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Mechanism of hypocoagulability in proton-irradiated ferrets

Gabriel S. Krigsfeld¹, Alexandria R. Savage¹, Jenine K. Sanzari¹, Andrew J. Wroe², Daila S. Gridley², and Ann R. Kennedy¹

¹Department of Radiation Oncology, University of Pennsylvania, Philadelphia, PA

²Department of Radiation Medicine , Radiation Research Laboratories , Loma Linda University and Medical Center , Loma Linda , CA , USA

Abstract

Purpose—To determine the mechanism of proton radiation-induced coagulopathy.

Material and methods—Ferrets were exposed to either solar particle event (SPE)-like proton radiation at a predetermined dose rate of 0.5 Gray (Gy) per hour (h) for a total dose of 0 or 1 Gy. Blood was collected pre- and post-irradiation for a complete blood cell count or a soluble fibrin concentration analysis, to determine whether coagulation activation had occurred. Tissue was stained with an anti-fibrinogen antibody to confirm the presence of fibrin in blood vessels.

Results—SPE-like proton radiation exposure resulted in coagulation cascade activation, as determined by increased soluble fibrin concentration in blood from 0.7 – 2.4 at 3 h, and 9.9 soluble fibrin units ($p < 0.05$) at 24 h post-irradiation and fibrin clots in blood vessels of livers, lungs and kidneys from irradiated ferrets. In combination with this increase in fibrin clots, ferrets had increased prothrombin time and partial thromboplastin time values post-irradiation, which are representative of the extrinsic/intrinsic coagulation pathways. Platelet counts remained at pre-irradiation values over the course of 7 days, indicating that the observed effects were not platelet-related, but instead likely to be due to radiation-induced effects on secondary hemostasis. White blood cell (WBC) counts were reduced in a statistically significant manner from 24 h through the course of the seven-day experiment.

Conclusions—SPE-like proton radiation results in significant decreases in all WBC counts as well as activates secondary hemostasis; together, these data suggest severe risks to astronaut health from exposure to SPE radiation.

Keywords

Radiation; HZE particles; low dose rate; SPE radiation; hemostasis

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Correspondence: Dr Ann R. Kennedy, Department of Radiation Oncology, University of Pennsylvania, 195 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA 19104, USA. Tel: 1 (215) 898 0079. Fax: 1 (215) 898 1411. akennedy@mail.med.upenn.edu.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Proposed manned-missions by the National Aeronautics Space Administration (NASA) to the surface of Mars have led to concerns about the effects of a three-year mission on astronaut health (Badhwar et al. 1992), with the most serious concerns involving the potential biological consequences of exposure to space radiation and prolonged exposure to microgravity (Cucinotta et al. 2001, Mognato et al. 2009, Wilson et al. 2012). On a mission to Mars, astronauts will be exposed to background levels of highly energetic, charged particles known as HZE particles and solar particle event (SPE) radiation (Hellweg and Baumstark-Khan 2007). As humans are not normally exposed to these types of space radiations on Earth, relatively little is known about their biologic effects. Therefore, research programs have been developed to address the physiological responses to HZE particle and SPE radiation exposure. An SPE involves the release of particles (protons, electrons, heavy ions) with energies greater than 10 Milli-electronvolt/nucleon (MeV/n) from the sun through solar flares and/or coronal mass ejections (Turner and Baker 1998). The sun's solar cycle can be categorized by an 11-year cycle that transitions from the sun's 'active' to 'inactive' phases (Kim et al. 2009). During the active stage of the sun's cycle, SPE occur several times a day and in the inactive stage, approximately once every five days. Most of the SPE that occur during the active phase are considered 'soft' SPE, releasing low doses of low energy protons, which have limited penetrating ability. In a large SPE, higher energy protons with greater penetrating ability are present, often along with HZE particle radiation. Large SPE are relatively rare; but hazardous for astronauts, as the doses of radiation can be very high. SPE are spontaneous and the cumulative radiation dose that astronauts are expected to receive is impossible to predict prospectively. NASA's Permissible Exposure Limits (PEL) guidelines have been described in detail elsewhere (NASA 03-05-2007). Examples of NASA PEL for specific organ systems are as follows: Skin: 30-day limit – 1.5 Gray (Gy); blood forming organs (BFO): 30-day limit – 0.25 Gy. Potential astronaut doses have been estimated from modeling three different large historical SPE (Hu et al. 2009); all of the simulations for the August 1972, September 1989, and October 1989 SPE have led to estimates that the potential absorbed doses during extra vehicular activity (EVA) would be high for both skin and BFO, surpassing the NASA PEL. The estimated doses inside the spacecraft during the August 1972 SPE were 2.70 Gy to the skin and 0.46 Gy to the BFO (Hu et al. 2009). These estimates clearly indicate that astronaut exposure to the SPE doses expected during the August 1972 SPE would exceed the current NASA PEL for 30 days, even for an astronaut inside the spacecraft for the entire SPE. As examples, inside the spacecraft, exposure to the August 1972 SPE would result in astronaut exposure to a skin dose (2.70 Gy) which is > 1.7 times higher than the current NASA 30-day PEL for skin (1.50 Gy-Equivalent [Eq]) and a BFO dose (0.46 Gy) which is > 1.8 times higher than the current NASA 30-day PEL for BFO (0.25 Gy-Eq). EVA exposure to the August 1972 SPE would result in astronaut exposure to a dose (32.15 Gy) which is > 20 times higher than the current NASA PEL for skin (1.5 Gy-Eq) and to a dose (1.38 Gy) which is > 5 times higher than the current NASA 30-day PEL for BFO (0.25 Gy-Eq). These estimated EVA doses would also exceed the 1-year PEL for skin (3 Gy-Eq) and for BFO (0.5 Gy-Eq), and the career limit for skin (4 Gy-Eq); the career PEL for BFO has not been established. The dose estimations described by Hu et al. (2009) included only the doses expected from SPE

radiation during a specific SPE, and they did not include the doses expected from other types of radiation that an astronaut would be exposed to during space travel (e.g., on a mission to Mars). Currently, it is believed that through a worst-case scenario (such as the Carrington flare of 1859), astronauts may be exposed to internal doses ~2 Gy from SPE radiation (Stephens et al. 2005, Townsend 2005).

Particular research interest has been directed at evaluating the proton component of an SPE to determine the effects on the hematopoietic system and hemostasis. Hematopoiesis regulates cell types in the blood, while hemostasis is essential to prevent prolonged bleeding and to allow the repair of the blood vessel when an injury occurs. Hemostasis is categorized into primary and secondary hemostasis (Smith 2009). During primary hemostasis, platelets are recruited to the site of injury and form an initial 'plug' to prevent further exposure between the blood flow and the subendothelial layer. Secondary hemostasis, also known as coagulation (Furie and Furie 1988), involves the enzymatic activation of a number of proteins (known as Factors) that result in the conversion of fibrinogen to fibrin, which forms a fibrin clot at the site of injury; the contact pathway is also activated which perpetuates the conversion of fibrinogen to fibrin. Radiation-induced coagulopathy, particularly hemorrhaging, has been reported for numerous irradiated human populations, including the accidental whole body exposures in Norway and Brazil (Reitan et al. 1990, Valverde et al. 1990). In both instances, humans were exposed to doses ranging from 3 – 6 Gy of radiation and coagulopathies (including extensive hemorrhage) were reported. A variety of model systems have been employed to study the consequences of ionizing radiation exposure near doses that lead to mortality in 50% of an experimental group, known as an LD₅₀. Several species (including ferrets, dogs, and pigs) have LD₅₀ values that are similar to those of human populations, while the mice LD₅₀ values can range from 2 – 4× higher than that of humans (Morris and Jones 1988). At the LD₅₀ values, the hematopoietic syndrome leads to death from hemorrhaging or bacterial infection (Lorenz and Congdon 1954). In higher mammalian organisms, extensive hemorrhaging at doses near the LD₅₀ has been observed in pigs (Moroni et al. 2011), dogs (Winchell et al. 1964), and monkeys (Taketa et al. 1967); while at the LD₅₀ level in mice, bleeding is not normally observed and death occurs due to infection (Miller et al. 1951, Lorenz and Congdon 1954, Boone et al. 1956). Coagulation parameters in wild-type mice are very different from those in humans and other large animals. 'Humanized' mice have been developed; to result in immune system responses like humans, not only are human cells needed in these mice, but also human liver/liver cells must be supplied as well to supply the clotting factors necessary to result in coagulation factors/parameters like those of humans (Tatsumi et al. 2012).

Historically, ferrets have been used for studies in virology, toxicology, pharmacology, and are considered the best animal model system to mimic human radiation induced vomiting and retching (King 1988, Harding 1995). Ferrets have also been used previously as a hemostasis model for von Willenbrand Factor and Factor XIII studies (Hoogstraten-Miller et al. 1995, Reed and Hounig 1999). Aging ferrets develop coagulopathies, therefore, baseline hemostasis parameters for ferrets have been published (Benson et al. 2008). In our previous studies on blood coagulation parameters in ferrets, we collected blood samples from ferrets that were already being used in emesis studies funded by the National Space Biomedical Research Institute's (NSBRI) Center for Acute Radiation Research (CARR) grant (through

their ‘ tissue sharing ’ activities) and we reported that SPE-like proton radiation exposure resulted in adverse effects on blood coagulation in ferrets (Krigsfeld et al. 2012). Specifically, the SPE radiation exposure resulted in increased prothrombin time (PT) and activated partial thromboplastin time (aPTT) values associated with the extrinsic and intrinsic coagulation pathways (Krigsfeld et al. 2012). Mechanistic studies revealed, in part, that the increases in clotting time were due to the deficiency in a number of factors that were part of extrinsic and/or intrinsic coagulation pathways, including Factor II, V, VII, VIII, IX, X, XI, and XII. These factor deficiencies resulted in a clinically significant bleeding risk, as many of the ferrets had international normalized ratio (INR) values of 2.0. Many of these deleterious radiation effects on coagulation were determined to be radiation dose-rate specific.

Several differences exist between the radiation exposures received by astronauts and radiation therapy patients. In space, the astronauts exposed to SPE radiation receive a whole body exposure, while radiation therapy patients are exposed to a targeted/partial body exposure. Astronauts are also exposed to SPE proton radiation at lower dose rates than patients treated with radiation in the clinic; therefore, many of our studies have involved doses administered at different dose rates. In our studies, we have determined that ferrets exposed to SPE-like, low dose rate (0.5 Gy/hour [h]) proton radiation were significantly more compromised, in terms of blood clotting parameters, compared to ferrets exposed to high dose-rate proton radiation (0.5 Gy/minute [min]). For numerous biological endpoints, lowering the dose-rate of radiation exposure can lead to a sparing effect. For the ferret blood clotting parameters previously analyzed (Krigsfeld et al. 2012), lowering the dose rate had the opposite response.

While previous work suggested that SPE-like proton radiation exposure resulted in increased bleeding time values, the mechanism by which SPE radiation-induced hypocoagulability occurs has yet to be elucidated. We have hypothesized that the SPE-like proton radiation exposure results in the activation of the coagulation cascade that results in a hypocoagulable state. In the work described here, fibrin is measured in the blood and in tissue from irradiated ferrets. Soluble fibrin is found in blood after the activation of the coagulation cascade. A rapid soluble fibrin assay (rapid-SF) was developed at the Loma Linda University Medical Center (LLUMC) for whole blood to aid in early detection of disseminated intravascular coagulation (DIC) in emergency room, operating room, or transplant patients; implementation of the rapid-SF assay has been employed over the last decade at LLUMC (Hay and Bull 2002). This assay system allowed us to measure the activation of the coagulation cascade in irradiated or sham-irradiated ferrets in response to proton radiation exposures. We also address the potential for treating hypocoagulability by supplementing the ferrets with factors that were depleted in our previously reported studies in which ferrets were exposed to SPE-like proton radiation.

Materials and methods

Animals

Sixty de-scented ferrets (12 – 15 weeks of age, 0.5 – 0.8 kg; Marshall Farms, North Rose, NY, USA) were housed at the LLUMC Animal Care Facility. Ferrets were acclimated for 5

days, after which they were weighed and anesthetized with 2 – 3% isoflurane (Midwest Veterinary Supply, Burnsville, MN, USA) inhalation for jugular vein blood collection. Prior to irradiation, ferrets were randomly assigned into three cohorts of approximately 20 animals each: Untreated, treated with a vitamin K1 supplement, phytonadione (International Medication Systems, Limited, South El Monte, CA, USA), or treated with recombinant Factor IX, called Benefix (Wyeth, Philadelphia, PA, USA); each cohort was further divided into sham-irradiated or irradiated groups. The doses of medication used for the ferrets were as previously described (Moller and Tranholm 2010): 1 milligram per 0.5 milliliter of phytonadione (1 mg/0.5 ml) was administered through subcutaneous injection or 50 individual units (IU) per kilogram (kg) of BeneFIX were administered through intraperitoneal injection 30 min prior to irradiation. Ferrets were euthanized 7 days (d) post-irradiation; lung, liver, spleen, and kidney samples were fixed in formalin (Fisher Scientific, Pittsburgh, PA, USA) and transferred to 70% ethanol (EtOH; Decon Laboratories, King of Prussia, PA, USA). The Institutional Animal Care and Use Committees (IACUC) of LLUMC and the University of Pennsylvania (UPENN) approved all of the animal protocols and procedures used in these studies.

Radiation procedures/blood sampling

Ferrets were placed in aerated radiation chambers (16×24×9 centimeters [cm]) for the duration of the radiation exposure. Sham-irradiated ferrets were placed in chambers for the corresponding time needed to expose the irradiated ferrets. For the proton radiation exposures, a custom-designed double-scattering system was developed and built to allow for the delivery of a 50 cm diameter radiation field with a radiation flatness of 3.5% as previously described (Wroe et al. 2013). This system was installed on one of the research beam-lines available at LLUMC and was tuned to deliver an SPE-like proton radiation dose of 1 Gy at a dose-rate of approximately 0.5 Gy/h. An in-house clinical modulator wheel was used to deliver a uniform dose as a function of depth, while a range shifter degraded the beam to 110 MeV at the inside of the irradiation chamber as previously described (Coutrakon et al. 1991, Lesyna 2007). The large field of this system allowed for the irradiation of up to four ferrets at any one time.

Characterization of the proton beam was completed using radiographic film, radiochromic film and ionization chambers. Depth dose profiles for the 155 MeV protons were measured using Gafchromic film (EDR-2; Ashland, Wayne, NJ, USA) that was angled to minimize errors in density between the film and water. Depth dose profiles were also verified with a plane parallel ionization chamber (PTW Markus ionization chamber). Field flatness was established using radiochromic film (Kodak XOMAT-V) at the external surface of the animal cage. The dose at the prescription point (center of modulation) was calibrated using an Exradin T1 ionization chamber.

Rapid soluble fibrin assay

After irradiation, all ferrets were observed for 3 h in the radiation chamber; after which they were anesthetized, blood was drawn directly into a syringe containing 3.8% sodium citrate (Ricca Chemical Company, Arlington, TX, USA) at a 9:1 ratio. The syringe was inverted several times to ensure proper mixing. Blood samples were incubated at 37 ° Celsius (C) for

1 h with rotation to ensure proper mixing. Three drops of blood were discarded and then three drops of blood were added to a borosilicate tube containing protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) that was freshly prepared, as previously described by Hay and Bull (2002). The tube was gently mixed, placed in an oscillator and the time until precipitation was recorded. Results were reported as soluble fibrin units (SFU), which were calculated as described by Hay and Bull (2002).

PT/aPTT assays

For the PT/aPTT assays, blood was collected into vacutainer tubes containing 3.8% sodium citrate (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). Platelet-poor plasma was fractionated by centrifugation (3000 g for 15 min). Plasma was diluted 1:5 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (Invitrogen, Carlsbad, CA, USA) containing 0.1% bovine serum albumin (HBS-B; Fisher Scientific), and then further diluted 1:1 with HBS-B, and incubated for 1 min. An equal amount of TriniCLOT PT excel (Trinity Biotech, Wicklow, Ireland) was added to each sample and clotting times were measured using a STart[®] 4 semi-automated hemostasis analyzer (Diagnostica Stago, Parsippany, NJ, USA). For the aPTT analysis, a second aliquot of plasma was diluted 1:3 with HBS-B, and equal parts of TriniCLOT Automated APTT reagent, HBS-B, and diluted plasma were added to a cuvette and incubated for 3 min. 50 microliters (μ l) of calcium chloride (Fisher Scientific) were added to initiate clotting, and clotting times were measured using the STart[®] 4 semi-automated hemostasis analyzer.

Immunohistochemistry

Tissue sections from formalin fixed tissue were prepared on slides by the Pathology Core Laboratories at the Joseph Stokes Jr. Research Institute as part of the Children's Hospital of Philadelphia. Slides were incubated at 68 ° C for 45 min, after which they were placed in cassettes filled with xylene (Fisher Scientific) and placed on a horizontal shaker twice for 20 min. Samples were rehydrated by EtOH gradient submersion 100 – 50% for 2 min and washed. Antigen retrieval was performed by placing slides in 10 millimolar Sodium Citrate buffer (Fisher Scientific) and heating for 10 min, and the slides were then washed. Hydrogen peroxide solution (3%; Abcam, Cambridge, MA, USA) was loaded onto the tissue for 12 – 15 min, followed by several washing steps with 1 $\times$ Tris Buffered Saline (TBS; Fisher Scientific). Next, samples were incubated with Protein Block (Abcam) for 45 min, followed by rabbit anti-human fibrinogen (Abcam) for 3 h for liver samples and ~ 24 h for lung and kidney samples. Slides were washed with 1 $\times$ TBS and incubated with anti-rabbit-horseradish peroxidase (Abcam). After several 1 $\times$ TBS washes, 20 μ l of chromogen (Abcam) were added to 1 ml of substrate and 100 μ l of solution were added to tissue sections and the reaction was stopped by the addition of water. Samples were dehydrated, cover slipped, and analyzed with an Olympus BX43 microscope with an Infinity 1 digital camera.

Complete blood cell counts

Blood draws were performed prior to the radiation exposure and at 3 h, 24 h and 7 d post-irradiation. Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes

(Becton, Dickinson, and Company) and kept at room temperature. A complete blood count (CBC) with differential analysis was performed using a Bayer Advia 120 Hematology Analyzer (Antech Diagnostics, Lake Success, NY, USA) within 24 h of collection.

Statistical analysis

The data were analyzed to confirm a normal distribution using PRISM 5.0 (Graphpad, La Jolla, CA, USA). The data were further analyzed to determine whether there were statistically significant differences between treatment groups using the Student's paired *t*-test (InStat v. 3.1 software, Graphpad). The results are presented as the mean $\pm$ standard error of the mean (SEM) and differences between treatment groups were considered statistically significant at $p < 0.05$.

Results

SPE-like proton radiation increases the soluble fibrin concentration in blood

Prior to radiation exposure, the mean soluble fibrin concentration was 0.7 ± 0.0 SFU for 10 animals; however, at 3 h post-irradiation with 1 Gy of SPE-like proton radiation, an increase in soluble fibrin concentration was observed, with an average of 2.4 ± 0.8 SFU for 10 ferrets (Figure 1). The concentration of soluble fibrin continued to increase in a statistically significant manner, with an average concentration of 9.9 ± 3.7 SFU at 24 h ($p < 0.05$) for 10 ferrets. By 7 d post-irradiation, the blood coagulation phenotype was normal, as the average soluble fibrin concentration returned to basal levels for six ferrets. To determine whether or not the increase in soluble fibrin was due to factors other than radiation, a cohort was exposed to sham-irradiation (0 Gy). In these animals, the average pre-irradiation soluble concentration was 1.1 ± 0.4 SFU; the SFU in the blood and remained at this low level over the course of 7 d post-sham-irradiation.

Fibrin clots were present in irradiated tissue

In order to confirm the activation of the coagulation cascade, tissue samples were stained with an anti-fibrinogen antibody to visualize fibrin clots in blood vessels. The ferrets irradiated with SPE-like protons exhibited positive staining for fibrin in several blood vessels from the livers, as shown by the arrows in Figure 2A; the livers from sham-irradiated ferrets, however, lacked positive staining for fibrin, as shown in the bottom panel of Figure 2A. In Figure 2B, a blood vessel in the liver of a ferret exposed to SPE-like proton irradiation and a blood vessel in a sham-irradiated ferret are shown. Many of the blood vessels in the irradiated ferrets were occluded with fibrin clots (which were identified with positive staining for fibrin), whereas the blood vessels from sham-irradiated ferrets were negative (i.e., they did not indicate positive staining for fibrin). Positive staining for fibrin was not observed in any tissues evaluated from the sham-irradiated ferrets at all time points, including: liver, lung, kidney, and spleen (Table I). Positive fibrin staining was confirmed in 100% of liver tissue taken from the 3 irradiated ferrets as well as in 2 out of 3 lung and kidney tissues at 3 h post-irradiation. Positive staining for fibrin was observed at 24 h post-irradiation for liver, lung, and kidney tissue in all three irradiated ferrets at this time-point. Minimal to no positive fibrin staining was observed in spleen sections taken from all irradiated ferrets.

SPE-like proton radiation affects complete blood cell counts

Blood samples were taken from the ferrets for CBC counts. Platelets were of particular interest in these measurements, as platelet numbers are critical in primary hemostasis. Prior to SPE-like proton radiation exposure, the average platelet count was $477,000 \pm 97,000$ cells/ μ l ($n = 8$); this concentration is adequate for generation of a platelet ‘plug’, as the normal value for ferrets ranges from 300,000 – 750,000 cells/ μ l. No statistically significant changes in platelet counts were observed over the course of 7 d after radiation exposure; the 7 d post-irradiation average platelet count was $391,000 \pm 67,500$ cells/ μ l (Figure 3A).

While platelet counts are unaffected by radiation exposure within 7 d after exposure, other blood cell counts were reduced by radiation (Figures 3B – D). Prior to radiation exposure, eight ferrets had an average white blood cell (WBC) count of $9.8 \pm 1.6 \times 10^3$ cells/ μ l. At 24 h post-irradiation, WBC counts for a total of 6 ferrets were decreased in a statistically significant manner to an average of $2.6 \pm 0.3 \times 10^3$ cells/ μ l when compared to their pre-irradiation WBC counts ($p < 0.05$). At 7 d, the differences in the WBC counts in the irradiated ferrets compared to the pre-irradiated ferrets were of borderline statistical significance, with an average WBC count of $3.6 \pm 0.4 \times 10^3$ cells/ μ l ($p < 0.1$, $n = 6$).

Lymphocytes are particularly sensitive to ionizing radiation. The preirradiation average lymphocyte count in eight ferrets was $6.0 \pm 1.4 \times 10^3$ cells/ μ l (Figure 3C). At 3 h post-irradiation, the average lymphocyte counts in seven ferrets decreased to $0.8 \pm 0.1 \times 10^3$ cells/ μ l, which represented a statistically significant decrease of 87% when compared to preirradiation counts ($p < 0.05$). Average lymphocyte counts decreased further at 24 h to $0.4 \pm 0.0 \times 10^3$ cells/ μ l ($p < 0.05$). By 7 d, the average lymphocyte counts began to increase to $1.2 \pm 0.2 \times 10^3$ cells/ μ l; however, the differences between the average lymphocyte counts in the irradiated ferrets vs. the pre-irradiated control ferrets were statistically significant, indicating that the lymphocyte counts remained low at this time. The average neutrophil counts for eight ferrets prior to irradiation were $3.1 \pm 0.4 \times 10^3$ cells/ μ l. At 3 h post-irradiation, the average neutrophil count in seven ferrets increased in a statistically significant manner to $5.7 \pm 0.8 \times 10^3$ cells/ μ l (Figure 3D). At 24 h, the average neutrophil count in seven ferrets decreased to $1.9 \pm 0.3 \times 10^3$ cells/ μ l, or 61% of the pre-irradiation average count ($p < 0.05$). At 7 d post-irradiation, neutrophil counts remained relatively low for six ferrets, with an average neutrophil count of $2.1 \pm 0.5 \times 10^3$ cells/ μ l; however, this differences between these 7 d neutrophil counts and the pre-irradiation neutrophil counts were not statistically significantly.

Effects of phytonadione and BeneFIX on SPE-induced hypocoagulability

Our previous studies indicated factor deficiencies after SPE-like proton irradiation, which included deficiencies in Factors II, V, VII, VIII, IX, X, XI, and XII, as well as statistically significant increases in bleeding times (PT/aPTT values) in ferrets exposed to SPE-like (1 Gy) proton radiation exposures (Krigsfeld et al. 2012). The results of experiments in irradiated ferrets with and without treatment with phytonadione and BeneFIX are shown in Figures 4A and 4B. The first column of data in Figure 4A gives the PT results for ferrets exposed to SPE-like (1 Gy) proton radiation (and their pre-irradiation values), indicating that SPE-like proton exposure results in a statistically significant increase in ferret PT values. Irradiated ferrets treated with phytonadione exhibited improvement in their PT values

compared to irradiated ferrets that were not treated with phytonadione. The PT values increased from 24.3 ± 0.9 (in pre-irradiated ferrets) to 25.7 ± 1.2 in (9) irradiated ferrets treated with phytonadione (Figure 4A); this was not a statistically significant increase. BeneFIX treatment also had a beneficial effect on PT values. The pre-irradiation PT values were 26.2 ± 1.9 and at 3 h post-irradiation, the PT values were 25.5 ± 1.5 (Figure 4A) in irradiated ferrets treated with BeneFIX; the differences between these values were not statistically significant. The first column of data in Figure 4B gives the aPTT results for ferrets exposed to 1 Gy of SPE-like proton radiation (and their pre-irradiation values), indicating that SPE-like proton exposure results in a statistically significant increase in ferret aPTT values. For ferrets treated with phytonadione, the aPTT values indicated an increase from 31.8 ± 2.0 (in pre-irradiated ferrets) to 35.1 ± 0.8 in (9) irradiated ferrets treated with phytonadione, $p < 0.1$ (Figure 4B). These results indicate that phytonadione did not lead to improvement in the aPTT values in irradiated ferrets. The aPTT values were positively affected by BeneFIX treatment in the irradiated ferrets; the aPTT values were 27.8 ± 2.9 (at pre-irradiation) and 30.0 ± 2.1 at 3 h post-irradiation (Figure 4B), and the differences between these values were not statistically significantly. Thus, BeneFIX treatment clearly improved clotting values in the irradiated ferrets.

Discussion

The experiments described here were designed to address the mechanism by which an SPE-like proton exposure of 1 Gy results in a decrease in factor concentrations and an increase in PT/aPTT values in ferrets. As described in the Introduction, a dose of 1.38 Gy has been estimated by Hu et al. (2009) as the dose that astronauts could have received to the BFO from EVA during that SPE. We have hypothesized that an SPE-like proton radiation exposure activates the coagulation cascade, resulting in the consumption of the various factors. Activation of the coagulation cascade within 24 h of radiation exposure was confirmed through a soluble fibrin assay and positive staining for fibrin clots in the lungs, livers, and kidneys of irradiated ferrets. As the platelet counts remained relatively unchanged over the course of 7 d, these data suggest that activation of hemostasis occurred due to effects on coagulation and not from a decrease in the number of peripheral platelets.

The SPE-like proton radiation activation of the coagulation cascade results in the consumption of clotting factors which, in turn, leaves the animal deficient in clotting factors. Soluble fibrin is present in the blood when the clotting cascade has been activated and this has been reported to be a marker of DIC (Hay and Bull 2002). DIC is a serious, life-threatening condition in which clotting and bleeding are occurring at the same time, and it is often fatal due to multiple organ failure. In order to confirm the results of the soluble fibrin assay indicating that the coagulation cascade was activated, immunohistochemistry techniques were employed to detect fibrin clots in isolated tissues. Sham-irradiated ferret tissue lacks positive staining for fibrin in blood vessels of livers, lungs, and kidneys, while positive staining for fibrin was observed at 3 and 24 h post-irradiation in the livers, lungs, and kidneys of irradiated animals. Positive staining for fibrin is directly correlated with activation of the coagulation cascade, as fibrin clots are not present in blood without the enzymatic cleavage of fibrinogen to fibrin. The positive staining results confirm the soluble fibrin assay results and give further evidence that the clotting cascade has been activated by

SPE proton radiation exposure. Mechanistically, activation of the clotting cascade decreases the bioavailability of the factors in the blood of irradiated ferrets and results in increased PT/aPTT values, as was reported in our previously published studies (Krigsfeld et al. 2012).

Potential ramifications of dysregulation of the coagulation cascade may be further exacerbated by significant decreases in blood cell counts (Figure 3B – D). Prolonged decreases in WBC, neutrophil, and lymphocyte counts leave the irradiated ferrets at risk for infection that would further overwhelm hemostasis and potentially lead to a DIC phenotype, as is observed in patients with sepsis. Indeed, it is well known that endotoxin (lipid A portion) released by Gram-negative bacteria activates certain factors of the intrinsic coagulation cascade such as Factor XII that, in turn, initiates fibrin formation and thereby also increases the risk for development of DIC.

To counteract the factor deficiencies that resulted in increased PT/aPTT values, two countermeasures were evaluated: Phytonadione (Vitamin K) and BeneFIX (recombinant Factor IX). It is noteworthy that treating irradiated ferrets with BeneFIX did improve the ferret clotting times by reducing them back to pre-irradiation values. SPE-like proton radiation led to increased clotting times in the irradiated ferrets, and treatment with BeneFIX led to a reduction in the bleeding times that were essentially equivalent to those of the non-irradiated control ferrets. Treatment with BeneFIX increases the concentration of Factor IX, a factor depleted post-irradiation. Thus, treatment with BeneFIX could have beneficial effects on coagulation when administered after the radiation exposure.

Additionally, the use of phytonadione as a potential countermeasure for the increased clotting times in irradiated ferrets was investigated. Vitamin K is essential for the post-translational modification of a glutamate amino acid to a carboxylated-glutamate that is necessary for Factor II, VII, IX, and X (Furie and Furie 1988). In these experiments, phytonadione had a minor beneficial effect on PT values in irradiated ferrets, but did not have effects on the aPTT values. Treatment of DIC with clotting factors or other components of plasma has been discussed elsewhere (Levi et al. 2009).

We have concluded that SPE-like proton radiation results in hypocoagulability that is due to the activation of the clotting cascade and the consumption of factors involved in coagulation. SPE-like proton radiation effects also include leucopenia and severe lymphocytopenia, which, in combination with the effects of hemostasis, could have major health consequences. In addition, our results indicate that BeneFIX can serve as a potential countermeasure for the increased bleeding times in ferrets caused by exposure to SPE-like proton radiation.

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Figure 1.

Fibrin concentrations in the blood are elevated after ferret exposure to a dose of 1 Gy of SPE-like proton irradiation. Effects of sham-irradiation and SPE-like proton irradiation on fibrin levels, which are expressed in terms of soluble fibrin units (SFU) for 10 ferrets at 3 h, 24 h, and seven ferrets at 7 d and sham-irradiated time-points. Exposure to SPE-like proton irradiation results in increased fibrin concentrations at 3 h ($\# p < 0.1$) and 24 h ($*p < 0.05$), compared to individual pre-irradiation SFU using a paired *t*-test. The y-axis is represented by an arbitrary concentration of relative SFU that directly correlates with the concentrations of soluble fibrin present in the blood. Error bars indicate the standard error of the mean for $n = 3$ independent experiments.

Figure 2.

Fibrin clots can be observed in the liver blood vessels from irradiated ferrets, but are not observed in liver blood vessels from sham-irradiated ferrets. In this Figure, representative images are shown of isolated liver tissue taken from a ferret irradiated with SPE-like protons at 3 h post-irradiation or from a sham-irradiated ferret. Tissue sections are stained with an anti-fibrinogen antibody (to identify fibrin clots) or a rabbit polyclonal IgG used as negative control. Negative controls are included to represent the lack of non-specific antibody binding. (A) Liver from a ferret exposed to SPE-like proton radiation and stained with anti-fibrinogen antibody contains fibrin clots in a number of blood vessels, while the negative control image (bottom left panel) does not indicate the existence of fibrin clots; sham-

irradiated ferret livers have clear blood vessels. Arrows indicate blood vessels (in sham and irradiated animals). The absence of fibrin clots (in sham-irradiated animals) is also indicated in (A). Image represents 40×magnification. (B) A 200×magnified image indicates positive staining for a fibrin clot in a liver blood vessel of an irradiated ferret as compared to the lack of a fibrin clot in a blood vessel from a sham-irradiated ferret. Fibrin clots are only present in irradiated ferret blood vessels . This Figure is reproduced in color in the online version of *International Journal of Radiation Biology* .

Figure 3.

Effects of SPE-like proton radiation on blood cell counts in ferrets. *P*-values were determined using a paired t-test. * $p < 0.05$. Error bars indicate the standard error of the mean for $n = 2$ independent experiments. (A) Platelet counts remained normal throughout the course of the study ($n = 6 - 8$ ferrets), (B) Ferrets exposed to proton irradiation exhibited a decrease in white blood cell counts compared to pre-irradiation values at time-points of 3 h, 24 h, and 7 d ($n = 6 - 8$ ferrets), (C) Ferrets exposed to proton irradiation had a statistically significant decrease in lymphocyte counts compared to pre-irradiation values at all time points evaluated ($n = 6 - 8$ ferrets), (D) Ferrets exposed to proton irradiation had a statistically significant increase in neutrophil counts at 3 h post-irradiation compared to pre-irradiation values; however, the neutrophil counts were reduced in a statistically significant manner compared to pre-irradiation values at 24 h ($n = 6 - 8$ ferrets). At 7 d, the differences between the neutrophil counts in irradiated ferrets compared to their pre-irradiation values were not statistically significant.

Figure 4.

BeneFIX mitigates the statistically significant increase in PT and aPTT values in ferrets exposed to 1 Gy SPE-like proton irradiation. Plasma was assayed for PT/aPTT prior to radiation exposure (preirradiation), or at 3 h post-irradiation. *P*-values were determined using a paired t-test. **p* < 0.05. Error bars indicate the standard error of the mean for *n* = 3 independent experiments. (A) A statistically significant increase in PT values was observed with exposure of ferrets to a dose of 1 Gy, *p* < 0.05, *n* 9 ferrets. Pre-treatment with phytonadione or BeneFIX followed by radiation exposure results in a lack of a statistically significant increase in PT values, *p* > 0.05, *n* = 6 – 9 ferrets, (B) Pre-treatment with BeneFIX followed by radiation exposure results in a lack of hypocoagulability (*p* > 0.05) as measured

by aPTT values, $n = 6$ ferrets. Phytonadione treatment did not mitigate the radiation-induced increase in aPTT values, # $p > 0.1$, $n = 9$ ferrets.

Table I

Presence of fibrin clots in tissue of irradiated ferrets. A summary of tissues evaluated for fibrin clotting and the number of ferrets reporting positive staining for fibrin in blood vessels of various organs at 3 h and 24 h post-irradiation is shown. The numbers reported are out of a total of three animals and the numbers in brackets are percentages of ferrets exhibiting positive staining for fibrin clots.

Group	Liver	Lung	Kidney	Spleen
Sham-irradiation	0/3 (0%) ^I	0/3 (0%)	0/3 (0%)	0/3 (0%)
3 h post-irradiation	3/3 (100%)	2/3 (66%)	2/3 (66%)	1/3 (33%)
24 h post-irradiation	3/3 (100%)	3/3 (100%)	3/3 (100%)	0/3 (0%)

^I Number in brackets is percentage of animals exhibiting positive staining for fibrin clots.