

Changes in cell cycle, apoptosis and necrosis following the establishment of a ^{125}I brachytherapy model in the spinal cord in Banna mini-pigs

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Abstract. Brachytherapy is regarded as the most effective method in the treatment of metastatic spinal tumors since little damage is caused to surrounding healthy tissue. However, this method may cause radiation myelopathy if an overdose occurs. In the present study, we established a Banna mini-pig ^{125}I spinal cord implantation model to provide a tool for the study of how to reduce these types of side effects. Cell cycle alteration, apoptosis and necrosis of spinal cord neurons in the presence of various doses and durations of ^{125}I brachytherapy were also investigated. The pigs were randomly divided into four groups, A, B, C and D. In group A, four ^{125}I seeds (total radioactivity, 4.0 mCi) were implanted into the dura mater of the spinal canal at the level of T13. In groups B and C, eight ^{125}I sources (total radioactivity, 8.0 mCi) were inserted at the same location. Groups A and C were raised for up to 8 months and group B for only 2 months. Neurons from the swine spinal cord at the T13 level were collected and cell cycle analysis was performed. Apoptosis and necrosis were tested by a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. The Banna mini-pig brachytherapy model was successfully established. Radiation myelopathy was closely associated with radiation dose and duration, more neurons were blocked in the G2 and S phases as dose and time increased, and an increase in apoptosis and necrosis was detected. Ratios of apoptosis and necrosis were reduced as lower doses and shorter durations of radiation were applied. Our results demonstrate that the Banna mini-pig is an ideal

animal to study ^{125}I brachytherapy. Low-dose and short-term brachytherapy may effectively decrease apoptosis and necrosis in spinal cord cells in Banna mini-pigs.

Introduction

Radiotherapy is one of the most effective treatments for metastatic spinal tumor. Pain alleviation was observed in 80-90% of patients following treatment (1-3). The most common complication of radiotherapy in the treatment of metastatic spinal tumors is radiation myelopathy, which is caused by radiation damage and results in neuron apoptosis and necrosis. The tolerance of spinal cord neuron cells to radiation is 40-50 Gy every four or five weeks, and if an overdose occurs, this may lead to radiation myelopathy (4). In animal models, radiation myelopathy is reportedly closely correlated to the form, dose and duration of radiotherapy, host immune status and the duration of disease (5).

In brachytherapy (a form of radiotherapy), a radiation source is permanently placed inside or next to the treatment locus. Although ^{125}I brachytherapy is an improved method for killing tumor cells locally and protecting healthy tissues, there are some negative effects, including radiation damage to the tissue surrounding the seeds, which may lead to complications (6,7). Appropriate animal models contribute to experimental research for the improvement of brachytherapy on metastatic spinal tumors. Establishment of a specific mammalian animal model that mimics the human clinical situation is crucial. Such a model may be a useful tool for clinicians to improve treatment efficacy and reduce the side effects. However, only certain rodent species have been reported to fulfill this task thus far, and their numbers are too small to simulate the real human physiological situation accurately (5).

A Banna mini-pig model was used in this study to simulate spinal interstitial brachytherapy, and aimed to study the cell-based radiation damage that was caused by ^{125}I . The Banna mini-pig spinal cord is similar to that of humans in terms of anatomical structure; therefore, it is a useful model to investigate the myelopathy pathology and the relationship between radiation dose and duration, and tissue damage. We implanted

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^{125}I seeds to spinal dura mater at the T13 level of mini-pigs. We then studied the dose- and time-dependent radiation damages to the healthy cells. The cell cycle alteration, apoptosis and necrosis ratio in spinal cord neuron cells were also examined.

Materials and methods

Radiation source and reagents. Brachytherapy seeds iodine-125 (BT-125-1) were purchased from Shanghai Xinke Medicine Ltd (Shanghai, China). Apparent radioactivity was 1.00 mCi/seed and the half life of these was 60.1 days. Prior to purchase, the ^{125}I seeds were randomly selected for activity testing in order to confirm the seed container integrity and apparent activity of the seeds.

X-ray computed tomography (CT) was purchased from Siemens, Germany; the digital subtraction angiography (DSA) was purchased from Philips, The Netherlands; and treatment planning systems (TPSs) were purchased from Hejie Medical Instruments (Tianjin, China). The CRC-15R calibrator was purchased from Capintec Inc. (Ramsey, NJ, USA).

Propidium iodide (PI), RNAase Triton X-100 and Trypsin were purchased from Sigma (St. Louis, MO, USA). The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) kit was from Millipore (Temecula, CA, USA).

Animal. Twenty healthy, adult, female Banna mini-pigs were selected for the experiment. The animals were provided and raised by the Animal Center at Kunming Medical College (China). The weights of the animals ranged from 20 to 25 kg (average 22.7 kg). The mini-pigs adapted to the laboratory environment for 1 week prior to modeling. This housing facility is a barrier housing facility, and is in keeping with national standards (Laboratory Animal-Requirements of Environment and Housing Facilities). The care of laboratory animals and the animal experimental surgery conformed to the Chinese Administration Rule of Laboratory Animal.

Grouping. The pigs were randomly divided into four groups: A, B, C and D, with 6 pigs in groups A, B and C, and 2 in group D. In group A, four brachytherapy seeds were implanted into the spinal dura mater at the T13 level. The pigs were raised for eight months (equal to 4 half-lives of ^{125}I). In group B, eight brachytherapy seeds were inserted into the same location. The pigs were monitored for two months (equal to 1 half-life of ^{125}I). In group C, eight seeds were treated similarly and pigs were monitored for eight months (4 half-lives of ^{125}I). Group D acted as an age-matched normal control, without ^{125}I implantation.

Radiation dose calculations. This study adopted Monte Carlo-aided dosimetry to calculate the accurate radiation dose that the mini-pig spinal surface received during the whole of the brachytherapy process. Briefly, we calculated the initial dose (termed $D_{(0)}$) immediately following implantation of the ^{125}I particles into the spinal cord at the T13 level. The formula used was $D_{(0)} = A_0 \times 1.27 \times \Lambda \times g_{(r)} \times F_{(r, \theta)} / r^2$, where A_0 is the particle initial radiation dose, which was tested by the CRC-15R calculator on the day prior to implantation. Λ is the constant parameter for ^{125}I , and in our study the value is 1.06; r is the distance between the spinal surface to the ^{125}I particles, which was obtained from magnetic resonance imaging (MRI)-detecting data; $g_{(r)}$ is the

radial dose functions; and $F_{(r, \theta)}$ is the anisotropy constant, the detailed data of the calculations were utilized according to previously published methods (8,9). After D_0 was confirmed, the radiation dose received by the spine was calculated using the formula $D_{(T)} = D_{(0)} \times T_{1/2} \times 1.443 \times [1 - e^{-T \times 0.693/T_{1/2}}]$. $D_{(T)}$ refers to the total received dose within the time interval T , and e is a natural constant.

Surgical procedures. Our preliminary data revealed that the T13 level was the best locus for surgery (data not shown). The mini-pigs were anaesthetized with sodium pentobarbital through the ear vein, and were then inserted in prone positions followed by skin preparation and sterilization. Digital subtraction angiography was used to precisely localize the surface projection of the T13 vertebra body and the vertebral pedicle. Following the template, a syringe needle, mounted with a 20 to 30 degree angle to the coronal plate, was inserted into the pedicle of the vertebral arch, where it connects vertebra, and was inserted into the T13 anterior spinal canal without damaging the dura mater. Meglumine diatrizoate was used to confirm the location of the needle. ^{125}I seeds were then implanted into the spinal canal (i.e., between the dura mater and anterior canal). Following the surgery, DSA was used to confirm the location of the ^{125}I seeds at the T13 level.

Cell phase analysis. Neuron cells in the T13 level of the spinal cord were collected and stored in Eppendorf tubes and diluted at a density of 1.0×1.0^6 per $100 \mu\text{l}$. The cell type was identified as neuronal cells by histological staining (data not shown). The cells were treated with 70% alcohol for DNA precipitation and stored at -20°C (for less than a week). Cells were re-suspended in cell cycle buffer [0.4 ml phosphate-buffered saline (PBS), 0.5 mg RNase, and $0.5 \mu\text{l}$ Triton-X100] following removal of the alcohol. The cells passed through nylon mesh filtration prior to being applied to Falcon 2052 tubes. PI ($10 \mu\text{l}$ of 5 mg/ml) was incubated with the cells for 10 min. The cells were then applied to flow cytometry (Beckman-Coulter, Brea, CA, USA) for analysis. For each sample, data were collected from 1×10^4 cells and analyzed using Coulten-cycle software. Experiments were repeated at least three times.

TUNEL assay. Digoxigenin-11-dUTP forms hetero-oligomers with dUTP at the 3-OH terminus of double-strand or single-strand DNA in apoptotic cells catalyzed by the TdT enzyme. FITC and PI fluorescent dyes labeled with dUTP were used to distinguish apoptotic, necrotic and normal cells by flow cytometry. TUNEL assays were performed to examine the rates of apoptosis and necrosis due to varying dose and durations of brachytherapy.

Data analysis. Standard statistical software (SPSS version 11.0; SPSS, Inc., Chicago, IL, USA) was used for data analysis. The Student's t-test and χ^2 test were used for variable and attribute data respectively. $P < 0.05$ was considered statistically significant. The data were expressed as the means \pm standard deviation (SD).

Results

Confirmation of ^{125}I seeds at the spinal T13 level by DSA and CT scanning. Following surgery, the Banna mini-pigs were

Table I. Cell cycle distribution of spinal cord cells following treatment with various doses and durations of radiation (% , mean \pm SD).

Group	n	G0/G1phase	S phase	G2/M phase
A	6	95.33 \pm 2.16	2.10 \pm 0.26	2.03 \pm 0.19
B	6	84.42 \pm 2.25	8.35 \pm 0.15	7.78 \pm 0.38
C	6	81.00 \pm 1.41	10.40 \pm 1.25	8.43 \pm 0.27
D	3	99.21 \pm 0.56	0.45 \pm 0.34	0.34 \pm 0.17

The differences between any two groups were statistically significant, $P < 0.05$; results are expressed as the mean \pm SD. SD, standard deviation.

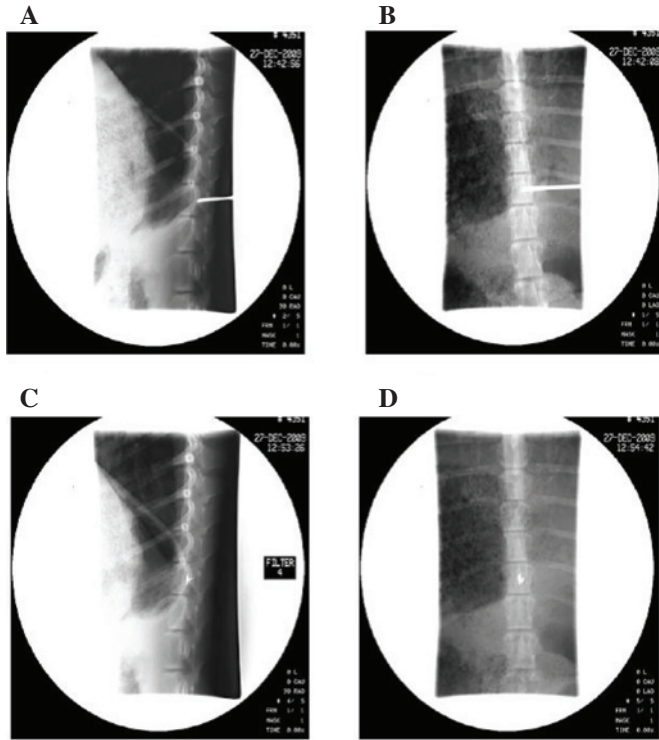


Figure 1. DSA scanning image of ^{125}I seeds in Banna mini-pig spinal cord. (A) Image of ^{125}I seeds injection from the lateral position in the process of surgery; (B) image of ^{125}I seeds injection from the normal position in the process of surgery; (C) lateral image of ^{125}I at the T13 level of the spinal cord following surgery; (D) normal image of ^{125}I in the T13 of the spinal cord following surgery. DSA, digital subtraction angiography.

consecutively treated with antibiotics for 3 days to avoid infection. CT-scanned data proved that ^{125}I seeds had been precisely inserted at the T13 target level, and that the procedure complied with TPS requirements. DSA images of one pig in group A were randomly selected to reveal the location of the ^{125}I seeds (Fig. 1).

Radiation dose measurement. Using CT scanning, radiation dose distribution was determined through the axial, sagittal and coronal planes of the spinal cord. Based on the formula mentioned in Materials and methods, the average radiation doses of the T13 level of the spinal cord were obtained for each group. The average radiation dose for group A was 10.14 \pm 0.087 Gy, group B was 14.05 \pm 0.61 Gy and group C was 18.53 \pm 1.4 Gy.

Cell cycle analysis. The focus of the present study was neuronal cells in the gray matter of the spinal cord, which mainly included the ventral horn and dorsal horn cells. These cells are more sensitive to radiation and induce myelopathy. Cells in the gray matter were carefully collected for cell cycle analysis. The effect of dose and duration of radiation on cell cycle distribution were investigated. As the amounts of brachytherapy seeds and the duration of radiation increased, compared to group D, a marked changed was observed in the cell cycle distribution of the spinal cord cells in groups A, B and C. The average ratios of cells in the G0/G1 phase were 95.33 \pm 2.16% in group A, 84.42 \pm 2.25% in group B and 81.00 \pm 1.41% in group C. The average ratios of spinal cord cells in the S phase were 2.10 \pm 0.26% in group A, 8.35 \pm 0.15% in group B and 10.40 \pm 1.25% in group C. The average ratios of spinal cord cells in the G2/M phase were 2.03 \pm 0.19% in group A, 7.78 \pm 0.38% in group B and 8.43 \pm 0.27% in group C. The differences between any two groups were statistically significant ($P < 0.05$, Table I, Fig. 2). Our data suggest that ^{125}I brachytherapy substantially affected the cell cycle distribution of spinal cord cells. The ratio of cells in the G0/G1 phase decreased, while that in the G2/M phase increased significantly as the radiation dose and time increased.

Apoptosis and necrosis in spinal cord cells. The apoptotic and necrotic ratio of the spinal cord cells exhibited a dose- and duration-dependent trend. When the number of brachytherapy seeds and radiation time was extended, the apoptosis and necrosis rates in the spinal cord cells increased significantly. The average apoptosis rate was 1.18 \pm 0.11% in group A, 6.78 \pm 0.38% in group B and 17.88 \pm 1.02% in group C. The average necrosis rate was 0.48 \pm 0.21% in group A, 0.80 \pm 0.05% in group B and 2.43 \pm 0.29% in group C. In group D, no obvious apoptosis or necrosis was observed. Differences between any two groups were statistically significant ($P < 0.05$, Table II and Fig. 3). The results indicate that, as the dose and time of brachytherapy increased, the survival of cells was reduced, whereas the apoptotic and necrotic cells significantly increased. We also monitored the behavioral changes of the mini-pigs as radiation accumulated. No obvious abnormality was noted in the mini-pigs of group A. One pig in group B had hair loss on the left hind leg and clumsy tail movement. Two pigs in group C exhibited slow movement in the hind legs, and one pig exhibited incontinence.

Discussion

^{125}I brachytherapy was introduced into radiation therapy in the 1970s, earlier documents regarding this method can be

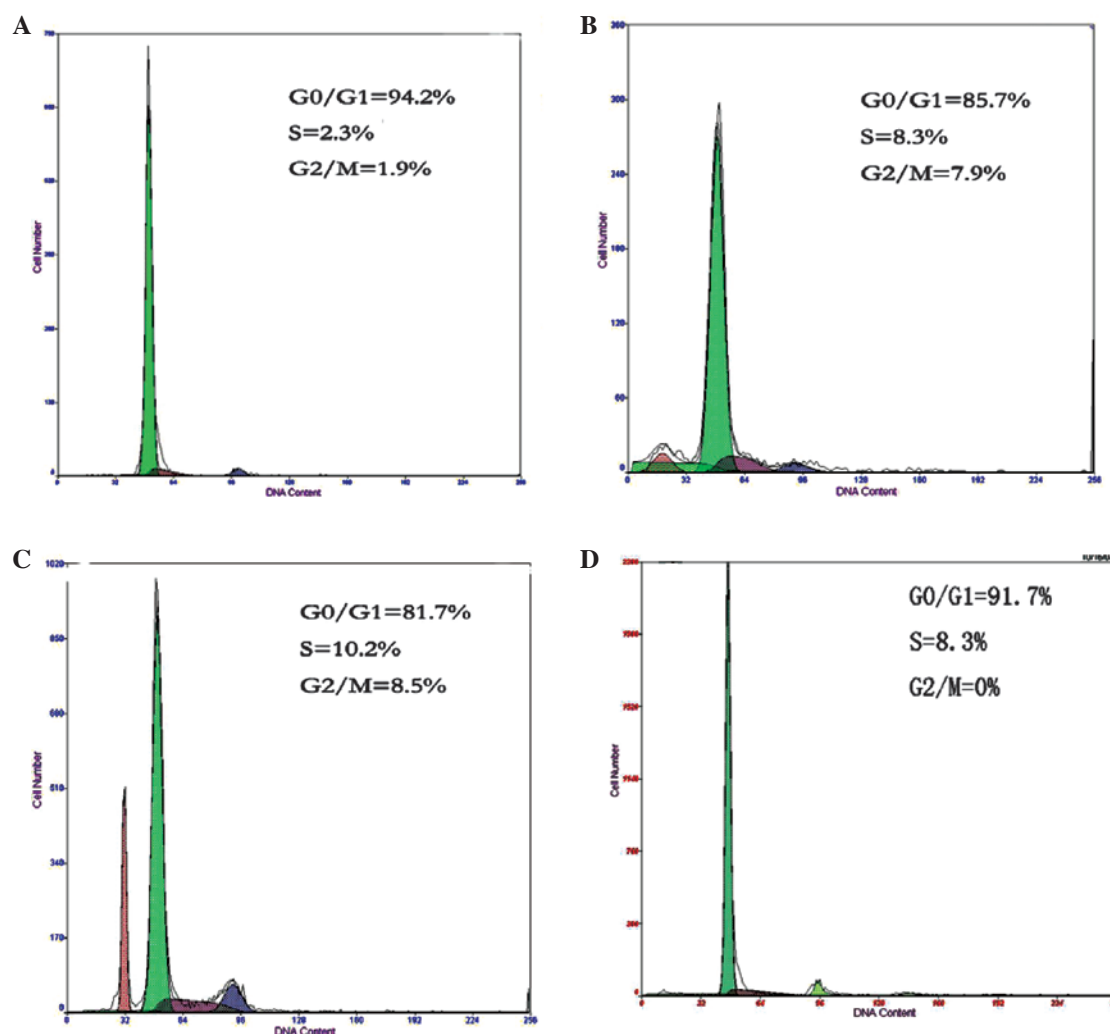


Figure 2. The cell cycle distribution of the spinal cord cells following treatment with varying doses of radiation. (A, B and C) The cell cycle distributions of the spinal cord cells in one pig for groups A-C, respectively, is shown. From left to right, the four peaks refer to the ratios of cells under apoptosis and necrosis (pink peak), cells in the G0/G1 phase (green peak), cells in the S phase (purple peak) and cells in the G2/M phase (blue peak).

Table II. The ratio of apoptosis and necrosis following varying doses of radiation (% , mean \pm SD).

Group	n	Apoptosis	Necrosis
A	6	1.18 \pm 0.11	0.48 \pm 0.21
B	6	6.78 \pm 0.38	0.80 \pm 0.05
C	6	17.38 \pm 1.02	2.43 \pm 0.29
D	3	0.12 \pm 0.11	0.05 \pm 0.04

The differences between any two groups were statistically significant, $P < 0.05$; results are expressed as the mean \pm SD. SD, standard deviation.

traced back to 1979 when clinical practitioners treated prostate cancer patients with ^{125}I implantation (10). This method has been widely applied in prostate, brain (11) and lung cancer treatment (12), and has been proven to be effective for the inhibition of cancer progression.

In the present study, we successfully established a Banna mini-pigs model to investigate the side effects of brachy-

therapy. The Banna mini-pig has a similar spinal structure to the human. Thus, our study may provide a valuable tool for use in brachytherapy for the treatment of metastatic spinal cancer in an animal model.

In conventionally fractionated radiotherapy (1.8-2.0 Gy per fraction), the tolerance of the spinal cord is only two thirds compared to that of regular tissues with regard to irreversible damages (13). Chronic progressive radiation myelopathy developed in patients after 0.5 to 2 years of treatment (13-17). Van den Aardweg *et al* previously evaluated the effects of local irradiation on various lengths of the spinal cord in mature pigs (37-43 weeks) (18). In that study, the effective dose 50 (ED50) values for chronic progressive radiation myelopathy were found to be 27.02 ± 0.36 , 27.68 ± 0.57 and 28.28 ± 0.78 Gy on a field length of 10, 5 and 2.5 cm, respectively, with a single high dose of radiation (25-32 Gy). In another study on the canine brain (19), the geographically circumscribed radiation from ^{125}I seeds was accompanied by increased permeability in blood-brain barrier (BBB), which may persist for more than 1 year following insertion of the ^{125}I seed. This altered BBB function was probably responsible for the cerebral edema associated with ^{125}I brachytherapy (19). It was reported that the high dose

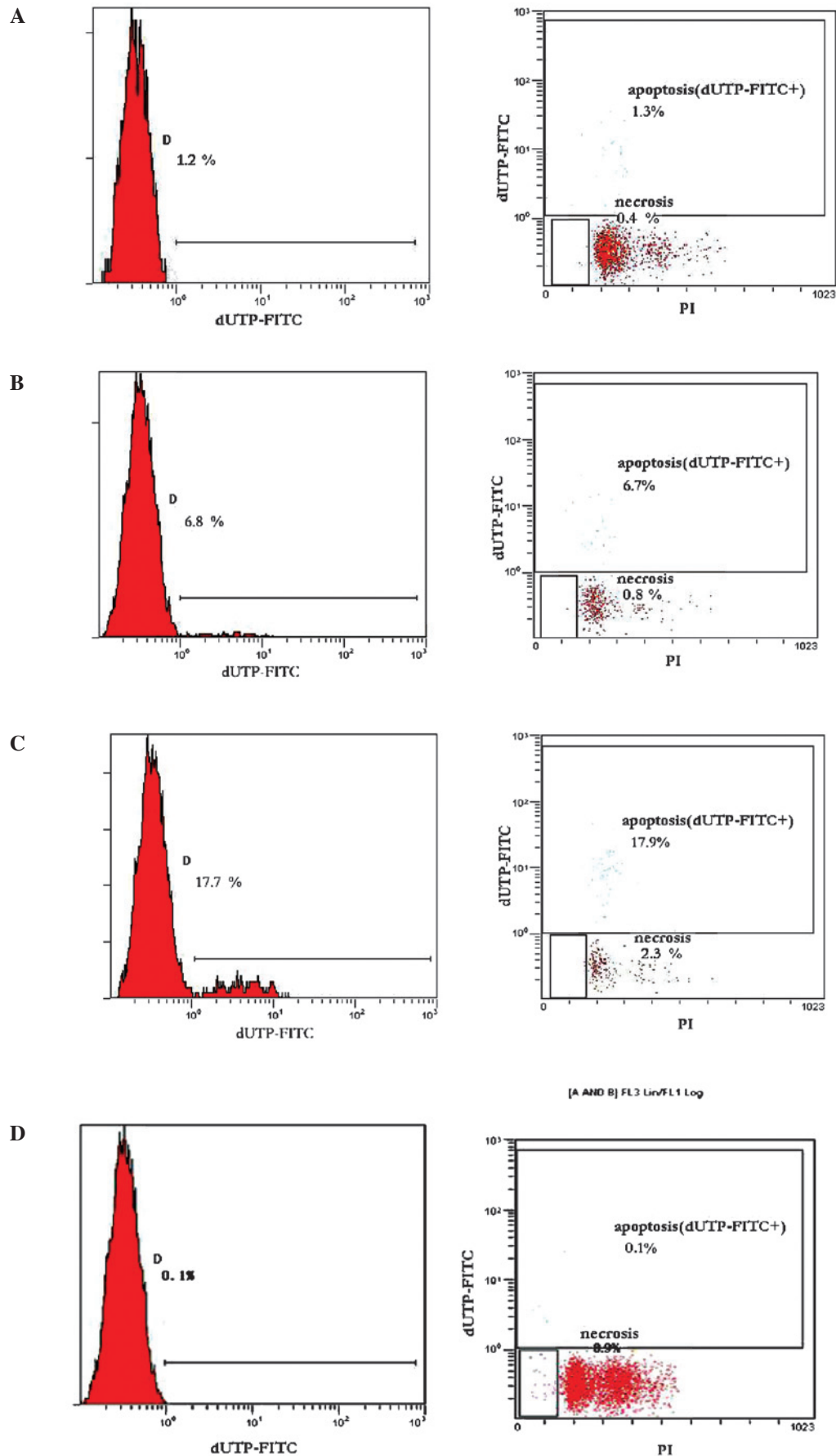


Figure 3. The rate of apoptosis and necrosis following various doses and durations of radiation measured by flow cytometry. (A, B and C) The rates of apoptosis and necrosis of the spinal cord cells for one pig of groups A-C, respectively, is shown. dUTP-FITC(+) represents apoptosis, while PI(+)/dUTP-FITC(-) represents necrosis. PI, propidium iodide.

radiation more efficiently treated brain tumors; at the same time, however, more damage was induced to the normal nerve tissues, resulting in a debilitating cognitive decline (20). We examined the changes of cell cycle distribution in spinal cord cells following radiation seed implantation with flow cytometry. Our results revealed that the ratio of spinal cord cells in the G2 and S phases increased as the radiation accumulated in mini-pigs. These data suggest that the cell cycle was blocked in the G2 and S phases after radiation. The cells in the G2 and S phases are more sensitive to radiation (21), and therefore more tumor cells were eliminated, while the apoptosis of normal cells also increased, which may lead to radiation myelopathy.

We analyzed the ratios of apoptosis and necrosis in spinal cord cells with TUNEL assay by flow cytometry. Our results demonstrated that the ratios of apoptosis and necrosis in spinal cord cells increased significantly as the dose and duration of radiation increased. Additionally, the mini-pigs exhibited behavioral signs of radiation damages. As the dose enhanced gradually, mini-pigs in group A exhibited signs of pain and sickness. Hair loss in the left hind leg and clumsy tail movement were observed in one pig from group B. Two pigs had paralysis of the hind legs in group C. After one half-life of ^{125}I (i.e., 2 months), all the animals were normal. After four half-lives, three pigs had slow movements of the hind legs. Our data indicate that higher doses caused greater damage to spinal cord cells and increased the chance of inducing radiation myelopathy, which develops chronically and irreversibly.

In conclusion, radiation myelopathy is closely correlated to the dose and duration of brachytherapy. A low dose and short-term radiation effectively reduces the apoptosis and necrosis of spinal cord cells, thus eliminating the occurrence of radiation myelopathy. Our results demonstrate that brachytherapy may cause damage to normal tissues, and that the dose and duration of brachytherapy requires careful calculation to treat metastatic spinal tumors.

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