Pulmonary toxicity in a rabbit model of stereotactic lung radiation therapy: Efficacy of a radioprotector

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ABSTRACT

This study aimed to assess the efficacy of the radioprotector amifostine in limiting radiation toxicity in a rabbit model of lung stereotactic body radiation therapy (SBRT) by correlating contrast-enhanced magnetic resonance angiography (ce-MRA), computed tomography (CT), and helium-3 (He-3) magnetic resonance imaging (MRI) with histopathology. Multiple MRI techniques were tested to obtain complementing physiologic information. Thirteen rabbits received SBRT to the right lower lobe of the lung. Specifically, 4 received 3 × 11 Gray (Gy), 6 received 3 × 11 Gy and 50 mg/kg of amifostine pre-SBRT, and 3 received 3 × 7, 3 × 9, or 3 × 13 Gy. Imaging was performed at baseline and 4, 8, 12, and 16 weeks post-SBRT. Ce-MRA perfusion difference between lungs in the irradiated group at 16 weeks post-treatment was statistically significant (P = .04) whereas the difference in the irradiated + amifostine group was not (P = .30). Histologically observed low red blood cell (RBC) count and CT hypodensity suggests changes were primarily related to perfusion; however, structural changes, such as increased alveolar size, were also present. No changes in He-3 MRI lung ventilation were observed in either group. Although radiation-induced injury detected in rabbits as CT hypodensity contrasted with increased density observed in humans/rodents, the changes in ce-MRA and CT were still significantly reduced after the addition of amifostine to SBRT. Use of CT and selected MRI techniques helped to pinpoint primary physiologic changes.

KEYWORDS amifostine, lung radiation, radioprotector

INTRODUCTION

Radiotherapy in combination with chemotherapy is the standard of care for patients with non-resected, advanced-stage lung tumors. However, the outcome of treatment is unsatisfactory, with a local control rate of approximately 30%. The use of higher doses has proved to be essential to significantly improve the treatment outcome [1, 2], but high radiation dose is associated with severe radiation toxicities to surrounding normal organs. To make radiotherapy more tolerable and reduce the injury to these normal organs, numerous radioprotectors have been experimented with. Among them, amifostine ([(s-2(3-aminopropylamino)ethyl-phosphorothioic-acid)] is the only Food And Drug Administration (FDA)-approved drug for protection from radiation [3]. Amifostine is a thiophosphate molecule, which is enzymatically hydrolyzed by alkaline phosphatase to an active thiol metabolite at the tissue site [4]. This metabolite is responsible for the radioprotective action of the compound by scavenging free radicals and detoxifying other reactive metabolites at the cellular level [5]. Partial lung irradiation in rats been shown to cause an increase in inflammatory cytokines, which are responsible for elevated free radicals and subsequent DNA damage [6]. The selective protection offered to normal cells compared with tumor cells is not completely understood; however, it has been proposed that the higher alkaline phosphatase activity and higher pH in normal cells facilitates more rapid uptake of the radioprotector [7]. Amifostine has been clinically tested by several

Received 18 February 2014; accepted 22 April 2014.

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groups, with a strong consensus demonstrating the favorable protection of normal cells [8]. In addition, there is a dependence on the levels of tissue oxygenation, which reduces the effectiveness of this drug in tumor cells [9–11]. Although the primary use of amifostine is to protect patients with head and neck cancer in radiotherapy, its capabilities were also tested in patients undergoing radiotherapy for lung cancer. Several non-randomized clinical trials have demonstrated the capability of amifostine to reduce the severity of lung injury after radiotherapy [12–13]. Rat studies have shown increased computed tomography (CT) density after radiation treatment, presumably due to fibrosis and pneumonitis [14]. In addition, radiation-induced fibrosis has been investigated in rats using hyperpolarized helium-3 (He-3) magnetic resonance (MR) imaging [15]. Moreover, amifostine was shown to increase the threshold of the radiation dose that leads to loss of lung function [16]. However, the side effects and inconveniences caused to patients by drug administration limits the wide application of amifostine in radiotherapy. Lung radiotherapy on a conventional schedule requires drug administration on a daily basis for weeks. Dose escalation using conventional fractionation will unavoidably lengthen the treatment and increase the dosage of amifostine and subsequent side-effects.

An alternative method to deliver much higher doses to the lung tumor in fewer fractions is by stereotactic body radiotherapy (SBRT). In clinical administration of SBRT, the entire treatment dose of 40–60 Gray (Gy) is delivered in 3–5 fractions, resulting in an extremely high biologic equivalent dose (BED). SBRT has been demonstrated to be a highly effective treatment for stage-I non-small cell lung cancer (NSCLC), with reports of over 90% control in small localized lung tumors in patients [17–18]. Although severe toxicity associated with these treatments is infrequent due to the small size of lesion, complications including radiation pneumonitis and esophagitis can occur with larger or centrally located tumors [19–21]. Application of a radioprotector, such as amifostine, may reduce the incidence and severity of toxic reactions while taking advantage of fewer fractions of treatment and, thus, lower dosage of the drug.

Although amifostine was investigated extensively in conjunction with conventionally fractioned radiotherapy, the efficacy of amifostine in SBRT has not been reported. An animal model is, therefore, necessary to investigate the feasibility. We have previously established an animal SBRT model using rabbits and tomotherapy [22]. In that model, a small volume (∼1.6 cc) of the rabbit lung was treated to 60 Gy in 3 fractions. The model, without irradiating the entire thoracic space, is a close simulation of human SBRT treatment. In this study, the same model is adapted to investigate the efficacy of amifostine in SBRT.

**MATERIALS AND METHODS**

**Subject Distribution and Stereotactic Lung Radiosurgery**

Thirteen healthy, New Zealand, white, female rabbits (∼3.5 ± 0.2 Kg; ∼6 MO; Burleson Inc, Morrisville, North Carolina, United States) were used and distributed randomly into 3 subgroups, as shown in Table 1. Rabbits were tested for potential diseases when acquired from an accredited supplier and isolated in individual cages during the entire study. Animals were monitored daily by experienced veterinary personnel for any signs of disease, stress, or discomfort. Weekly body weight and temperature measurements were performed and the strict guidelines from the local Animal Care and User Committee were followed, as approved by the study protocol. Prior to each of the radiation and imaging procedures described further, the animals were anesthetized with an injection of ketamine–xylazine (50 mg/kg–5mg/kg; Butler Schein Animal Health, Inc., Dublin, Ohio, United States).

A pilot study was first conducted to determine the threshold radiation dose to produce detectable injury using a time-resolved contrast-enhanced magnetic resonance angiography (ce-MRA) technique. Four rabbits received doses in 3 fractions of 7, 9, 11, or 13 Gy within a week, administered 3–4 days apart, at the anatomic location illustrated in Figure 1. A threshold dose of 3 × 9 Gy was determined from the pilot study; thus, 9 rabbits were irradiated with 3 × 11 Gy within a week. Six of the rabbits received 50 mg/kg of the radioprotector (Amifostine, Med-Immune, Gaithersburg, Maryland, United States).

**TABLE 1. Subject Distribution**

<table>
<thead>
<tr>
<th>Radiation Dose [Gy]</th>
<th>Radioprotector [mg/kg]</th>
<th>Number of animals</th>
<th>Subgroup</th>
</tr>
</thead>
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<tr>
<td>3 × 7</td>
<td>0</td>
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<td>Dose escalation</td>
</tr>
<tr>
<td>3 × 9</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 × 11</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 × 13</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 × 11</td>
<td>0</td>
<td>3 + 1*</td>
<td>Control</td>
</tr>
<tr>
<td>3 × 11</td>
<td>3 × 50</td>
<td>6</td>
<td>Radioprotector</td>
</tr>
</tbody>
</table>

*One rabbit is common between the dose escalation and control subgroups.
FIGURE 1. Top row: Treatment plan (radiation dose, $3 \times 11$ Gy) superimposed on 3-dimensional CT images (A: axial; B: coronal, and C: sagittal view). Different colors represent percentage of dose delivered, with red at the center being 100% of the dose; green and blue are 75% and 50%, respectively. Exemplary imaging results from 3 animals at 8-weeks post-treatment, including: CT (second row), first-pass ce-MRA (third row), high-resolution ce-MRA (fourth row), and He-3 ventilation images (bottom row). The effects of radiation treatment in the right lung of the irradiated rabbit are clearly depicted by the hypodense area (white arrow) in the CT image, and the matching perfusion defects (white arrow) in the first-pass ce-MRA and high-resolution ce-MRA images (white arrow) (right column). Irradiation + amifostine rabbits showed no noticeable defect in CT, first-pass ce-MRA or high-resolution ce-MRA (left and center column). Ventilation was homogeneous in all animals.
intravenously 20 minutes before each stereotactic radiation dose. A single cylindrical target (∼1.6 × 1.6 × 1.6 cm³) was irradiated using helical tomotherapy (TomoTherapy, Madison, Wisconsin, United States) as described previously [22].

**MR Perfusion Imaging**

Time-resolved, first-pass ce-MRA and high-resolution ce-MRA were performed using a 1.5-Tesla clinical scanner (Sonata, Siemens Medical Solutions, Malvern, Pennsylvania, United States) at baseline and 4, 8, 12, and 16 weeks post radiotherapy. The time-resolved technique allowed visualization of small perfusion delays caused, for example, by shunting of pulmonary capillary flow. The high-resolution ce-MRA showed narrowing of larger vessels as well as non-transitory perfusion deficits. All acquisitions were performed using a standard Siemens birdcage head-coil. The animals were anesthetized but breathing spontaneously.

For both techniques, Three Dimensional Fast-Low-Angle-Shot (3D-FLASH) pulse sequences were used with the parameters listed in Table 2. Two seconds after the beginning of the first-pass pulse sequence, a 3-cc intravenous injection of a gadolinium chelate (0.5 cc/kg, Omniscan, GE/Nycomed, Oslo, Norway) was administered. Each image was subtracted automatically from the corresponding image acquired during the first measurement. Maximum intensity projection (MIP) images corresponding to each of the subsequent time-resolved measurements were generated for perfusion visualization and evaluation. Immediately after the first-pass ce-MRA acquisition, the high-resolution ce-MRA data set was acquired.

In order to determine the repeatability of the first-pass ce-MRA technique, 3 of the animals were scanned twice within a week, and the perfused areas of their lungs were analyzed by the same blinded reviewer and compared quantitatively.

**Computed Tomography Imaging**

All animals had a CT scan of their lungs within 1 week of each MRI session (baseline and 4, 8, 12 and 16 weeks post-SBRT), performed using a Philips Brilliance 16-slice CT scanner (Philips Healthcare, Best, Netherlands). All images were obtained with an in-plane resolution of 0.59 × 0.59 mm² and a slice thickness of 1.5 mm.

**MR Ventilation Imaging**

Hyperpolarized He-3 is a gaseous MRI contrast agent that provides unique ways to evaluate lung structure and function in vivo [23–25]. Ventilation imaging, wherein the spin-density of He-3 gas is imaged following inhalation and suspended respiration, permits assessment of the distribution of inhaled gas. Areas of the lung for which ventilation is reduced or absent appear as regions of lower or no MR signal, respectively. He-3 was polarized in a commercial system (IGI9600, MITI, Durham, North-Carolina, United States) via the spin-exchange method, as described previously [26].

Ventilation imaging was performed on the same MR scanner described earlier for the MR perfusion studies, and used a gradient-echo-based pulse sequence.

Each animal was anesthetized and scanned with He-3 at baseline and 4, 8, 12 and 16 weeks post radiation treatment. At each time point, 3 contiguous coronal images covering the entire lung volume were acquired with a resolution of 1.8 × 1.8 × 20 mm³ and repetition time (TR)/echo time (TE) was 12/6.8 ms.

**Histopathology**

At week 16, the animals were euthanized and their lungs and trachea were harvested in a single block through an anterior midline incision parallel to the sternum. Lungs were preserved in a formalin bath for 24 hours at constant pressure. Subsequently, tissue samples from 6 different regions of each lung were carefully excised. Mean chord length (MCL) measurements were used to quantify alveolar size difference. Preparation of the microscope slides with the histologic sections, as well as the method and algorithm used to analyze the MCL values of the lung alveoli, have been described previously [27].

**Data Analysis**

Data from the first-pass ce-MRA acquisitions was analyzed on a Siemens image-analysis workstation using

<table>
<thead>
<tr>
<th>TABLE 2. 3D FLASH Pulse Sequence Parameters</th>
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<tbody>
<tr>
<td><strong>First-Pass MRA</strong></td>
</tr>
<tr>
<td>Matrix</td>
</tr>
<tr>
<td>Voxel [mm³]</td>
</tr>
<tr>
<td>Flip angle [°]</td>
</tr>
<tr>
<td>Slices per slab</td>
</tr>
<tr>
<td>Bandwidth [Hz/pixel]</td>
</tr>
<tr>
<td>TR/TE [ms]</td>
</tr>
<tr>
<td>Measurements</td>
</tr>
<tr>
<td>Acquisition time [s]</td>
</tr>
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the Argus software package. The irradiated area of the perfused right lung and the whole left lung were traced manually by a blinded reviewer on the first MIP image that showed contrast reaching the bottom of the lower lobe of the left lung. After manual tracing, the Argus software was used to automatically perform a temporal analysis; the software corrected the positions of the selected regions-of-interest for any shifts due to respiratory motion.

CT images were analyzed qualitatively by visual assessment of any changes in the tissue densities, and quantitatively by manually tracing each lung and determining the corresponding mean and standard deviation in Hounsfield units (HU) using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, United States).

The ventilation MR images were analyzed qualitatively by visual assessment of any ventilation defects and quantitatively by calculating the total volume of the lung as described previously [24,25]. The lung volume calculation used an algorithm developed in-house with MATLAB software (MathWorks, Natick, Massachusetts, United States) [24,25].

For the various metrics calculated, the Mann–Whitney two-tailed U-test [28] was used to assess the significance of changes over time and differences between the right and left lung. For all statistical comparisons, the threshold for significance was set to \( P = .05 \).

RESULTS

MR Perfusion Imaging

The ce-MRA repeatability studies performed in 3 animals yielded a non-statistically significant mean percentage change (±standard deviation) of 5.7 ± 1.6% and 7.6 ± 10.9% for the right and left lung, respectively (\( P = .13 \)).

The ratios (irradiated + amifostine/irradiated) of the mean areas of perfused lung tissue for all animals and time points are shown in Figure 2. The difference in mean perfused areas between the right and left lung was statistically significant (\( P = .04 \)), whereas that for the irradiated + amifostine group was not (\( P = .30 \)). Furthermore, the very small lung perfusion defects in the irradiated area of irradiated + amifostine group in comparison with obvious perfusion defects shown in the irradiated group (Figure 1) might also be an indication of the radioprotective effect of this substance.

Computed Tomography Imaging

The ratios (irradiated + amifostine/irradiated) of the mean HU values for all animals and time points are shown in Figure 3. At the final time point, the difference of the HU values between the right and left lungs was marginally statistically significant for the irradiated group (\( P = .06 \)) or for the irradiated + amifostine group (\( P = .68 \)); however, a clear trend was observed for the irradiated group.

MR Ventilation Imaging

Ventilation images were obtained for all animals at each time point. In all cases and at each time point, homogeneous ventilation was observed and no ventilation defects were detected (Figure 1). At baseline, the mean lung volumes calculated from the ventilation images were 162 ± 31 cm\(^3\) for the irradiated group and 139 ± 37 cm\(^3\) for the irradiated + amifostine group. The mean lung volumes remained approximately constant during the entire study, with values for the irradiated and irradiated + amifostine groups, respectively, of 146 ± 11 cm\(^3\) and 135 ± 30 cm\(^3\) at 4 weeks post, 154 ± 13 cm\(^3\) and 140 ±
FIGURE 3. Ratios (irradiated + amifostine/irradiated) of the mean Hounsfield units (HU) versus study time calculated from the CT data (radiation dose, 3 × 11 Gy). Error bars indicate the standard error of the mean (SEM) HU for each time point for N = 10 independent experiments. At baseline, the values for the right and left lungs were identical. At 4 weeks post treatment, the right lung ratio value showed a 17% decline, whereas the left lung ratio increased by 12%. The difference in the ratio values between right lung and left lung at the final time point was 16%, with left lung ratio values identical to that at baseline. (RL, right lung; LL, left lung).

14 cm³ at 8 weeks post, 133 ± 40 cm³ and 142 ± 17 cm³ at 12 weeks post, and 146 ± 6 cm³ and 160 ± 29 cm³ at 16 weeks post therapy.

Histopathology
The MCL value for the areas that received radiation (lower lobe, right lung) in the irradiated group was 37.0 ± 12.8 μm, whereas that for the whole left lung of the irradiated group was 24.3 ± 1.7 μm. The difference in MCL values between the 2 lungs was statistically significant (P < .05). The corresponding MCL values for the irradiated + amifostine group were 33.6 ± 4.1 μm (lower lobe, right lung) and 22.0 ± 4.3 μm (left lung). These differences between the 2 lungs were also statistically significant (P < .05). Examples of changes in the lung tissue samples are presented in Figure 4. Changes in the alveolar microstructure were also assessed during the histologic analysis. In animals from the irradiated group, the irradiated areas presented mild alveolar wall destruction as compared with other areas of the same lung or with the corresponding area of the left lung in the same animal. These changes in the alveolar structure could also be observed and quantified in CT images (Figures 1, 3, 4) as areas of lower tissue density. None of the animals showed any symptomatic side effects associated with radiation toxicity.

DISCUSSION
Lung SBRT is shown to be a promising treatment for early-stage lung cancer with high local control rates and relatively low complication rates. However, to treat larger and more centrally located tumors, radiation-induced pneumonitis can be a rate-limiting factor [19–21]. No histologic hallmarks of pneumonitis were observed in this study. However, this may have been due to the short follow-up post treatment. Previous studies of SBRT using rodent animal models have reported pneumonitis developing within 12–16 weeks post treatment [29]. It is possible that the V/Q mismatch observed in the rabbits is indicative of early pneumonitis. Clinical studies using CT have shown that patients with interstitial pneumonia had high V/Q mismatch areas that corresponded to cystic air spaces [30].
Multiple SBRT clinical trials report treating patients with total doses of 40–60 Gy in 3–5 fractions [17, 31, 32], and, thus, our total experimental dose of 33 Gy represents the 55%–75% isodose-lines of patients treated clinically with SBRT. These isodose-lines can encompass large normal lung volumes in patients undergoing SBRT, especially if tumors are large and located centrally or in the lower lobes of the lung. Therefore, any radioprotector that can reduce radiation-induced lung injury in this clinically relevant dose range could reduce the toxicity for patients who meet the current eligibility criteria for lung SBRT, and could potentially expand the eligibility criteria to larger and/or more numerous lung tumors.

To improve the local control rate for these patients using SBRT, normal tissue needs to be better spared from the aggressive regimen. Amifostine as an FDA-approved radioprotector was tested in several limited lung trials, including 1 with daily doses up to 3.5 Gy, but its efficacy in highly hypofractionated treatment with daily doses of 8 Gy or higher was not demonstrated [13]. To explore the feasibility of protecting lungs from radiation in an SBRT regimen, an animal study was conducted. One difficulty in performing this study was the lack of a small, tumor-type-defined target volume capable of receiving a uniform radiation dose in these conventional animal experiments. To overcome this difficulty, we showed that precise dose delivery to a sub-volume of the rabbit lung is achievable using helical tomotherapy [22]. Using this platform, we studied the efficacy of amifostine in a manner more closely emulating human treatments. We determined that the minimum dose for radiation-induced lung injury identifiable by MR and CT imaging within a few weeks was approximately 3 × 9 Gy fractions in our rabbit model. We chose 3 × 11 Gy fractions to ensure that all control (irradiated) animals would have a detectable radiation-induced lung injury while also minimizing complications associated with radiation toxicity. Additional studies using higher, more clinically relevant doses (up to 60 Gy in 3–5 fractions) will be required to understand the value of amifostine in patients.

Consistent with previous reports, it was shown that, compared with ventilation, lung perfusion changes are more sensitive to the SBRT dose. Endothelial cells ablated by radiation may contribute to the remarkable decrease in perfusion as a characteristic response to the SBRT dose [32, 33]. Vascular damage and changes observed in rabbits without amifostine within this study are similar to those found in previous studies completed on rats without the use of radioprotectors [34]. Early changes in the vasculature is a well-known mechanism of radiation damage to the tissue [35]. Most importantly, amifostine is shown to be effective in reducing the severity of lung tissue injury, measured by both the serial perfusion and CT measurements. Previous studies have also shown that amifostine acts as a free-radical scavenger that protects the tissue from cytotoxic effects of ionizing radiation [36, 37].

Despite the positive results provided in this work, there are limitations which preclude extrapolation to human patients at this time. Although the difference between irradiated + amifostine and irradiated groups is significant, the statistical power is relatively low due to the limited sample size tested. A larger scale animal study, possibly based on mice, and a state-of-the-art small-animal irradiator such as the small animal radiation research platform (SARRP) would allow for more rigorous testing than that provided by this study [38]. Moreover, additional study arms, like that presented by Vujaskovic and colleagues, including a lung tumor model and a sham control group will be necessary to determine the full efficacy of amifostine with SBRT in a clinical scenario [39, 40]. Nevertheless, our study shows the feasibility of amifostine use for radiation protection in rabbits treated with SBRT above the threshold for radiologic appreciation. The potential clinical benefit of amifostine protection during SBRT could result in improved tumor control probability for an expanded patient population. The fewer doses required for amifostine in SBRT treatment would further facilitate its clinical application.

**CONCLUSION**

Our data from CT and multiple MR-based techniques showed the beneficial effect that a clinically used radioprotector, in this case amifostine, may have in the reduction of early radiotherapy toxicity. Specifically, our rabbit model of SBRT-induced lung injury showed that 50 mg/kg of intravenous amifostine significantly reduced radiation-induced perfusion deficits in the lung following 3 × 11 Gy fractions. The dose of 50 mg/kg intravenously is lower than that reported in preclinical animal trials, which administered 200 mg/kg of amifostine intraperitoneally [36]. In this study, we also found no significant changes in ventilation, which is consistent with our previous study which used doses as high as 3 × 20 Gy [22]. For the early detection of pulmonary radiation injury, ce-MRA showed good correlation with CT and sensitivity at least as high as CT, which is the gold standard clinical modality for lung cancer. Although more work is needed to translate this data to the clinical application of amifostine use in SBRT,
we are encouraged by the potentially correlative perfusion protection we observed.

Declaration of interest: This work was supported in part by a local Cancer Center grant (CCSG) and by Siemens Medical Solutions. Sponsors had no involvement in the study design, data collection and analysis, interpretation of data or with this manuscript.

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