Spinal cord biological safety of image-guided radiation therapy versus conventional radiation therapy☆

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Abstract
Tumor models were simulated in purebred Beagles at the T9-10 levels of the spinal cord and treated with spinal image-guided radiation therapy or conventional radiation therapy with 50 or 70 Gy total radiation. Three months after radiation, neuronal injury at the T9-10 levels was observed, including reversible injury induced by spinal image-guided radiation therapy and apoptosis induced by conventional radiation therapy. The number of apoptotic cells and expression of the proapoptotic protein Fas were significantly reduced, but expression of the anti-apoptotic protein heat shock protein 70 was significantly increased after image-guided radiation therapy compared with the conventional method of the same radiation dose. Moreover, the spinal cord cell apoptotic index positively correlated with the ratio of Fas/heat shock protein 70. These findings indicate that 3 months of radiation therapy can induce a late response in the spinal cord to radiation therapy; image-guided radiation therapy is safer and results in less neuronal injury compared with conventional radiation therapy.

Key Words
Image-guided radiation therapy; conventional radiation therapy; spinal cord; neurons; apoptosis; Fas; heat shock protein 70; biological safety; vertebral body; tumor

Research Highlights
(1) This study used dog T9-10 to simulate a vertebral body invaded by tumor.
(2) Results of pathology, ultrastructure, neuronal apoptosis, Fas and heat shock protein 70 expression showed that image-guided radiation therapy is biologically safer than conventional radiation therapy.

Abbreviations
IGRT, image-guided radiation therapy; HSP70, heat shock protein 70

INTRODUCTION
The control and treatment of vertebral column tumors remain difficult. Image-guided radiation therapy (IGRT) has been extensively used in clinical tumors because of its pronounced physical advantages[11]. Spinal IGRT is a four-dimensional radiation therapy technology. Based on three-dimensional radiation, IGRT also considers movement of the vertebral column metastatic tumor during treatment and displacement errors of interval procedures, such as changes in radiation therapy dose distribution induced by respiratory movement, peristaltic movement, daily setup error, target area
contraction, as well as the influence of treatment procedures. Prior to and during treatment, the vertebral column metastatic tumor and spinal cord are monitored in real-time using advanced imaging equipment, and treatment conditions are adjusted according to organ position changes to allow the radiation to follow the tumor, thereby preventing radiation-induced spinal cord injury\(^2\). However, no basic animal studies have documented the safety of spinal IGRT\(^3\).

In this study, we conducted spinal IGRT on dogs and compared to conventional radiation therapy to evaluate the safety of IGRT on spinal cord neurons through observing spinal cord neuronal pathology, ultrastructural changes, apoptotic index, and the expressions of the proapoptotic protein Fas and the anti-apoptotic protein heat shock protein 70 (HSP70).

**RESULTS**

**Quantitative analysis of experimental animals**

A total of 40 Beagles were selected and subjected to spinal IGRT at the T9-10 levels \((n = 20)\) or conventional radiation therapy \((n = 20)\) receiving dosages of 50 or 70 Gy, with 10 dogs for each dose. The 40 dogs were included in the result analysis.

**Pathological changes in injured spinal cord tissues at the T9-10 levels**

Hematoxylin-eosin staining showed that after conventional radiation therapy, a large number of apoptotic cells were observed, some neurons lost their polarized morphology, Nissl bodies were disrupted or disappeared, and nuclei contracted, fragmented, or dissolved; moreover, the structure of some cells was obscure, and the nuclear and plasma membranes were disintegrated. In the IGRT groups, most neurons displayed normal appearance, with reversible injuries, and few apoptotic cells were observed; however, the polar appearance had disappeared in some neurons, Nissl bodies were lightly stained, and nuclei were swelling and located laterally (Figure 1).

**Ultrastructural changes of injured spinal cord tissue**

Transmission electron microscopy revealed the following in the conventional radiation therapy groups: sparse cytoplasm, swollen mitochondria, broken or vacuolated cristae, expanded rough endoplasmic reticulum, degranulation, widened nuclear membrane space, pyknotic or disintegrated nuclei, pyknotic and condensed chromatin at the nuclear membrane, crescent or ring-shaped morphologies and apoptotic cells. While in the IGRT groups, the following was observed: the appearance of most neurons, cell organs and nuclei were normal, although some neurons had mildly swollen mitochondria (Figure 2).

**Cell apoptosis in injured segments of spinal cord**

TUNEL staining showed that the nuclei of normal spinal...
neurons were stained blue, but those of apoptotic neurons were brown (Figure 3). Statistical analysis showed that with increasing radiation dose, the apoptotic index increased ($P < 0.01$); and under the same radiation dose, the apoptotic index was significantly reduced in the IGRT groups compared with the conventional radiation therapy groups ($P < 0.01$; Table 1).

Fas and HSP70 expression in the injured segments of spinal cord

Fas-positive products, stained brown-red or brown-yellow, were observed in the cytoplasm and nuclei of neurons in the anterior horn of the spinal cord, but the nuclei were either mildly or had no staining (Figure 4). HSP70-positive products, stained brown-red or brown-yellow, were mainly observed in the cytoplasm and nuclei of neurons in the anterior horn of the spinal cord (Figure 5).

Immunohistochemistry showed that with increasing radiation dose, Fas expression was increased, but HSP70 expression decreased ($P < 0.01$); under the same radiation dose, Fas expression was less in the IGRT groups compared with conventional radiation therapy groups ($P < 0.01$), but HSP70 expression was greater than in the conventional radiation therapy groups ($P < 0.01$; Table 2).

### Table 1  Apoptotic index (%) of cells in the spinal cord tissue following image-guided radiation therapy and conventional radiation therapy

| Group                        | Radiation dose (Gy) | 50            | 70            |
|------------------------------|--------------------|---------------|---------------|
| Conventional radiation therapy | 7.7±1.0            | 12.0±1.1*     |               |
| Image-guided radiation therapy | 0.9±0.6a           | 2.5±0.9ab     |               |

Results are expressed as mean ± SD of 10 dogs in each group at each radiation dose. *$P < 0.01$, vs. conventional radiation therapy group; **$P < 0.01$, vs. 50 Gy group (t-test). Cell apoptotic index (%) = mean absorbance of TUNEL-positive cells × percentage of TUNEL-positive cells × 100%.
Table 2  Fas and heat shock protein 70 (HSP70) expression (percentage of positive area) in the spinal cord tissue following image-guided radiation therapy and conventional radiation therapy

| Group          | Fas       | HSP70     |
|----------------|-----------|-----------|
|                | 50 Gy     | 70 Gy     | 50 Gy     | 70 Gy     |
| Conventional radiation therapy | 19.8±5.0  | 21.9±7.1  | 2.7±0.6   | 1.6±0.3   |
| Image-guided radiation therapy   | 2.5±1.2   | 4.1±1.4   | 10.7±1.1  | 8.4±1.0   |

Results are expressed as mean ± SD of 10 dogs in each group at each radiation dose. *P < 0.01, vs. conventional radiation therapy group; **P < 0.01, vs. 50 Gy group (t-test). Fas and HSP70 expression was represented by the mean percentage of the positive area in the field of view. Larger area indicates greater protein expression.

Correlation of Fas, HSP70 and Fas/HSP70 with cell apoptosis in the injured spinal cord tissue

Pearson correlation analysis showed that spinal neuron apoptotic index was positively correlated with Fas expression (r = 0.498, P < 0.05; y = -1.239 + 0.491x) and the ratio of Fas/HSP70 (r = 0.981, P < 0.05; y = 1.423 + 0.796x), but negatively correlated with HSP70 (r = -0.696, P < 0.05; y = 5.827–1.395x).

DISCUSSION

A large number of studies have examined radiation-induced cell injury using in vitro cultured cells[3]; however, large mammals have much greater advantages for performing these analyses. This study used Beagles as subjects, which have similar anatomic and tissue structures for skeleton, muscle and nerves as human, and exhibit similar pathological and clinical manifestations of spinal cord injury[4]. To guarantee precise stereotaxis and position fixation during IGRT, we simulated the commonly used human stereotactic facial mask by designing a body membrane fixation frisket for a dog to maintain the spinal cord position during IGRT and allow precise target region during radiation[5-6].

Radiation myelopathy, also called radiation myelitis, is a disease caused by neuronal degeneration and necrosis due to radiographic exposure in combination with various other factors. The occurrence of radiation myelopathy is associated with exposure of the spinal cord to large-dose radiation[3]. Baumann et al[7] found that 2 years after completion of radiation treatment, with daily fractions of approximately 200 cGy, the development of radiation myelopathy was expected in 1% of the patients receiving 50 to 55 Gy; which increased to 5% in patients receiving 55 to 60 Gy. Thus, the tolerance of normal spinal cord is 40–50 Gy 4–5 times per week, and doses larger than this value may result in a higher risk of radiation myelopathy. To observe the biological safety of spinal IGRT on dog spinal cord neurons, we used 50 and 70 Gy, thereby exceeding the tolerance dose, in dogs. Results showed that the degree of injury to neurons because of IGRT was significantly lower than conventional radiation therapy. Moreover, the cell injury after IGRT was mostly reversible, whereas irreversible apoptosis occurred following conventional radiation therapy, consistent with previous findings[4]. In addition, apoptosis after conventional radiation therapy indicates that radiation at this dose can induce radiation myelitis, but IGRT can protect spinal cord tissues around the vertebral body. Generally, apoptosis resulted from radiation-induced DNA injury occurring several hours after radiation[3]. In the present study, after 3 months of radiation, evident cell apoptosis was present in the conventional radiation therapy group, indicating that delayed apoptosis of spinal cord neurons occurred following treatment.

Fas is a cell apoptotic signaling molecule, which rapidly triggers apoptosis after binding of its ligand FasL[8-11]. Results from the present study showed that neuronal apoptosis mediated by Fas occurred after radiation, and Fas expression and cell apoptosis exhibited a dose-effect relationship with radiation. HSP70 can improve cell tolerance to stressors, thereby preventing stress-induced apoptosis[12-14]. Results showed that HSP70 expression significantly inhibited neuronal apoptosis caused by radiation.

HSP70 can mediate apoptosis signal transduction through the Fas pathway. It has been shown that an increase in Fas expression can inhibit HSP70 expression, thereby promoting apoptosis; in contrast, high HSP70 expression can inhibit Fas-mediated apoptosis, thereby promoting cell survival. Thus, it is hypothesized that the ratio of these proteins, Fas/HSP70, determines whether a cell undergoes apoptosis in response to apoptotic signal stimulation. In the present study, the neuronal apoptotic index positively correlated with Fas expression and HSP70 ratio, but negatively correlated with Fas/HSP70 expression, demonstrating that the ratio of Fas/HSP70 is a major factor for the fate of cell apoptosis.

Results of analyses of cell morphology, apoptosis and apoptosis-related protein expression demonstrated that IGRT is safer than conventional radiation therapy. IGRT can outline a target of any shape for radiation, protect sensitive normal tissues inside or outside of the target regions, increase regional control rate, thereby preventing complications in normal tissues. The present
study provides evidence for the clinical dose of IGRT and duration of treatments for improving regional control of radiation. However, as the spinal cord is a tissue of late response, we only observed the spinal cord exposed to radiation for 3 months. Further studies could prolong the duration of observation and investigate ideal methods for outlining a target region and protecting normal tissue for better treatment of vertebral column tumors.

MATERIALS AND METHODS

Design
A randomized, controlled, animal study.

Time and setting
The experiments were performed at the Key Laboratory of Xinjiang Medical University and Tumor Hospital of Xinjiang Medical University in 2012.

Materials
A total of 40 purebred male, adult Beagles, aged 1–1.5 years, weighing 12.0 ± 1.5 kg, body length 60 ± 5 cm, were provided by the Laboratory Animal Institute of Sichuan Academy of Medical Sciences (license No. SCXK (Chuan) 2004-15). The dogs were separately housed at 20–23°C, with humidity of 40–60%, and allowed free access to food and water. The animal procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[15].

Methods
Radiation patterns
The 40 dogs were randomly assigned to groups, and T9-10 levels were confirmed as the target regions for radiation. The target region was accurately outlined using CT (CT Imaging System, Cleveland, OH, USA) assisted by computer and radiation procedures were formulated. The dogs were intravenously anesthetized and placed in the dog body membrane fixation mould, and subjected to IGRT and conventional radiation therapy (Figure 6), receiving 5 or 7 Gy (i.e. 50 or 70 Gy in total) each time, 300 cGy/min, once a day, 10 times over a 14 day period (Monday-Friday only).

Sampling and histological observation of T9-10 segments
Three months after radiation, the dogs were anesthetized and sacrificed, and T9-10 spinal cord was harvested under a microscope, immediately fixed in 10% formalin and 2.5% glutaraldehyde, stored in liquid nitrogen, paraffin embedded, and sectioned into 4 μm-thick sections, followed by routine hematoxylin-eosin staining. Spinal cord histological changes were observed by light microscopy (Leica, Wetzlar, Germany).

Transmission electron microscopy of injury spinal cord ultrastructure
Samples were washed with buffer solution, prefixed with 2.5% glutaraldehyde for 2 hours, post-fixed with 1% osmic acid for 2 hours, washed with PBS, dehydrated with an increasing gradient of alcohol and acetone, embedded, dried by baking overnight at 37°C, and sectioned at 50 nm thickness. The sections were observed and images were obtained by transmission electron microscopy (Hitachi, Hokkaido, Japan).

TUNEL detection for cell apoptosis
Spinal cord neuronal apoptosis was detected by the TUNEL method with an in situ apoptosis detection kit (Boehringer Mannheim, Berlin, Germany). The sections were dewaxed, hydrated, followed by in situ end labeling according to manufacturer’s instruction. The sections were visualized by dianaminobenzidine (Boehringer Mannheim), and mounted with neutral gum. Ten fields of view with dense apoptotic cells from each section were selected using BT-2000 color pathological imaging analysis system (Hubei Botai Electronic Technology, Wuhan, Hubei, China) to calculate neuronal apoptotic index (%) = mean absorbance of TUNEL-positive cells × percentage of TUNEL-positive cells (percentage of positive cells in total cells) × 100%[14].

Immunohistochemistry for Fas and HSP70 expression
Streptavidin biotin peroxidase complex method was used to perform immunohistochemistry according to the kit. Briefly, paraffin sections were dewaxed, hydrated, incubated with the primary antibody, rabbit anti-dog Fas or HSP70 monoclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 2 hours,
followed by the secondary antibody, goat anti-rabbit IgG (1:100; Santa Cruz Biotechnology) at 37°C for 1 hour. Ten fields of view were randomly selected from two anterior horns of each section and the positively stained area was measured automatically using the BT-2000 color pathological imaging analysis system. The mean percentage of positive staining out of the total area was used as Fas and HSP70 expression.

**Statistical analysis**
Data were analyzed using SPSS version 19.0 (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Intergroup differences were compared utilizing t-test (α = 0.05), and the correlation of spinal cord Fas, HSP70 and Fas/HSP70 with cell apoptosis was analyzed using Pearson correlation analysis. A value of \( P < 0.05 \) was considered statistically significant.

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**Conflicts of interest:** None declared.

**Ethical approval:** This study received permission from the Animal Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University, China.

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