Chapter IX: Acute Radiation Effects

(K) Effects of Total Body Irradiation on the Pregnant Female, Fetus and Placenta

Michael W. Epperly[1], Lora Rigatti[2], and Joel S. Greenberger[1]

[1]Dept. of Radiation Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA
[2]Dept. of Animal Medicine, D.L.A.R.-Veterinary Services, University of Pittsburgh, Pittsburgh, PA

Abstract:

The acute and late effects of irradiation on the developing fetus are of great concern for study in the fields of Environmental Health, Clinical Medicine, and Radiation Counter-Terrorism. Prior research describing the teratogenic effects of irradiation on the developing human fetus, as well as animal model studies in the embryo have led to policies and procedures in community hospitals and larger hospital systems that minimize the effect of irradiation on the fetus. While research in the radiobiology of fetal and placental damage has been sparse since the end of the Cold War, much work has been carried out on the effects of infectious agents, chemical toxins including: the effects of alcohol and cigarette smoke, diet, and other environmental agents on the developing human fetus. This section of the web-based textbook will focus on current knowledge about acute and chronic ionizing radiation effects on the developing fetus. We will discuss current knowledge about fetal age, and time of exposure, or the effects that are specific to placenta compared to fetus. We will describe direct fetal irradiation effects, as well as indirect effects through irradiation of the mother. We will describe methods for study of fetal mouse irradiation and analysis of effects of administered mitigators. We will describe methods for study of fetal mouse irradiation and analysis of effects of administered mitigators. Of critical importance is the development of systems by which to test the effects of new radiation countermeasures on pregnant women, and how to be certain that a radiation mitigator, which is safe in adults will also be safe for the developing embryo.

Introduction:

The effects of ionizing irradiation on fetal development and publications in this area rely upon classic studies that were carried out over two time intervals: early studies during the initial discovery of x-rays and radiation emitting isotopes around the turn of the 20th Century, and a second series of experiments and publications from international radiation biology laboratories carried out during the Cold War (1945 – 1989). Several basic radiobiological principles have endured. One principle is the fragility to irradiation of the early gestational embryo (first trimester in humans) leading to cell death and spontaneous abortion. A second concept is that of teratogenesis and fetal abnormalities in embryos irradiated to low doses during early gestation, as well as during mid or late gestation and resulting in genetic, physical, physiological, neurocognitive, and/or developmental defects. Based on current knowledge, pharmaceutical companies bringing new drugs through the development stage, including animal model studies, and early clinical trials, have focused on critical analysis of safety to both mother and fetus during pregnancy. Several categories of antimicrobial and anticancer agents have been noted to be particularly risky to the fetus, as well as for pregnant women. Many clinical care providers require periodic pregnancy tests/or discussion of the risks of any drug or combination of drugs on the developing fetus. In many cases, evaluations may include statements such as "safety in pregnancy is not established". Delineation of the risks of care providers to the patient and her family have been a mainstay of the informed consent process.

One example, of the effect of concern for fetal developmental effects, is the analysis of data from the 1950s and use of the sedative antidepressant drug, Thalidamide, which resulted in fetal limb abnormalities leading to the removal of the drug from the market. The mechanism of these fetal abnormalities from Thalidamide has recently been shown to involve endothelial cells in the developing fetus. In the 1990s, Thalidamide and analogs returned to clinical use for treatment of multiple myeloma and other malignancies involving cancer cell interactions with reticuloendothelial (endothelial-like) cells in the marrow microenvironment. A guiding principle for current drug discovery research is the concept that any indication for specific use of a pharmaceutical in a specific disease category must include concern for potential teratogenic and other harmful effects to the developing fetus.

Historical Perspective

There have been two periods during which the radiobiology of fetal development was intensively studied. The first was after the discovery of ionizing irradiation in the publication of the first x-ray machine by Roentgen in 1895, followed shortly by the discovery of radiation emitting isotopes by Marie and Pierre Curie (Radium), and a second interval of intense research on fetal radiobiology, during the early decades of the "Cold-War" 1945 – 1975.

Early publications on ionizing irradiation effects in the fetus are summarized in most radiobiology textbooks. Principles well known to students of radiobiology, clinicians, and radiation safety officers include understanding that the early gestational fetus is most vulnerable to lethality of ionizing irradiation and can cause termination of pregnancy (spontaneous abortion). A second principle is that exposure to even low doses of ionizing irradiation to early

gestation, as well as mid-gestational, and late gestational fetus may be teratogenic (cause physical or neurocognitive abnormalities of growth and development) (Table 1).

Radiation counter-terrorism research in the modern era, must emphasis a need to understand the effects on the developing fetus of any new countermeasure against ionizing irradiation. In the event of an accidental or willful irradiation event including dispersal of radiation emitting isotopes, a subgroup of the population in the geographic area of dispersal will include pregnant women. Therefore, a radiation mitigator drug must be safe for the mother, as well as the unborn child. Understanding many principles of radiobiology of the developing fetus includes an answer to the following, as well as other questions:

1. Will an ingested or inhaled or injected radioisotope cross the placenta?
2. In which fetal organs will the dispersed isotope concentrate?
3. How will the half-life of the isotope determine lethal or teratogenic effects?
4. What teratogenic effects can be expected?
5. What effects of irradiation to the developing fetus may not be detectable until after birth, later life, or potentially when the irradiated child reaches adulthood?
6. Will irradiation effects on the developing fetus affect his/her fertility as an adult?
7. Perhaps most importantly, will administration of a radiation countermeasures be deleterious to the developing fetus and/or exacerbate any irradiation damage to the fetus that might not otherwise be measurable in the mother?

This chapter will focus on methodologies for carrying out research in the important area of radiobiology of the fetus. Animal models will be discussed, and the importance of the placenta and understanding effects of irradiation on the fetus will be highlighted. This section is particularly relevant given the importance of the placenta and understanding the pathophysiology of the effects of other toxic agents to the developing fetus including: alcohol, cigarette smoke, toxic metals, drugs including opiates, Human Papilloma Virus (HPV), Human Immunodeficiency Virus (HIV), Cytomegalovirus (CMV), and Zika Virus.

A section will be devoted to basic methodologies of studying fetal damaging effects of ionizing irradiation including references to the methodologies described elsewhere in this web-based textbook. These methods include: real time polymerase chain reaction for quantitation of RNA transcripts, Luminex assay for quantitation of proteins, histopathology techniques for visualizing irradiation effects on specific cell populations within a tissue in situ, assays for neurocognitive effects on the newborn and young adult, measure of developmental retardation effects of irradiation on the fetal nervous system, and quantitating effects of ionizing irradiation on interaction in utero between maternal and fetal physiology.

Basic Principles in Radiobiology of the Developing Fetus

Ionizing irradiation is particularly damaging to dividing cells. The basic principles discussed in Chapter 1 hold true in the developing fetus. However, the rapidly developing cell populations in the fetus include those, which are non-proliferative in the adult, most prominently the central nervous system. Depending upon the animal model, gestational age will determine the target

organs, which display ionizing irradiation effects at a particular time. This timeline is shown for mice with gestation time of 21 days in Table 1.

During the Cold War (1945-1989), irradiation experiments were carried out looking at acute toxicity to large animals including: pigs, cattle, dogs, and non-human primates. Military exercises carried out for management of irradiation casualties that were anticipated during a nuclear war focused on irradiation effects in adults, and little attention was directed to the potential for irradiation exposure of the fetus in pregnant females. Experience from nuclear accidents in both radioisotope production facilities (weapons production) and in nuclear power plants, led to evidence that exposure of pregnant humans to irradiation followed specific principles with respect to fetal outcomes.

Radiation of early gestational embryos leads to abortion, mid-gestational irradiation may cause major defects in the developing target organ systems including: central nervous system, heart, and great vessels, and irradiation of the late term fetus may be initially tolerated, but be detected later as cognitive or reproductive defects in the adult. There is relatively little information with respect to fetal outcomes on the interaction of commonly used pharmaceutical agents by pregnant women during the time of irradiation exposure to the fetus. Most drugs that are potentially teratogenic in animal models never get to the stage of clinical use, but the potential for a given drug exacerbating effects of irradiation has not been addressed. These questions become relevant again in the development and application of new radiation mitigator drugs, which would be distributed uniformly to large numbers of radiation victims of radiation counter-terrorism scenario including pregnant women.

Ionizing Irradiation Effects on the Placenta

The placenta functions as both a metabolic and an immune system for the developing fetus. Maternal and fetal circulation interact through a rich vascular network in the placenta (4). Vascular interactions provide support for the developing fetus by providing all maternal metabolic functions (liver, kidney, nutritional transfer, and metabolic processing). The placenta also separates from the mother, the independent fetal functions (development of the new fetal hematopoietic system, development of the fetal central nervous system, and the fetal cardiovascular systems).

The immunological functions of the placenta also provide an intricate balance allowing the maternal humoral and cellular immune functions to protect the developing fetus until the independent fetal lymphohematopoietic system becomes independent. Since the ionizing irradiation sensitivity of lymphoid cells in both the maternal and developing fetal lymphohematopoietic systems is expected, there has been interest in the damage response capacity of the fetal independent from the placental systems. In the case of the maternal total body irradiation response, there are principles well established in other chapters of this textbook regarding bone marrow and intestinal radiosensitivity, which will clearly apply. While little is known about the total body radiation response of the fetal intestinal and lymphohematopoietic systems with respect to the development of an independent metabolic network, it is well-established that the mid-gestational fetus moves rapidly to an independent metabolic system.

Even before intestinal uptake of nutrients, renal and hepatic clearance of toxic substances by metabolism, there is clearly fetal liver accumulation of glycogen. There are also fetal storage systems for calcium metabolism (developing skeleton), and metabolic reserves (hepatic glycogen, fat stores, gluconeogenesis). While ionizing irradiation affects several developing organ systems moving toward fetal independence during mid and late gestation, the mechanism of each organ specific effect is largely unknown. Ionizing irradiation is clearly known to damage the placenta, which has a unique pattern of stress response gene expression (1).

Methodologies for Study of Ionizing Irradiation Effects on the Fetus

There has been much research on the murine model systems for quantitating fetal development.

Generation of homologous recombinant deletion (knockout) and transgenic mouse strains have provided opportunities to study mechanism of the "neonatal lethal" genotype. For example, a genetic absence of a critical DNA repair gene in the Fanconi Anemia (FA) pathway, in Fancd2-/- mice was not associated with lethality of the developing embryo, and bypass pathways were available during growth and development to allow live birth of knockout mice, producing no detectable quantities of this gene product. However, in the case of homologous recombinant deletion of the acid aldehyde dehydrogenase-2 (ADH2) gene, these animals died during gestation (2-3).

An analysis of the mechanism of death revealed that the developing fetus critically required the ADH2 protein to counteract lethal DNA strand breaks that could not be, otherwise, repaired in the absence of the FancA gene product. During early gestation, maternal ALDH2 function would be provided by transplacental transfer of gene product and facilitate removal of aldehydes form the developing fetus, so that this toxic reactive oxygen species could not afflict lethal DNA double strand breaks, which could not be repaired in the absence of the Fancd2 gene product. There are many other examples of the molecular cause of fetal lethality in utero, based on time of gestation when the genetic effect is expressed, and which, therefore, could be attributed to a genetic cause of a particular metabolic defect (Table 1).

New Concerns for Fetal and Maternal Radiation Biology

A concern for effects of low level irradiation has led to the widespread application of radon tests in the real estate market alerting potential homeowners to the hazards of continuous low level irradiation. In hospitals and clinics, signage alerts women to the possibility of damaging effects of x-rays, CT scans, and other investigational radiographic modalities on the developing fetus. These concepts are manageable in the peacetime environment. While the fetus has protective mechanisms against scar formation from wounding, the fetal response to ionizing irradiation damage is different. The concepts of radiation damage and response breakdown in the event of a radiation accident or willful dispersal of ionizing irradiation agents. While chronic low dose irradiation may be safe, there is little experimental data to support the designation of a maximum safe dose or dose rate. Experimental models for low dose or low dose rate effects in pregnant females are not readily available.

This section of the web-based textbook will review methodologies that are currently used to analyze the effects of irradiation on the developing fetus, with particular attention to the placenta. A separate section of this chapter will deal with the methods for evaluating the effects of a new radiation protector or mitigator drug or combination of drugs on the developing fetus and how modern biochemical and molecular biologic tools can be used to supplement data on histopathology of fetal development, behavioral analysis of cognitive and motor development after birth, and an analysis of the effects of new radiation mitigators on these processes.

Analysis of Irradiation Effects on the Developing Fetus (animal models):

As shown in Table 1, the mid-gestation fetal mouse irradiation (E13.5) was often chosen for TBI studies with pregnant female mice based on a gestation time of 21 days from conception to birth the E13.5 mouse serves as a model for study of irradiation effects on the developing brain, heart, and lungs (Table 1). Mice irradiated at E13.5 show a clear decrease in fetal movement measured at E20. The radiation mitigator drug, JP4-039 (2-3), given at E14.5 to the mother I.V. clearly ameliorated the visible decreased fetal movement in utero at E20. A focus on the E13 time point was also chosen based on predicted effects of development stages at this time point on heart and blood vessels, and on the central nervous system (Table 1).

Ionizing irradiation effects on the placenta and similarity of some damage effects to some in utero virus or drug effects.

The effects of ionizing irradiation on the mouse and human placenta have been described (1-4).

How to Study Radiation Countermeasure Drug or Other Agent Amelioration Effects on Radiation Damage Including Radiation Isotope Transport Across the Placenta.

Methodology has been published on the ways to study virus effects on the placenta, and how virus influences the fetus including CMV, Zika Virus, HPV, and others.

Effect of Irradiation on the Developing Mid-Gestational Embryo:

Studies on irradiated E13.5 fetal mice revealed effects of 3 Gy TBI on liver glycogen, brain development (cerebral, cerebellar, and hippocampus) cellularity.

Knowledge About Whether the Amelioration Effect of a Radiation Mitigator On the Fetus is Associated with Drug Crossing the Placenta.

Early studies with MnSOD-PL facilitated understanding of direct effects on the fetus, or indirect effects through the irradiated and drug treated mother (1). Irradiation at E13.5 decreases fetal birthweight, fetal length, and also the damage to the placenta. The gross anatomic irradiation damage to fetus brain, as well as gross changes in liver, may be ameliorated by a mitigator drug such as JP4-039 (2-4).

Effect of Ionizing Irradiation on E13.5 Fetal Mice:

Research on irradiation effects on the fetus largely dissipated after the Cold War with a decreased concern for massive thermonuclear war. Both the former Soviet Union and United States Radiation Biology Programs focused on more clinically related biology. The state of the literature during the Cold War and in previous studies is well summarized in a recent textbook "Radiobiology for the Radiologist" Hall and Gaccia, Editors, Lippincott. A summary of fetal radiation biology in the current state of knowledge is quite interesting and predictable.

The developing fetus in animal models, as well as in humans, goes through stages of tissue development consistent with embryogenesis. Examples from the mouse model are highly instructive, because most work has been done in rodents (Table 1).

Specific Methods for Studying Irradiation Effects on the Development of the Fetus: Collection and Storage of Plasma, Intestine, and Bone Marrow for Histopathology.

Mice are irradiated as described (1-3), and sacrificed at serial time points after irradiation. Plasma is collected according to published methods. Intestine (ilium) is removed and placed on dry ice to freeze prior to storage at -80 degrees Celsius. Bone marrow (femur and tibia) is removed, centrifuged at 2000 rpm at 4-6 degrees Celsius for 10 minutes, with the supernatant discarded, leaving a concentrated pellet, and is stored at -80 degrees Celsius.

Histopathology

Fixation in Hematoxylin & Eosin and sectioning have been discussed in detail (1).

Rt-PCR and Proteomics Methods (Preparation of Plasma, Intestine, and Bone Marrow)

RNA assay by rt-PCR is well described (5). Plasma is thawed on ice to room temperature, and vortexed, to remove particulate prior to micropipetting for dilution for the Luminex Protein Assay (6). 4 milligrams of intestine was dissected, weighed, and then homogenized in 1000 mL of 0.1% phosphate-buffered saline (PBS), and stored at -80 degree Celsius. Prior to use in the Luminex Protein Assay, intestine homogenate is thawed to room temperature and centrifuged as previously described. The bone marrow pellet is reconstituted via serial pipetting and homogenized in 500 mL of 0.1% PBS, and stored at -80 degrees Celsius. Prior to use in the Luminex Protein Assay, the bone marrow homogenate is thawed to room temperature and centrifuged as previously described.

Protein Immunoassay

Two different protein assays are run for each fetal tissue to study a mitigator drug (example: JP4-039) (2-3) (plasma, brain, intestine, bone marrow) and experimental group (9.25 Gy, 9.25 Gy + JP4-039 (2-3, 6), 9.25 Gy. Single Plex Magnetic Bead Kit, as well as a 32 Multiplex Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA) that tested protein concentrations for Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF, and others (6).

Each kit (MCYTOMAG-70K, TGFBMAG-64K-01) provides reagents for use in the Luminex Protein Immunoassay, which included 1 vial of Mouse Cytokine/Chemokine Standard, 2 vials Mouse Cytokine/Chemokine Quality Controls, 1 vial Serum Matrix, 1 96-Well Plate, 30 mL Assay Buffer, 60 mL 10X Wash Buffer, 1 3.2 mL bottle Mouse Cytokine Detection Antibodies, 1 3.2 mL bottle Streptavidin-Phycoerythrin. The 32 Multiplex kit (MCYTOMAG-70K) provides 1 3.5 mL Pre-Mixed 32-plex Beads. The TGFB1 kit (TGFBMAG-64K-01) provides the above reagents, as well as 5 mL Sample Diluent, 1.0 mL 1.0 N HCl, 1.0 mL 1 N NaOH, and 1 3.5 mL Anti-TGFB1 Bead.

Preparation of Reagents for Immunoassay (6)

Preparation of Beads, Quality Controls, Wash Buffer, and Serum Matrix

Antibody-immobilization beads are sonicated for 30 seconds and then vortexed for 1-minute prior to use. Quality Control 1 and 2 were reconstituted with 250 microliters of Assay Buffer. 60 mL 10X Wash Buffer is diluted 1:10 with 540 mL deionized water.

For TGFB1, Serum Matrix (for plasma samples) is reconstituted in 1.0 mL deionized water and 4.0 mL Assay Buffer and allowed to sit for 10 minutes at room temperature. 0.1 mL of reconstituted Serum Matrix is then micropipetted and diluted in 0.5 mL Assay Buffer, for a final dilution of 1:30.

For 32 Multiplex, Serum Matrix is reconstituted in 2.0 mL of Assay Buffer, and allowed to sit at room temperature for 10 minutes.

Preparation of Standards

Mouse Cytokine Standard is reconstituted in 250 microliters Assay Buffer, inverted to mix, vortexed, and then transferred to a polypropylene microfuge tube and labeled as “Standard 6.” Serial dilutions are performed, by which 50 microliters of Standards 6, 5, 4, 3, and 2, is micropipetted to a microfuge tube containing either 200 microliters of Assay Buffer (for 32 Multiplex), or 150 microliters of Assay Buffer (for TGFB1), until Standard 1 are obtained (6).

Dilution and Treatment of Samples

For both 32 Multiplex and TGFB1, plasma samples required a dilution. 32 Multiplex plasma samples are diluted 1:2 by combining 25 microliters of plasma with 25 microliters of Assay Buffer. TGFB1 plasma samples are diluted 1:5 by combining 12.5 microliters of plasma with 50 microliters Sample Diluent. Following dilution, 25 microliters of each 1:5 diluted sample is plated on a 96-Well Plate and treated with 2.0 microliters of 1.0 N HCl, and is tested to ensure pH was below 3.0. The plate is sealed and covered with aluminum foil, and shaken at room temperature for 15 minutes. The acid-treated plasma samples are then further diluted 1:6 by combining 10 microliters of acid-treated plasma with 50 microliters of Assay Buffer, leading to a final dilution of 1:30.

Intestine and bone marrow samples for the TGFB1 Immunoassay are also required an acidification step. 25 microliters of each tissue sample was plated onto a 96-Well Plate, treated with 2.0 microliters of 1.0 N HCl, and are tested to ensure pH was below 3.0. The plate was sealed and covered with aluminum foil, and shaken at room temperature for 15 minutes. The acid-treated samples are then neutralized with 2 microliters 1.0 N NaOH prior to addition to the sample well (6).

Luminex Immunoassay Procedure

All plasma, intestine, bone marrow, brain, liver, and other organ samples are prepared as previously described for tissue removal and sectioning (6).

32-Multiplex Immunoassay

First, 200 microliters of Wash Buffer is added to each well of the plate. The plate was then sealed and covered on a plate shaker for 10 minutes at room temperature. Wash buffer is then removed via suction.

25 microliters of each prepared Standard and Control are added to the appropriate well, followed by 25 microliters of Assay Buffer to the Background (0 pg/ml Standard) and sample wells. 25 microliters of either Serum Matrix (for plasma) or 0.1% PBS (for intestine and bone marrow, or other organs) is added to each Background, Standard, and Control well. 25 microliters of sample is then added to each Sample well. The bottle of Pre-mixed Beads is vortexed and then 25 microliters is added to each well. The plate is then sealed and covered and incubated overnight at 2-8 degrees Celsius.

The next day, the well contents are removed via suction, and the plates are washed twice with 200 microliters of Wash Buffer, as described in the first step. 25 microliters of Detection Antibodies are then added to each well, and the plate was sealed, covered, and incubated for 1 hour on a plate shaker at room temperature. Following incubation, 25 microliters of Streptavidin-Phycoerythrin was added to each well, and the plate is again sealed, covered, and incubated for 30 minutes on a plate shaker at room temperature. Following incubation, well contents are removed via suction, and the plates are washed twice with 200 microliters of Wash Buffer as previously described. Following the two washes, 150 microliters of Wash Buffer is added to each well, the plate is placed on a plate shaker for 5 minutes, and then read on Luminex (6).

TGFB1 Immunoassay

First, 200 microliters of Assay Buffer is added to each well of the plate. The plate was then sealed and covered on a plate shaker for 10 minutes at room temperature. Wash buffer is then removed via suction (6).

25 microliters of each prepared Standard and Control is added to the appropriate well, followed by 25 microliters of Assay Buffer to the Background (0 pg/ml Standard) and sample wells. 25

microliters of either Serum Matrix (for plasma) or 0.1% PBS (for intestine and bone marrow) is added to each Background, Standard, and Control well. 25 microliters of sample is then added to each Sample well. The bottle of Pre-mixed Beads is vortexed and then 25 microliters is added to each well. The plate is then sealed and covered and incubated overnight at 2-8 degrees Celsius.

The next day, the well contents are removed via suction, and the plates are washed twice with 200 microliters of Wash Buffer, as described in the 32-Multiplex Immunoassay. 25 microliters of Detection Antibodies are then added to each well, and the plate is sealed, covered, and incubated for 1 hour on a plate shaker at room temperature. Following incubation, 25 microliters of Streptavidin-Phycoerythrin is added to each well, and the plate is again sealed, covered, and incubated for 30 minutes on a plate shaker at room temperature. Following incubation, well contents are removed via suction, and the plates are washed twice with 200 microliters of Wash Buffer as previously described. Following the two washes, 100 microliters of Wash Buffer is added to all wells, the plate is placed on a plate shaker for 5 minutes, and then read on Luminex.

Protein Concentration Determination

Intestine and bone marrow homogenates are tested for protein concentration using the manufacturer's software program. BioRad protein detection assay is diluted to 1:5 in deionized water. Each sample and standard (2mg/ml, 1mg/ml, 0.75mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0mg/ml) is run plated on a 96-Flat Bottom Well Plate, in triplicate with 5 microliters of sample/standard combined. 250 microliters of the 1:5 diluted protein detection assay is added to each well and run for analysis at a wavelength of 595 nm.

The average of each sample and standard is calculated, and a standard curve is formulated based on the averages of the standards. This standard curve is used to obtain the actual protein concentrations of each sample, which is used standardize the Luminex Immunoassay results from "pg/ml" to "pg/mg protein."

Data Analysis

Standardization of Fetal Compared to Maternal Tissues Including: Intestine and Bone Marrow Results

Luminex reported the protein concentrations in pg/ml, but given the varying concentrations of protein per sample, a protein concentration assay is run on each sample, as previously described. For each intestine and bone marrow sample, the Luminex result for that sample is divided by the determined protein concentration to give standardized concentrations of pg/mg protein, as that is more accurate than pg/ml. Student's unpaired t-tests are used to compare each group that received mitigating drugs' time point to its corresponding time point in the control group, as well as all days in each experimental group (including the control) to Day 0 (non-irradiated baseline) of the control group (6).

Videography of the Fetus in the Uterus

To determine whether irradiation induces defects in placental circulation and/or fetal development, and which have been shown to induce fetal death either at the time of birth or within the first 24 hrs after birth, a technique was used to examine fetal movement in the uterus. Female pregnant mice can be anesthetized on day E21, the day of expected completion of gestation and birth and the abdomen opened with a midline incision. Camera videography can be carried out to examine fetal movement in the uterus.

Unirradiated fetal mice demonstrate significant movement, measured in number of movements over 10 seconds. Each individual fetal animal can be studied on the videogram, and a mean and standard error of movement was calculated based on the number of movements over a 10, 20, or 30 second interval. The movement of fetal mice that had been irradiated at E13.5, when the video is taken at E21, should show decrease in movement. In addition, the uterus should show a blanching, suggestive of poor circulation, and the fetal mice may be smaller. In fetal mice that had been irradiated on day E13.5, and then the mother given JP4-039/F14 intravenously at E14.5 (or another mitigator), there may be some improvement in fetal movement, although not to the level seen in the control unirradiated mice. These measurements may be helpful since, as described above, newborn mice may die on day of birth or within 24 hrs, and thus, the physiologic functions of these animals are difficult to study.

Physiologic Functioning of the Fetal Liver, Cardiovascular System, and Central Nervous System

Newborn mice alive within the first 24 hrs of birth can be studied for the histopathology of various organs. It may be obvious that there are changes in glycogen deposition in the fetal liver of irradiated animals, and even decreased in animals that had received JP4-039 after irradiation. Control fetal mice (pups) on the day of birth showed relatively high levels of glycogen indicative of good nutrition and storage of nutrients. The depletion of glycogen is an indication of fetal stress, and this has been documented elsewhere. The cardiovascular system, which is known to be developing during day E13.5 (the day of irradiation). The central nervous system effects may be significant in documenting multiple defects in irradiated fetal mice, irradiated on day E13.5. These data may be classified in several categories: fetal mice with decrease in the cerebral cortex, cerebellum, and hippocampus with respect to numbers of neurons in layers of neurons. In animals that were born of irradiated mothers and survived for several weeks, one may detect hydrocephalus and misshapen skulls due to accumulation of fluid in the developing fetal brain. These defects may not seen to a significant magnitude in animals that received JP4-039 or another mitigator via the maternal circulation on E13.5.

Studies of Radiation Effects on the Placenta

There have been detailed analysis of the placenta in a previous publication (4). However, an effect of JP4-039 on placental weight may be observed. Placental weight at the time of birth was decreased in 3.0 Gy (E13.5) irradiated fetal mice, may be ameliorated by JP4-039 given at day E14.5. Controls for these experiments should include pregnant female mice given 3 Gy irradiation and F14 alone, or F14 containing the non-mitochondrial targeted 4-Amino-Tempo, on

E14.5. These animals may not demonstrate amelioration of the irradiation effect as was seen in those pregnant females given JP4-039/F14 on day E14.5.

Lung Development in Fetal Mice and Lung Function in Newborns

One may determine the cause of death in newborn mice either dying at the time of birth or within 24 hrs after birth. These studies require placing video cameras in the mouse quarters and focusing on watching maternal birth, and the number of live births. This procedure is necessary, because it is well known that mice often will not accept damaged or defective newborn babies, and these can be removed from the cage such that observation only once per day could lead to a false impression that mice had not been born, whereas in fact they had been born and either eaten or destroyed by the mother. The video camera placement has been used for other studies of cause of death in mice. In prior studies, video cameras were placed in the cages of NOS1-/- mice that were found dead with no known cause of death. The video camera allowed detection of Grand Mal seizures causing central nervous system mediated death and led to further studies linking the esophagus paraspinal sensory nervous system to brain induced seizures. The video camera technique may be important in fetal mouse studies to determine that the mice were born and were dying within 24 hours.

Newborn mice may be observed for several factors including lung development. It may be determined by video camera that the mice are breathing and histopathology of animals removed immediately before death may demonstrate that the lungs are indeed inflated.

GI Tract Function in Newborn Mice (nursing)

Video camera studies allow detection of the initiation of nursing by newborn mice from 3 Gy total body irradiated mothers on E13.5. If mice began to nurse, and there is detection in mice removed after interrupted nursing that there is a milk plug in the stomach, this indicates proper nursing function and processing of maternal milk in the stomach. However, the milk plugs in survivors of 3 Gy irradiation at E13.5 may be smaller. Photographs and histopathology of the milk plugs demonstrated that the quantity of milk was decreased. The video camera observations allow determination if the newborn fetal mice stopped nursing and ceased to continue accumulating milk plugs. Observations of defective nursing behavior may correlate with defects in histopathology of the cerebrum, cerebellum, and hippocampus. Video camera observations may be critical, because shortly after the cessation of nursing, maternal mice may respond to the defective behavior of newborns and kill them.

Early Gestational Damage Effects

Histopathology of removed irradiated fetal mice on the day of birth allowed detection of abnormalities in brain, liver, and other organs. These are mid-gestational effects. Studies of early gestational effects would require total body irradiation experiments on E7 earlier pregnant animals, and these studies will undoubtedly require lower total body irradiation doses since it is known that early gestational mice suffer greater damage from total body irradiation than those that have matured further.

Newborn mice should be measured for weight carried out with the standard gram scale and multiple measurements with collection of mean and standard error have been utilized for these studies. Fetal development also requires measurement of fetal length. This is carried out by measurements in millimeters from the tip of the nose to the tip of the tail in newborn mice. Mice irradiated at E13.5 show decrease in fetal weight, and there may be normalization of fetal weight in animals that received JP4-039.

Preparation and Delivery of Radiation Mitigator Drugs

The GS-nitroxide (JP4-039) is prepared according to the published methods (2-3). Briefly, the drug is dissolved in an F14 emulsion (2) to obtain a concentration of 4 mg/ml. The F14 emulsion has previously been described (2).

Synthesis of JP4-039, a peptide isostere-linked 4-amino-Tempo nitroxides, can be performed as described previously (3). Drug is expended in F14 emulsion for administration. F14 is an emulsion developed as an in vivo vehicle for JP4-039, which was originally used topically to mitigate irradiation damage to the skin (2). This formulation consists of a mixture of 10% sesame oil (Sigma-Aldrich, St. Louis, MO, USA), 5% soy phophatidyl choline (Avanti Polar Lipids, Alabaster, AL, USA), and 85% Dulbecco's phosphate-buffered saline (Lonza, Walkersville, MD, USA). During the preparation, the active ingredient (JP4-039) is mixed with other components before making the emulsions. Sonication was performed for 1-2 hours using a continuous model at 17-20 W output with ice water cooling and a stream of nitrogen blowing on top. JP4-039 is administered I.V. in 100 microliters containing 400 microgram drug. In all experiments, JP4-039 was delivered 24 hours after total body irradiation. Studies of other mitigator drugs on the fetus and mother may require entirely different methods for preparation and delivery (5).

References:

1. Epperly Michael W, Smith Tracy, Zhang Xichen, Greenberger Benjamin, Komanduri Paavani, Wang Hong, and Greenberger Joel S. Modulation of in utero total body irradiation induced newborn mouse growth retardation by maternal manganese superoxide disumutase-plasmid liposome (MnSOD-PL) gene therapy. Gene Therapy, 18: 579-583, 2010.

2. Goff Julie P, Epperly Michael W, Shields Donna, Wipf Peter, Dixon Tracy, and Greenberger Joel S. Radiobiologic effects of GS-nitroxide (JP4-039) in the hematopoietic syndrome. In Vivo, 25: 315-324, 2011.

3. Rwigema Jean-Claude M, Beck Barbara, Wang Wei, Doemling Alexander, Epperly Michael W, Shields Donna, Franicola Darcy, Dixon Tracy, Frantz Marie-Celine, Wipf Peter, Tyurina Yulia, Kagan Valerian E, Wang Hong, and Greenberger Joel S. Two strategies for the development of mitochondrial-targeted small molecule radiation damage mitigators. Int. J. Radiat. Oncol. Biol. Phys.,, 80(3): 860-868, 2011.

4. Kanter David, O'Brien Matthew B, Shi Xiao-Hua, Chu Tianjiao, Mishima Takuya, Beriwal Sushil, Epperly Michael W, Wipf Peter, Greenberger Joel S, and Sadovsky Yoel. PL-13-10114/the impact of ionizing radiation on placental trophoblasts. Placenta, PL13-10114, 1-7, 2014.

5. Kalash Ronny, Epperly Michael W, Goff Julie, Dixon Tracy, Sprachman Melissa M, Zhang Xichen, Shields Donna, Cao Shaonan, Wipf Peter, Franicola Darcy, Berhane Hebist, and Greenberger Joel S. Amelioration of irradiation pulmonary fibrosis by a water-soluble bi-functional sulfoxide radiation mitigator (MMS350). Radiat. Res., 180: 474-490, 2013.

6. Steinman Justin, Epperly Michael, Hou Wen, Willis John, Wang Hong, Fisher Renee, Liu Bing, Bahar Ivet,, McCaw Travis, Kagan Valerian, Bayir Hulya, Yu Jian, Wipf Peter, Li Song, Huq M Saiful, and Greenberger Joel S. Improved total-body irradiation survival by delivery of two radiation mitigators that target distinct cell death pathways. Radiat. Res., 189(1): 68-83, 2018.

Table 1: Time table of sequence of organ development in fetal mouse.

Gestation day	Embryo Development and Theiler Stage Summary
E1	Fertilization; Dividing egg stage 2-4 cells. Zona pellucida present. First cleavage occurs at about 24 hours.
E2	Morula (early to fully compacted) 4-16 cells. Zona pellucida present. Usually found in the oviduct towards the utero-tubal junction.
E3	Blastocyst with 16-40 compacted cells. Zona pellucida present. Embryo progresses from morula to the blastocyst. Early evidence of the blastocoelic cavity. In the blastocyst stage (zona-intact) there is a distinct inner cell mass and an outer layer of trophectoderm cells. Usually located in the uterine lumen.
E4	Blastocyst. Zona pellucida absent. Invariably located within the uterine lumen. Attachment of blastocyst. Blastocyst implants, first evidence of embryonic endoderm cells covering the blastocoelic surface of the inner cell mass.
E5	Nodal signaling from the epiblast induces distal visceral endoderm (DVE) formation that will establish embryo anterior–posterior axis.
E6	Stage 9a: Advanced egg-cylinder stage with the first evidence of an embryonic axis. Clear morphological distinction between the embryonic and extra-embryonic ectoderm. The ectoplacental cone is further invaded by maternal blood and the original lumen of the uterine crypt has disappeared. Stage 9b: Advanced Endometrial Reaction. Late in this stage gastrulation begins, producing the first mesodermal cells.
E6.5	Heart - earliest heart precursors are 50 founder cells located on both sides of the midline in the epiblast of early gastrula stage embryos.
E7	Stage 10a Amnion: Tissue at the posterior end of the primitive streak bulges into the pro-amniotic cavity and forms the amniotic fold. Stage 10b Amnio: In the mesoderm of the posterior amniotic fold small cavities coalesce to form a single cavity, the exocoelom. Stage 10c Amnion: The allantoic bud first appears, gastrulation continues and the node becomes visible.
E7.5	Stage 11a Neural Plate, Presomite stage: The amniotic cavity is now sealed off into three distinct cavities - the amniotic cavity, the exocoelom and the ectoplacental cleft. The neural plate is defined anteriorly and the head process is developing. In the midline, subjacent to the neural groove, the notochodal plate is visible. Stage 11b Neural Plate, Presomite stage: The allantoic bud elongates. Stage 11c Neural Plate, Presomite stage: The rostral part of the neural plate begins to enlarge to form the head folds. The neural groove is visible. Stage11d Neural Plate, Presomite stage: Head folds continue to enlarge and the foregut pocket begins to form. Heart - migration of anterior lateral plate mesoderm towards midline; forms a linear heart tube.
E8	Stage 12a: First Somites Unturned embryo with first appearance of somite pairs 1-4 somites. The allantois extends further into the exocoelom and the maxillary components of the 1st branchial arch become prominent. The preotic sulcus is visible in the 2-3 somite embryo. The cardiogenic plate begins to form and the foregut pocket is clearly visible. Stage 12b: First Somites Unturned embryo with first appearance of somite pairs 5-7 somites. The headfolds are particularly prominent and neural closure occurs in the region

	of the 4th and 5th somites, extending in both directions from this site. The otic placode appears at 9 somite pair stage (between E8.5 to 8.75). The optic placodes are first evident and become indented to form the optic pits. The heart rudiment develops rapidly. The allantois contacts the chorion at the end of this stage.
E8.5	Stage 13: Turning of the embryo. This is a short period with turning initiated in embryos with 6-8 pairs of somites and usually completed in embryos with 14-16 pairs of somites. The first branchial arch has maxillary and mandibular components but the maxillary process is not visible until later (TS16). A second branchial arch is now evident. There is evidence of regionalization of the heart and the neural tube is closed from a point opposite the outflow tract to the proximal part of the tail. Neural: Formation and closure of anterior neuropore. The rostral extremity of the neural tube closes in embryos with usually about 15-18 somite pairs and defines this stage. The otic pit becomes progressively more indented but not closed; the mandibular process of the 1st branchial arch is clearly visible. The 3rd branchial arch becomes visible late in the stage. Limb: An increasingly prominent ridge on the lateral body wall, approximately at the level of the 8th-12th somite, indicates the site of the future forelimb bud. Absent: forelimb bud. Heart: Heart tube undergoes looping. The second heart field (pharyngeal mesoderm cells) contributes to parts of the right ventricle, the interventricular septum, the venous pole, and the base of the outflow tract.
E9.5	Stage 14: Formation of posterior neuropore, forelimb bud. The posterior neuropore forms and the condensation of the forelimb bud become apparent near the 8th-12th somite pairs. A distinct condensation of the hind limb bud appears just at the end of the stage.The forebrain vesicle subdivides into telencephalic and diencephalic vesicles. Stage 15: Closure of posterior neuropore. Hind limb bud and tail bud. The hind limb bud becomes visible at the level of the 23rd-28th somites. The tail bud appears as a short stump and the 3rd and 4th branchial arches are distinctly concave. Rathke's pouch and the nasal processes start to form. At the end of this stage the posterior neuropore begins to close. Absent: thin and long tail. Integumentary System Development Neural Crest Development: Melanoblasts precursors derived from the neural crest.
E10	Stage 17: Deep Lens Indentation The most obvious distinguishing features are the deepening of the lens pit, with a narrowing of its outer pore-like opening, and the first appearance of the physiological umbilical hernia. The 1st branchial arch is conspicuously divided into maxillary and mandibular components. There is advanced development of the brain tube and the tail elongates and thins. Hearing and Balance: Formation of vestibular (otic) ganglion cells (E10-12). Heart: Cardiac neural crest cells migrate to the outflow tract and endocardial cushions to form the the outflow tract septum (systemic and pulmonary separation). Renal System Development: metanephric mesenchyme present, an area of intermediate mesoderm caudal to the mesonephros and adjacent to a widening of the ‘nephric duct, metanephric portion’ that gives rise to the metanephros.
E10.5	Integumentary System Development Neural Crest Development : Neural crest melanoblasts upregulate early specific markers (Mitf, Tyrosinase, Dct, Kit) Hearing and Balance: Afferent processes of vestibular ganglion cells invade the macula utricle and saccule and cristae of the semicircular canals.

E11	Closure of Lens Vesicle. The primary externally recognizable feature is the progressive closure of the lens vesicle. The somites in the cervical region are no longer visible and the rapid growth of the brain is striking. The nasal pits start to form. Palate Development: palatal shelves protrude from bilateral maxillary processes. Hearing and Balance: Efferent nerve endings first approach hair cells (E11-12) Genital System: gonad between E11 to E12 differentiates from a bipotential to sexually-differentiated state, based upon transcriptome analysis. Cardiovascular System: Heart outflow tract supported by the right ventricle, characteristic bend dividing the outflow tract into proximal and distal portions and the distal portion extends to the margins of the pericardial cavity Tooth Development: placode stage Mammary Development: the thickening and stratified ectodermal milk line breaks up into individual placodes and the underlying mammary mesenchyme begins to condense. Heart: Progressive septation of the outflow tract and septation of the atria and ventricles.
E11.5	Aorta-Gonad-Mesonephros (AGM) region is a site of hematopoietic stem cell (HSC) development prior to colonization of the embryonic liver.
E12	Stage 20: Earliest signs of fingers. The 'handplate' (anterior footplate) is no longer circular but develops angles which correspond to the future digits. The posterior footplate is also distinguishable from the lower part of the leg. It is possible to see the pigmentation of the pigmented layer of the retina through the transparent cornea. The tongue and brain vesicles are clearly visible. Genital System: (E11-E12) gonad differentiates from a bipotential to sexually-differentiated state, based upon transcriptome analysis.
E12.5	Genital system: (12.5, somite 30 stage) cell migration finished. Coelomic epithelium basement membrane thickens to form the tunica albuginea. Leydig cells arise. Genital system: (12.5-13.5) Oocytes in the ovary enter first meiotic prophase. These arrest later around birth at the diplotene stage (also known as dictyate). Joints : (12.5 -13.5) interzone forms in cartilage of digits which is the precursor to synovial joint formation. Tooth Development: bud stage Palate Development: (E12.5-E14) palatal shelves grow vertically along the developing tongue. Heart: Progressive septation of the outflow tract and initiation of atrioventricular canal septation.
E13	Stage 21: Anterior footplate indented, marked pinna. The distal borders of the anterior and posterior footplates are now indented and the digit widths and locations can be discerned. The 'elbow' and 'wrist' are now identifiable. The pinna rapidly develops and forms a crest at right angles to the head. Five rows of vibrissae are visible as well as a prominant hair follicle over the eye and another over the ear. The lens vesicle has lost its lumen. The physiological umbilical hernia is prominent. Ovary : (E13.5-16.5) ovarian cord structure formation required for oocyte development[19] Hearing and Balance - Peak hair cell mitosis in crista ampullaris, maculae of saccules, and utricles (E13-17) Gastrointestinal tract - differential gene expression of some selected markers during development (E10.5 and E13.5) of the mouse gastrointestinal tract.[20]
E13.5	Liver: (E13.5-15.5) single layer of hepatoblasts forms close to the portal mesenchyme

	(ductal plate) and expresses bile duct-specific cytokeratins. Heart: Bilaterally asymmetrical aortic arch system, completely septated outflow tract and ventricles and remodeling of the atrioventricular cushions.
E14	Stage 22: Fingers separate distally. Individual 'fingers' are visible in the anterior footplate and there are deep indentations between the 'toes' which are not yet separated.The long bones of the limbs are present and there are hair follicles in the pectoral, pelvic and trunk regions. The pinna is turned forwards and the umbilical hernia is conspicuous. Thyroid ultimobranchial body (UBB) and thyroid primordium fuse and give rise to calcitonin-producing C cells and thyroglobulin-producing follicular cells, respectively. Tooth Development - cap stage
E14.5	Palate Development: palatal shelves elevate, meet, and fuse at the midline, to form an intact palate shelf. Lymph Node development initiated Heart: Atrial septation complete.
E15	Stage 23: Toes separate. The 'toes' separate and are clearly divergent, not becoming parallel until later. Hair follicles are present in the cephalic region but not at the periphery of the vibrissae. The pinna covers more than half of the external auditory meatus and the eyelids are still open. Absent: nail primordia, 'fingers' 2-5 parallel. Hearing and Balance: Afferent synaptogenesis with hair cells begins Tooth Development: bell stage
E15.5	Mammary Development E15.5 - mammary epithelium begins to proliferate at the tip and the primary sprout pushes through the mammary mesenchyme towards the underlying fat pad.
E16	Stage 24: Reposition of umbilical hernia 'Fingers' 2-5 are nearly parallel. Nail primordia are visible on the 'toes'. The eyelids have fused in most cases by the end of the stage and the pinna almost completely covers the external auditory meatus. The umbilical hernia is disappearing and there is a corresponding increase in the size of the peritoneal sac. Absent: 'fingers' and 'toes' joined together. Heart: (E15.5 (TS24) - E18.5 (TS27)) Definitive external prenatal configuration achieved, Atrioventricular valve leaflets are being modified, Coronary arteries are being modified.
E16.5	Liver: ductal plate becomes partially bi-layered.
E17	The skin has thickened and formed wrinkles and the subcutaneous veins are less visible. The 'fingers' and 'toes' have become parallel and the umbilical hernia has disappeared. The eyelids have fused. Whiskers are just visible. Absent: ear extending over auditory meatus, long whiskers
E17.5	Liver: bilayered ductal plate remodelled with focal dilations between the two cell layers. Prostate: urogenital sinus epithelial cells grow into mesenchyme to form prostate buds.
E18	Long whiskers: The whiskers that were present at stage 25 are longer and the skin has thickened. The pinna is larger and such that virtually none of the lumen of the auditory meatus is visible. The eyes are barely visible through the closed eyelids. Hearing and Balance - Morphological differentiation into Type I and Type II hair cells. Integumentary System Development - Langerhans cells (LCs) precursors recruited into the epidermis in a single wave around E18 and acquired a dendritic morphology.
E18.5	Mammary Development: elongating duct has now grown into the fat pad and has branched into a small ductal system. Cells of the mammary mesenchyme have formed the nipple, which is made of specialized epidermal cells.

E19	New born mouse