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# Effects of Targeted Proton Radiation on Spinal Cord in a Porcine Model: A Pilot Study

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**Abstract.** *Aim: To determine whether proton radiation can be used to treat chronic intractable pain. The focus of this study was on the biological effects of spinal cord irradiation. Materials and Methods: Proton radiation (0-25 Gy, single fraction) was applied to the spinal cord within L3-L5 of Yucatan mini-pigs (n=20). Skin reaction, body mass and behavior were monitored. At euthanasia, blood and spinal cord were analyzed. Results: Skin morbidity was mild and overall health for the 5-20 Gy-treated groups was good based on behavior and weight gain up to 8.5-9 months post-exposure. The 25 Gy-treated animals developed hind limb weakness at 2.5-3 months and were euthanized. Radiation had a significant effect on white blood cell count ($p<0.05$), with the 25 Gy-treated mini-pigs having the highest number of all three major leukocyte populations. A few differences were also noted for erythrocyte parameters, but the blood chemistry panel was normal. Apoptosis in the targeted portion of the spinal cord was elevated in the 20- and 25 Gy-treated groups versus 0 Gy ($p<0.05$) based on the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. There was a trend ($p<0.1$) for a radiation effect on glial fibrillary acidic protein expression, with the highest value being found after 25 Gy. Histology showed no difference between 0 versus 25 Gy. Conclusion: The data demonstrated that a small segment of the spinal cord can be readily targeted using proton radiation; doses ranging from 5-20 Gy were well-tolerated in an animal model with radiosensitivity similar to humans. Future studies with a pain model should use ≤15 Gy.*

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Nervous-tissue trauma is frequent in combat personnel, but also occurs in the general population. In the United States, there are 1.2 million individuals with spinal cord injury and approximately 12,500 new cases each year based on 2014 data from National Spinal Cord Injury Statistical Center (https://www.nscisc.uab.edu/PublicDocuments/fact_figures_docs/Facts202013.pdf). A substantial proportion of these patients develop neuropathic pain, a condition that is among the most excruciating of all chronic pain syndromes and for which management is a challenging problem. Drugs, surgery (*e.g.* cordotomy, segmental cordectomy), transcutaneous and transcranial stimulation, acupuncture, physical therapy, and cognitive/behavior therapy all too often fail to provide an acceptable level of pain control (1-5).

Chronic intractable pain can also occur after injury to non-neural tissues, *i.e.* often referred to as nociceptive pain. Although the mechanisms underlying nociceptive hypersensitivity remain unclear, excitatory mechanisms of the spinal dorsal horn have been implicated (6). Indeed, the mechanisms underlying chronic intractable pain, regardless of the initiating event, are complex and remain poorly understood. It has been suggested that neuropathic and nociceptive pain could be viewed as different points on the same continuum due to overlap in mechanisms and treatment modalities (7).

Given the tremendous impact on quality of life when treatment is ineffective, there is urgent need for new treatment approaches that will improve outcome. High healthcare cost and loss of productivity are, of course, additional concerns. Studies using irradiation of injured spinal cord in rat models have reported beneficial effects under some conditions (8, 9).

In a rat neuropathic pain model, gamma-knife irradiation of the injured sciatic nerve resulted in histological and behavioral improvement (10). Although these data are encouraging, much more research is needed using protons. To date, the great majority of studies have been carried out using photons (*e.g.* x-rays, γ-rays) and rodents that are well-

known to be more radioresistant than humans. Technical advances will soon make it possible to use stereotactic proton radiation therapy at the Loma Linda University Medical Center (LLUMC) to treat disorders related to the spinal portion of the central nervous system (CNS).

The primary objectives of this pilot study were to determine biological effects after proton irradiation of a specific, pre-determined portion of the spinal cord and the dose at which neurological deficits occur. Since protons release most of their energy where they stop (Bragg peak) and can be delivered to the intended target with high precision, risk for collateral tissue damage is decreased compared to photon radiation such as x-rays and γ-rays. A porcine model was selected because its anatomy, physiology and radiosensitivity are well-known to be very similar to humans (11-13) and it is approved by the Food and Drug Administration for testing DNA damaging agents. Support for the use of mini-pigs in toxicity research has also been expressed in the RETHINK European FP6 Project (14). It must be emphasized here, that the current study was preformed to develop procedures and acquire data on dose-dependent biological consequences after highly targeted proton irradiation of a portion of the spinal cord using a large animal model. Accurate information on proton radiation-induced effects within the spinal cord is essential prior to moving on to clinical studies. The long-term goal is to determine if a low dose of a highly focused proton beam will disrupt abnormal signaling associated with chronic pain while causing little or no side-effects.

## Materials and Methods

*Animals and study design*. All procedures with live animals were approved by the LLU Animal Care and Use Committee (IACUC protocol #8110022) and the Animal Care and Use Review Office (ACURO proposal 11042001) of the United States government. Male Yucatan mini-pigs ~5 months of age and weighing between 20-25 kg were ordered from S & S Farms (Ramona, CA, USA). The animals were maintained in our Animal Care Facility (ACF) under standard conditions for ~1 week after receipt to recover from shipping stress prior to study initiation. Their body weight was determined at arrival and at monthly intervals thereafter. Changes in behavior (feeding, aggressiveness, activity level), appearance and onset of neurologic signs were closely monitored throughout the study. Euthanasia was humanely and rapidly performed when the animal exhibited neurological deficits or at the end of the study period, *i.e.* 8.5-9 months after irradiation, by intravenous injection of pentobarbital (1 ml/10 lbs, 390 mg/ml), followed by dissection of the diaphragm to ensure that euthanasia was successful. All procedures with live animals were carried out in close collaboration with our veterinarian and ACF staff.

*Proton irradiation*. Prior to irradiation, treatment planning was carried out for each mini-pig using magnetic resonance (MR) and computed tomographic (CT) imaging as for patients, *i.e.* implementing the Odyssey® computer system (PerMedics, Inc., San Bernardino, CA, USA). Mini-pig alignment was achieved using 2-dimensional orthogonal X-rays as is also carried-out for patients. However, for both planning and subsequent proton irradiation, the mini-pigs were intramuscularly (*i.m.*) injected with Telazol (4 mg/kg)/ketamine (2 mg/kg)/xylazine (2 mg/kg) for sedation prior to administration of isoflurane (1-3%) anesthesia and supine (belly-up) placement into a specially designed stereotactic holder to minimize movement due to breathing. Once stabilized, *i.m.* yohimbine (0.1 mg/kg) was given to reverse xylazine effects. The Telazol, ketamine, xylazine and yohimbine were all obtained from MWI Veterinary Supply, Inc. (Aurora, CO, USA). Normal body temperature was maintained using a thermoregulated water-heating pad. The proton synchrotron (Fermi National Accelerator Laboratory, Batavia, IL, USA) at LLUMC was used to deliver radiation (passive modulated spread-out Bragg peak, 1.5 Gy/min) bilaterally in a single fraction to the full thickness of the spinal cord within the L3-L5 region of the spinal column. The L3-L5 level was selected because it covers the majority of entries of the sciatic nerve and is easily identifiable in mini-pigs using conventional imaging techniques due to spinal column widening at L4. Doses were 0, 5, 10, 15, 20 and 25 Gy; there were 20 animals in total, with 3-4 per group. Immediately after irradiation the animals were transported back to their original housing in the ACF and observed until fully recovered from anesthesia. The 0 Gy control mini-pigs were handled in the same as the irradiated animals, including all procedures related to anesthesia and recovery; the only exception was that they were not transported to the proton treatment facility for positioning in a stereotactic holder.

Table I. *Body weight (kg) of mini-pigs over the course of time. Mini-pigs were weighed at arrival (month 0) and then approximately once each month thereafter. Proton irradiation of the spinal cord within L3-L5 of the spinal column was carried out approximately 1 week after arrival. The data are means±standard error of means for n=3-4 animals/group. Mini-pigs that received 25 Gy developed hind limb weakness and were euthanized 2.5-3 months after irradiation. There were no differences in body weight associated with radiation.*

| | Radiation dose | | | | | |
|---|---|---|---|---|---|---|
| Months | 0 Gy | 5 Gy | 10 Gy | 15 Gy | 20 Gy | 25 Gy |
| 0 | 25.0±0.0 | 22.6±0.3 | 22.6±0.8 | 21.3±0.7 | 24.0±0.5 | 23.8±0.7 |
| 1 | 28.2±0.1 | 22.9±1.0 | 20.8±1.9 | 21.8±0.3 | 25.8±0.2 | 24.9±0.0 |
| 2 | 29.5±0.1 | 25.4±2.2 | 25.4±1.4 | 25.2±0.2 | 28.0±1.5 | 27.9±0.4 |
| 3 | 34.4±0.3 | 30.7±1.5 | 25.9±2.9 | 30.4±1.2 | 29.4±7.1 | 28.2±0.7 |
| 4 | 36.9±1.1 | 32.1±1.9 | 27.5±3.1 | 31.3±1.9 | 34.2±2.4 | -- |
| 5 | 42.1±0.2 | 33.1±2.0 | 29.3±2.2 | 32.6±3.6 | 37.5±2.6 | -- |
| 6 | 41.3±0.6 | 36.9±0.8 | 32.9±1.9 | 34.4±2.8 | 38.7±2.9 | -- |
| 7 | 43.8±0.3 | 38.9±1.6 | 35.6±2.0 | 37.3±3.1 | 41.1±3.1 | -- |
| 8 | 44.0±0.2 | 40.2±0.8 | 36.3±2.0 | 41.7±5.5 | 44.7±2.9 | -- |
| 9 | 47.7±0.7 | 40.8±1.7 | 37.4±1.6 | 44.0±5.2 | 45.4±2.8 | -- |

*Complete blood count (CBC)*. At the time of euthanasia, a blood volume of approximately 1.5 ml was collected from the cephalic vein in syringes containing [K2]-ethylenediaminetetra-acetic acid. A CBC analysis was performed using an automated hematology VetScan HMII machine (Abaxis, Inc., Union City, CA, USA). It included white blood cell (WBC) count and three-part differential

Table II. *White blood cell (WBC) count and major leukocyte populations in mini-pigs at the time of sacrifice. Collection of blood from the cephalic vein was carried out at 8.5-9 months after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. The only exception was the group that received 25 Gy, which were euthanized at 2.5-3 months post-irradiation due to hind limb weakness. Data were obtained using an automated VetScan HMII hematology analyzer. The presented means±standard error of means are for n=3 animals per group.*

| | Radiation dose | | | | | |
|---|---|---|---|---|---|---|
| Test | 0 Gy | 5 Gy | 10 Gy | 15 Gy | 20 Gy | 25 Gy |
| WBC ($10^9$/l)[a] | 8.5±0.3 | 8.9±0.2 | 9.6±1.4 | 8.7±0.5 | 12.1±3.2 | 15.4±0.6[a]* |
| Lymphocytes ($10^9$/l) | 5.0±0.2 | 5.6±0.2 | 4.8±0.4 | 5.3±0.7 | 5.4±0.5 | 6.8±1.8 |
| Monocytes ($10^9$/l) | 0.13±0.05 | 0.09±0.01 | 0.22±0.12 | 0.24±0.07 | 0.09±0.01 | 0.41±0.24 |
| Granulocytes ($10^9$/l) | 3.4±0.4 | 3.2±0.1 | 4.6±1.7 | 3.2±0.2 | 6.6±3.6 | 8.2±1.3 |
| Lymphocytes (%) | 58.5±3.1 | 63.0±1.0 | 53.1±10.6 | 60.0±5.1 | 52.2±15.0 | 43.7±10.2 |
| Monocytes (%) | 1.7±0.4 | 1.0±0.1 | 2.1±0.8 | 2.8±1.0 | 0.8±0.1 | 2.6±1.5 |
| Granulocytes (%) | 40.1±3.0 | 36.0±1.1 | 44.8±9.9 | 37.2±4.1 | 47.0±15.0 | 53.6±9.0 |

[a]One-way ANOVA: $p<0.05$. *$p<0.1$ *vs*. 0 Gy, 5 Gy and 15 Gy.

(lymphocytes, monocytes, granulocytes), as well as a series of red blood cell (RBC) and platelet (PLT) parameters.

*Chemistry panel*. A chemistry panel was assayed on blood from the 0 Gy- and 25 Gy-treated mini-pigs using the same samples that were used to determine CBC. These data were obtained with a VetScan Chemistry Analyzer (Abaxis); an Abaxis Comprehensive Rotor was used to run the Comprehensive Diagnostic Chemistry panel, *i.e*. a reagent rotor that is 8 cm in diameter and 2 cm in height. A standard series of parameters were rapidly quantified, including albumin, blood urea nitrogen, phosphorus, total protein, *etc*.

*Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay*. This assay was performed on 6-mm sections of spinal cord (within L3-L5) including dorsal horn using standard procedures. Briefly, paraffin sections were deparaffinized in Histo-Clear and then pemeabilized in 0.3% Triton X-100. Sections were evaluated using the DeadEnd™ fluorometric TUNEL staining kit (no. 3250; Promega Corp., Madison, WI, USA). Three randomly selected fields on each of two sections of spinal cord sample in white matter or dorsal horn were examined using an Olympus IX81 microscope (Olympus America Inc., Center Valley, PA, USA). TUNEL-positive cells were identified by green fluorescence, the nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

*Immunohistochemical staining for glial fibrillary acidic protein (GFAP)*. Immunofluorescence staining for astrocyte activation and reactive gliosis in spinal cord and dorsal horn was performed using antibody to GFAP. Series of 6 mm sections within the L3-L5 region were incubated with anti-GFAP (1:400, clone GA5, cat# MAB360; Millipore, Billerca, MA, USA) at 48˚C for 2 h followed by Alexa Fluor® 488 donkey anti-mouse IgG (H+L) conjugated secondary antibody (1:200, cat# REF A21202; Life Technologies, Grand Island, NY, USA) for 2 h at room temperature and counterstained with DAPI. To determine GFAP immunoreactivity, fluorescence intensity was measured on three randomly selected fields on each of two sections in white matter or dorsal horn and calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/Java). Once the green channel was separated from blue channel in an image, fluorescence intensities from the areas of interest were measured using the integral/density feature in the ImageJ program and data were extracted and averaged within the group. Fluorescence was averaged across 3-4 animals per group.

*Morphology*. This procedure was carried-out on a subset of spinal cord sections that included samples from the 0 Gy- and 25 Gy-treated groups. The standard hematoxyin and eosin (H&E) staining method was used and evaluation was preformed in a blinded manner by an LLUMC pathologist. Briefly, two spinal cord segments within the L3-L5 region of the spinal column were fixed in 4% paraformaldehyde overnight. The samples were rinsed thoroughly in tap water and dehydrated in graded alcohol prior to paraffin embedding then a series of sections (6 mm thickness) were made for staining. After deparaffinization in Histoclear (Fisher Scientific, Pittsburgh, PA, USA), sections were rehydrated in alcohol series and stained with H&E to assess tissue and cell morphology.

*Statistical analyses*. Quantitative data were analyzed using one-way analysis of variance (ANOVA) with radiation as an independent variable. A two-way ANOVA was performed for body mass with radiation and time as the independent variables. The non-parametric Tukey test was used to determine difference between sets of two groups and obtain means and standard errors of the mean (SEM). A *p*-value of less than 0.05 was selected to indicate significant difference; $p<0.1$ was accepted indicating a trend.

## Results

*Body mass and overall health status*. The data for body mass for the various groups at monthly time points are presented in Table I. As expected, there were no obvious differences in body weight related to radiation dose. The irradiated animals presented some dermal morbidity at the site of proton beam entry within about 3-5 days after the procedure. However, there were no obvious signs of pain or discomfort related to the skin reaction even after the maximum dose of 25 Gy. The skin reaction resolved uneventfully and none of the animals

Table III. *Red blood cell (RBC) and platelet (PLT) parameters for mini-pigs at the time of sacrifice. Blood was collected from the cephalic vein at 8.5-9 months after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. However, the group that received 25 Gy was euthanized at 2.5-3 months post-irradiation due to hind limb weakness. Data were obtained using an automated VetScan HMII hematology analyzer. The presented data are means±standard error of means for n=3 animals per group.*

| | Radiation dose | | | | | |
|---|---|---|---|---|---|---|
| Test | 0 Gy | 5 Gy | 10 Gy | 15 Gy | 20 Gy | 25 Gy |
| RBC ($10^{12}$/l) | 5.96±0.35 | 6.24±0.31 | 6.67±0.18 | 6.64±0.42 | 6.54±0.20 | 7.88±1.10 |
| HGB (g/dl) | 12.4±0.5 | 12.0±0.3 | 12.0±0.2 | 12.8±0.5 | 12.3±0.2 | 13.8±2.2 |
| HCT (%) | 35.1±1.5 | 33.6±1.7 | 34.6±0.4 | 35.0±1.3 | 33.4±1.0 | 39.1±5.3 |
| MCV (fl)[a] | 59.0±1.5 | 54.0±2.3 | 51.7±1.3* | 53.0±1.5 | 51.3±0.9* | 50.0±1.2* |
| MCH (pg)[a] | 20.7±0.3 | 19.3±0.4 | 18.0±0.8* | 19.4±0.6 | 18.8±0.4 | 17.4±0.4* |
| MCHC (g/dl) | 35.1±0.4 | 35.8±1.5 | 34.7±0.9 | 36.7±0.2 | 36.8±0.5 | 35.1±0.8 |
| RDW (%) | 17.0±0.5 | 19.9±0.3 | 19.6±1.0 | 18.6±0.3 | 19.1±0.2 | 21.2±0.7 |
| PLT ($10^9$/l) | 355.3±55.3 | 504.0±20.4 | 444.0±43.6 | 395.0±23.5 | 340.0±62.1 | 461.3±61.6 |
| MPV (fl) | 10.3±0.1 | 10.9±0.5 | 10.6±0.6 | 10.6±0.4 | 10.2±0.5 | 9.5±0.2 |
| PDW (%) | 39.3±0.2 | 39.8±0.9 | 40.2±1.1 | 39.3±0.8 | 38.9±1.1 | 38.3±0.6 |

HGB, Hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution width; MPV, mean platelet volume; PDW, platelet distribution width. [a]One-way ANOVA: $p<0.05$. *Significantly lower *vs.* 0 Gy, $p<0.05$.

required treatment. Figure 1 shows residual hyperpigmentation on the skin of a mini-pig 3 weeks after 25 Gy irradiation. The only mini-pigs that exhibited neurological deficits were the three that had received 25 Gy of proton irradiation approximately 2.5-3 months previously. Two of these animals exhibited hind limb weakness and were found to be acutely immobile; the third animal showed mild weakness of the pelvic limbs and a stiff gait. The 25-Gy animals were humanely euthanized immediately after deficits were noted. All remaining mini-pigs that were irradiated had no obvious signs of pain and all had normal mentation (energy/activity level), appetite and bowel movement throughout the study.

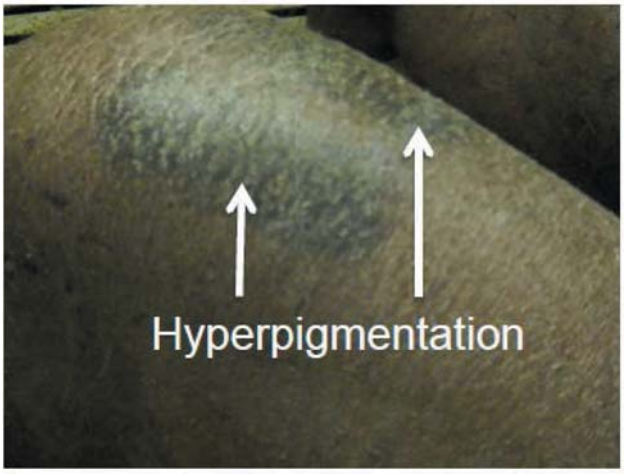


Figure 1. *Residual hyperpigmentation on skin of mini-pig approximately 3 weeks after 25 Gy proton irradiation. Two oblique posterior beams were used to target the spinal cord within the L3-L5 region of the spinal column. Skin reaction appeared within a few days after irradiation, but resolved uneventfully and required no treatment.*

*WBC counts and three-part differential*. The WBC counts and three-part differential data are shown in Table II. Based on one-way ANOVA, there was a significant radiation effect on WBC count ($p<0.05$), with the 25 Gy-treated mini-pigs having the highest number of leukocytes. The Tukey test revealed that there was a trend for increased WBC in blood from the 25 Gy-treated animals *versus* those that received 0 Gy, 5 Gy or 15 Gy ($p<0.1$). There were no statistically significant differences regarding lymphocyte, monocyte and granulocyte numbers or percentages.

*RBC and PLT parameters*. The RBC and PLT data are presented in Table III. Similarly to the WBC counts, the 25 Gy-treated animals had the highest number of RBCs and both hemoglobin and hematocrit were relatively high, although statistical support was lacking. However, there was a significant effect on the mean corpuscular volume (MCV; one-way ANOVA: $p<0.05$). The MCV was lower in the groups that received 25 Gy, 20 Gy or 10 Gy when compared to the 0 Gy controls ($p<0.05$). There was a significant effect on mean corpuscular hemoglobin (MCH; one-way ANOVA: $p<0.05$). Groups that received either 25 Gy or 10 Gy had lower MCH values compared to 0 Gy animals ($p<0.05$). Values for mean corpuscular hemoglobin concentration and PLT counts, volume and distribution width were essentially equivalent among groups.

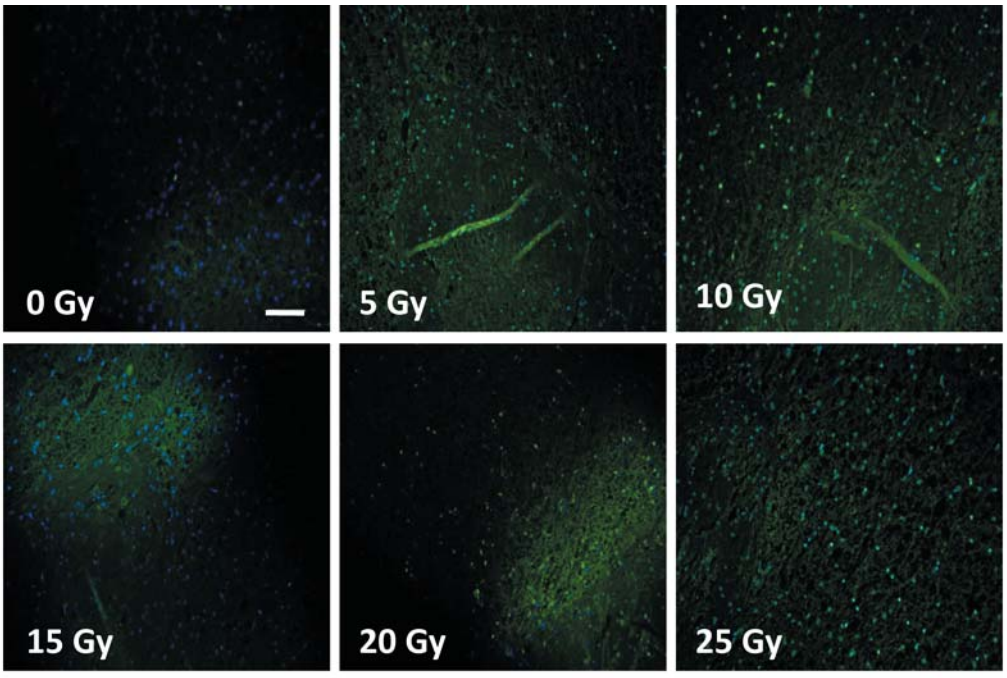


Figure 2. *Representative images of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in spinal cord sections, including dorsal horn, from mini-pigs. The samples were obtained after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. TUNEL positivity (green) is associated with apoptotic cell death. Scale bar=50 μm.*

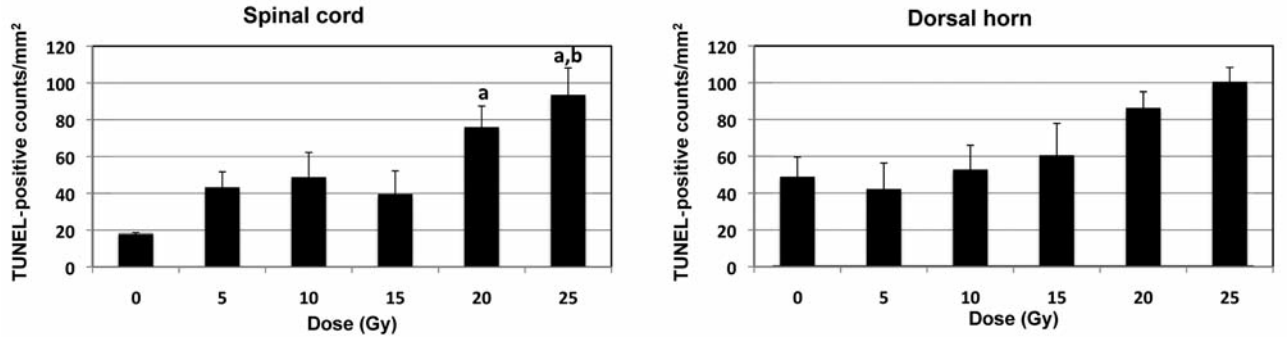


Figure 3. *Results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay on spinal cord sections from mini-pigs. The samples were obtained after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. There were 3-4 animals/group. The TUNEL assay detects DNA fragmentation that is associated with apoptotic cell death. For the TUNEL-positive cell counts/mm$^2$ in the spinal cord (upper panel), one-way analysis of variance (ANOVA) resulted in a value of p=0.006 for the effect of radiation. For the dorsal horn (lower panel), one-way ANOVA gave a value of p=0.060. a: p<0.05 vs. 0 Gy; b: p<0.05 vs. 15 Gy.*

*Chemistry panel*. There were no significant differences or trends related to radiation for any of the items assayed in the chemistry panel. The means±SEMs for the 0 Gy *vs*. 25 Gy animals are shown in Table IV.

*Apoptosis based on TUNEL assay*. Figure 2 shows representative images of TUNEL-positive cells in spinal cord sections from each of the six groups obtained after proton irradiation. The dorsal horn is included in the images. The quantitative data (mean±SEM) are shown in Figure 3. For the TUNEL-positive cell counts/mm$^2$ in the spinal cord (upper panel in Figure 3), the values ranged from 17.8±0.9 (0 Gy-treated group) to 93.5±14.7 (25 Gy-treated group). Statistical analysis based on ANOVA resulted in a value of $p$=0.006 for TUNEL-positive cell density in the spinal cord. Follow-up with post-hoc Tukey's test revealed significant

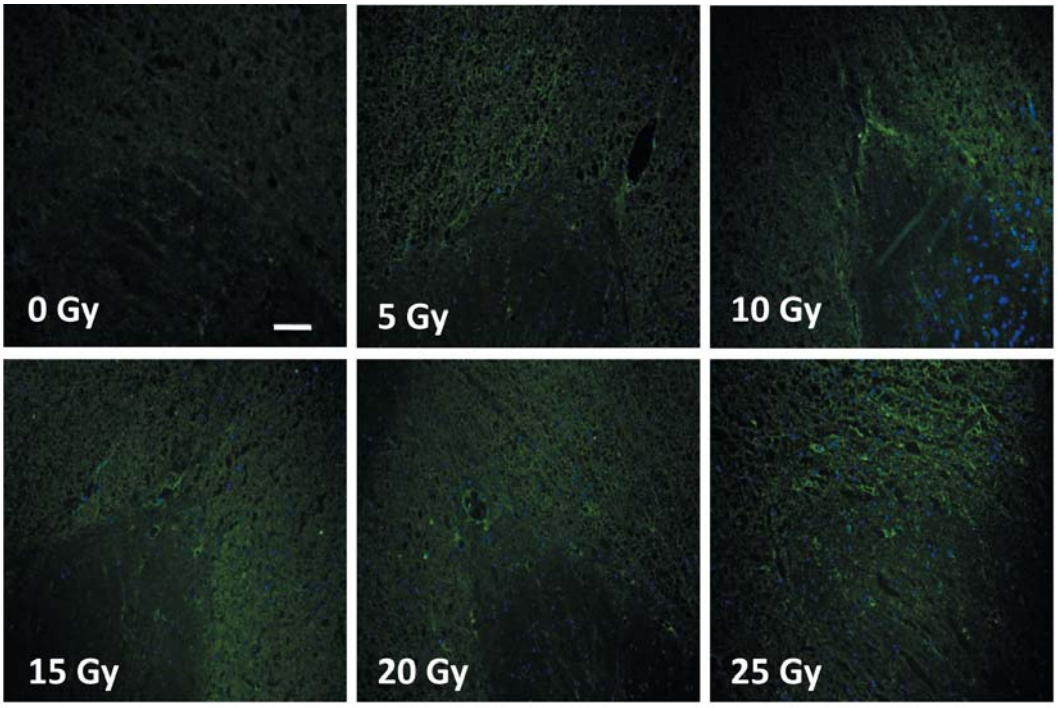


Figure 4. *Representative images of fluorescence intensity for glial fibrillary acidic protein (GFAP) in spinal cord sections, including dorsal horn, from mini-pigs. The samples were obtained after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. GFAP is produced by astrocytes in response to central nervous system injury, a process referred to as reactive astrogliosis. Green indicates GFAP positive. Scale bar=50 μm.*

difference between 0 Gy *versus* 20 Gy and 25 Gy ($p<0.05$) and 15 Gy *versus* 25 Gy ($p<0.05$). The one-way ANOVA for TUNEL-positive cell density in the dorsal horn did not reach statistical significance (lower panel in Figure 3), although a strong trend was noted ($p=0.060$). TUNEL-positive values in the dorsal horn ranged from 42.3±14.1 (5 Gy-treated group) to 100.5±7.8 cell counts/mm$^2$ (25 Gy-treated group); the 0 Gy-treated group had 49.0±10.7 cell counts/mm$^2$.

*GFAP expression*. Figure 4 shows representative images of fluorescence intensity for GFAP positivity after 0 Gy and proton doses that ranged from 5 Gy to 25 Gy. The dorsal horn is included in the images. The upper panel in Figure 5 shows that the average fluorescence intensity in the spinal cord±SEM ranged from 5.6±0.8 (0 Gy group) to 19.3±2.9 (25 Gy group); one-way ANOVA resulted in $p=0.055$ for radiation effect. GFAP positivity in the dorsal horn is summarized in the lower panel in Figure 5. Values here ranged from 4.5±1.3 (0 Gy group) to 25.4±6.7 (15 Gy group). One-way ANOVA resulted in $p=0.083$.

*Histology based on H&E staining*. There was no appreciable neuronal depopulation, gliosis, myelin abnormalities or

Table IV. *Comparison of chemistry parameters in blood from mini-pigs that received 0 Gy versus 25 Gy proton irradiation of the spinal cord (L3-L5 region). Blood was collected from the cephalic vein at the time of euthanasia. A Comprehensive Diagnostic Chemistry panel was obtained using a VetScan Chemistry Analyzer. Data are the means±standard error of means for n=3 animals per group. There were no statistical trends or significant differences between the two groups.*

| Test | Radiation dose | |
|---|---|---|
| | 0 Gy | 25 Gy |
| Albumin (g/dl) | 4.1±0.1 | 4.0±0.4 |
| Alkaline phosphatase (U/l) | 51.3±13.2 | 59.7±8.5 |
| Alanine transaminase (U/l) | 62.7±5.4 | 67.7±2.2 |
| Amylase (U/l) | 1220.3±293.9 | 998.0±114.3 |
| Total bilirubin (mg/dl) | 0.37±0.03 | 0.33±0.03 |
| Blood urea nitrogen (mg/dl) | 14.5±0.3 | 15.7±3.2 |
| Calcium (mg/dl) | 11.0±0.03 | 11.0±0.35 |
| Phosphatase (mg/dl) | 6.5±0.4 | 7.2±0.3 |
| Creatinine (mg/dl) | 1.1±0.2 | 0.8±0.1 |
| Glucose (mg/dl) | 82.3±3.5 | 90.0±3.5 |
| Sodium (mmol/l) | 140.7±1.7 | 145.0±3.6 |
| Potassium (mmol/l) | 4.2±0.03 | 4.9±0.8 |
| Total protein (g/dl) | 7.0±0.4 | 7.4±0.3 |
| Globulin (g/dl) | 2.9±0.4 | 3.4±0.2 |

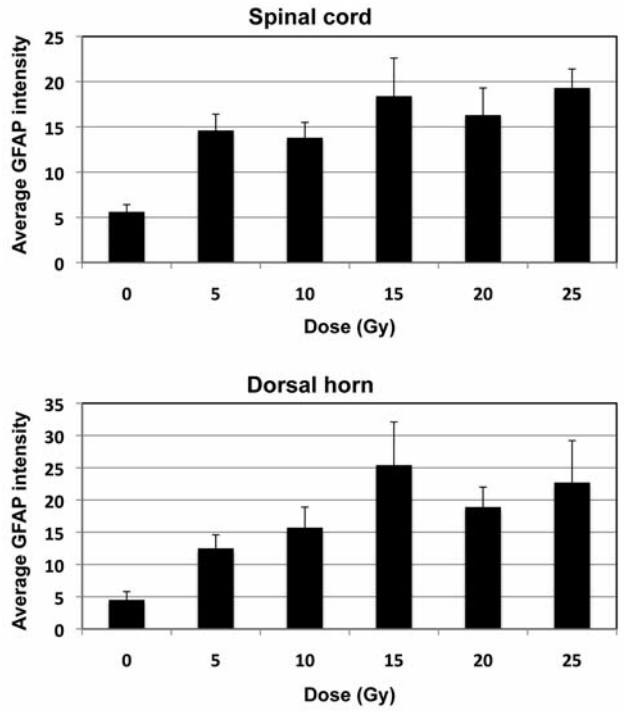


Figure 5. *Results of the assay for glial fibrillary acidic protein (GFAP) on spinal cord sections from mini-pigs. The samples were obtained after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. There were 3-4 animals/group. GFAP is produced by astrocytes in response to central nervous system injury (reactive astrogliosis). One-way ANOVA approached significance for both the spinal cord (upper panel) and dorsal horn (lower panel), i.e. p=0.055 and p=0.083, respectively.*

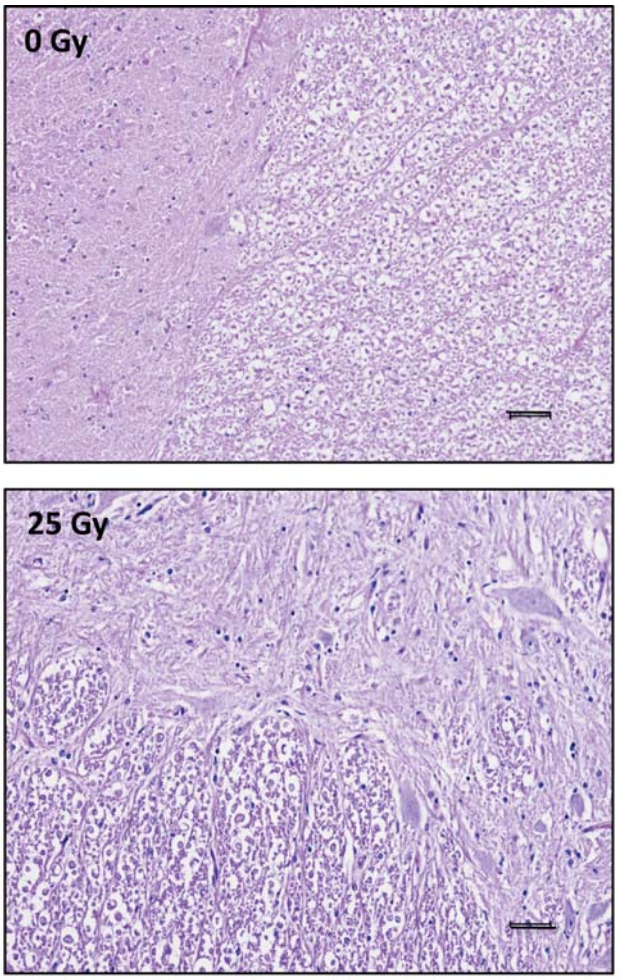


Figure 6. *Representative images of spinal cord sections from mini-pigs after staining with hematoxylin and eosin. There were no abnormalities noted in the samples obtained from animals after proton irradiation of the spinal cord within the L3-L5 region of the spinal column. Scale bars=50 μm.*

pathologic cellular infiltrate. Figure 6 shows representative examples of spinal cord sections from mini-pigs that received 0 Gy and 25 Gy.

## Discussion

The skin reaction that appeared within a few days after irradiation resolved uneventfully with no obvious signs of discomfort. More importantly, there were no neurological deficits noted in the animals that received 5-20 Gy up to 8.5-9 months post-exposure and their overall health remained very good based on behavior, food intake and weight gain. This was not entirely surprising since the spinal cord has long been considered as being highly radioresistant compared to the great majority of other tissues/organs, *e.g.* hematopoietic system, skin and gastrointestinal tract. However, based on qualitative observation, the dose of 25 Gy led to stiff gait and hind limb weakness at 2.5 to 3 months after exposure.

The great majority of pre-clinical studies evaluating radiation effects on the spinal cord have used rodent models (15, 16) and, to our knowledge, there are essentially no reports with which our data can be directly compared to. A study in Yucatan mini-pigs compared spinal cord radiosensitivity after uniform *versus* laterally non-uniform dose distributions targeting the C5-C8 region (17). Radiation was delivered using an image-guided linear accelerator. Spinal cord doses ranged from 17.5 to 24.1 Gy; the end-point was change in gait within 1 year. There was no change for either dose distribution when the spinal cord dose was 17.8 Gy or less, but all animals that received 21.8 Gy or more had deficits (17). A similar study of mini-pigs with emphasis on paralysis noted that the single-session tolerance dose delivered to left spinal nerves of the C5-C8 region was within the 19.0 to 24.1 Gy range (18). This latter study also showed extensive degeneration and fibrosis in spinal nerves of animals that received 24.9 Gy.

In the present study, blood was collected at the time of euthanasia for CBC and chemistry analyses, laboratory tests that are routinely carried-out on patients under a wide range of conditions. The WBC count increased steadily with increasing radiation dose, with the 25 Gy-treated group having the highest values. The numbers of all three major leukocyte types and percentages of monocytes and granulocytes were relatively high in the 25 Gy-treated group. Overall, this indicates that the 25 Gy dose of radiation induced a relatively potent inflammatory response that was detectable in the blood circulation at the same time that neurological deficits were noted. Inflammation, although often thought of as acute and transient, can sometimes persist for extended periods of time. In a rat study of traumatic spinal cord injury, infiltration of all three major leukocyte types remained detectable throughout the entire follow-up time of 180 days (19). Tissue damage due to radiation can also result in a chronic inflammatory response (20, 21). The only other blood parameters significantly affected in our study were the MCV and MCH, both of which were lower in one or more of the irradiated groups *versus* those not irradiated.

Possible radiation effects on TUNEL and GFAP in spinal cord samples included focus on the dorsal horn because it receives sensory information from various parts of the body. Although the molecular and cellular mechanisms underlying chronic pain are not yet completely understood, loss of signal inhibition in the dorsal horn is a key event in its initiation and maintenance (22, 23).

TUNEL positivity in the spinal cord was consistently higher in the irradiated groups compared to the unirradiated group, although statistical significance was obtained only after 20 Gy and 25 Gy. Similar results were obtained in the dorsal horn and a trend for a radiation dose effect was noted.

The TUNEL assay detects DNA fragmentation in cells that results from apoptotic signaling cascades by labeling the terminal end of nucleic acids. Genetic alterations due to stressors such as ionizing radiation can affect the cell's apoptotic machinery that is linked to many pathologies, including neurodegeneration (24). In the literature, apoptosis is often described as a rapidly occurring form of cell death after radiation exposure. It is known, however, that radiation-induced damage can result in a prolonged inflammatory response that includes production of unstable radicals with potential to damage DNA. In addition to unstable oxygen and nitrogen species produced by activated pro-inflammatory microglia and infiltrating leukocytes, certain radiation-induced cytokines such as tumor necrosis factor-α can induce generation of unstable oxygen radicals, and thus can contribute to DNA damage (25). Indeed, the consequences of radiation exposure include dynamic processes that can persist for extended periods of time (26). Based on these and other reports, we opted to include the TUNEL assay in our study.

GFAP immunoreactivity was consistently higher in both the spinal cord and dorsal horn in all irradiated groups compared to those treated with 0 Gy. GFAP is produced by astrocytes in response to CNS injury, a process often referred to as reactive astrogliosis during which the cells undergo morphological, biochemical and functional changes (27). In addition, reactive astrogliosis results in an abnormal increase in the number of astrocytes due to destruction of nearby neurons and an inhibitory glial scar that limits axonal regeneration (in severe cases), as well as the production of chemokines that contribute to inflammation (28, 29). GFAP is the classical marker for reactive astrogliosis. Radiation is known to activate microglia that, in turn, contribute to the process of reactive astrogliosis and expression of GFAP (30, 31).

Collectively, the data presented herein are unique. Indeed, studies evaluating proton radiation effects in large animal models are very few. Our data demonstrated that targeting a small segment of the spinal cord was readily accomplished with the proton beam. Increases in WBC counts and TUNEL and GFAP positivity after irradiation were noted, but significance was obtained primarily with the highest doses used. Future studies in a pain model should focus on proton radiation doses that are 15 Gy or less.

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