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Hypertension Predisposes to Radiation Toxicity in a Rat Model of Localized Intestinal Irradiation

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Abstract

Purpose: Vascular disease is believed to be a risk factor for radiation injury in normal tissues, including the intestine. Clinical studies evaluating the relationship between hypertension and radiation enteropathy have been inconclusive, possibly because most hypertensive subjects were receiving blood pressure reducing treatment. This study assessed the association between uncontrolled hypertension and intestinal radiation injury in a clinically relevant rat model.

Methods and materials: Spontaneously Hypertensive Rats (SHR) and normotensive Wistar Kyoto (WKY) rats received localized fractionated X-radiation of a small bowel loop. At 1, 2 and 6 weeks after radiation exposure, intestinal loops were assessed for structural, cellular and molecular signs of radiation toxicity.

Results: In the early post-irradiation phase, hypertensive rats showed decreased collagen deposition, decreased intestinal wall thickness and a higher perforation rate. In the intermediate phase, the Radiation Injury Score (RIS) and the subserosal thickness were increased in SHR, possibly due to decreased Matrix MetalloProteinase (MMP) activity.

Conclusions: Hypertensive rats sustain more acute and intermediate term intestinal radiation injury than WKY control rats. Decreased MMP activity may play a role in the pathogenesis of delayed reactive fibrosis in hypertensive animals. These findings support the notion that uncontrolled hypertension may be risk factor for the development of both acute and delayed intestinal radiation injury.

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Introduction

Intestinal radiation injury is one of the most common complications during and after radiotherapy for tumours of the abdomen and pelvis. It is, therefore, one of the most important dose limiting factors. Radiation enteropathy occurring during or within 3 months after treatment is classified as early or acute radiation toxicity. It is characterized by disruption of the epithelial barrier and mucosal inflammation. Acute radiation injury may cause severe morbidity during treatment and may even require treatment interruption or alteration. Late intestinal toxicity refers to enteropathy that presents at least 3 months, but sometimes even as late as several years, after treatment. Its main characteristics are intestinal wall fibrosis and vascular sclerosis. Late radiation-induced intestinal injury can severely reduce quality of life in cancer survivors.

Various clinical studies have suggested that co-morbidities associated with vascular injury, such as smoking, diabetes and hypertension predispose to radiation-induced enteropathy [1-3]. However, several studies were not able to identify a history of hypertension as a risk factor for intestinal radiation injury [4-6]. While interpreting these results, it has to be kept in mind that a history of hypertension does not necessarily mean that patients indeed had high blood pressure during radiotherapy. Probably most of the patients were receiving antihypertensive drugs, of which some have been suggested to reduce intestinal radiation toxicity [7], and thus blood pressure was not at all or only marginally elevated. Hence, it is difficult to evaluate the role of high blood pressure in the development of radiation enteropathy using the results of these clinical studies.

Since it is unethical to study the effect of untreated uncontrolled hypertension on the development of intestinal radiation injury in a clinical setting, we used a rat model. Spontaneously Hypertensive Rats (SHR) are the most widely used animal model of human essential hypertension. The present study assessed the intestinal radiation response at 1, 2 and 6 weeks after irradiation in SHR and normotensive Wistar Kyoto rats (WKY). The results demonstrated that SHR sustain more severe radiation injury than normotensive controls. Decreased collagen deposition and intestinal wall thickness in the early phase after radiation exposure may have predisposed the SHR rats to the development of intestinal perforations. In the later phase, however, there were signs of increased fibrosis in the SHR. SHR may be prone to late fibrosis due to decreased Matrix Metalloproteinase (MMP) activity in this phase. These results support the hypothesis that hypertension may be a risk factor for the development of early as well as delayed intestinal radiation injury and emphasize the importance of controlling clinical hypertension in patients scheduled to undergo radiation therapy.

Materials and methods

Animals

A total of 48 WKY rats and 50 SHR (Harlan, Indianapolis, IN) were used for this experiment. At the start of the experiment animals had a body weight of 175-200 gram. Animals were individually housed in conventional cages under standardized conditions with controlled temperature and humidity and a 12-12 hour day-night light cycle. Animals had had free access to tap water and rat chow (Formulab Chow 5008, Purina Mills, St. Louis, MO).

The experimental protocol was reviewed and approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee.

Blood pressure monitoring

To confirm the presence of hypertension in SHR, blood pressure was monitored for a period of 24 hours in unrestrained conscious rats using telemetry as described previously [8]. Rats used to confirm the hypertension model did not undergo surgery or radiation exposure.

Experimental radiation enteropathy model

A previously described surgical model for fractionated localized irradiation of the small intestine was used [9, 10] . Briefly rats were anesthetized and orchiectomized. A 4-cm loop of ileum, located approximately 10-15 cm from the ileocecal junction, was fixed into the left part of the scrotum. This procedure creates an artificial “scrotal hernia” containing a segment of small intestine which can be irradiated locally. Since the bowel loop technically remains in the abdominal cavity, this procedure does not induce any structural of functional intestinal changes.

In order to distinguish potential surgery-induced differences from radiation-induced differences, rats were randomly selected to be irradiated or to serve as unirradiated controls.

After a 4 week postoperative recovery period, the bowel loops in the “scrotal hernias” of the rats in the radiation group received localized, fractionated irradiation. The rats were irradiated with fraction of 4.8 Gy for 9 consecutive days (9 x 4.8 Gy) to a total dose of 43.2 Gy. We opted for this fractionated radiation scheme with an EQD2 (α/β10) of 53.3Gy and an EQD2 (α/β3) of 67.4Gy as with this schedule induction of acute and delayed intestinal injury was expected to be similar as after abdominal irradiation in a clinical setting. Radiation was performed with a Seifert Isovolt 320 X-ray machine (Seifert, X ray Corporation, Fairview Village, PA), which was operated at 250 kVp and 15 mA with 3mm Al added filtration (half-value layer 0.85 mm Cu, dose rate 4.49 Gy/min). Dosimetric considerations have been described elsewhere [9].

Rats were euthanized 1, 2 or 6 weeks after the last day of irradiation. Unirradiated rats were euthanized at the same dates. The first 2 time points are representative for acute radiation injury, the last time point for intermediate term/delayed radiation injury. After opening of the scrotal hernia the intestinal loop was macroscopically assessed for the presence of intestinal perforations. Irradiated and unirradiated intestinal samples were obtained from each rat and fixed in methanol-Carnoy’s solution (methanol: choloroform: glacial acetic acid, 6:3:1) for histological and immunohistochemical studies, or snap frozen in liquid nitrogen and stored at -80°C for enzyme activity studies. Rats were euthanized prematurely if they showed signs of severe discomfort and weight loss and were not expected to live more than one day.

Quantitative histopathology and morphometry

Radiation Injury Score. The severity of radiation injury was assessed in H&E stained sections using the Radiation Injury Score (RIS) system [10,11]. Seven histopathologic parameters of radiation injury (mucosal ulcerations, epithelial atypia, thickening of subserosa, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion) were assessed and graded according to severity from 0 to 3. The sum of the scores for the individual alterations constitutes the RIS. This scoring system has been extensively used and validated in our laboratory. All specimens were evaluated in a blinded fashion by two

separate researchers.

Mucosal surface area. Intestinal mucosal surface area is a well validated, sensitive parameter of intestinal radiation injury[12] . Mucosal surface area was measured in vertical sections using a projection/cycloid method as described by Baddeley et al [13].

Thickness of intestinal wall. Early (Reactive) and delayed intestinal fibrosis are reflected by increased intestinal subserosal thickness and increased intestinal wall thickness. Subserosal thickening is a more specific marker of reactive fibrosis than intestinal wall thickness. Wall thickness is a measure of both reactive fibrosis and intestinal smooth muscle cell hyperplasia. Wall thickness (submucosa, muscularis externa, and subserosa) and subserosal thickness were measured with an eyepiece linear microruler. Five measurements, 500 µm apart, were obtained per specimen. The mean wall thickness of each sample was used as a single value for statistical analysis.

Immunohistochemistry

Immunohistochemical staining for collagen I and collagen III was performed using the standard avidin-biotin complex technique as described previously [14,15] . Quantitative assessment of immunoreactivity was performed using computerized image analysis (Image-Pro Plus, Media Cybernetics, Silver Substance Pring, MD) as described in detail and validated previously. Areas positive for the immunoreactivity of extracellular matrix-associated collagen I and collagen III were measured in 20 fields (40X objective) per section.

Matrix metalloproteinase activity assay

To measure gelatinase and collagenase activity in intestinal tissue samples the Molecular Probes EnzCheck Gelatinase/Collagenase Assay Kit (Life technologies, Carlsbad, CA) was used. Tissue homogenate of each sample was used in separate assays to determine gelatinase and collagenase activity. DQ- gelatin, degradable by all MMPs, or DQ-collagen I, degradable by MMP-1, 8, and 13, were used as substrates. The assay was performed according to the manufacturer's instructions. Black 96 well plates were loaded with a 200 µl reaction volume containing 25 µl 20% w/v tissue homogenate, 100 µl of either DQ-gelatin (25 µ/ml) or DQ-collagen I (µg/ml) dissolved in 1x reaction buffer and 75 µl 1x reaction buffer. The reaction was allowed to proceed for 24 hours at 18°C. Fluorescence was measured after 24 hours at 495/515 nm using a Synergy HT plate reader (Bio-Tek, Winooski, VT). Gelatinase and collagenase activity was quantified using a collagenase standard curve (0-10 U/ml).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 17.0 (IBM, Armonk, NY). Data are presented as the mean ± Standard Error of the Mean (SEM). Non-parametric independent –samples Mann-Whitney U Tests were used for all outcome parameters except for perforation rates, and differences were considered statistically significant when the p-value was less than 0.05. Chi-square statistics were used to analyse the perforation rates. Analyses were performed on samples from animals that survived until the planned date of euthanization. Since more than half of the SHR did not survive until the planned euthanasia date at 6 weeks, a second analysis was performed including the rats that had to be euthanized between week 2 and week 6.

Results

Blood pressure

We confirmed that SHR have much higher blood pressure than WKY rats (Figure 1). SHR rats showed significantly higher diastolic as well as systolic blood pressure compared to WKY rats (mean 188/137mmHg v. 132/95 mmHg).

Symptoms

In the early as well as in the intermediate phase, symptomatically, SHR seemed to be more affected by localized intestinal irradiation than WKY rat. Many of the SHR showed an enlarged, swollen scrotum. Of the 11 SHR that were scheduled to be euthanized 6 weeks after radiation exposure, 7 had to be euthanized before the end of the experiment because of their worsening clinical condition. In the WKY group only 2 rats had to be euthanized prematurely. Rats were euthanized prematurely if they showed signs of severe discomfort and weight loss and were not expected to live more than one day. Unirradiated animals did not show the above mentioned symptoms.

Intestinal perforations

Unirradiated animals did not develop perforations. In the early phase after irradiation, significantly more SHR developed intestinal perforations than WKY rats (Table 1). One week after irradiation none of the control rats had developed a perforation, whereas 88% of the SHR had at least one intestinal perforation (p=0.002). At two weeks post-irradiation 33% of the WKY rats versus 83 % of the SHR had developed at least one perforation (p=0.08). No perforations were found in animals that were euthanized 6 weeks after radiation exposure. However, when we also include animals that were planned to be euthanized at 6 weeks, but were actually euthanized at an earlier time point because of their worsening condition, more perforations are seen in the SHR group (40% v. 0%). However, the difference was not statistically significant (p=0.09).

Histopathology and morphometry

Consistent with the increased perforation rate, there was a significant difference in wall thickness at one week after irradiation (Figure 2). SHR showed decreased wall thickness compared to the control rats (p=0.01). Furthermore there was a borderline statistically significant decrease in subserosal thickness at one week (p=0.06).

After 6 weeks, however, increased subserosal thickness (p=0.01) and a trend towards increased wall thickness (p=0.08) was found in SHR. Moreover, after 6 weeks SHR showed higher RIS than WKY rats (p=0.01) (Figure 3). No significant differences in mucosal surface area were observed. No differences in wall thickness, subserosal thickness, mucosal surface area or radiation injury score were observed in the unirradiated groups.

Collagen I and III

Post-irradiation immunoreactivity levels of collagen I were decreased in the SHR group at one and two weeks (p = 0.03 and p = 0.002, respectively) (Figure 4). Collagen III immunoreactivity levels showed a similar pattern, however, the decrease was only significant at one week after irradiation (p = 0.03) (Figure 4 and 5). At 6 weeks after irradiation no significant differences were found in intestinal collagen I and collagen III staining. A slightly higher collagen III deposition at 1 week (mean ± SEM 60±4 v.

48±3 x10^6 pixel units/µm^2, p=0.03) and collagen I deposition at 6 weeks (mean±SEM 211±5 v. 189±6 x10^6 pixel units/µm^2, p=0.03) was observed in the unirradiated WKY group compared to the SHR group.

Matrix metalloproteinase activity

When analyzing the intestinal samples of animals that survived until the 6 week euthanasia date, a trend towards decreased gelatinase and collagenase activity the SHR group was observed (p = 0.1 and p=0.1, respectively). This trend became highly significant (p= 0.002 and p = 0.002, respectively) when the analysis was performed on the samples of all animals that were planned to be euthanized at week 6, thus including the rats that had to be euthanized prematurely (Figure 5). At the earlier time points, no differences were observed.

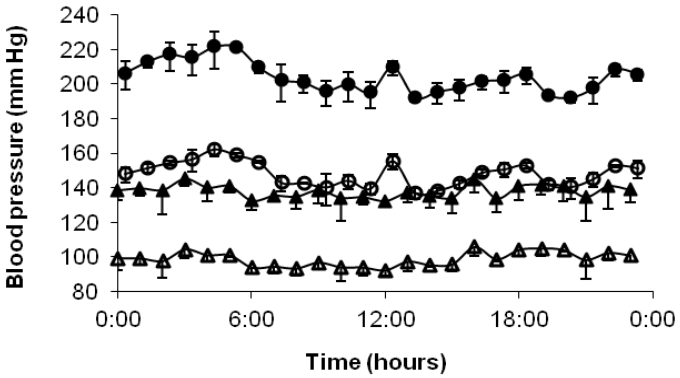

Figure 1: SHR have a higher blood pressure that WKY rats.
Mean hourly values for diastolic (open symbols) and systolic (closed symbols) blood pressure in male Spontaneously Hypertensive Rats (SHR) (n=2) and male Wistar Kyoto (WKY) rats (n=3).
Mean ± SEM.

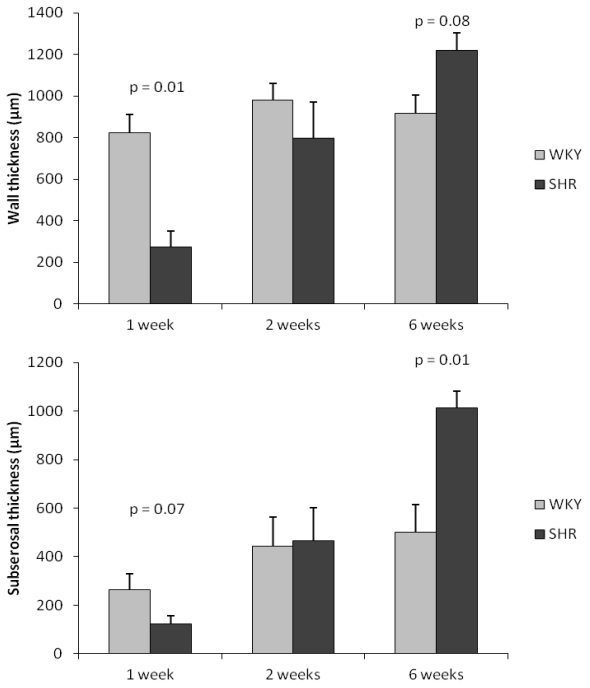

Figure 2: In the early post-irradiation phase SHR show decreased wall thickness compared to WKY. In contrast, after 6 weeks, subserosal thickness is increased in SHR compared to WKY, Intestinal wall thickness (A) and subserosal thickness (B) in irradiated intestine from WKY (filled bars) and SHR (open bars) rats at 1, 2 and 6 weeks after irradiation.
Mean ± SEM.

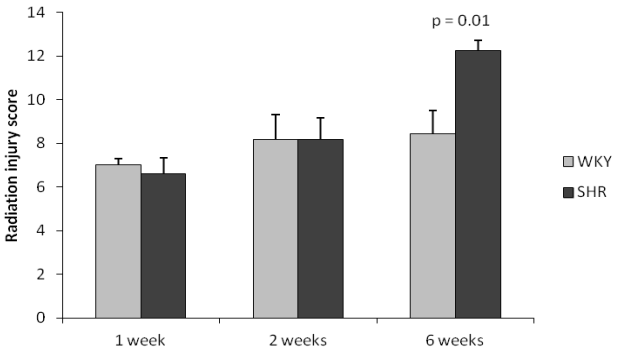

Figure 3: SHR show a higher Intestinal radiation injury score (RIS) 6 weeks after radiation exposure.
RIS in WKY rats (filled bars) and SHR (open bars) at 1, 2, and 6 weeks after irradiation.
Mean ± SEM.

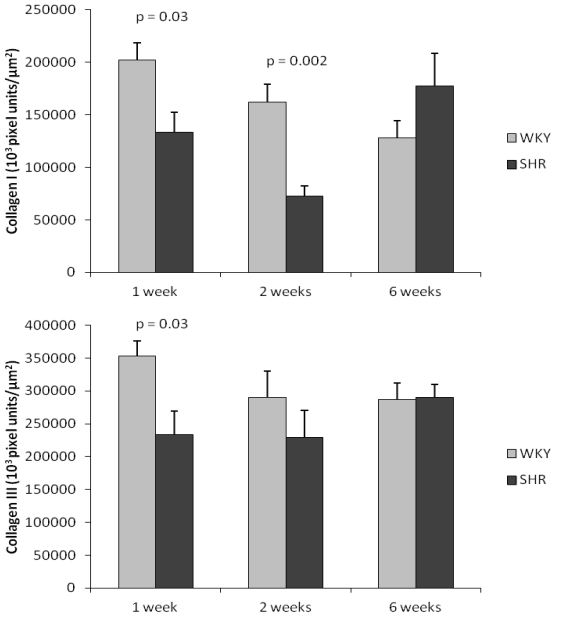

Figure 4: Decreased collagen III in the early post-irradiation phase in SHR compared to WKY.
Collagen immunoreactivity in irradiated intestine from WKY (filled bars) and SHR (open bars) rats at 1, 2 and 6 weeks after irradiation. Computerized image analysis was used to determine (A) collagen I and (B) collagen III immunoreactivity.
Mean ± SEM.

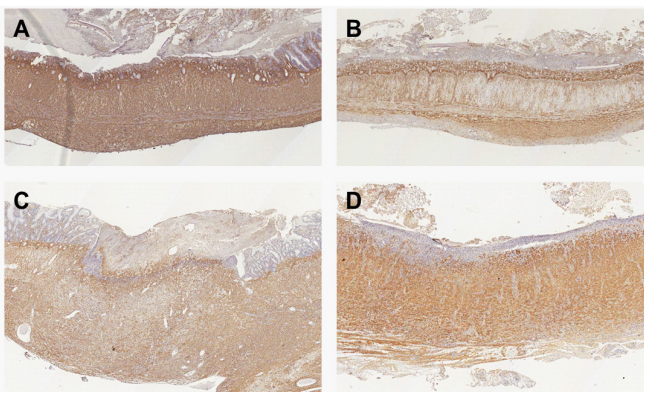

Figure 5: Immunohistochemical evidence for decreased collagen III presence in SHR at 1 weeks after radiation exposure.
Collagen III staining in intestinal specimens form WKY and SHR at 1 week after irradiation (A and B, respectively) and 6 weeks after irradiation (C and D, respectively).

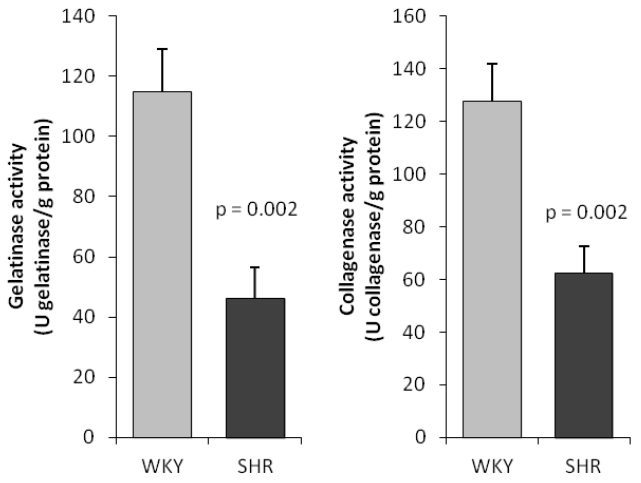

Figure 6: Decreased collagenase and gelatinase activity in SHR compared to WKY at 6 weeks post-irradiation.
Collagenase (A) and gelatinase (B) activity (U/ml) in WKY rats (filled bars) and SHR (open bars) planned to be euthanized at 6 weeks after radiation exposure, thus including animals that had to be euthanized prematurely.
Mean ± SEM

Table 1: In the early post-irradiation phase SHR show a higher rate of intestinal perforations after radiation exposure than WKY.

	WKY	SHR
1 Weeks	0 (0%)	7 (88%)
2 Weeks	2 (33%)	5 (83%)
6 Weeks	0 (0%)	2 (0%)

Discussion

To assess whether uncontrolled high blood pressure predisposes to the development of radiation enteropathy, we compared intestinal radiation injury after localized irradiation of the gut between hypertensive rats, SHR, and normotensive WKY rats. Hypertensive rats exhibited more severe acute and intermediate term radiation toxicity.

The male SHR rat model is one of the most commonly used hypertension models [16]. SHR are genetically predisposed to the development of hypertension. Abnormal gene expression of components in the renin-angiotensin system appears to be of particular importance in this respect. It had been shown that the genetic predisposition and environmental factor induce hypertension in SHR by shifting the balance between endothelial production of nitric oxide (NO) and Reactive Oxygen Species (ROS) more towards ROS synthesis [17, 18]. Decreased NO availability and increased superoxide production is also seen in human subjects with hypertension [19]. The vasculature of SHR shows similar structural and morphological characteristics as the vasculature of human hypertensive subjects, such as increased deposition of extracellular matrix in resistance vessels, caused by altered activity of Matrix Metalloproteinases (MMPs) [20].

With this study we show that SHR rats are more susceptible to the development of early and intermediate term radiation enteropathy. Since many of the structural vascular changes in SHR and human hypertensive subjects depend on altered MMP activity and increased MMP activity is a well-recognized modulator of intestinal injury [21-23], we hypothesized that the decreased intestinal wall thickness and collagen I and collagen III deposition that were seen in the early post-radiation phase in SHR were caused by increased MMP activity. However, we did not find a difference in gelatinase or collagenase activity in the early post-irradiation phase. In contrast, there seemed to be decreased MMP activity in the late post-irradiation phase in SHR. These results could explain why SHR are more prone to the development of delayed reactive intestinal fibrosis. Moreover, as decreased MMP activity may contribute to vascular dysfunction and thereby aggravate radiation injury. This needs further investigation.

The mechanisms underlying the increased acute radiation injury in the SHR group remain to be elucidated. Other groups have suggested that SHR rats may develop more intestinal radiation injury, because of increased apoptosis in jejunal crypts due to higher sympathetic activity [24]. It is, however, questionable whether the results of these experiments are also valid for our study. The experiments were done with a single low dose (0.5-2 Gy) of total body radiation instead of localized fractionated intestinal radiation with a dose more comparable to doses used during radiation therapy. In our experiments we did not find a difference in mucosal surface area between the SHR and the WKY group.

Microvascular changes and endothelial dysfunction have been shown to play an important role in the development of intestinal radiation injury [25]. Hence, future experiments are needed to determine the role of microvascular dysfunction and the pre-existent NO/ROS imbalance in hypertensive tissue in the development of radiation injury. It can be hypothesized that hypertensive tissue has decreased buffer capacity to cope with the radiation induced oxidative stress. In SHR endothelial function can be improved by supplementing the Nitric Oxide Synthase (NOS) co-factor tetrahydriobiopterin [26, 27], indicating that there are inadequate supplies of tetrahydriobiopterin in SHR rats. Tetrahydriobiopterin may also play an important role in the prevention of radiation injury[28]. It may function as a radioprotector by preserving endothelial NOS (eNOS) activity and preventing eNOS uncoupling that leads to increased oxidative stress. Therefore, inadequate tetrahydriobiopterin supplies may predispose SHR rats to the development of radiation injury.

A potential limitation of our study is the fact that with the used model we can't distinguish whether the observed differences in radiation response are mainly dependent on the presence of high blood pressure itself or on genetic differences between SHR and WKY causing high blood pressure. Further research is needed to answer this question and to investigate whether blood pressure reducing therapies ameliorate radiation injury.

Conclusions

In summary, compared to normotensive rats, hypertensive rats develop more intestinal radiation injury both in the early and the intermediate phase after localized irradiation of the intestine. Decreased MMP activity may play a role in the pathogenesis of delayed reactive fibrosis in hypertensive animals. These results support the hypothesis that high blood pressure at the time of irradiation may be risk factor for the development of radiation injury and point to the need for further mechanistic studies.

Acknowledgements

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