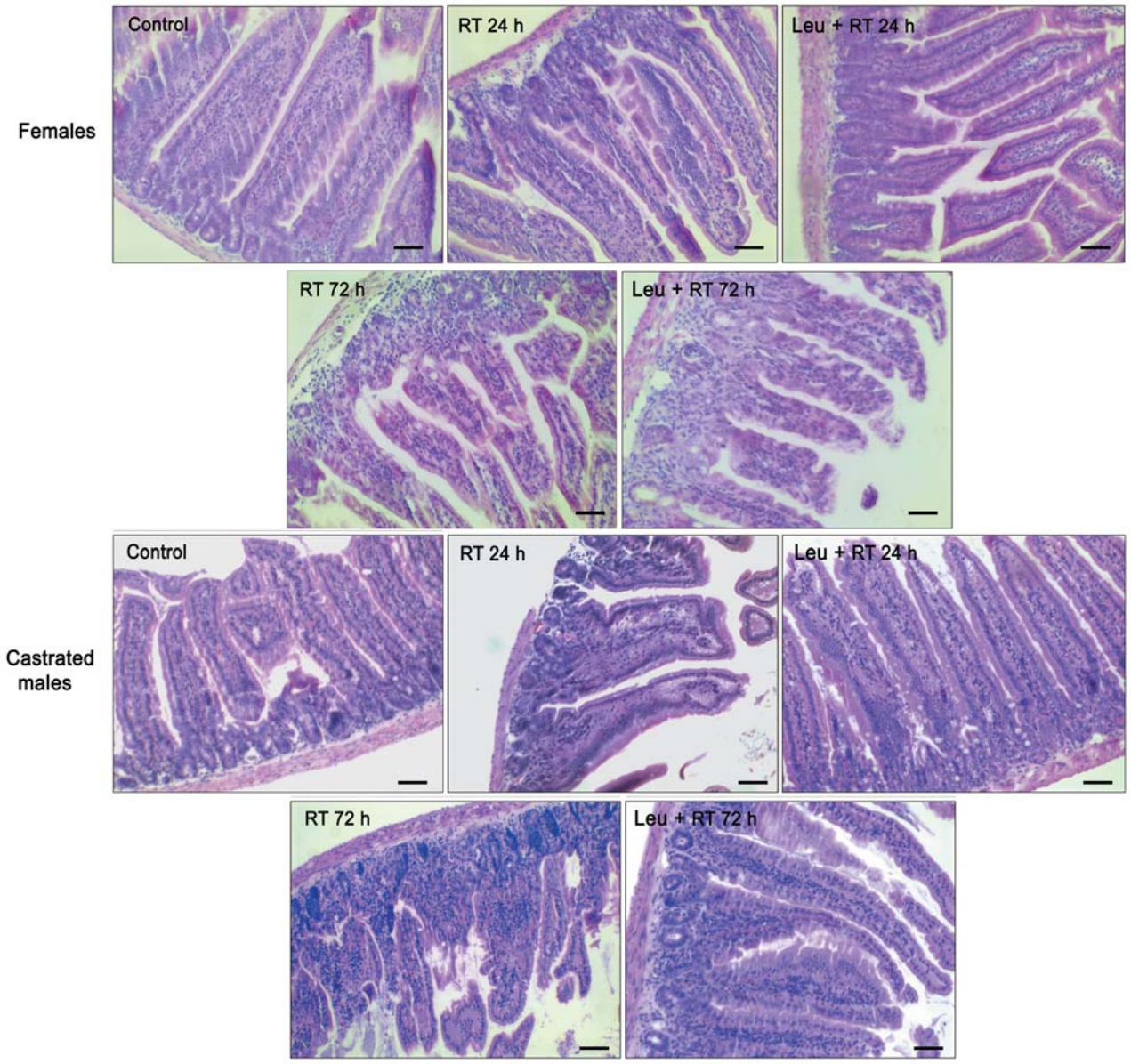

Figure 2. *Leuprorelin (Leu) reduced histological signs of radiotherapy (RT)-induced damage to the jejunum in female and castrated male mice. Full-thickness images of jejunal wall of female (upper panels) and castrated male (lower panels) mice treated with saline (Control), 12 Gy RT or Leu plus RT, 24-72 h after irradiation. Bars=100 μm.*

Despite several studies on the timing of ADT and shrinkage rate, the histological and molecular effects of ADT on irradiated bowel remain uninvestigated. The aim of the study was to evaluate whether ADT exerted any effect on irradiated bowel in a murine model of radiation-induced intestinal damage.

Materials and Methods

Animals and experimental protocol. Male and female C57BL/6J mice (Charles River Laboratories, Milan, Italy) were housed at controlled temperature (22±2˚C), lighting (12 hours) and humidity (60±10%). Some of the male mice underwent castration by bilateral orchiectomy. Mice were anesthetized with Avertin® (25 ml/kg of a 1.25% w/v 2,2,2-tribomoethanol solution, *i.p.*) and testes were removed at laparotomy. Animals were allowed to recover for 2 weeks before further treatments. Male (castrated/intact) and female mice underwent 12 Gy total body irradiation (TBI), as described elsewhere (12, 13), with or without 3 months pretreatment with leuprorelin acetate (0.054 mg/kg/month *i.p.*; Enantone®; Takeda Italia, Rome, Italy). To test the effect of the inhibition of LH release, a group of intact male mice was pretreated with the LHRH

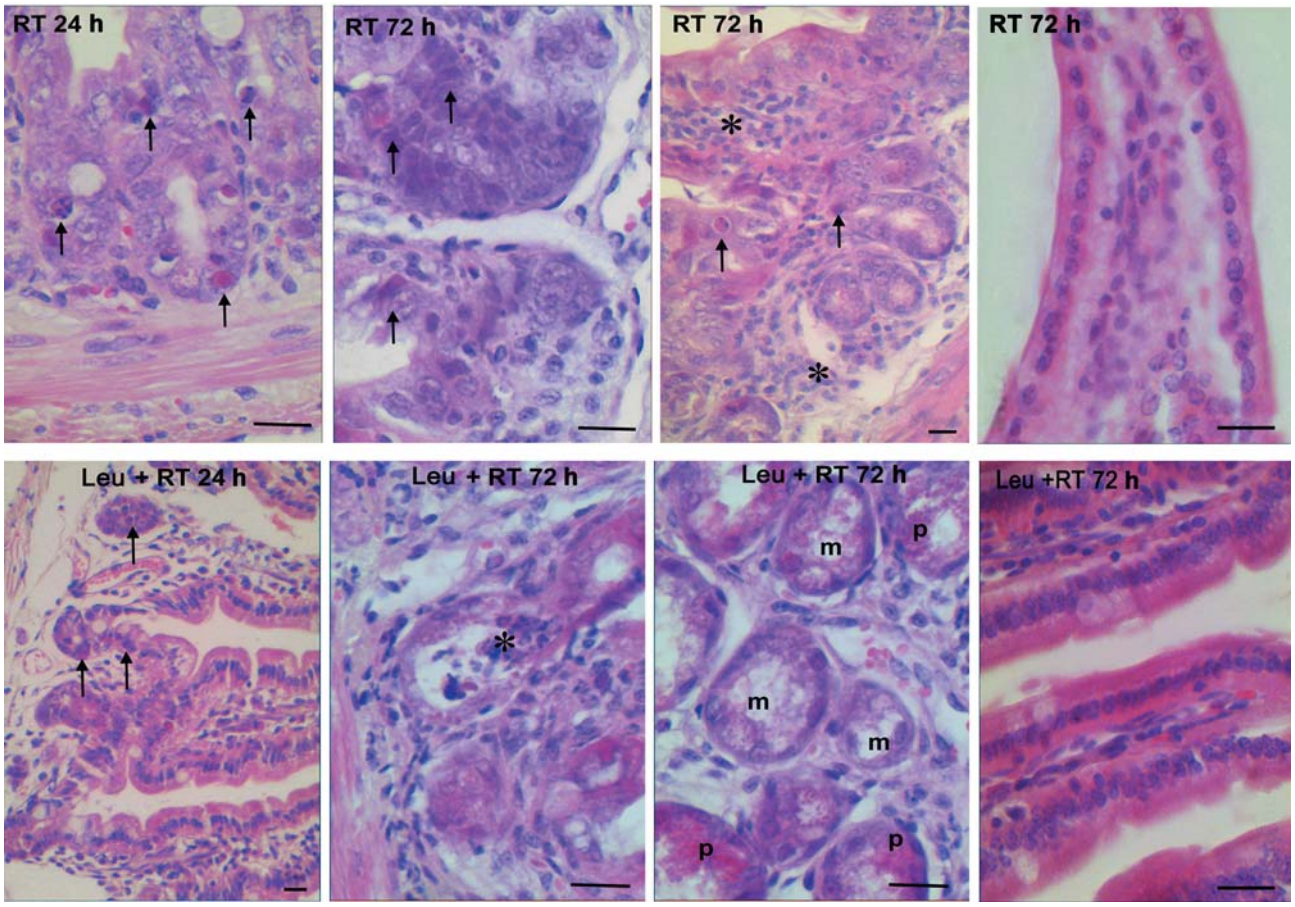

Figure 3. *Representative images of the protective effect of leuprorelin in jejunal wall of irradiated male mice. Representative images of jejunal wall of intact male mice treated with 12 Gy radiotherapy (RT) or Leu plus RT, 24-72 h after irradiation. Arrows: Apoptotic cells, asterisks: apoptotic/necrotic tubular glands, m: muciparous cells, p: serozymogenic Paneth cells. Bars=20 μm.*

antagonist Cetrorelix acetate (0.5 mg/kg/day *s.c.*; Sigma-Aldrich, St. Louis, MO, USA) for 7 days before TBI. Non-irradiated mice were used as control. After 24-72 hours, mice were sacrificed and jejunum and colon segments collected.

Histology and assessment of nitrotyrosine. Representative 5 μm-thick sections were cut from formalin-fixed, paraffin-embedded jejunum and colon and stained with hematoxylin-eosin for histology or used for immunohistochemistry.

Nitrotyrosine immunohistochemistry was performed on samples from intact male mice as described elsewhere (14). Photomicrographs of tissue were taken using a digital photomicroscopy apparatus with a ×20 objective. On digitized images, measurements of surface area and optical density (OD) of nitrotyrosine-immunostained tissue was obtained using the ImageJ 1.33 software (US National Institutes of Health, Bethesda, MD, USA) upon selection of a threshold to include only the immunolabeled tissue surface area. Values are reported as arbitrary units (surface area×OD×10^{-6}).

Microcolony survival assay. The degree of regeneration of jejunal crypts from intact male mice was assessed by microscopic examination of slides prepared for histological analysis, as previously described (15).

Real-time polymerase chain reaction (PCR). RNA extraction, retrotranscription and gene expression measurement by real-time PCR (TaqMan) were performed on samples from intact male mice as previously reported (16). The amount of target gene (transforming growth factor-beta 1 (*Tgfb1*), interleukin 6 (*Il6*), B-cell lymphoma 2 (Bcl2)-associated X protein (*Bax*), transformation-related protein 53 (*Trp53*), collagen type I/III (*Col1, Col3*), matrix metalloproteinase-2 and -13 (*Mmp2*, *Mmp13*); Applied Biosystems, Foster City, CA, USA), normalized to that of an endogenous reference (glyceraldehyde 3-phosphate dehydrogenase, *Gapdh*), was given by $2^{-\Delta\Delta Ct}$ calculation (17).

Western blot analysis. Western blot was performed on samples from intact male mice as described elsewhere (16) using anti-BCL2, anti-nuclear factor kappa B (NFκB), anti-TGFβ (1:400) and anti-GAPDH (1:1500) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistics. Statistical analysis was performed using SPSS software 12.0 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance

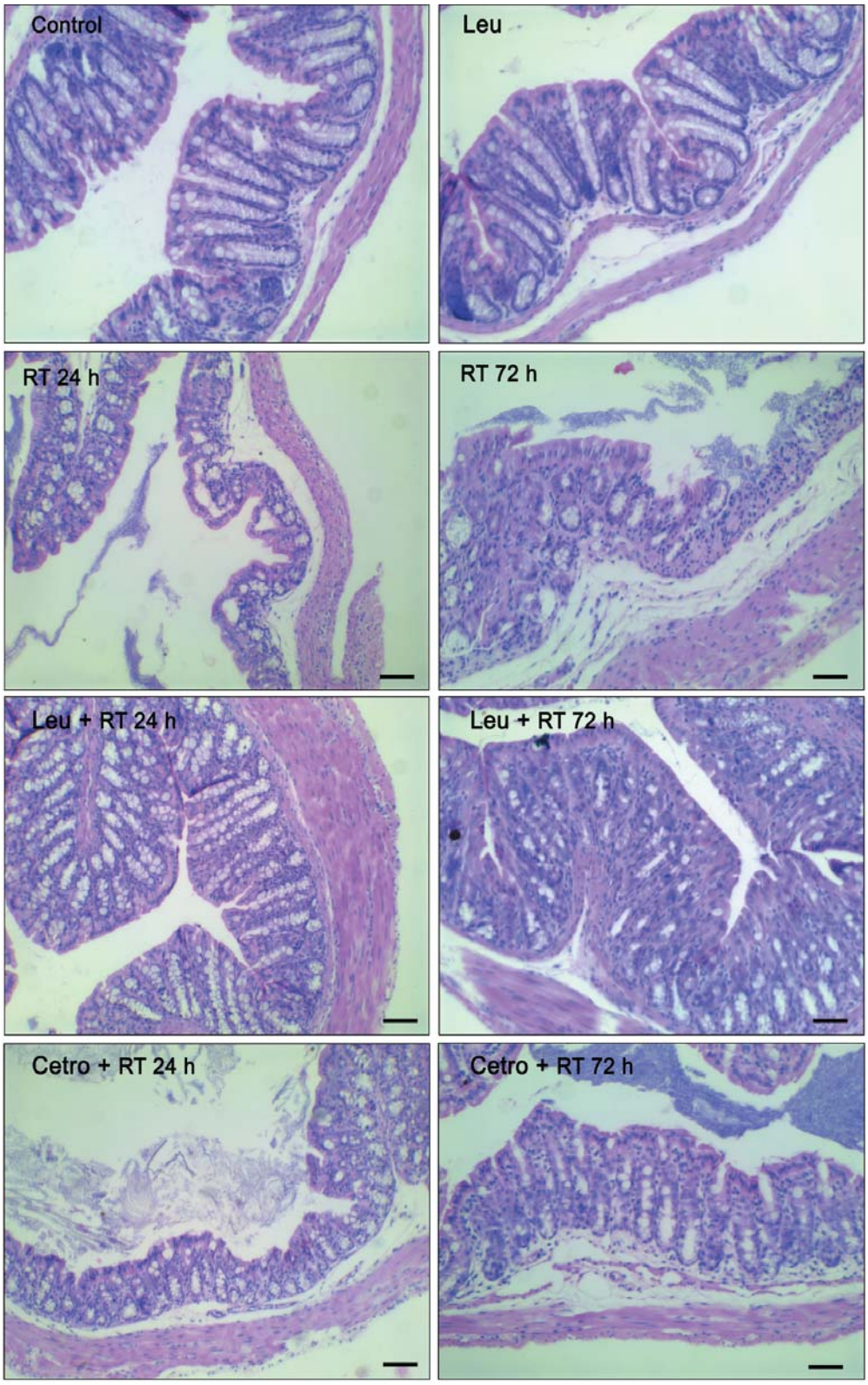

Figure 4. *Leuprorelin (Leu) reduced histological signs of damage to colon induced by radiotherapy (RT) in male mice. Representative full-thickness images of colon wall of intact male mice treated with saline (Control), Leu, 12 Gy RT, Leu plus RT or Cetrorelix (Cetro) plus RT, 24 h or 72 h after irradiation. Bars=100 μm.*

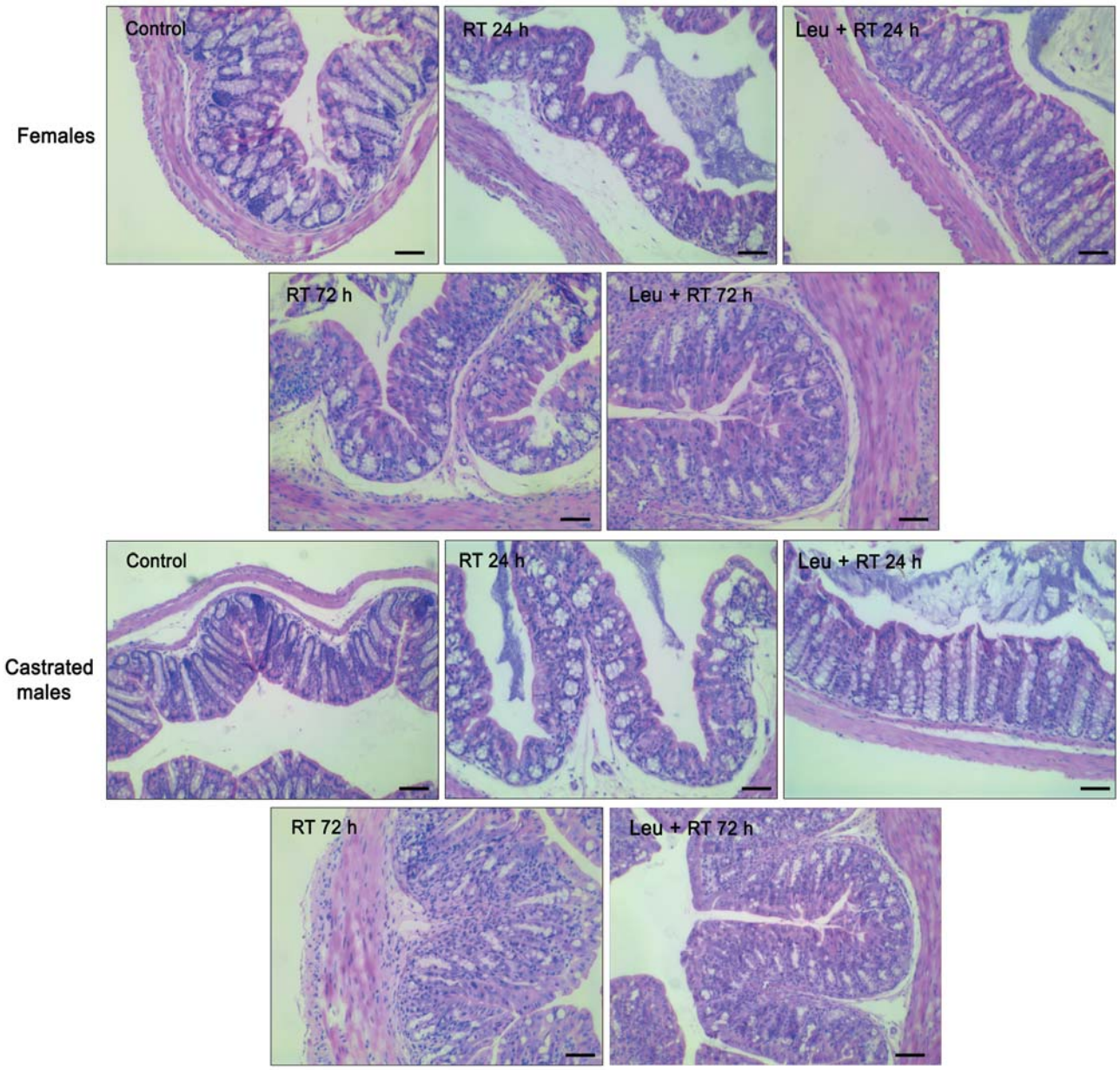

Figure 5. *Leuprorelin reduced histological signs of damage to colon induced by radiotherapy (RT) in female and castrated male mice. Representative full-thickness images of colonic wall of female (upper panels) and castrated male (lower panels) mice treated with saline (Control), 12 Gy RT, or Leu plus RT, 24 h or 72 h after irradiation. Bars=100 μm.*

was applied, a *p*-value less than 0.05 (two-sided test) was considered statistically significant. Data are expressed as the mean±SEM.

Results

Histological analysis. Histological analysis of jejunal specimens of male mice (Figure 1) showed that control mice exhibited normal features, with long villi lined with a continuous columnar epithelium containing numerous goblet cells and well-developed tubular glands composed of muciparous cells and serozymogenic Paneth cells. RT caused a time-dependent hypoplasia of tubular glands, occurrence of a marked inflammatory infiltrate and shortening of villi, with loss of normal histological structure. Leuprorelin dampened the RT-induced changes, both 24 and 72 h after RT, resulting in histological features similar to those of controls. Cetrorelix showed a substantially lower protective activity as compared to leuprorelin. The ability of leuprorelin

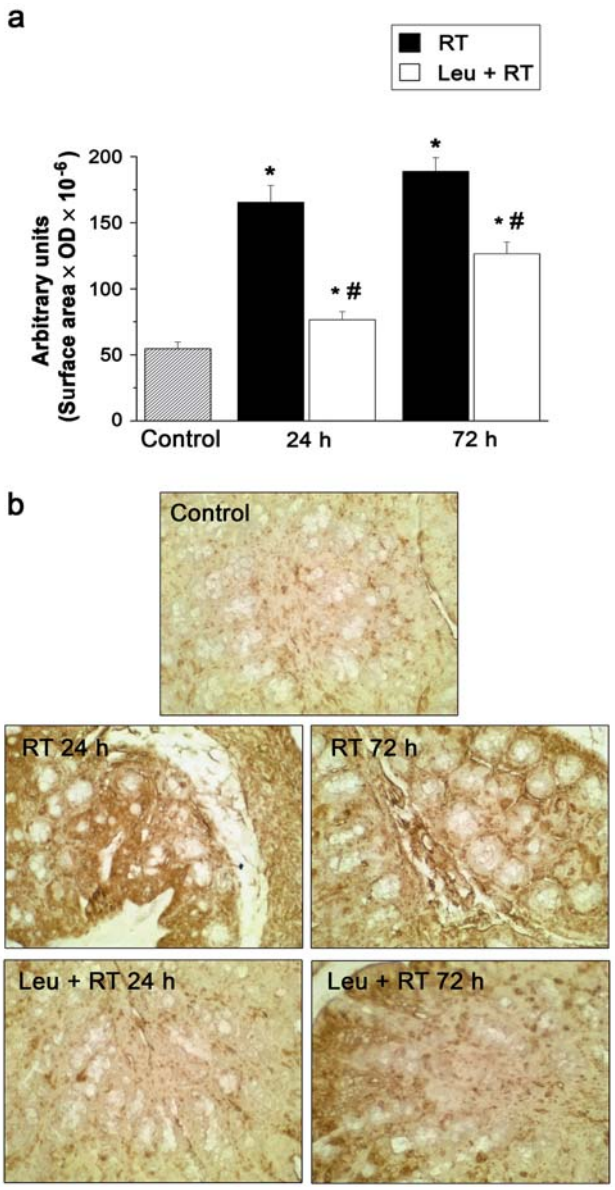

Figure 6. *Effect of leuprorelin (Leu) on radiotherapy (RT)-induced nitrotyrosine levels in the jejunal wall. a: Nitrotyrosine levels were determined in jejunum specimens of intact male mice treated with saline (Control), 12 Gy RT or Leu+RT, 24-72 hours after irradiation. Results are shown as arbitrary units (mean); error bars indicate the standard error of the mean (SEM) for three independent experiments. *$p<0.05$ vs. control; #$p<0.05$ vs. RT. b: Representative images of nitrotyrosine-immunostained jejunum sections. Bars=50 μm.*

to reduce RT-induced jejunal damage was also confirmed in female and castrated male mice (Figure 2).

The protective effect of leuprorelin on jejunal mucosa was better-appreciated in images at higher magnification (Figure 3). RT caused edema of the villous stroma, thinning of columnar epithelial cells, with disappearance of striated border; numerous apoptotic cells were detected in tubular glands and the staminal ring (arrows in Figure 3); a marked inflammatory infiltrate was present, especially around the glands (asterisks in Figure 3). Conversely, columnar epithelium of mice pre-treated with leuprorelin mostly exhibited normal features, with continuous striated border and intercalated goblet cells. Tubular glands still showed apoptotic cells (arrows), especially at the 24-h time point, while the inflammatory infiltrate in the mucosal/submucosal stroma was markedly reduced. Although some tubular glands appeared apoptotic/necrotic, others contained a normal cell population of muciparous and serozymogenic Paneth cells.

These findings were confirmed in colon sections of intact male (Figure 4), castrated male and female mice (Figure 5): RT caused mucosal ulceration, reduction of goblet cells, shortening or total disappearance of microvilli, submucosal edema and diffuse inflammatory infiltrate, leading to mucosal structure disruption after 72 h. Leuprorelin maintained mucosal histological architecture similar to that of controls, the only remarkable alteration being the occurrence of apoptotic cells in the gland wall.

The analysis of colon sections from Cetrorelix-pretreated mice again showed a weaker protective effect of Cetrorelix against radiation-induced intestinal damage compared to leuprorelin (Figure 4).

Microcolony survival assay. Microcolony assay was performed to evaluate leuprorelin radioprotective effect on radiation-induced damage to crypts. RT time-dependently reduced the number of regenerating crypts in the jejunum compared to healthy controls (control=93.3±2.9; RT at 24 h=65.4±3.3, $p<0.001$; RT at 72 hours=33.2±1.1, $p<0.001$). Leuprorelin hindered the reduction of crypt number at both time points. In particular, 24 h after irradiation, the number of regenerating crypts in leuprorelin-pre-treated mice was comparable to that of controls (Leu+RT at 24 h=93.8±2.7, $p<0.001$ *vs.* RT; Leu+RT at 72 h=56±2.7, $p<0.001$ *vs.* RT, $p<0.001$ *vs.* control).

Evaluation of nitrotyrosine. Excess superoxide anion and nitric oxide induced by irradiation react to form peroxynitrite, which causes DNA damage, membrane lipid peroxidation and cell injury. RT-induced nitroxidative injury to jejunal tissue was determined by immunostaining for nitrotyrosine, a marker of protein nitrosylation by peroxynitrite (Figure 6). In cross sections of jejunal tissue from control mice, nitrotyrosine immunoreactivity was very weak and scanty. RT significantly increased the area of nitrotyrosine-immunoreactive tissue at both time points ($p<0.05$ *vs.* control). Leuprorelin markedly reduced RT-induced nitrotyrosine immunoreactivity ($p<0.05$).

Molecular analysis. We assessed the modulation of markers of fibrosis (Tgfb1,Col1, Col3, Mmp2 and Mmp13), inflammation (Il6 and Nfkb) and apoptosis (Trp53, Bax and

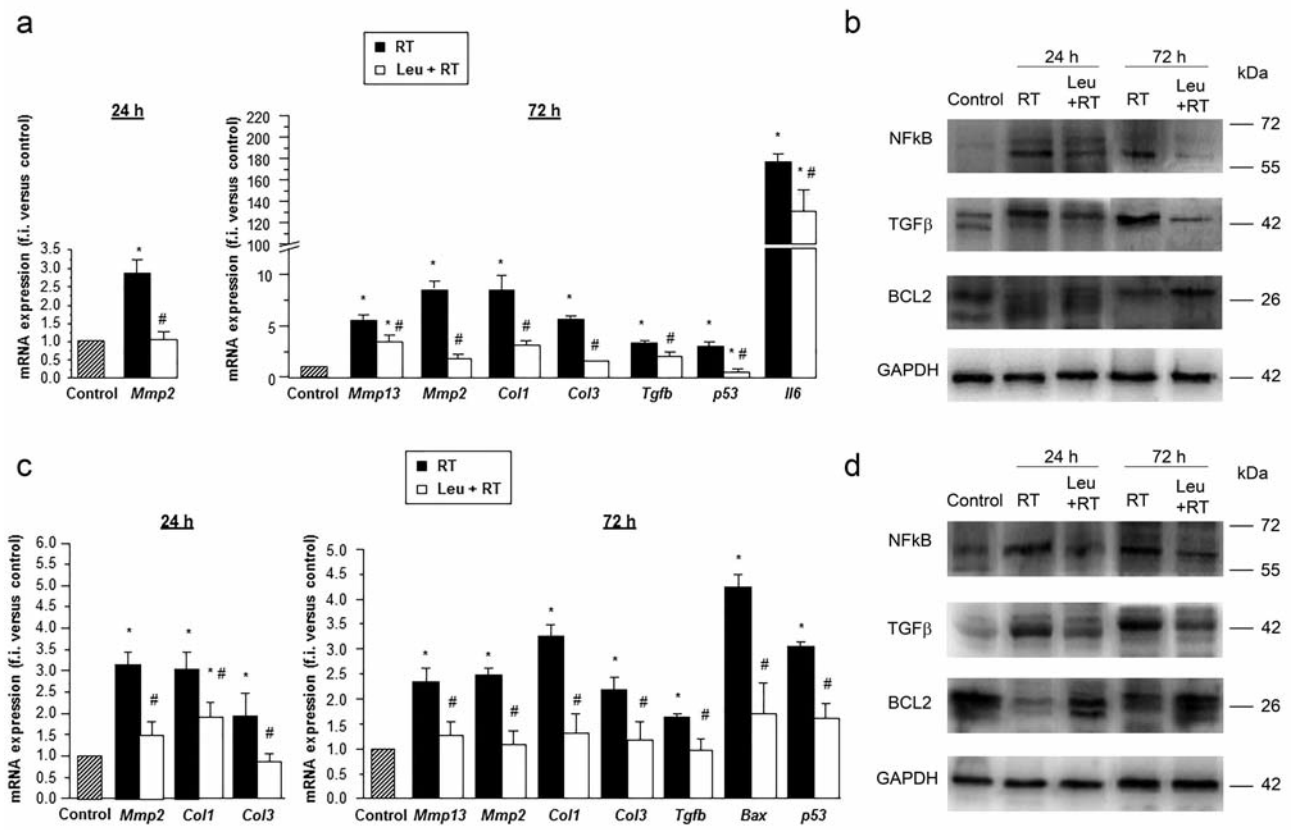

Figure 7. *Leuprorelin (Leu) reduced radiation (RT)-induced overexpression in markers of fibrosis, inflammation and apoptosis in jejunum and colon. mRNA expression of matrix metalloproteinase-2 (Mmp2), and -13 (Mmp13), collagen 1 (Col1), and -3 (Col3), transforming growth factor-beta (Tgfb), transformation-related protein 53 (Trp53), interleukin 6 (Il6) and B-cell lymphoma 2 (Bcl2)-associated X protein (Bax) in jejunum (a) or colon (c) tissues of intact male mice treated with saline (control), 12 Gy RT or Leu plus RT, measured as fold increase (f.i.) vs. expression in control (taken as 1). Error bars indicate the SEM for 10 independent experiments. *p<0.05 vs. control, #p<0.05 vs. RT. Jejunum (b) and colonic (d) tissue homogenates from intact male mice treated with saline (Control), 12 Gy RT or Leu plus RT were immunoblotted with specific antibodies to assess nuclear factor-kappa B (NFκB), TGFβ and BCL2 protein expression at 24-72 h after irradiation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.*

Bcl2) by real-time PCR and western blot analysis of jejunal and colonic tissues of intact male mice.

In jejunal specimens, RT significantly increased gene expression of Mmp2 at 24 h after irradiation, and of *Mmp2*, *Mmp13*, *Col1*, *Col3*, *Tgfb, Trp53* and *Il6* 72 h post-irradiation ($p<0.05$ *vs.* control, Figure 7a). In colonic tissues, RT significantly increased gene expression of *Mmp2*, *Col1* and *Col3* at 24 hours post-irradiation, and *Mmp2*, *Mmp13*, *Col1*, *Col3*, *Tgfb*, *Trp53* and *Bax* at 72 hours post-irradiation ($p<0.05$ *vs.* control, Figure 7c). Leuprorelin significantly reduced radiation-induced overexpression of the above genes in both jejunum and colon ($p<0.05$ *vs.* RT).

In samples of jejunum (Figure 7b) and colon (Figure 7d), RT caused enhanced protein expression of NFκB and TGFβ, and a reduced expression of the anti-apoptotic protein *BCL2 vs.* controls. Leuprorelin reduced RT-induced overexpression of these markers of inflammation and fibrosis, at both time points. RT-induced Bcl2 down-regulation was inhibited in jejunal tissues at only 72 hours post-irradiation, while in colonic tissues at both 24 and 72 hours after RT.

Discussion

On the basis of clinical evidence of decreased RT-induced intestinal toxicity in patients with prostate cancer undergoing ADT before RT (10), we used a previously developed murine model of radiation-induced intestinal toxicity (12, 13) to evaluate whether ADT exerted a protective effect against radiation-induced bowel injury and to elucidate the mechanisms involved.

As ADT we used the LHRH agonist leuprorelin, administered using a therapeutic protocol similar to that for patients with prostate cancer (18). In mice pre-treated with leuprorelin, we observed a marked reduction of RT-induced

jejunal and colonic injury, as demonstrated by histological analysis, and confirmed by microcolony assay.

It is well-known that ionizing radiation triggers a cascade of molecular events that eventually lead to destruction of intestinal tissue. The activation of the transcription factor NFκB induces the local release of pro-inflammatory cytokines, such as IL6 and TNFα, which are involved in intestinal damage (19).

The fibrogenic cytokine TGFβ, in particular, is one of the key mediators of radiation-induced intestinal fibrosis, as it induces collagen and extracellular matrix deposition (20, 21), stimulates the expression of the fibrosis marker α-smooth muscle actin (22), and the synthesis of MMPs, shown to promote tissue injury and inflammation in several gastrointestinal diseases (23). The damage induced then leads to apoptosis of the mucosal cells, thus further amplifying destruction of intestinal tissue (19).

Hence, the protective activity of leuprorelin against intestinal damage can be explained, at least in part, by the inhibitory effect we observed on the radiation-induced expression of tissue markers of fibrosis, inflammation and apoptosis. In order to elucidate the mechanisms underlying the radioprotective effect of leuprorelin, we evaluated whether this was hormone-mediated or not. Leuprorelin causes its anticancer effect by interfering with the normal pulsatile release of LHRH from the hypothalamus. During the first 1-3 weeks of treatment, there is an increase of testosterone but subsequently down-regulation of LH secretion occurs, eventually resulting in low testosterone levels typical of castration (24). LHRH and its receptor have been detected in rodent digestive tract (25) and *LHRH* mRNA has been shown to be expressed in human intestine (26). Thus, we can suppose that LHRH may have a functional regulatory role in the gut. Clinical and experimental evidence supports the hypothesis that the beneficial effects of leuprorelin on radiation injury are hormone-mediated. Leuprolide has been shown to reduce symptoms in patients with irritable bowel syndrome and chronic intestinal pseudo-obstruction (27, 28), and it has been hypothesized to act through the down-modulation of LH secretion (29). Indeed, gonadotropins and gonadal hormones have known antagonistic effect on gastrointestinal motility (30, 31). In order to exclude a concomitant influence of gonadal hormones on bowel radioprotection, we repeated the experiment in castrated male and female mice. Since we did not observe any significant differences among the groups, we can exclude a role for gonadal hormones on the observed protective effect.

To test the effect of the inhibition of LH release we used the LHRH antagonist Cetrorelix, but we only observed a weak protective effect on irradiated bowel, lower than that obtained with leuprorelin. This difference may be due to the potent gonadotropin-lowering activity and the peculiar mechanism of action of LHRH antagonists.

There is evidence that LHRH agonists are able to modulate oxidative stress, *e.g.* by the inhibition of nitric oxide synthesis and peroxynitrite generation (32-34). We observed that leuprorelin significantly reduced radiation-induced nitrotyrosine immunostaining in intestinal mucosa, thus we can hypothesize that its radioprotective effect involves the reduction of tissue oxidative stress.

Conclusion

Our findings provide a mechanistic background to clinical observations of improved intestinal tolerance in patients undergoing ADT before RT. The radioprotective effect appears to be related to the reduction of oxidative stress in intestinal tissues expressing LHRH receptors.

Compliance with Ethical Standards

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Animal experiments were approved by the animal Ethics Committee of the University of Florence (protocol n° 56/2009). This article does not contain any studies with human participants performed by any of the Authors.

Conflicts of Interest

The Authors declare that they have no conflict of interest. The Authors declare that they have full control of all primary data and that they agree to allow the journal to review their data if requested.

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