Radiation-induced cardiopulmonary dysfunction
van der Veen, Sjoerdjte Johanna

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
ACE INHIBITION ATTENUATES RADIATION-INDUCED CARDIOPULMONARY DAMAGE

Sonja J. van der Veen¹,², Ghazaleh Ghobadi¹,²#, Rudolf A. de Boer³, Hette Faber¹,², Megan V. Cannon³, Peter W. Nagle¹,², Sytze Brandenburg⁴, Johannes A. Langendijk², Peter van Luijk², Robert P. Coppes¹,²

¹Department of Cell Biology, ²Department of Radiation Oncology, ³Department of Experimental Cardiology, University Medical Center Groningen, University of Groningen, ⁴Kernfysisch Versneller Instituut, University of Groningen, Groningen, the Netherlands.

# Current address: Department of Radiation Oncology, the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands

Radiother Oncol. 2015 Jan;114(1):96-103. doi: 10.1016/j.radonc.2014.11.017
In thoracic irradiation, the maximum radiation dose is restricted by the risk of radiation-induced cardiopulmonary damage and dysfunction limiting tumour control. We showed that radiation-induced subclinical cardiac damage and lung damage in rats mutually interact and that combined irradiation intensifies cardiopulmonary toxicity. Unfortunately, current clinical practice does not include preventative measures to attenuate radiation-induced lung or cardiac toxicity. Here, we investigate the effects of the ACE inhibitor captopril on radiation-induced cardiopulmonary damage.

Method: After local irradiation of rat heart and/or lungs captopril was administered orally. Cardiopulmonary performance was assessed using biweekly breathing rate measurements. At 8 weeks post-irradiation, cardiac hemodynamics were measured, CT scans and histopathology were analysed.

Results: Captopril significantly improved breathing rate and cardiopulmonary density/structure, but only when the heart was included in the radiation field. Consistently, captopril reduced radiation-induced pleural and pericardial effusion and cardiac fibrosis, resulting in an improved left ventricular end-diastolic pressure only in the heart-irradiated groups.

Conclusion: Captopril improves cardiopulmonary morphology and function by reducing acute cardiac damage, a risk factor in the development of radiation-induced cardiopulmonary toxicity. ACE inhibition should be evaluated as a strategy to reduce cardiopulmonary complications induced by radiotherapy to the thoracic area.

Keywords: Thoracic tumours, Radiation-induced cardiac toxicity, Radiation-induced lung toxicity, ACE inhibition, Captopril
Abstract

Background and Purpose: In thoracic irradiation, the maximum radiation dose is restricted by the risk of radiation-induced cardiopulmonary damage and dysfunction limiting tumour control. We showed that radiation-induced sub-clinical cardiac damage and lung damage in rats mutually interact and that combined irradiation intensifies cardiopulmonary toxicity. Unfortunately, current clinical practice does not include preventative measures to attenuate radiation-induced lung or cardiac toxicity. Here, we investigate the effects of the ACE inhibitor captopril on radiation-induced cardiopulmonary damage.

Material and Methods: After local irradiation of rat heart and/or lungs captopril was administered orally. Cardiopulmonary performance was assessed using biweekly breathing rate measurements. At 8 weeks post-irradiation, cardiac hemodynamics were measured, CT scans and histopathology were analysed.

Results: Captopril significantly improved breathing rate and cardiopulmonary density/structure, but only when the heart was included in the radiation field. Consistently, captopril reduced radiation-induced pleural and pericardial effusion and cardiac fibrosis, resulting in an improved left ventricular end-diastolic pressure only in the heart-irradiated groups.

Conclusion: Captopril improves cardiopulmonary morphology and function by reducing acute cardiac damage, a risk factor in the development of radiation-induced cardiopulmonary toxicity. ACE inhibition should be evaluated as a strategy to reduce cardiopulmonary complications induced by radiotherapy to the thoracic area.

Keywords: Thoracic tumours, Radiation-induced cardiac toxicity, Radiation-induced lung toxicity, ACE inhibition, Captopril
Introduction

Thoracic tumours are among the most common human malignancies. The treatment of choice often includes radiation therapy. Unfortunately, the radiation dose that can be safely administered to the tumour is limited by the risk of radiation-induced toxicity of the surrounding tissues. The radiation dose emitted to the heart following radiotherapy is of particular concern. Specifically, the prevalence of late onset heart failure has been reported including accelerated atherosclerosis, pericardial and myocardial fibrosis, conduction abnormalities, and injury to cardiac valves. Both incidence and severity of radiation-induced heart toxicity (RIHT) increase with higher radiation doses, larger volumes exposed, a younger age at time of exposure, and greater time elapse following treatment. In addition, the absolute radiation-related risk for a major cardiac event is far greater with pre-existing cardiac risk factors or ischemic cardiac disease in women treated for breast cancer. Interestingly, even with the most advanced photon techniques most women still receive doses of greater than 1-5 Gy to the heart.

Interestingly, the risk of radiation-induced lung toxicity (RILT), another potentially life-threatening side effect of radiotherapy of thoracic tumours, is enhanced when pre-existing cardiac disease exists and the heart is co-irradiated. RILT traditionally is divided into an early inflammatory phase, termed “radiation pneumonitis”, and a late fibroproductive phase, termed “fibrosis”. Recently, in a preclinical model, we found that, in concert with inflammation, radiation-induced vascular remodelling played a major role in the aetiology of early RILT. It was shown that lung irradiation induced early pulmonary vascular remodelling which resulted in pulmonary hypertension and right ventricle (RV) hypertrophy which eventually lead to cardiopulmonary dysfunction. Additionally, heart irradiation may cause an increased left ventricular (LV) end-diastolic pressure, perivascular fibrosis and consequential pulmonary edema. Although RIHT is regarded as a late side effect of radiotherapy, these recent preclinical and clinical findings show that radiation can also affect hearts function early after irradiation, albeit without apparent clinical symptoms. As such, both lung and heart irradiation can cause pulmonary and cardiac toxicity through different mechanisms which consequentially lead to an intensified loss of cardiopulmonary performance after combined irradiation. Our experimental set-up presented herein allows for accurate proton irradiation of specific lung sub-volumes and the heart, and provides a model where early, previously unnoticed subclinical cardiac damage can be visualized. Using the interaction between lung and cardiac damage, we are able to study the physiological mechanism and potential mitigation of early subclinical cardiac damage that is potentially responsible for the development of late symptomatic damage.

Inhibition of the renin-angiotensin system (RAS) seems to be an alluring strategy for attenuating radiation-induced cardiopulmonary dysfunction. There is overwhelming evidence implicating ACE inhibitors in the protection from adverse cardiac remodelling and heart failure development. Moreover, it is known that RAS plays a role in cardiac remodelling and interstitial fibrosis. Interestingly, preclinical studies indicate that suppression of the RAS may ameliorate radiation-induced toxicity in different organs like the kidneys, the central nervous system and lungs. However, in the latter study...
interaction with cardiac damage after whole thorax irradiation was not assessed. Therefore, in the current study we aim to investigate the affect of ACE inhibition on early radiation-induced heart damage.

Materials and methods

See supplementary data for complete materials and methods.

Animals
The animals used in the experiments were adult male albino Wistar rats of the Hsd/Cpb:WU strain. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Local lung and heart irradiation
To induce cardiopulmonary damage, rat hearts, lungs, or both were irradiated (single fraction) using the shoot-through technique with 150 MeV protons from the cyclotron at the Kernfysisch Versneller Instituut, as published previously \textsuperscript{9,11,23,24}. Rats were given captopril in the drinking water. The animals (sham) treated with captopril were observed for 3 months after irradiation to determine cardiopulmonary function, structure and molecular changes as described below.

Breathing rate assay
To assess response of cardiopulmonary function to radiation, breathing rate (BR) was measured before and every two weeks after the irradiations up to week 12, as previously described \textsuperscript{10,23}.

CT imaging
Local density changes were assessed using CT imaging 8 weeks after irradiation. The CT images were analysed with our recently developed highly-sensitive CT analysis method \textsuperscript{25}.

Cardiac hemodynamic measurements
To investigate the cardiac physiological changes early after heart and/or lung irradiation, LV or RV hemodynamic measurements were carried out 8 weeks after irradiation. For LV measurements the recorded parameters included LV end-diastolic and end-systolic pressure (LVEDP and LVESP), dp/dtmax (maximum rate of LV pressure change), dp/dtmin (minimum rate of LV pressure change) and Tau (LV relaxation constant). For RV hemodynamic measurement the right ventricle pressure (RVP) and pulmonary artery pressure (PAP) were recorded.

Histopathology
Sections of the rat lungs, LV, RV and intraventricular septum (IVS) were stained with Haematoxylin & Eosin (HE) and Masson’s Trichrome (MT) and analysed morphologically for signs of parenchymal and vascular damage or cardiac damage, respectively.
Quantitative polymerase chain reaction (QPCR)
Total RNA was extracted from the heart LVs and mRNA levels of ANP and BNP were measured by QPCR.

Statistical analysis
Data are expressed as mean values (means +/- SEM). Statistical analyses were performed using the two-way repeated measures ANOVA followed by the post-hoc Tukey or Bonferroni correction for multiple comparisons and the log-rank (Mantel-Cox) to compare distributions of two groups. Correlational analyses were performed using the Pearson correlation coefficient. In all statistical analyses, significance was defined as $P<0.05$.

Results

Captopril decreases structural and functional damage in irradiated hearts
To study the influence of ACE inhibition on early radiation-induced cardiac damage, we first investigated the effect of captopril on cardiac structure and function. Rat hearts were irradiated (HIR) (Sup. Fig. S1A), thereafter captopril was administered. We assessed the effect of captopril on irradiation-induced LV perivascular and interstitial fibrosis. Eight weeks after irradiation, perivascular fibrosis developed in the LV, but this was significantly decreased with captopril treatment (Fig. 1A and B). Moreover, captopril decreased the LV interstitial fibrosis score in HIR as well as in un-irradiated animals (Fig. 1C and D). Perivascular/interstitial fibrosis is known to be an important factor in the pathophysiological process contributing to diastolic dysfunction by impairing cardiac relaxation. Therefore, we next investigated whether the observed decrease in LV perivascular and interstitial fibrosis by captopril treatment translated into an improved cardiac diastolic function. Indeed, HIR was associated with increased LVEDP and the LV relaxation constant, Tau, which were both normalized by captopril treatment (Fig. 1E and F). These effects are independent of any changes in systemic blood pressure, maximum and minimum rates of LV pressure change (dP/dt max and dP/dt min) or changes in LV weight, since these parameters were comparable between captopril and control-treated rats (Sup. Fig. S2). The secretion of brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) are stimulated by excessive myocardial stretch. Therefore we assessed BNP and ANP mRNA levels in the irradiated LV by qPCR. HIR lead to increased ANP and BNP mRNA, which appeared to be lowered by captopril treatment, albeit not significantly (Sup. Fig. S3A and B).
Captopril reduces acute cardiac damage

Quantitative polymerase chain reaction (QPCR)

Total RNA was extracted from the heart LVs and mRNA levels of ANP and BNP were measured by QPCR.

Statistical analysis

Data are expressed as mean values (means +/- SEM). Statistical analyses were performed using the two-way repeated measures ANOVA followed by the post-hoc Tukey or Bonferroni correction for multiple comparisons and the log-rank (Mantel-Cox) to compare distributions of two groups. Correlational analyses were performed using the Pearson correlation coefficient. In all statistical analyses, significance was defined as \( P < 0.05 \).

To study the influence of ACE inhibition on early radiation-induced cardiac damage, we first investigated the effect of captopril on cardiac structure and function. Rat hearts were irradiated (HIR) (Sup. Fig. S1A), thereafter captopril was administered. We assessed the effect of captopril on irradiation-induced LV perivascular and interstitial fibrosis 15. Eight weeks after irradiation, perivascular fibrosis developed in the LV, but this was significantly decreased with captopril treatment (Fig. 1A and B). Moreover, captopril decreased the LV interstitial fibrosis score in HIR as well as in un-irradiated animals (Fig. 1C and D).

Perivascular/interstitial fibrosis is known to be an important factor in the pathophysiological process contributing to diastolic dysfunction 15,17,26. Therefore, we next investigated whether the observed decrease in LV perivascular and interstitial fibrosis by captopril treatment translated into an improved cardiac diastolic function. Indeed, HIR was associated with increased LVEDP and the LV relaxation constant, Tau, which were both normalized by captopril treatment (Fig. 1E and F). These effects are independent of any changes in systemic blood pressure, maximum and minimum rates of LV pressure change (dPdtmax and dPdtmin) or changes in LV weight, since these parameters were comparable between captopril and control-treated rats (Sup. Fig. S2).

The secretion of brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) are stimulated by excessive myocardial stretch 27. Therefore we assessed BNP and ANP mRNA levels in the irradiated LV by qPCR. HIR lead to increased ANP and BNP mRNA, which appeared to be lowered by captopril treatment, albeit not significantly (Sup. Fig. S3A and B).

Figure 1: Captopril decreases structural and functional damage in irradiated hearts. (A and B) Immunohistochemical staining and morphological quantification of perivascular fibrosis and interstitial fibrosis (C and D) in irradiated hearts shows a significant reduction after captopril treatment. Masson trichrome staining was performed: red stain = muscle fiber, blue stain = collagen. Scale bar: 100 µm. Data are presented as means +/- SEM; n ≥ 3. (E) Heart irradiation induces LV diastolic dysfunction. This was shown by increased left ventricle end-diastolic pressures (LVEDP). Captopril treatment decreased the elevated pressures after HIR. Data are presented as means +/- SEM; n ≥ 4. (F) HIR leads to an increased LV relaxation constant Tau. Treatment with captopril leads to a significant decrease of Tau after HIR. Data are presented as means +/- SEM; n ≥ 4. (G) Early cardiopulmonary dysfunction was assessed by increase in breathing rate (BR). LIR induces increased BR; captopril treatment did not affect BR. LHIR enhanced BR increase compared to LIR, and captopril attenuated early radiation-induced cardiopulmonary dysfunction in LHIR. Data are presented as mean area under BR increase curve from week 0 to 8 after irradiation: means +/- SEM; n=9. ***\( P < 0.001 \), **\( P < 0.01 \) compared with control. ##\( P < 0.001 \), ###\( P < 0.01 \), ##\( P < 0.05 \) comparison between +/- captopril treatment.
ACE inhibition ameliorates early radiation-induced cardiopulmonary dysfunction only when the heart is co-irradiated

Since captopril significantly protects against radiation-induced structural and functional cardiac changes, we hypothesized that it should also exert protective effects on the lung. Therefore, we next investigated the effect of captopril on cardiopulmonary dysfunction early after irradiation using our non-invasive heart and lung interaction rat model.

We measured BR as a measure of cardiopulmonary function in rats irradiated on the heart, lung, both heart and lung, and treated the animals with captopril including a sham-treated group (see Sup. Fig. S4). Captopril treatment following administration of 50% 20 Gy lung irradiation (LIR) (Sup. Fig. S1C) resulted in a BR increase of ~40 breaths per minute (bpm) at 6-8 weeks post-irradiation that was similar to the sham-treated animals (Sup. Fig. S5) and as previously described. Indeed, analysis of the area under the curve of the BR increase from week 0 to 8 after irradiation indicated (Fig. 1G) that captopril did not influence the control and radiation-induced BR increase after LIR.

Next, we investigated the effect of ACE inhibition on BR after cardiac irradiation. As observed in our previous experiments, HIR does not significantly affect the BR nor does HIR with captopril treatment (Fig. 1G and Sup. Fig. S5B). However, after combined heart and 50% lung irradiation (LHIR) (see Sup. Fig. S1B), captopril significantly reduced the enhanced BR observed after untreated LHIR (Fig. 1G and Sup. Fig. S5B). As such, the enhanced cardiopulmonary dysfunction after inclusion of the heart in the irradiation field was completely diminished after captopril treatment leading to a BR level comparable to the LIR animals. In summary, captopril only ameliorates radiation-induced BR increase when the heart is co-irradiated.

Captopril treatment reduces lung damage measured by CT after (co-)irradiation of the heart

Thoracic irradiation leads to structural changes of heart and lungs. Therefore, we assessed the affect of captopril on these structural changes by using our recently developed CT analysis method which is highly sensitive for non-invasive assessment of thoracic structural changes. Consistent with the improved cardiopulmonary function, the severe structural changes of the heart and lungs observed after LHIR were significantly reduced after captopril treatment (Fig. 2A and B), and again, this was only observed when the heart was co-irradiated. These structural changes are due to, for example, pleural or cardiac effusion, pulmonary edema, infiltration or fibrosis. Therefore, to further investigate how captopril improves cardiopulmonary function and structure, we visually assessed the presence or absence of effusion. After opening the thorax and diaphragm of the animals, no animals from the LIR group but most of the HIR and LHIR animals presented with effusion in their pleural cavity. Moreover, none of the HIR animals treated with captopril showed signs of pleural effusion. A similar observation was made for pleural effusion after LHIR and captopril treatment (Fig. 2C). Several animals also presented with pericardial effusion in combination with pleural effusion. LHIR led to pericardial effusion in 6 out of 9 irradiated animals. After captopril treatment, only 1 out of 9 animals presented with pericardial effusion (Fig. 2C). Pleural and pericardial effusion, known side effects of thoracic irradiation, were clearly diminished by ACE inhibition.
50% lung irradiation (LHIR) (see Sup. Fig. S1B), captopril significantly reduced the enhanced with captopril treatment (Fig. 1G and Sup. Fig. S5B). However, after combined heart and lung, both heart and lung, and treated the animals with captopril including a sham-treated control and radiation-induced BR increase after LIR.

Next, we investigated the effect of ACE inhibition on BR after cardiac irradiation. As previously described, indeed, analysis of the area under the curve of the BR increase 6-8 weeks post-irradiation that was similar to the sham-treated animals (Sup. Fig. S5) and since captopril significantly protects against radiation-induced structural and functional dysfunctions, early after irradiation using our non-invasive heart and lung interaction rat analysis method which is highly sensitive for non-invasive assessment of thoracic remodelling/occlusion, pulmonary artery pressure (PAP) and RV hypertrophy in the non-treated animals. Consistent with our previous experiments, LIR, HIR and LHIR treated animals. Consistent with our previous experiments, LIR, HIR and LHIR.

Thoracic irradiation leads to structural changes of heart and lungs. Therefore, we assessed for pleural effusion. A similar observation was made for pleural effusion after LHIR and HIR. From the LIR group but most of the HIR and LHIR animals presented with effusion in their pleural cavity. Moreover, none of the HIR animals treated with captopril showed signs of pulmonary edema, infiltration or fibrosis. Therefore, to further investigate how captopril fibrosis after HIR (Fig. 3A, B and D). After LHIR, captopril treatment significantly reduced features may explain the decrease of pleural effusion following captopril treatment. Taken together, captopril treatment decreases RILT, but consistent with the previous findings, this effect is observed only when the heart is (co-)irradiated.

Captopril treatment decreases RILT only when the heart is (co-)irradiated

We already showed that early radiation-induced damage to cardiac structures is reduced with captopril treatment which may indirectly lead to less pleural effusion. To study this in more detail we next analysed lung morphology. Morphologic analysis showed that captopril treatment lead to significantly less alveolar inflammation, infiltrates and early fibrosis after HIR (Fig. 3A, B and D). After LHIR, captopril treatment significantly reduced inflammation, infiltrates and interstitial edema in the lungs (Fig. 3A, B and C). These features may explain the decrease of pleural effusion following captopril treatment. Taken together, captopril treatment decreases RILT, but consistent with the previous findings, this effect is observed only when the heart is (co-)irradiated.

ACE inhibition does not protect the pulmonary vasculature after thoracic irradiation

Previously, we found that radiation-induced pulmonary vascular remodelling is an important factor in the development of RILT resulting in pulmonary hypertension and RV hypertrophy that eventually leads to cardiopulmonary dysfunction. In addition to parenchymal damage, lung irradiation caused vascular damage which also influenced cardiopulmonary function. To this end, we studied the effect of captopril on early radiation-induced pulmonary vascular damage. We analysed pulmonary vascular remodelling/occlusion, pulmonary artery pressure (PAP) and RV hypertrophy in the non-and captopril-treated animals. Consistent with our previous experiments, LIR, HIR and LHIR.
Captopril reduces acute cardiac damage

**Figure 3:** Lung morphology after LIR, HIR and LHIR with or without captopril treatment. (A) LIR, HIR and LHIR all lead to a significant increase in alveolar inflammation. Captopril significantly reduced inflammation after HIR and LHIR. (B) LIR, HIR and LHIR lead to increased alveolar infiltrates. Captopril treatment significantly decreased infiltration after HIR. (C) HIR and LHIR lead to interstitial edema. Captopril treatment significantly decreased interstitial edema. (D) Early pulmonary fibrosis presented after HIR and LHIR, which was significantly reduced by captopril. Data are presented as means +/- SEM; n ≥ 4; ***P<.001, **P<.01, *P<.05 compared with control.

lead to vascular remodelling/occlusion in- and out-of the irradiated field indicating a global vascular response in the lungs, while HIR caused vascular remodelling only in the irradiated field (Fig. 4A and B). Captopril treatment did not decrease vascular remodelling. Consistent with this, no differences in PAP or RV hypertrophy were observed (Fig. 4C and D, Sup. Fig. S6). It therefore appears that although captopril ameliorates cardiopulmonary dysfunction after combined heart and lung irradiation, this cannot be explained by a protective effect on the pulmonary vasculature which is equally damaged in animals with or without captopril.

In summary, these data indicate that captopril solely protects the heart from radiation damage. Captopril treatment attenuated radiation-induced pleural and pericardial and cardiac fibrosis resulting in an improved LVEDP and Tau. In addition, captopril reduced RILT, but only when the heart was co-irradiated.
Captopril reduces acute cardiac damage

**Figure 4:** ACE inhibition does not protect the pulmonary vasculature after thoracic irradiation. (A) Vascular remodelling is present in- and out- of the irradiated field after LIR, HIR and LHIR with or without captopril treatment. (B) Quantification shows a significant increase of luminal occlusion in the irradiated fields of all the groups. Captopril treatment does not change the level of luminal occlusion. The outcome from each group was subtracted from the control group and then presented in the graphs. (C) Captopril does not prevent the development of right ventricle (RV) hypertrophy. The increased workload of the RV leads to right ventricle hypertrophy (RVH) after LIR and LHIR. Captopril showed no affect on the development of RVH in these groups. (D) Captopril had no affect on pulmonary artery pressure after LIR and LHIR. LIR and LHIR lead to an elevated pulmonary artery pressure; no effect of captopril was observed. Data are presented as means +/- SEM; n ≥ 4; ***P<.001, **P<.01, *P<.05 compared with control.
Chapter 04 | Captopril reduces acute cardiac damage

Discussion

In the current study we show that ACE inhibition attenuated early radiation-induced heart damage and consequently prevented the influence of dose to the heart on lung toxicity. Specifically, we show that captopril prevented LV perivascular fibrosis and the elevations in LVEDP induced by heart irradiation, thus, preventing LV diastolic dysfunction and consequent pulmonary damage. RIHT after cancer therapy is generally believed to be a late side effect. However, evidence from our studies 15 as well as other preclinical 30,31 and clinical 32,33 studies suggest that radiation may already exert an early affect on the heart. Although this early affect is subtle and subclinical, it may have detrimental consequences when combined with lung irradiation. Apart from this, it may exert late effects, such as ischemic heart disease/accelerated atherosclerosis, pericardial and myocardial fibrosis, conduction abnormalities, and injury to cardiac valves 4,5. In our experimental model, captopril reduced the heart component of thoracic irradiation-induced damage, diminishing its effect on total early cardiopulmonary damage which only became clinically discernible when both heart and lung were irradiated.

The ACE inhibitor, captopril, was selected based on previous radiation studies where it was proven to be the most successful thus far in reducing radiation damage to multiple organs 20,34-36.

However, an attenuating effect on the hearts function after irradiation its affect on the heart has not been reported. This is interesting because many studies have shown that cardiac dysfunction other than caused by radiation can be attenuated by ACE inhibition. Previous studies showed that ACE inhibition has a protective effect on the lung after thoracic irradiation. However, in these studies the heart was always co-irradiated. In the current study, high precision protons were used instead of photons, which enabled selective irradiation of only the lungs, or virtually only the heart. This helped to elucidate that ACE inhibition does not reduce RILT directly, but rather indirectly by reducing acute heart damage which lead to secondary reductions in pulmonary function loss, inflammation, edema and fibrosis. Apart from their hypotensive action, ACE inhibitors are known to have other properties such as anti-inflammatory action 38. Further, it has been suggested that the sulfhydryl group in the molecular structure of captopril confers in a free radical scavenger activity, and this can account in part for its radioprotection 39. It might act as an antioxidant to reduce inflammatory reactive oxygen species and thus mitigate radiation-induced toxicity. In this study it was found that captopril reduced fibrosis in rat hearts early after irradiation, which in the field of cardiology research is a known property of captopril 40,41.

Reduction of early radiation-induced cardiac fibrosis and consequently cardiac diastolic dysfunction may be of importance for the development of late cardiac damage. Interestingly, radiation-induced myocardial fibrosis that occurs much later can be reduced by captopril 42. Given that in our model we found ACE inhibition to improve not only cardiac structure, but also function early after irradiation, implicates an important therapeutic application of ACE inhibitors in the field of thoracic radiation oncology that warrants further investigation.

Although many differences exist between our animal studies and radiotherapy in the clinic, for example, single dose versus fractionation, the presence of patient related co-
morbidities, patient pulmonary/cardiac status, and concomitant chemotherapy, there are indications that the same mechanisms might play a role in humans. Both preclinical and clinical studies showed that Ag II may initiate cardiac perivascular fibrosis and diastolic dysfunction \(^{43,44}\). Therefore, reduction of fibrosis by ACE inhibition in rats may show reduction in the human heart as well. Moreover, treatment with ACE inhibitors during thoracic irradiation has already shown positive effects, albeit without underpinning the mechanism. Acute respiratory complications and post-radiotherapy radiological changes such as lung consolidation were less likely to develop in lung cancer patients who were treated with ACE inhibitors \(^{37,45}\). As in preclinical studies, the dose to the heart was not taken into account in these clinical studies. We therefore propose that retrospective studies be initially performed to compare radiation-toxicity in patients who were or were not receiving ACE inhibitors during their radiation treatment where the heart had been included in the radiation field. This may probably show an even greater protective effect of ACE inhibition when doses to the heart are assessed. To further translate our findings to clinical practice, clinical studies should be performed to investigate whether ACE inhibition exerts the same protective effect on acute radiation-induced heart damage and consequently on RILT as shown in our preclinical study presented herein. Treatment with ACE inhibitors should start directly after irradiation in order to protect against acute heart damage that potentially bears consequences during the later stages of irradiation damage. Since the occurrence of a subsequent cardiac event increases by 7.4% with each gray of radiation to the heart \(^{3}\), and patients treated for thoracic cancers still receive relevant doses to the heart, it is therefore of great clinical importance to find strategies to reduce this toxicity. Interestingly, ACE inhibitors have been found to not change the radiosensitivity of lung tumour cell lines \(^{46}\) and even reduce the risk of colorectal cancer \(^{47}\). To conclude, ACE inhibition reduces RILT by ameliorating acute cardiac damage. ACE inhibition such as with the clinically-approved agent, captopril, may be a promising strategy to reduce early cardiopulmonary complications induced by radiotherapy to the thoracic area in patients receiving a considerable dose to the heart.
References


Supplementary Data

Material and Methods

Animals
The animals used in the experiments were adult male albino Wistar rats of the Hsd/Cpb:WU strain, bred in a specific pathogen free colony (Harlan-CPB, Rijswijk, The Netherlands). Animals were housed 4 per cage in our central animal facility of the University Medical Center Groningen under a 12 h light - 12 h dark cycle, and were fed rodent chow (RMH-B, Hope Farms, Woerden, and The Netherlands) and water ad libitum. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Local lung and heart irradiation
To induce cardiopulmonary damage, rat hearts, lungs, or both were irradiated (single fraction) using the shoot-through technique with 150 MeV protons from the cyclotron at the Kernfysisch Versneller Instituut, as published previously 1-4. In short, the shoot-through technique only employs high-energy protons and no lower energy (Bragg peak) protons. This results in a very uniform dose distribution in the longitudinal direction (±1%) and sharp lateral field edges (20–80% isodose distance: 1 mm) (Sup. Fig. S1), which ensures no dose deposit in the shielded parts of the lung. The irradiation ports were designed using computed tomography (CT) scans of animals of the same age and weight as previously described 5.

Positioning of the animals and dosimetry were adapted from procedures used previously for parotid gland irradiations in our laboratory 6. Rats, weighing 280 g – 320 g were anaesthetized with intraperitoneal injection of Ketanest (36 mg/kg) and Rompun (6 mg/kg) and positioned in a polymethylmethacrylate holder hanging vertically by their upper incisor teeth fitted in a groove of a positioning rod just behind the appropriate collimator. The hanging position did not influence the density distribution in the lung tissue as determined by CT-density measurements.

Rats were given captopril in the drinking water with a daily dose of 30-60 mg/kg body mass, comparable with that given clinically. The animals (sham) treated with captopril were observed for 3 months after irradiation to determine cardiopulmonary function, structure and molecular changes as described below.

Breathing rate assay
To assess response of cardiopulmonary function to radiation, breathing rate (BR) was measured before and every two weeks after the irradiations up to week 12, as previously described 3-5.

After two training sessions, a breathing rate (BR) at rest was recorded for each rat which took place less than a week before irradiation. As described earlier 7-8, an unrestrained animal was placed in a 1500 ml airtight but transparent tube of a whole-body plethysmograph connected to a pressure transducer. The frequency of pressure changes...
inside the tube was recorded and displayed on a calibrated chart as breaths per minute (bpm). A mean BR of an animal was then calculated from minimum of 4 steady regions of the recording lasting ≥ 15 seconds. If the measurement required more than 5 minutes to obtain, the animal was let out of the tube and rested to prevent anxiety as well as drop of oxygen inside the tube. A mean BR of a dose group (bpm) with its standard error (SEM) was calculated from the means of individual animals at each time point. The increase of BR, relative to the mean BR in weeks 0–2 after irradiation, was used as an indicator of early loss of pulmonary function.

*CT imaging*

Local density changes were assessed using CT imaging (pixel size = 0.37 x 0.37 mm², slice thickness = 1 mm) 8 weeks after irradiation. The same three randomly chosen rats from each group were anesthetized with an intraperitoneal injection of Ketanest (36 mg/kg) and Rompun (6 mg/kg) and scanned in a specially designed CT holder 9. The CT images were analysed with our recently developed highly-sensitive CT analysis method 10.

*Cardiac hemodynamic measurements*

To investigate the cardiac physiological changes early after heart and/or lung irradiation, LV or RV hemodynamic measurements were carried out 8 weeks after irradiation.

For LV hemodynamic measurement, a pressure catheter was introduced via the carotid artery and guided through the aorta into the LV to record the hemodynamic parameters by monitoring the LV pressure-volume loop (n=9 per group). The recorded parameters included LV end-diastolic and end-systolic pressure (LVEDP and LVESP), dp/dtmax (maximum rate of LV pressure change), dp/dtmin (minimum rate of LV pressure change) and Tau (LV relaxation constant).

For RV hemodynamic measurement, a fluid-filled catheter with the pressure transducer was induced via right external jugular vein into the RV cavity and guided into the pulmonary artery under pressure waveform monitoring to record the right ventricle pressure (RVP) and pulmonary artery pressure (PAP) (n=4 per group). All procedures were carried out while the rats were anesthetized (isoflurane) and ventilated with room air. After completion of the measurements, the thorax was opened and heart and lungs were examined in situ for overt abnormalities, such as pericardial and pleural effusion. Heart and lungs were then excised for further histological analysis. The heart was divided into atria, ventricles and intraventricular septum (IVS) and weighed separately to assess LV and RV hypertrophy (LVH and RVH). RVH was assessed by measuring the ratio of RV weight to IVS plus LV weight.

*Histopathology*

Specimens of the LV, RV and IVS were separately fixed in 4% formaldehyde in PBS (pH 7.3). The lungs were inflated by intratracheal infusion of 4% buffered formaldehyde under a hydrostatic pressure of 20cm H₂O. The trachea was tied and the entire specimen was immersed in 4% buffered formaldehyde for overnight fixation. Samples were taken from irradiated and shielded lung (at a distance of at least 3 mm from the irradiated field edge) 8 weeks post-irradiation. The heart and lung samples were embedded in paraffin.
Subsequently, the lung and heart tissue sections (5 respectively 3 μm thick) were stained with Haematoxylin & Eosin (HE) and Masson’s Trichrome (MT) and analysed morphologically for signs of parenchymal and vascular damage or cardiac damage, respectively, as described hereunder.

Using HE staining, pulmonary parenchymal inflammation and infiltration was scored from 0-3 and 0-2 on the basis of the number of inflammatory cells and the number of infiltrates in the lung parenchyma, respectively. For interstitial edema, using HE staining, scores from 0-3 were given reflecting the severity of the damage to the interstitial tissue and the amount of affected tissue with/without interstitial infiltrate. For fibrosis, MT staining was used. Pulmonary parenchymal fibrosis was scored 0-3 based on the amount of deposited collagen.

To measure cardiac perivascular and interstitial fibrosis, MT staining was performed on paraffin sections for all experimental animals (n ≥ 3-4). Perivascular fibrosis was quantified by calculating the ratio of the fibrosis area surrounding the vessel to the total vessel area using Scanscope, Aperio Technologies, Vista, CA, USA. For this quantification, at least 20 vessels were examined and depending on the amount of vessels the average results per group were obtained from at least 4 rat LVs as described before11. To measure interstitial fibrosis, whole stained sections were scanned (tissue FACS) and fibrosis score for the entire section was calculated (Scanscope, Aperio Technologies, Vista, CA, USA).

For morphometric analysis of vascular dimensions, lung sections were stained with Verhoeff’s elastic stain and analysed according to a protocol described by van Albada et al.12. In short, 40 randomly chosen pulmonary arterioles <50 μm were assessed at 400x magnification using an image analysis system (CZ KS400; Imaging Associates, Bicester, UK). Two different vascular areas were defined: outer vessel area, luminal area. The outer vessel area was defined as the area within the external elastic lamina. The area within the internal elastic lamina was defined as the luminal area. Areas were transformed into diameters and subsequent wall thickness was defined by subtracting the external diameter from luminal diameter. Occlusion was calculated in these pulmonary vessels according to the following formula: (outer vessel diameter - luminal diameter)/(outer vessel diameter). Pulmonary arterioles were excluded from measurement if the ratio of longest and shortest diameter exceeded 2, an incomplete circular shape or a collapse of more than one quarter of the vessel wall.

Quantitative polymerase chain reaction (QPCR)

Total RNA was extracted from the LVs using the Stratagene Absolutely RNA Miniprep kit and cDNA was generated subsequently using the M-MLV Reverse Transcriptase kit (Invitrogen) according to the manufacturers’ protocols. Biorad IQTM SYBR® Green Supermix was used to perform amplifications in a Bio-Rad IQ iCycler machine as described by the manufacturer. The following primers were used to amplify ANP and BNP: 5’-TTTGGAGGACAAGATGCCT and 5’-CCCAATCCACTCTGGGCT respectively 5’-AGCTGTTGGACCGTCTACGA and 5’-TTGCAGCCAGCCACTGA. The mRNA levels were normalized using 36B4 (ribosomal) primers with the sequences: 5’-GTTGCCTCAGTGCTCACTC and 5’-GCAGCGCAAATGCAGATGG.
Statistical analysis
Data are expressed as mean values (means +/- SEM). Statistical analyses were performed using the two-way repeated measures ANOVA followed by the post-hoc Tukey or Bonferroni correction for multiple comparisons and the log-rank (Mantel-Cox) to compare distributions of two groups. Correlational analyses were performed using the Pearson correlation coefficient. In all statistical analyses, significance was defined as $P < 0.05$.

Supplementary Fig. 1: Isodose contours and anatomy. Colored areas indicate dose, the solid and dashed contour indicate the outlines of heart and lung, respectively.
Supplementary Fig. 2: Left ventricular hemodynamics of non-irradiated and HIR animals with and without captopril treatment. Overall, the morphologic differences were not accompanied by hemodynamic disturbances, suggesting early remodelling, not overt heart failure. (A) dPdtmax corrected for LV end-systolic pressure (LVESP). (B) dPdtmin corrected for LVESP. (C) Systemic systolic blood pressure. (D) Systemic diastolic blood pressure. (E) Left ventricular weight, relative to body weights.
Supplementary Fig. 3: Irradiated hearts have increased LV expression of ANP and BNP mRNA levels, which are not reduced by captopril. (A), (B) Heart irradiation leads to increased ANP and BNP mRNA levels in the LVs. Captopril treatment decreased ANP and BNP mRNA levels after HIR, however not significantly. Data are presented ±SEM; n = 9.

Supplementary Fig. 4: Experimental set-up. 50% rat lungs (LIR), heart (HIR) or both (LHIR) were irradiated to 20 Gy. Captopril treatment was started immediately after irradiation until 12 weeks post-irradiation (PI). To assess cardiopulmonary function, biweekly breathing rate measurements were performed until 12 weeks PI. At 8 weeks, the peak of early radiation induced cardiopulmonary dysfunction, hemodynamic measurements, CT scans, cardiac remodelling analysis, histologic analysis and qPCR were performed. BR: breathing rate, PI: post-irradiation, L: lung, H: heart, IR: irradiation.
Supplementary Fig. 5: Breathing rate (BR) increase until 12 weeks after LIR, HIR and LHIR with or without captopril treatment. (A) No difference between the BR increase of the LIR animals treated with or without captopril was observed. (B) HIR does not alter increased BR nor does HIR with captopril. HLIR lead to an enhanced BR increase, captopril decreases the BR increase similarly to the BR level of LIR rats. Data are presented ±SEM; n = 9.
Supplementary Fig. 6: Right ventricle hemodynamics after LIR, HIR and LHIR with or without captopril treatment. (A) Systolic, diastolic and mean right ventricle pressures. (B) Mean right ventricle pressures. (C) Systolic, diastolic and mean pulmonary artery pressures. (D) Mean pulmonary artery pressures. Data are presented ±SEM; n = 9.
Supplementary references


