Diagnostic Imaging[1], Department for Companion Animals and Horses, University of Veterinary Medicine, Vienna, Austria; Applied and Translational Radiobiology (ATRAB)[2], Department of Radiation Oncology, Medical University of Vienna, Vienna, Austria; Platform Radiooncology and Nuclear Medicine[3], Department for Companion Animals and Horses, University of Veterinary Medicine of Vienna, Vienna, Austria

Effects of thalidomide on urothelial radiation-induced alterations of reactive oxygen species, iNOS and endothelial PECAM-1

J. Raiber[1,2]*, M. Kleiter[3a], J. Kowaliuk[2b], E. Ludewig[1], C. Rowan[1c], S. Sarshahi[2] and W. Dörr[2d]

ORCID:
a) 0000-0002-1168-3973
b) 0000-0002-8796-4309
c) 0000-0003-2573-1734
d) 0000-0003-1477-6849

Received February 7, 2022
Accepted October 5, 2022
Published October 27, 2022

Keywords: malondialdehyde, NF-κB, irradiation, urinary bladder.

Schlüsselwörter: Malondialdehyd, NF-κB, Bestrahlung, Harnblase.

Summary

We have previously demonstrated in a mouse model that single dose irradiation induces a biphasic activation of nuclear factor-kappa B (NF-κB) and that the NF-κB inhibitor thalidomide can suppress this upregulation and improve functional bladder impairment. This follow-up study investigated the effect of thalidomide on irradiation-induced reactive oxygen (ROS) and induced NO-synthase (iNOS) and platelet-endothelial cell adhesion molecule 1 (PECAM-1) expression during the early radiation response in the same mice.

We used immunohistochemistry to investigate the formation of malondialdehyde as a ROS biomarker with iNOS expression in the urothelium of formalin-fixed paraffin-embedded bladder specimens. We also analysed the expression of PECAM-1 in endothelial cells of the *lamina propria*. The irradi-

Zusammenfassung

Wirkungen von Thalidomid auf strahlungsinduzierte Änderungen von reaktiven Sauerstoffspezies, iNOS und endothelialem PECAM-1 im Urothel

Einleitung

In unserer vorausgegangenen Studie in einem Mausmodell wurde nachgewiesen, dass Einzeldosisbestrahlung den Transkriptionsfaktor NF-κB im Urothel der Harnblase biphasisch aktiviert und dwie Verabreichung des NF-κB Inhibitors Thalidomid diese Aktivierung unterdrückt und damit die frühe strahleninduzierte Zystitis lindert. Ziel dieser Folgestudie in denselben Mäusen war es, die Wirkung von Thalidomid auf reaktive Sauerstoffspezies (ROS), induced NO-Synthase (iNOS) und platelet-endothelial cell adhesion molecule 1 (PECAM-1) in der frühen Phase der strahleninduzierten Zystitis nach einmaliger Bestrahlung zu untersuchen.

Material und Methoden

Die Bildung von Malondialdehyd als Biomarker für ROS wurde immunhistochemisch gemeinsam mit der urothelialen Expression von iNOS in formalinfixierten und Paraffin-eingebetteten Blasenproben bestimmt. Zusätzlich wurde die Expression von PECAM-1 in den Endothelzellen der *Lamina propria* evaluiert. Zuerst wurde der Effekt einer Einzeldosisbestrahlung am Tag 0 mit 23 Gy untersucht und im Anschluss wurde die modulierende Wirkung einer Thalidomidbehandlung von Tag 1–15 auf alle untersuchten Marker erfasst.

Ergebnisse

Einzeldosisbestrahlung führte zu einer vermehrten Bildung von Malondialdehyd in der ersten Phase der frühen Strahlenreaktion der Harnblase. Die Behandlung mit

*E-Mail: jutta.raiber@gmx.de

ation-induced effects on ROS generation, iNOS, and PECAM-1 expression post-single-dose irradiation of 23 Gy on day 0 were evaluated and their modulation by thalidomide treatment from day 1–15 assessed.

Irradiation induced an increased formation of malondialdehyde in the first phase of the early radiation response. Thalidomide administration had no significant effect on this biomarker formation. Irradiation resulted in a biphasic increase of iNOS and PECAM-1 expression. Thalidomide treatment significantly suppressed iNOS expression, shifted PECAM-1 expression to an earlier peak and provoked an extended staining fraction in the second phase of the early radiation response.

We conclude that iNOS suppression contributes to the established therapeutic effect of the NF-κB inhibitor thalidomide, improving bladder dysfunction and increasing radiation tolerance. We suggest that PECAM-1 contributes to the therapeutic effect by cross signaling with the adherens junction protein β catenin. The ameliorating effect of thalidomide does not seem to stem from substantially altering ROS generation.

Thalidomid hatte keinen signifikanten Effekt auf diesen ROS Biomarker. Die Bestrahlung führte zu einer biphasischen Erhöhung der iNOS und PECAM-1 Expression. Thalidomid konnte die erhöhte iNOS Expression signifikant unterdrücken und bewirkte weiters eine frühe Aktivierung der PECAM-1 Expression und einen erhöhten Prozentsatz PECAM-1 positiver Endothelzellen am Ende der Frühphase.

Schlussfolgerung

Die Unterdrückung von iNOS durch den NF-κB Inhibitor Thalidomid trägt zum bereits nachgewiesenen therapeutischen Effekt von Thalidomid auf die frühe strahleninduzierte Zystitis mit verbesserter Blasenfunktion und erhöhter Strahlentoleranz bei. Weiters schließen wir aus unseren Ergebnissen, dass PECAM-1 an der lindernden Wirkung von Thalidomid durch eine Interaktion mit dem Adherens Junction Protein β Catenin beteiligt ist. Thalidomid konnte die Bildung von Malondialdehyd nicht effektiv unterdrücken und eine Modulation von ROS scheint beim therapeutischen Effekt von Thalidomid keine Rolle zu spielen.

Abbreviations: AJ = adherens junctions; DMSO = dimethyl sulfoxide; EC = endothelial cells; ERC = early radiation cystitis; FFPE = formalin-fixed paraffin-embedded tissue; Gy = Gray; IHC = immunohistochemistry, immunohistochemical analysis; iNOS = induced NO-synthase; μm = micrometer; NF-κB = factor kappa light chain enhancer of activated B cells; PECAM-1 = platelet-endothelial cell adhesion molecule 1; RNS = reactive nitrogen species; ROS = reactive oxygen species

Introduction

Pelvic solid tumours - such as rectal, endometrial, prostate and bladder tumours - are commonly treated with radiotherapy. The urinary bladder is a critical organ at risk of radiation-induced acute and chronic side effects (Jaal & Dörr 2006a). Irradiation of the bladder induces the generation of reactive species, an inflammatory response (Kawamura et al. 2018) and a depletion of superficial umbrella cells (Jaal & Dörr 2006a), which has been suggested to result in a leaking urothelium (Hu et al. 2000, 2002; Jaal & Dörr 2006b).

Reactive species can be subdivided into reactive oxygen species (ROS) and reactive nitrogen species (RNS) and result in oxidative and nitrosative stress (Jones 2008). As ROS have short half-lives and high responsiveness, direct analysis is not possible and stable degradation products are used as biomarkers (Dröge 2002). Radiation-induced generation of ROS activates NF-κB, which can further contribute to the development of oxidative-nitrosative stress (Livolsi et al. 2001). NF-κB translocates to the nucleus and activates proinflammatory genes that produce cytokines, chemokines and cell adhesion molecules such as platelet-endothelial cell adhesion molecule 1 (PECAM-1). PECAM-1 is expressed on endothelial cells, platelets and leucocytes and enables leukocyte immigration into the tissue (Vollmar et al. 2013).

Early radiation injury results in early radiation cystitis (ERC) and clinical signs in humans typically start during treatment and resolve several weeks after radiotherapy (Joiner 2009). Patients suffer from an inflammatory tissue reaction with clinical symptoms such as increased micturition frequency and urgency, dysuria, nycturia and haematuria. The only option to alleviate the clinical side effects is the reduction of the radiation dose, which leads to lower tumour control and survival rates (Mallick et al. 2015). Identifying substances able to ameliorate radiation side effects without lowering radiation dose would substantially improve cancer treatment.

Previous work suggests that the sedative, tranquilizer and antiemetic drug thalidomide has potential for use in this regard (Franks et al. 2004). Thalidomide is an inhibitor of NF-κB, a key proinflammatory signaling molecule in the ERC (Kowaliuk et al. 2020). We have shown that single dose irradiation in a mouse model induces a biphasic NF-κB activation in the urothelium of the urinary bladder. Treatment with thalidomide inhibits NF-κB activation in ERC, with a decrease in the fraction of early responding animals and with increased radiation tolerance (Kowaliuk et al. 2020).

In this follow-up study, on the same bladder specimens, we investigated whether thalidomide modulates the irradiation-induced oxidative stress biomarker malondialdehyde as well as induced NO-synthase (iNOS) and PECAM-1 and whether the modulation contributes to the known improvement of bladder dysfunction.

Material and methods

Animals and housing

We used FFPE-fixed and archived bladder specimens that we collected during our initial study as described (Kowaliuk et al. 2020). In the initial study, female C3H/Neu mice were group-housed under pathogen-free conditions on wood bedding at a controlled temperature (22 ± 2°C) and a 12/12 h light-dark cycle. Fresh water and mouse diet (sniff Spezialdiäten GmbH, Soest, Germany) were provided *ad libitum*. The experiments were performed following the current animal welfare legislation with the approval of the authorities (Federal Ministry of Education, Science and Research, ref. number BMWF-66.009/0148-WF/II/3b/2014).

Irradiation

The pelvic irradiation in the initial study has been described (Dörr & Beck-Bornholdt 1999; Kowaliuk et al. 2020). Percutaneous local X-ray irradiation was performed with a YXLON MG325 X-ray device (YXLON International GmbH, Hamburg, Germany) operating at 200 kV and 20 mA. Animals were positioned in dorsal recumbency under general anaesthesia (pentobarbitone-sodium, 60 mg/kg intraperitoneal). The bladder was emptied by a transurethral catheter and irradiated with a single dose of 23 Gy using a field size of 0.9 cm². This dose causes a functional bladder impairment in 90 % of irradiated animals (Kowaliuk et al. 2020; Krischak et al. 2021).

Experimental design

Archived bladder specimens from two control groups (non-irradiated and irradiated control) and an early thalidomide treatment group (day 1–15 post-irradiation) were used. The group design has been described in our previous studies. Only bladder specimens from the early thalidomide treatment group were assessed for the present study as our previous work has shown the strongest treatment effects when thalidomide is given in this period (Kowaliuk et al. 2020; Krischak et al. 2021). The non-irradiated control group received neither irradiation nor thalidomide. Five animals were included and bladder specimens evaluated from days 1, 6, 18, 24 and 30 post-irradiation. The irradiated control group was irradiated on day 0 without further thalidomide treatment. In the early thalidomide treatment group, irradiation was performed on day 0 and thalidomide was administered intraperitoneally (100 mg/kg, dissolved in 80 % dimethyl sulfoxide, DMSO) from day 1–15 post-irradiation. In the irradiated control and the thalidomide treatment group, bladder specimens from 5 animals per day were collected from days 1–30 (n=150 for each group) (Table 1).

Tab. 1: Experimental design / Versuchsdesign

Treatment groups	Irradiation	Thalidomide	Number of samples d1–d30
Non-irradiated control	none	none	5
Irradiation control	d0	none	150*
Treatment	d0	daily d0–d15	150*

d = day; * = 5 samples per day, 5 mice per day and group / d = Tag; * = 5 Proben pro Tag, 5 Mäuse pro Tag und Gruppe

Immunohistochemical staining and analysis

We have described the technique of bladder collection and fixation (Kowaliuk et al. 2020; Krischak et al. 2021). Perfusion and immersion paraformaldehyde fixation was used to allow rapid tissue fixation (Roti-Histofix 4 % acid-free, pH 7, Roth, Germany). After 48 hours of fixation at room temperature, samples were halved, embedded in paraffin and stored at 4 °C.

For this study, tissue sections of 5 μm were cut by a rotary microtome (Leica Biosystems, Germany), placed on adhesive microscope slides and fixed in an incubator (BD115, Fa. Binder GmbH, Tuttlingen, Germany) for 12 hours at 37 °C. To prepare tissue sections for standard immunohistochemical analysis (IHC), slides were deparaffinized and rehydrated. For antigen retrieval, all samples were boiled in sodium citrate buffer (pH 6) in the microwave for 20 minutes at 600 Watt. To reduce background staining, slides were further incubated with 3 % H_2O_2 for 10 minutes and then placed in sample blocking solution (1 ml TBS, 15 μl Goat Normal Serum) for 60 minutes.

The following primary antibodies (Ab) were used for immunohistochemical analysis: a polyclonal goat Ab (BioRad AHP797) to detect malondialdehyde-protein adducts, a polyclonal rabbit Ab (ab15323) to detect iNOS (Ye et al. 2014) and polyclonal rabbit (Proteintech11265) for PECAM-1 detection (Yao et al. 2019). Preliminary staining was performed to establish the optimal antibody concentration for IHC analysis. The final primary antibody dilutions were 1:750 for malondialdehyde, 1:200 for iNOS and 1:150 for PECAM-1. Negative controls omitting the primary Ab were performed to exclude non-specific binding of the secondary Ab.

Primary antibodies were applied overnight at 4 °C with the determined Ab-specific dilution, then slides were incubated at room temperature with the secondary antibody for 60 minutes, ABC rabbit kit (Vector Laboratories, Burlingame, CA) solution for 30 minutes and DAB (3,3`-Diaminobenzidine, Vector Laboratories) for 10 seconds. Finally, all slides (manual or automated) were stained with haematoxylin 50 for 3 minutes and fixed with Entellan (Entellan®new, Merck KGaA, Darmstadt, Germany).

Immunohistochemical analysis was performed with a light microscope (Motic B3 Professional Series,

Deutschland GmbH, Wetzlar, Germany). A semiquantitative, arbitrary scoring system from 0–3 with 0.5 increments was used to rate the staining intensity of urothelial and endothelial cells. Three randomly chosen optical fields were scored at 400x magnification by the same observer (JR). Score 1 was defined as a weak cytoplasmic staining intensity (Zhang et al. 2017), score 2 as moderate and score 3 as strong staining intensity. When the staining intensity was deemed to be between categories, 0.5 increments were made. For PECAM-1, the fraction of positive-staining endothelial cells in three-vessel sections was established in addition to the staining intensity using an optical grid (staining fraction).

Statistical analysis

The staining intensities in the treatment group were compared to those in the irradiation control group to assess the effect of thalidomide on malondialdehyde, nitrotyrosine, iNOS and PECAM-1. We used a two-way ANOVA and Tukey-HSD *post-hoc* test differences in staining intensity. A p-value lower than 0.05 was regarded as statistically significant. Data from 15 samples collected over three-day intervals (5 specimens per day) were used to assess a treatment effect over time while preserving sufficient statistical power to test hypotheses. Because of the death of some animals during the initial study (Kowaliuk et al. 2020), some 3-day periods included fewer than 15 bladder specimens. Statistical analysis was performed with STATA 15 and graphical formatting with R Version 4.0.4.

Results

All bladder specimens of the non-irradiated control group (n=5) showed an IHC staining intensity score of 1 for the assessed antigens at each time point, so there were detectable baseline levels of the oxidative-stress biomarker malondialdehyde and iNOS in the urothelium. We also observed constitutive PECAM-1 expression in endothelial cells of the *lamina propria* with a staining fraction of 20–30 %.

Due to the low number of samples in this group, no further statistical testing was performed. Changes in the staining intensity of all antigens could be visualized in the irradiated control group and the treatment group. A two-way ANOVA test and Tukey-HSD *post-hoc* test were performed to compare them.

Malondialdehyde-protein adducts

Effect of irradiation

Single-dose irradiation induced an early increase in the formation of malondialdehyde protein adducts starting at day 3 and reaching a peak around day 9. Staining intensity decreased until day 15 to about the same level as on day 3. Subsequently, only a slight decrease in staining intensity was observed until day 30 (Fig. 1).

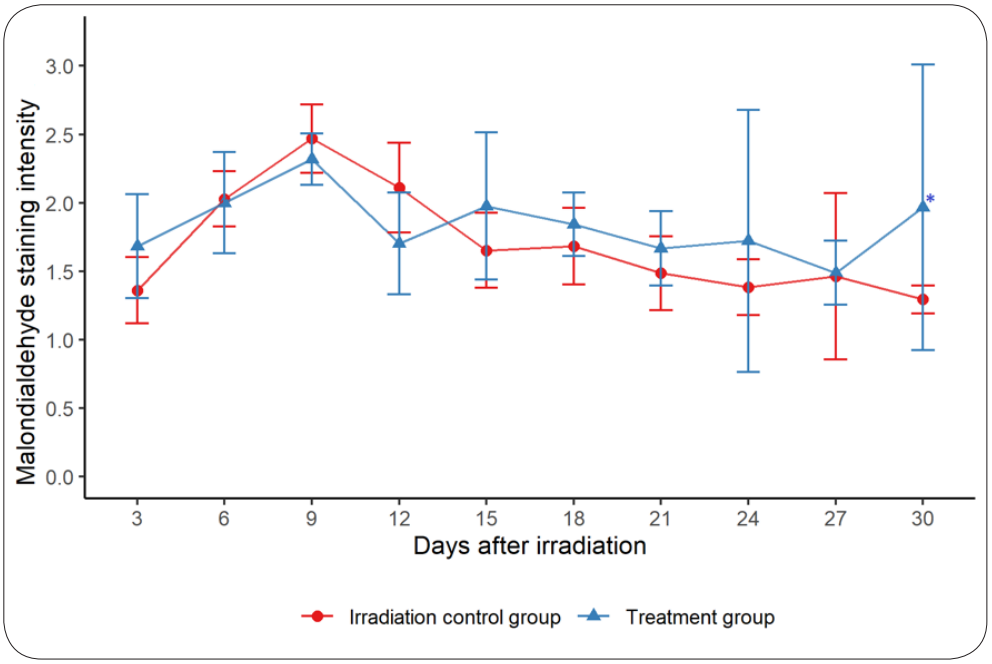

Fig. 1: Effect of irradiation on day 0 on the urothelial formation of malondialdehyde and its modulation by thalidomide from day 1 to 30 per 3-day period. Data points represent the average immunohistochemical staining intensity score from 15 samples (5 samples per day). Three representative fields of the urothelium were quantified by an arbitrary scoring system. Values are represented as means; bars indicate the 95% confidence interval. * shows p-value ≤ 0.05 of the two-way ANOVA and Tukey-HSD *post-hoc* test comparing the treatment groups with the irradiation control group. Two groups were examined: the irradiation control group (red) with single-dose irradiation of 23 Gy and the treatment group (blue) with single-dose irradiation and thalidomide administration from day 1 to 15. / Wirkung der Bestrahlung am Tag 0 auf das Vorkommen von Malondialdehyd im Urothel und seine Beeinflussung durch Thalidomid von Tag 1 bis 30 pro 3-Tages-Zeitraum. Die Datenpunkte stellen den durchschnittlichen immunhistochemischen Färbeintensitätsgrad aus 15 Proben (5 Proben pro Tag) dar. Drei repräsentative Felder des Urothels wurden durch ein objektives Scoring-System quantifiziert. Werte werden als Mittelwerte dargestellt; Balken zeigen das 95%-Konfidenzintervall an. * zeigt den p-Wert ≤ 0,05 der two-way ANOVA- und des Tukey-HSD *post-hoc* Tests, mit denen die Behandlungsgruppen mit der Bestrahlungskontrollgruppe verglichen werden. Zwei Gruppen wurden untersucht: die Bestrahlungskontrollgruppe (rot) mit Einzeldosisbestrahlung von 23 Gy und (2) die Behandlungsgruppe (blau) mit Einzeldosisbestrahlung und Thalidomidverabreichung von Tag 1 bis 15.

Effect of thalidomide administration

The treatment group exhibited a near-identical profile to that of the irradiation control group: an early increase on day 3 with a peak on day 9, followed by a subsequent decrease in staining intensity. After day 15, a mild reduction in the intensity score was seen until day 27. Apart from on day 30, where the statistical spread was high, there was no significant difference between the irradiation control group and the treatment group in any 3-day period (Fig. 1). For the non-significant effects, the p-values were between 0.13 and 0.91.

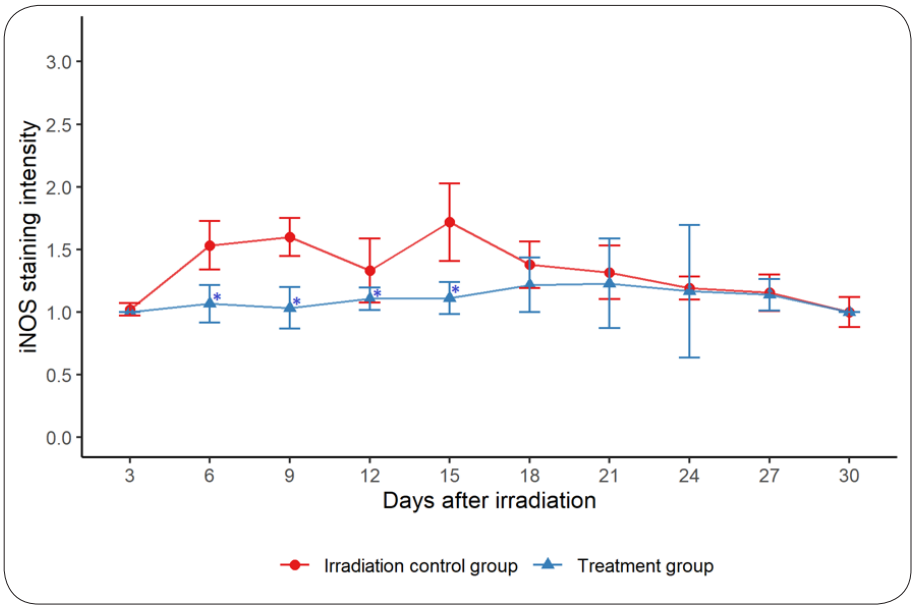

Fig. 2: Effect of irradiation on day 0 on urothelial iNOS level and its modulation by thalidomide from day 1 to 30 per 3-day period. Data points represent the average immunohistochemical staining intensity score from 15 samples (5 samples per day). Three representative fields of the urothelium were quantified by an arbitrary scoring system. Values are represented as means; bars indicate the 95% confidence interval. * shows p-value ≤ 0.05 of the two-way ANOVA and Tukey-HSD *post-hoc* test comparing the treatment groups with the irradiation control group. Two groups were examined: the irradiation control group (red) with single-dose irradiation of 23 Gy, (2) treatment group (blue) with single-dose irradiation and thalidomide administration from day 1 to 15. / Wirkung der Bestrahlung am Tag 0 auf iNOS im Urothel und seine Beeinflussung durch Thalidomid von Tag 1 bis 30 pro 3-Tages-Zeitraum. Die Datenpunkte stellen den durchschnittlichen immunhistochemischen Färbeintensitätsgrad aus 15 Proben (5 Proben pro Tag) dar. Drei repräsentative Felder des Urothels wurden durch ein objektives Scoring-System quantifiziert. Werte werden als Mittelwerte dargestellt; Balken zeigen das 95%-Konfidenzintervall an. * zeigt den p-Wert ≤ 0,05 der two-way ANOVA und des Tukey-HSD *post-hoc* Tests, mit denen die Behandlungsgruppen mit der Bestrahlungskontrollgruppe verglichen werden. Zwei Gruppen wurden untersucht: die Bestrahlungskontrollgruppe (rot) mit Einzeldosisbestrahlung von 23 Gy und (2) die Behandlungsgruppe (blau) mit Einzeldosisbestrahlung und Thalidomidverabreichung von Tag 1 bis 15.

Induced NO-Synthase

Effect of irradiation

Irradiation induced a biphasic increase in iNOS expression with a first expanded peak between day 6 to day 9 and a second peak on day 15. A short decline in iNOS expression was observed between the two peaks on day 12. After day 15, there was a steeper decline in staining intensity until day 18, followed by a slow constant decrease to baseline levels on day 30 (Fig. 2).

Effect of thalidomide administration

In contrast to the irradiation control group, the treatment group showed no peak in iNOS expression. The staining intensity remained low over the entire study period, with a significant difference to the irradiation control between day 6 and day 15. On day 18, the treatment group and the irradiation control group were approximately the same, showing parallel iNOS expression curves with no substantial difference until day 30 (Fig. 2). For the non-significant effects, the p-values were between 0.15 and 1.00.

PECAM-1

Effect of irradiation

Staining intensity of the endothelial cells

Irradiation induced a biphasic increase in PECAM-1 expression with a first peak on day 12, followed by a decrease to baseline levels on day 15. Subsequently, the staining intensity increased slowly, reaching a late second peak on day 2, and then decreasing until day 30. Before day 6, no rise in expression was detected (Fig. 3A).

Staining fraction of the endothelial cells

The staining fraction in the irradiation control group showed a comparable time curve to the staining intensity. The percentage of stained endothelial cells started to increase on day 3, with a steep rise after day 6, reaching a peak on day 12. The staining fraction then decreased to the baseline level on day 15 before increasing to the second peak on day 27. The percentage of stained endothelial cells then decreased again until day 30 (Fig. 3B).

Effect of thalidomide administration

Staining intensity of the endothelial cells

In the thalidomide treatment group, an early increase in PECAM-1 expression was seen on day 3, reaching a peak on day 6, followed by a decrease until day 12. The expression then remained at this low level until day 18 and there was a small second peak on day

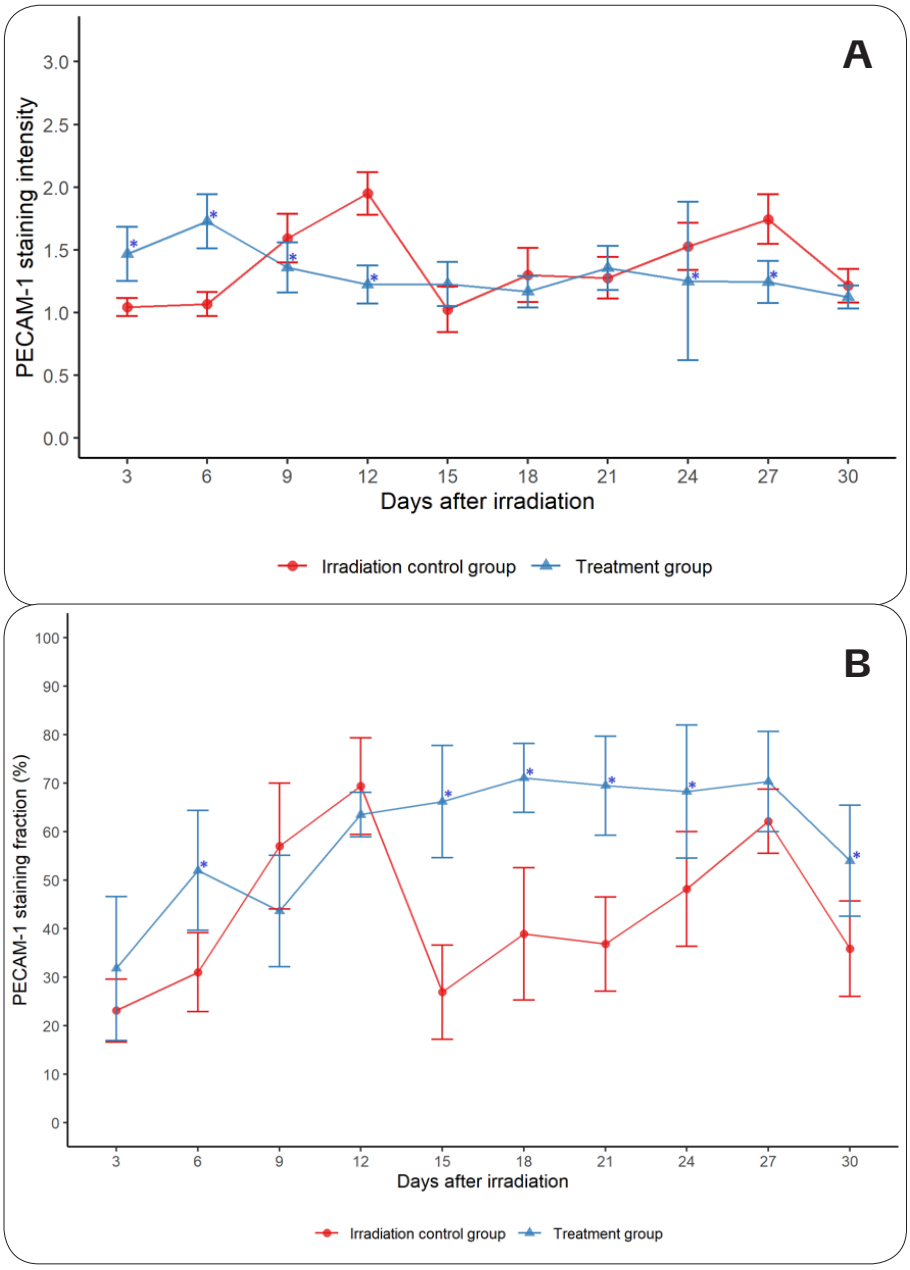

Fig. 3: Effect of irradiation on day 0 on endothelial PECAM-1 levels and its modulation by thalidomide from day 1 to 30 per 3-day period. Data points represent the average immunohistochemical staining intensity score (A) or the staining fraction (B) from 15 samples (5 samples per day). An arbitrary scoring system quantified three representative fields of the endothelium. The fraction of positively stained endothelial cells was established in three-vessel sections using an optical grid. Values are represented as means; bars indicate the 95% confidence interval. * shows p-value ≤ 0.05 of the two-way ANOVA and Tukey-HSD post-testing comparing the treatment groups with the irradiation control group. Two groups were examined: the irradiation control group (red) with single-dose irradiation of 23 Gy, (2) treatment group (blue) with single-dose irradiation and thalidomide administration from day 1 to 15. / Wirkung der Bestrahlung am Tag 0 auf das PECAM-1 Level im Urothel und seine Beeinflussung durch Thalidomid von Tag 1 bis 30 pro 3-Tages-Zeitraum. Die Datenpunkte stellen den durchschnittlichen immunhistochemischen Färbeintensitätsgrad (A) oder Anteil der gefärbten Zellen (B) aus 15 Proben (5 Proben pro Tag) dar. Drei repräsentative Felder des Endothels wurden durch ein objektives Scoring-System quantifiziert. Der Anteil der angefärbten endothelialen Zellen wurde durch ein optisches Raster ermittelt. Werte werden als Mittelwerte dargestellt; Balken zeigen das 95%-Konfidenzintervall an. * zeigt den p-Wert ≤ 0,05 der two-way ANOVA und des Tukey-HSD *post-hoc* Tests, mit denen (2) die Behandlungsgruppen mit der Bestrahlungskontrollgruppe verglichen werden. Zwei Gruppen wurden untersucht: die Bestrahlungskontrollgruppe (rot) mit Einzeldosisbestrahlung von 23 Gy, und die Behandlungsgruppe (blau) mit Einzeldosisbestrahlung und Thalidomidverabreichung von Tag 1 bis 15.

21 before the staining intensity decreased slightly until day 30. Significantly higher staining intensity was detected on days 3 and 6 and there was significantly lower staining intensity on days 9, 12, 24 and 27 compared to the irradiation control (Fig. 3A). For the non-significant effects, the p-values were between 0.08 and 0.46.

Staining fraction of endothelial cells

In the thalidomide treatment group, an early increase in the percentage of stained endothelial cells was found on day 3 and an early peak on day 6, followed by a decrease on day 9. The staining fraction then started to rise again and reached an extended plateau from day 15–27 before decreasing at the end of the study. In comparison to the irradiated control, a significantly higher staining fraction was detected at the first peak (day 6), during the plateau (day 15–24) and at the end of the study (day 30) (Fig. 3B). For the non-significant effects, the p-values were between 0.06 and 0.23.

Discussion

We investigated the effects of irradiation on the formation of malondialdehyde and the expression of iNOS and PECAM-1 during the ERC in an established mouse model before evaluating the modulating effects of thalidomide on these biomarkers.

Irradiation induces the generation of free radicals, reactive aldehydes and oxidative and nitrosative by-products. The generation of ROS/RNS is challenging to measure due to their short half-lives and high responsiveness (Dröge 2002; Koerdt et al. 2016).

Malondialdehyde is derived as a product of lipid peroxidation and serves as a biomarker for oxidative stress (Ayala et al. 2014). During lipid peroxidation, ROS -

mainly hydroxyl and hydroperoxyl radicals - react with free fatty acids to form malondialdehyde through activation of lipoxygenases, myeloperoxidases, cyclooxygenases and cytochrome P450 (Nikolic et al. 2007; Niki 2009) as well as by non-enzymatic mechanisms (Papac-Milicevic et al. 2016). Malondialdehyde forms adducts with cellular proteins and either free malondialdehyde or protein adducts can be used as ROS biomarkers (Tsikas 2017). In the present study, single-dose irradiation induced a steep peak of malondialdehyde formation in the first early radiation response phase, which was characterized by a biphasic pattern. The first phase lasts from day 1–15 and the second phase from day 16–30 (Jaal & Dörr 2006a). Enhanced ROS levels activate multiple inflammatory signalling pathways, leading to increased expression of NF-kB (Pooladanda et al. 2019; Akimov et al. 2020). Treatment with the NF-kB inhibitor thalidomide during the first phase of the early radiation response (day 1–15) had no significant effect on malondialdehyde protein adducts. However, other authors have shown that ROS levels or free malondialdehyde could be effectively reduced by NF-kB inhibition (Pooladanda et al. 2019; Akimov & Kostenko 2020). In these studies, ROS was not irradiation induced (endotoxin-induced, fluoride intoxication). It appears that a single high dose of radiation, as we used, provokes the generation of a large amount of ROS and that thalidomide cannot significantly alter this ROS production or further lipid peroxidation.

Ionizing radiation also activates the inducible isoform of nitric oxide synthase (iNOS). Nitric oxide synthases (NOS) exist in various isoforms and catalyse nitric oxide synthesis from L-arginine. The inducible form is mainly involved in inflammatory diseases (Speyer et al. 2003) and its activation results in the hyperproduction of nitric oxide (Mikkelsen & Wardman 2003; Ahlatci et al. 2014). Nitric oxide is an oxygen free radical and can diffuse into other cells and activate guanyl cyclase and cyclic guanosine monophosphate (cGMP), which targets a variety of downstream effector molecules (Vaupel et al. 2015). Nitric oxide hyperproduction is a known nitrosative stress inducer that results in increased concentrations of its later metabolites (Pooladanda et al. 2019; Akimov et al. 2020). One important nitrogen species is peroxynitrite, the product of the reaction of nitric oxide and superoxide, which can lead to the nitration of tyrosine on proteins (Ischiropoulos 2003; Pacher et al. 2007; Koerdt et al. 2016). iNOS expression is one of the pro-oxidant targets of NF-κB (Morris et al. 2003). In our study, irradiation induced a biphasic activation of iNOS, which could be effectively suppressed by thalidomide. This finding correlates with the results of our previous study, where we found an irradiation-induced biphasic NF-κB activation and downregulation of NF-κB by thalidomide (Kowaliuk et al. 2020). The regulation of iNOS expression by NF-κB and the potential modulation of iNOS via NF-κB signaling pathways has been investigated by others. Our results are in good concordance with the reports of various NF-κB-inhibitors in different tissues and diseases that found the same effect, namely that NF-κB-inhibition can suppress iNOS activity and nitrosative stress (Moreno et al. 2011; Pooladanda et al. 2019; Akimov et al. 2020).

In addition to the urothelium, vascular endothelial cells are severely affected in patients undergoing pelvic radiation therapy. Radiation injury to endothelial cells (EC) can contribute to dysfunction and structural damage to the urinary bladder (Soler et al. 2011). Platelet endothelial cell adhesion molecule-1 (PECAM-1) is an endothelial cell junction molecule highly expressed on the EC surface (Park et al. 2015). PECAM-1 has many functions and is an essential modulator of adhesion, inflammatory cell infiltration, endothelial permeability and endothelial cell proliferation (Biswas et al. 2003). We expected to see a proinflammatory involvement of PECAM-1 in the early radiation response phase. However, our results support the role of PECAM-1 in promoting β-catenin accumulation as described by others (Biswas et al. 2003). Adhesion and communication between EC is mediated by intercellular junctions, including adherens junctions (AJ) (Park et al. 2016). β-catenin is a component of adherens junctions and PECAM-1 is an important binding partner (Biswas et al. 2003). In the present study, irradiation induced a biphasic increase in PECAM-1 expression with a first peak on day 12 and a second peak on day 27. Interestingly, the same biphasic peaks on day 6 and day 24–27 were observed in our previous study for β-catenin in urothelial cells (Krischak et al. 2021). We can speculate that a similar β-catenin expression is provoked on the EC. Thalidomide treatment resulted in an early increase in PECAM-1 expression and shifted the peak to day 6. In our previous study, thalidomide treatment also resulted in an early peak of β-catenin expression on day 6 and an extended plateau from day 21–30 in urothelial cells (Krischak et al. 2021). In the present study, the PECAM-1 staining fraction in EC also showed an extended increase between days 18–27. When looking at staining intensity alone, the effect was less pronounced. Other studies have suggested that PECAM-1 localizes and sequesters β-catenin at the cell membrane (Ilan et al. 1999), although it can also promote the accumulation of transcriptionally active β-catenin and stimulate EC proliferation (Biswas et al. 2003).

The present study has some limitations. One of them is that malondialdehyde, iNOS and PECAM-1 were analysed by immunohistochemistry alone and not with other methods, such as next-generation sequencing, RNA microarray technology, mass spectrometry or western blotting. However, immunochemistry can generate comparable results to these techniques (Cepinskas et al. 2003). Another limitation is the small group size of the non-irradiation control group (five animals). Ideally, the sizes of the treated and control groups should be the same. Nevertheless, due to limited experimental resources, the sizes of control groups are often smaller in comparable experimental settings (Jaal & Dörr 2006a). Furthermore, the potential influence of selected factors is difficult to evaluate. Thalidomide was dissolved in DMSO for intra-

peritoneal injection as recommended by the manufacturer (Kowaliuk et al. 2020; Krischak et al. 2021). DMSO has anti-inflammatory potential via NF-κB inhibition (Rawls et al. 2017). Consequently, the dissolvent's small supportive therapeutic effect cannot be ruled out. Other factors that might have influenced the results are thalidomide dose, the beginning and duration of thalidomide treatment, the irradiation dose, the number of counted sections and the magnifications used.

Conclusion

Single dose irradiation induces the increased formation of the ROS biomarker malondialdehyde in the bladder urothelium of mice. It causes a biphasic increase of iNOS levels in the urothelial cells and of PECAM-1 levels in endothelial cells of the *lamina propria*. The NF-κB inhibitor thalidomide effectively suppresses iNOS activation. Thalidomide also shifts PECAM-1 levels to an earlier peak and provokes an increased and extended staining fraction in the second phase of the early radiation response. We conclude that iNOS suppression contributes to the established therapeutic effect of thalidomide, improving bladder dysfunction and increasing radiation tolerance. We suggest that PECAM-1 contributes to the therapeutic effect by cross signaling with the adherens junction protein β-catenin. However, the ameliorating effect of thalidomide does not seem to stem from substantially altering ROS generation.

Fazit für die Praxis:
Bei der Strahlentherapie von Tumoren im Bereich des Beckens wird häufig die Harnblase mit hohen Dosen exponiert, was eine frühe strahleninduzierte Zystitis verursacht. Klinisch manifestiert sich dies in Form von Inkontinenz, Dysurie und Pollakisurie.
Der Transkriptionsfaktor NF-κB ist zentral in die Pathogenese der frühen Strahlenreaktion der Harnblase involviert. Eine Behandlung mit dem NF-κB Inhibitor Thalidomid ist ein vielversprechender Ansatz zur Reduktion von Nebenwirkungen. Unsere Studie zeigt, dass bei der Linderung klinischer Symptome die medikamentelle Unterdrückung von iNOS und die Modulation von Adhäsionsmolekülen (PECAM-1) eine wichtige Rolle spielen.

References

Ahlatci A, Kuzhan A, Taysi S, Demirtas OC, Alkis HE, Tarakcioglu M, et al. Radiation-modifying abilities of *Nigella sativa* and thymoquinone on radiation-induced nitrosative stress in the brain tissue. Phytomedicine. 2014;21:740–744. DOI:10.1016/j.phymed.2013.10.023

Akimov OY, Kostenko VO. Role of NF-κB transcriptional factor activation during chronic fluoride intoxication in development of oxidative-nitrosative stress in rat's gastric mucosa. J Trace Elem Med Biol. 2020;61:126535. DOI:10.1016/j.jtemb.2020.126535

Akimov SA, Molotkovsky RJ, Kuzmin PI, Galimzyanov TR, Batishchev OV. Continuum Models of Membrane Fusion: Evolution of the Theory. Int J Mol Sci. 2020;21:3875. DOI:10.3390/ijms21113875

Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438. DOI:10.1155/2014/360438

Biswas P, Canosa S, Schoenfeld J, Schoenfeld D, Tucker A, Madri JA. PECAM-1 promotes β-catenin accumulation and stimulates endothelial cell proliferation. Biochem Biophys Res Commun. 2003;303:212–218. DOI:10.1016/s0006-291x(03)00313-9

Cepinskas G, Savickiene J, Ionescu CV, Kvietys PR. PMN transendothelial migration decreases nuclear NFkappaB in IL-1beta-activated endothelial cells: role of PECAM-1. J Cell Biol. 2003;161:641–651. DOI:10.1083/jcb.200212048

Dörr W, Beck-Bornholdt HP. Radiation-induced impairment of urinary bladder function in mice: Fine structure of the acute response and consequences on late effects. Radiat Res. 1999;151:461–467.

Dröge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002;82:47–95. DOI:10.1152/physrev.00018.2001

Federal Ministry of Education, Science and Research, ref. number BMWF-66.009/0148-WF/II/3b/2014

Franks ME, Macpherson GR, Figg WD. Thalidomide. Lancet. 2004;363:1802–1811. DOI:10.1016/S0140-6736(04)16308-3

Hu P, Deng FM, Liang FX, Hu CM, Auerbach AB, Shapiro E, et al. Ablation of uroplakin III gene results in small urothelial plaques, urothelial leakage, and vesicoureteral reflux. J Cell Biol. 2000;151:961–972. DOI:10.1083/jcb.151.5.961

Hu P, Meyers S, Liang F-X, Deng F-M, Kachar B, Zeidel ML, et al. Role of membrane proteins in permeability barrier function: Uroplakin ablation elevates urothelial permeability. Am J Physiol Renal Physiol. 2002;283:F1200–F1207. DOI:10.1152/ajprenal.00043.2002

Ilan N, Mahooti S, Rimm DL, Madri JA. PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated beta-catenin. J Cell Sci. 1999;112:3005–3014. DOI:10.1242/jcs.112.18.3005

Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun. 2003;305:776–783. DOI:10.1016/s0006-291x(03)00814-3

Jaal J, Dörr W. Radiation induced inflammatory changes in the mouse bladder: The role of cyclooxygenase-2. J Urol. 2006a;175:1529–1533. DOI:10.1016/S0022-5347(05)00658-0

Jaal J, Dörr W. Radiation-induced damage to mouse urothelial barrier. Radiother Oncol. 2006b;80:250–256. DOI:10.1016/j.radonc.2006.07.015

Joiner M. Basic clinical radiobiology. 4th ed. London: Hodder Arnold; 2009.

Jones DP. Radical-free biology of oxidative stress. Am J Physiol Cell Physiol. 2008;295:C849-C868. DOI:10.1152/ajpcell.00283.2008

Kawamura K, Qi F, Kobayashi J. Potential relationship between the biological effects of low-dose irradiation and mitochondrial ROS production. J Radiat Res. 2018;59:ii91–ii97. DOI:10.1093/jrr/rrx091

Koerdt S, Steinstraesser L, Stoeckelhuber M, Wales CJ, Rohleder NH, Babaryka G, et al. Radiotherapy for oral cancer decreases the cutaneous expression of host defence peptides. J Craniomaxillofac Surg. 2016;44:882–889. DOI:10.1016/j.jcms.2016.04.014

Kowaliuk J, Sarsarshahi S, Hlawatsch J, Kastsova A, Kowaliuk M, Krischak A, et al. Translational aspects of Nuclear Factor-Kappa B and its modulation by thalidomide on early and late radiation sequelae in urinary bladder dysfunction. Int J Radiat Oncol Biol Phys. 2020;107:377–385. DOI:10.1016/j.ijrobp.2020.01.028

Krischak A, Kowaliuk J, Sarsarshahi S, Dörr W, Kleiter M. Effect of irradiation on the expression of E-cadherin and β-catenin in early and late radiation sequelae of the urinary bladder and its modulation by NF-κB inhibitor thalidomide. Strahlenther Onkol. 2021;197:537–546. DOI:10.1007/s00066-021-01751-y

Livolsi A, Busuttil V, Imbert V, Abraham RT, Peyron JF. Tyrosine phosphorylation-dependent activation of NF-kappa B. Requirement for p56 LCK and ZAP-70 protein tyrosine kinases. Eur J Biochem. 2001;268:1508–1515. DOI:10.1046/j.1432-1327.2001.02028.x

Mallick S, Madan R, Julka PK, Rath GK. Radiation induced cystitis and proctitis - prediction, assessment and management. Asian Pac J Cancer Prev. 2015;16:5589–5594. DOI:10.7314/apjcp.2015.16.14.5589

Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003;22:5734–5754. DOI:10.1038/sj.onc.1206663

Moreno B, Jukes J-P, Vergara-Irigaray N, Errea O, Villoslada P, Perry VH, et al. Systemic inflammation induces axon injury during brain inflammation. Ann Neurol. 2011;70:932–942. DOI:10.1002/ana.22550

Morris KR, Lutz RD, Choi H-S, Kamitani T, Chmura K, Chan ED. Role of the NF-kappaB signaling pathway and kappaB cis-regulatory elements on the IRF-1 and iNOS promoter regions in mycobacterial lipoarabinomannan induction of nitric oxide. Infect Immun. 2003;71:1442–1452. DOI:10.1128/iai.71.3.1442-1452.2003

Niki E. Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med. 2009;47:469–484. DOI: 10.1016/j.freeradbiomed.2009.05.032

Nikolic DM, Cholewa J, Gass C, Gong MC, Post SR. Class A scavenger receptor-mediated cell adhesion requires the sequential activation of Lyn and PI3-kinase. Am J Physiol Cell Physiol. 2007;292:C1450–C1458. DOI:10.1152/ajpcell.00401.2006

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424. DOI:10.1152/physrev.00029.2006

Papac-Milicevic N, Busch CJ-L, Binder CJ. Malondialdehyde epitopes as targets of Immunity and the implications for atherosclerosis. Adv Immunol. 2016;131:1–59. DOI:10.1016/bs.ai.2016.02.001

Park S-H, Sorenson CM, Sheibani N. PECAM-1 isoforms, eNOS and endoglin axis in regulation of angionesis. Clin Sci (Lond). 2015;129:217–234. DOI:10.1042/CS20140714

Park S-H, Shin MJ, Kim DW, Park J, Choi SY, Kang Y-H. Blockade of monocyte-endothelial trafficking by transduced Tat-superoxide dismutase protein. Int J Mol Cell Med. 2016;37:387–397. DOI:10.3892/ijmm.2015.2444

Pooladanda V, Thatikonda S, Bale S, Pattnaik B, Sigalapalli DK, Bathini NB, et al. Nimbolide protects against endotoxin-induced acute respiratory distress syndrome by inhibiting TNF-α mediated NF-κB and HDAC-3 nuclear translocation. Cell Death Dis. 2019;10:81. DOI:10.1038/s41419-018-1247-9

Rawls WF, Cox L, Rovner ES. Dimethyl sulfoxide (DMSO) as intravesical therapy for interstitial cystitis/bladder pain syndrome: A review. Neurourol Urodyn. 2017;36:1677–1684. DOI:10.1002/nau.23204

Soler R, Vianello A, Füllhase C, Wang Z, Atala A, Soker S, et al. Vascular therapy for radiation cystitis. Neurourol Urodyn. 2011;30:428–434. DOI:10.1002/nau.21002

Speyer CL, Neff TA, Warner RL, Guo R-F, Sarma JV, Riedemann NC, et al. Regulatory effects of iNOS on acute lung inflammatory responses in mice. Am J Pathol. 2003;163:2319–2328. DOI:10.1016/S0002-9440(10)63588-2

Tsikas D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Anal Biochem. 2017;524:13–30. DOI:10.1016/j.ab.2016.10.021

Vaupel P, Schaible H-G, Mutschler E. Anatomie, Physiologie, Pathophysiologie des Menschen. 7th ed., Stuttgart: WVG Wissenschaftliche Verlagsgesellschaft; 2015.

Vollmar A, Zündorf I, Dingermann T. Immunologie. 2nd ed., Stuttgart: WVG Wissenschaftliche Verlagsgesellschaft; 2013.

Yao Y, Li Y, Song Q, Hu C, Xie W, Xu C, et al. Angiogenic Factor AGGF1-Primed endothelial progenitor cells repair vascular defect in diabetic mice. Diabetes. 2019;68:1635–1648. DOI:10.2337/db18-1178

Ye Z, Gorman AA, Uittenbogaard AM, Myers-Morales T, Kaplan AM, Cohen DA, et al. Caspase-3 mediates the pathogenic effect of *Yersinia pestis* YopM in liver of C57BL/6 mice and contributes to YopM's function in spleen. PLoS ONE. 2014;9:e110956. DOI:10.1371/journal.pone.0110956

Zhang F, Hao M, Jin H, Yao Z, Lian N, Wu L, et al. Canonical hedgehog signalling regulates hepatic stellate cell-mediated angiogenesis in liver fibrosis. Br J Pharmacol. 2017;174:409–423. DOI:10.1111/bph.13701

Please cite as:

Raiber J, Kleiter M, Kowaliuk J, Ludewig E, Rowan C, Sarshahi S, Dörr W. Effects of thalidomide on urothelial radiation-induced alterations of reactive oxygen species, iNOS and endothelial PECAM-1. Wien Tierarztl Monat – Vet Med Austria. 2022;109:Doc13. DOI:10.5680/wtm000013