

Citrulline as a Biomarker for Gastrointestinal-Acute Radiation Syndrome: Species Differences and Experimental Condition Effects

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Animal models of hematopoietic and gastrointestinal acute radiation syndromes (ARS) have been characterized in order to develop medical countermeasures. Acute radiation-induced decrease of intestinal absorptive function has been correlated to a decrease in the number of intestinal crypt cells resulting from apoptosis and enterocyte mass reduction. Citrulline, a non-coded amino acid, is produced almost exclusively by the enterocytes of the small intestine. Citrullinemia has been identified as a simple, sensitive and suitable biomarker for radiation injury associated with the gastrointestinal ARS (GI-ARS). Here we discuss the effect of radiation on plasma citrulline levels in different species including mice, Göttingen minipigs and rhesus nonhuman primates (NHPs) measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). The effects of experimental study conditions such as feeding and anesthesia were also examined on plasma citrulline levels in the NHPs. Both C57BL/6 mice and Göttingen minipigs were exposed to different partial body irradiation (PBI) doses from 13-17 Gy and 8-16 Gy, respectively, whereas NHPs were exposed to total body radiation doses from 6.72 to 13 Gy. Blood samples were taken at different timepoints and plasma citrulline levels were measured in the three species at baseline and following radiation. Basal plasma citrulline concentrations (mean \pm SEM) in mice and minipigs were 57.8 \pm 2.8 μ M and 63.1 \pm 2.1 μ M, respectively. NHPs showed a basal plasma citrulline concentration of 32.6 \pm 0.7 μ M, very similar to that of humans (~40 μ M). Plasma citrulline progressively decreased following radiation treatment reaching nadir values between Days 3.5 and 7. The onset of citrulline recovery was observed earlier at lower radiation doses, only partial citrulline recovery was noted at higher radiation doses in minipigs and NHPs but complete recovery was noted in mice at all doses. Anesthesia (i.e., ketamine and acepromazine) significantly decreased plasma citrulline levels by 35.5% (P=0.0017) compared to unanesthetized NHPs. In the postprandial state, citrulline concentrations were slightly but

significantly decreased by 12.2% ($P=0.0287$) in NHPs. These results suggest that plasma citrulline is affected by experimental conditions such as anesthesia and feeding.

INTRODUCTION

Development of effective medical countermeasures for the treatment of large populations in the event of deliberate and/or accidental radiological catastrophes requires well characterized animal models (1). Biomarkers are a cornerstone of safety and efficacy assessments when using animal models of acute radiation syndrome (ARS) and qualification of relevant endpoints is part of the foundations in biodefense research. At least four sub-syndromes that are associated with ARS are currently being actively investigated in the context of accidental ionizing radiation exposure. These include the delayed effects of acute radiation exposure (DEARE), both the hematopoietic syndrome (H-ARS) and the gastrointestinal syndrome (GI-ARS) which are specific targets for the development of novel medical countermeasures, and the cerebrovascular/central nervous system (CV/CNS) syndrome which occurs within a radiation exposure range considered incurable (2). Radiation-induced cell death, mostly due to DNA damage, results in functional alterations in various tissues. The H-ARS is characterized by leukopenia and thrombocytopenia, increased susceptibility to infection, hemorrhage and anemia (2, 3). Radiation-induced damage to the gastrointestinal tract is characterized by intestinal crypt cell apoptosis and mitotic cell death leading to loss of mucosal barrier function, villus atrophy, alterations in the normal intestinal bacterial flora, and enterocyte mass reduction (4). Owing to a high cell division rate, the small intestine is highly sensitive to radiation exposure and biomarkers of small-bowel radiation injury are valuable both for diagnostic purposes in the clinic, as well as for drug development of radiation injury medical countermeasures.

In humans, circulating citrulline levels can be used as a biomarker indicative of a functional intestine (5). Citrulline, a non-DNA coded amino acid, is a by-product of many different cellular enzyme reactions including glutamine metabolism in small bowel enterocytes (6, 7); however, it is not a component of either proteins or nutritional products (8). Citrulline is almost exclusively synthesized by the small intestine and is important in the subsequent synthesis of arginine, a critical precursor of vascular nitric oxide (NO) (9). Citrulline synthesis is abundant within the small intestine enterocytes because of the presence of high levels of the enzymes needed for synthesis (e.g., arginase II and ornithine carbamoyl transferase) coupled with the low activity of citrulline catabolic enzymes (e.g., argininosuccinate synthase and argininosuccinate lyase) (10). Citrulline is then released from the enterocytes into the circulation and primarily taken up by the kidney, avoiding liver metabolism (11). Thus, plasma citrulline concentration is highly proportional to intestinal enterocyte mass (12-14). Because of this relationship, citrullinemia has been identified as a simple assay for radiation-induced small bowel epithelial cell loss following irradiation (15, 16). In this context, it is important to explore what, if any, additional independent factors (other than radiation) may alter plasma citrulline levels.

Herein, we discuss the effect of radiation on plasma citrulline levels in different species (C57BL/6 mice, Göttingen minipigs and rhesus non-human primates (NHPs)) and the effect that experimental study conditions such as feeding and anesthesia can have on plasma citrulline levels, specifically in the rhesus model.

MATERIALS AND METHODS

Animal models, experimental environment and radiation exposure

All experimental procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC) and the Canadian Council on Animal Care guidelines for use of experimental animals. All protocols included strict euthanasia criteria reviewed and approved by the IACUC. All animals were monitored continuously (i.e., technical staff and veterinarians were available 24 hours a day) for development of any untoward clinical signs.

For all species, the animal room environment was maintained at a temperature of $21 \pm 3^{\circ}\text{C}$ with a relative humidity of $50 \pm 20\%$, a light dark cycle of 12 hours light/12 hours dark and 10 - 15 air changes per hour. Temperature and relative humidity were monitored continuously. For the radiation procedure, all animals were irradiated using a Cobalt-60 gamma source (Theratron® 1000). Prior to all animal irradiation, the radiation dose was calibrated using an acrylic phantom placed in the same experimental set up that was used for animal irradiation. Body measurements were taken to deliver midline to tissue dose. The measurements were taken using an ion chamber with a solid water phantom build-up. Dosimetry was obtained by a Farmer ionization chamber connected to an electrometer that was included in each radiation treatment session. Nanodots were also used but only as back-up in case the electrometer was not functional.

Mice (C57BL/6) were received from Jackson Laboratories and were acclimated for a minimum of 13 days prior to irradiation. A standard certified commercial chow (Harlan Teklad Certified Irradiated Global Rodent Diet #2918C) was provided to the animals *ad libitum*. Certified treats from Bio-Serv (yogurt drops) was provided as part of the animal enrichment program at least once a week. Acidified municipal tap water (which had been exposed to

ultraviolet light and purified by reverse osmosis) was provided to the animals *ad libitum*. Mice were irradiated with partial shielding (PBI) in subsets of up to 12 mice in a custom designed restrainer where their left pelvic limb (distal to the mid-femur; approximately 3%) was extended and maintained in position with an elastic band. The left pelvic limb was shielded with a cerrobend structure. The animals were not anesthetized during the irradiation and the animals were not acclimated to the irradiation devices. During the PBI, the animals were immobilized and the restraining devices and the irradiation source were not moving during irradiation. The dose was between 13-17 Gy at 0.60 Gy/min and the Cobalt-60 source was on top of the animals. Plasma citrulline levels were measured following irradiation or sham procedures. Whole blood was collected into EDTA-coated tubes at scheduled termination on Days 1, 3.5, 7, 10 and 30, and centrifuged at 1500 rpm for 10 min at 4°C. Plasma was harvested and stored at -70°C.

Göttingen minipigs were received from Marshall Farms and were acclimated for a minimum of 30 days prior to irradiation. A standard certified commercial chow (Harlan Teklad Certified Miniswine Diet #7037C) was provided to the animals twice daily in the morning and in the afternoon. Treats or fruits/vegetables were provided as part of the animal enrichment program. Municipal tap water (which had been exposed to ultraviolet light and purified by reverse osmosis) was provided to the animals *ad libitum*. The Göttingen minipigs were exposed to PBI at doses ranging from 8-16 Gy at a radiation dose rate of approximately 0.50 Gy/min. The shielded areas in minipigs were shielded with a cerrobend structure and included the head, thorax and pelvic limb (approximately 50% of bone marrow based on CT-Scan estimates in control animals). The animals were anesthetized (ketamine 15 mg/kg, IM) during the irradiation and the animals were not acclimated to the irradiation devices. In order to produce homogenous dose distribution, irradiation was divided in two parts: First, the animals received half of the dose by

right lateral irradiation followed by the second half of the dose delivered by left lateral irradiation. Blood samples were taken prior to irradiation and on Days 1, 3, 5, 7, 9, 15, 19, 20, 25, 30, 35, 40 and 45 into EDTA-coated tubes.

Rhesus macaques were acclimated for a minimum of 6 weeks prior to irradiation. A standard certified commercial chow (Harlan Teklad Certified Hi-Fiber Primate Diet #7195C) was provided twice daily in the morning and in the afternoon. Treats or fruits/vegetables were provided as part of the animal enrichment program. Municipal tap water (which had been exposed to ultraviolet light and purified by reverse osmosis) was provided to the animals *ad libitum*. NHPs were exposed to total body irradiation (TBI) doses ranging from 6.72-13 Gy delivered at 0.60-0.80 Gy/min. NHPs were anesthetized (ketamine hydrochloride 100 mg/mL and acepromazine 10 mg/mL at a dose of 0.1 mL/kg) during the irradiation and the animals were acclimated to the irradiation devices. In order to produce homogenous dose distribution, irradiation was divided in two parts: First, the animals received half of the dose by antero-posterior irradiation followed by the second half of the dose delivered by postero-anterior irradiation. Blood samples were collected prior to irradiation and between Days 1 to 10 for rhesus monkeys into EDTA-coated tubes. In non-irradiated NHPs, blood samples were obtained after a period at least 12 hours of fasting and within 2 hours after feeding from two cohorts of animals to assess the impact of food consumption on plasma citrulline levels. To assess the impact of anesthesia, blood samples were obtained for plasma citrulline from non-irradiated animals that were anesthetized (ketamine 9.09 mg/kg and acepromazin 0.9 mg/kg, intramuscular) and compared to plasma citrulline concentrations obtained from the same animals without anesthesia. Blood samples collected within three hours of the anesthetic regimen were considered from an anesthetized animal and were subsequently pooled. Blood samples collected

from animals not previously anesthetized or after the three hour window post-anesthetic regimen were considered for the 'no anesthesia' group.

A summary of the animals used in this comparative assessment is shown in Table 1.

Chemicals and Materials:

L-Citrulline (98% purity) was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). D7-Citrulline (L-Citrulline-2,3,3,4,4,5,5-d7, 99% purity) was purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). HPLC grade acetonitrile, methanol, 2-propanol, hydrochloric acid (1N) and formic acid (88%) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Type 1 water was used throughout the qualification.

Citrulline Detection in Mouse:

Citrulline in mouse plasma (K₃-EDTA) was quantified by hydrophilic interaction liquid chromatography (HILIC)-MS/MS (Biosimetry Diagnostic Core Laboratories, Little Rock, AR) as previously described (17).

Method Qualification for Citrulline Detection in Minipig and Rhesus:

A protein precipitation extraction method using a high performance liquid chromatographic mass spectrophotometric (LC-MS/MS) detection method for the determination of endogenous citrulline levels in rhesus monkey plasma (K₃EDTA) and Göttingen minipig plasma (K₂EDTA) was qualified. Calibration standards and quality control (QC) samples were prepared by spiking known concentrations of standards and QC samples in reverse osmosis filtered and deionized water. The calibration curve consisted of seven standards ranging from 1.00 to 50.0µg/mL (5.7

to 285.4 μM). The endogenous citrulline concentrations in pooled lots of non-irradiated rhesus plasma (K_3EDTA) and Göttingen minipig plasma (K_2EDTA) donors were quantified using the standard curve and QCs in reverse osmosis filtered and deionized water. QCs were prepared in plasma (rhesus and Göttingen minipig) by addition of three concentrations (17.1, 57.1 and 114.2 μM) to the pre-determined endogenous levels, using the appropriate spiking solutions. The plasma QCs were quantified versus standard curve and QCs in water. Any interference due to the presence of arginine was investigated to ensure assay detection of citrulline was selective and sensitive. Arginine was determined not to interfere with citrulline quantitation as spiked concentrations of arginine eluted at a different retention time than citrulline using the LC-MS/MS system in both minipig and NHP plasma.

Minipig and NHP Sample Preparation:

500 μL of Internal Standard (D7-Citrulline 500.0 ng/mL) was added to 20 μL of sample, followed by mixing and centrifugation for 10 minutes at 4000 rpm. Twenty (20) μL of supernatant was added to injection vials containing 400 μL of 80/20 (v/v) acetonitrile/water and thoroughly mixed.

Minipig and NHP Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS analyses were performed on an Applied Biosystems API4000 Mass Spectrometer coupled with a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan). Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. LC gradient separation was performed on a Zorbax HILIC Plus column (2.1x50mm, 3.5 μm) (Agilent Technologies, USA) operated at 40°C, with a flow rate of 0.500

mL/min. The retention time for both citrulline and the internal standard (IS) occurred at 2.35 minutes and the total run time was 5.0 minutes.

Detection was performed using the Multiple Reaction Monitoring (MRM) mode using the electro-spray ionization (ESI) technique in positive-ion mode with the following transitions: Citrulline (m/z 177.1 → 160.2) and D₇-Citrulline (m/z 183.0 → 166.0). MS sensitivity was decreased using the ¹³C isotope for Citrulline. The other state file parameters used were as follows: spray voltage +5000V; temperature 550°C; Collision (CAD) gas pressure 6psi; GS1 pressure 60psi; GS2 pressure 60psi; Curtain gas 30psi; Collision Energy (CE) 20eV; Entrance Potential (EP) 10V and Collision Cell Exit Potential (CXP) 12V.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between two groups were performed using a two-tailed unpaired Student's t test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered significant at P<0.05.

RESULTS

Citrullinemia in different species

Plasma citrulline levels were measured in the different species prior to irradiation and results were used as baseline values (Figure 1). The mean (\pm SEM) baseline plasma citrulline level in C57BL/6 mice (n=20) was $57.8 \pm 2.8 \mu\text{M}$ (range: 41.6 – 81.2 μM). In Göttingen minipigs (n=81), the mean (\pm SEM) baseline plasma citrulline level was $63.1 \pm 2.1 \mu\text{M}$ (range: 24.4 – 128.0 μM). In the NHPs (n=209, males and females), the baseline plasma citrulline level was found to be lower than that in other species with a mean (\pm SEM) value of $32.6 \pm 0.7 \mu\text{M}$ (range: 7.9 – 69.8 μM). Plasma citrulline levels from female NHPs were significantly lower than levels in males, $29.2 \pm 1.2 \mu\text{M}$ compared to $33.7 \pm 0.9 \mu\text{M}$ (P=0.0061), an observation not seen in minipigs. In humans, no significant difference in plasma citrulline levels between genders were reported as per Geigy Scientific Tables where values were $37.0 \pm 9.0 \mu\text{M}$ and $35.0 \pm 10.0 \mu\text{M}$ for the males and the females, respectively (18).

Plasma citrulline levels post-irradiation

Plasma citrulline levels decreased in a dose- and time-dependent manner after irradiation with or without partial bone marrow shielding. Plasma citrulline levels decreased rapidly in the first days following irradiation to reach a minimum level at Day 3.5 and 5 for the mice and the mini-pigs, respectively (Figure 2A and 2B). However, the nadir in minipigs does appear to shift to later timepoint at higher radiation dose levels (Day 7 at 12 and 16 Gy; Figure 2B). Plasma citrulline recovery to the normal (or pre-irradiation) range was more rapid for the lowest radiation dose administered to both species but complete citrulline pre-irradiation level recovery was noted in minipigs exposed to partial body irradiation at 16 Gy only 30 days following irradiation where 6

of the 7 animals survived to Day 30. In NHP, animals exposed to TBI at 6.72 Gy reached a plasma citrulline nadir at 3 days after irradiation and returned to baseline within Day 7 after exposure (Figure 2C). NHPs exposed to radiation dose levels ranging from 9.2-13 Gy reached a plasma citrulline nadir level at Day 7 with citrulline levels ranging from -62.8 to -84.8% compared to values prior to irradiation. Although a slight recovery was noted after Day 7, plasma citrulline levels remained low at Day 10 after irradiation with values ranging from 7.69–13.64 μM compared to baseline ranges between 31.77–43.55 μM in rhesus exposed to high dose TBI. The plasma citrulline levels decrease, compared to baseline, was radiation dose dependent in mice, minipigs and NHPs as shown in Figure 3 although the radiation dose dependence was time sensitive and in mice was more apparent during the recovery phase than at the nadir of the response.

Effect of feeding on plasma citrulline levels in rhesus NHPs

In various studies, blood samples for plasma citrulline levels were collected in rhesus either in a fasted state or after feeding. Plasma citrulline levels were slightly but significantly lower in animals that were fed prior to blood collection compared to the second cohort of animals when fasted with concentrations of $30.6 \pm 1.3 \mu\text{M}$ and $34.8 \pm 1.2 \mu\text{M}$, respectively (Figure 4). The difference in plasma citrulline levels in post-prandial animals compared to fasted animals without any effect from radiation was -12.2% ($P=0.0287$).

Effect of ketamine/acepromazine anesthesia on plasma citrulline levels in rhesus NHPs

Animals were considered anesthetized if the blood collection was done within 3 hours of an intramuscular injection with ketamine (9.09 mg/kg) and acepromazine (0.9 mg/kg). The study

results showed that anesthesia with ketamine/acepromazine significantly decreased plasma citrulline levels by -35.5% ($P=0.0017$). After anesthesia, plasma citrulline levels were 21.7 ± 2.4 μM compared to 33.6 ± 2.5 μM in the same unanesthetized animals (Figure 5).

DISCUSSION

Citrulline is a widely accepted biomarker in humans to quantify the enterocyte functional metabolic mass (19). In this study, the analysis of the plasma citrulline concentrations in mice, minipigs and NHPs highlights interesting species differences and assesses the impact of several experimental study conditions on this biomarker.

Healthy adult Caucasian subjects with normal intestinal mucosa and normal renal function have post-absorptive mean plasma citrulline levels of 40 μM (with a range between 20 and 60 μM) (6, 19, 20). Citrulline levels typically vary with age as well as with the ethnicity of the subject group. For example, citrulline levels were shown to increase in elderly subjects (>70 years) whereas it was reported to be lower in Asian subjects compared to Caucasians (19, 21). Unfortunately, in this study there was insufficient data to address the effects of age on plasma citrulline levels post-irradiation. Nevertheless, this represents an interesting consideration that should be assessed in future investigations. Plasma citrulline levels in mice, minipigs and NHPs are relevant as these species are commonly used as non-clinical models in the evaluation of citrulline as a GI biomarker. Recently, the minipig model has been increasingly considered to study radiation-induced GI-ARS (22, 23). The mean baseline citrulline level for NHPs in this study was $32.6 \pm 0.7 \mu\text{M}$, which is comparable to values reported in the literature (i.e., 10 to 80 μM) for this species. Basal citrulline levels in mice and minipigs were also similar to concentrations previously reported (17, 24-27). Plasma citrulline levels in NHPs were comparable to concentrations reported for healthy humans (6, 16, 19), whereas mice and minipigs presented mean concentrations that were generally higher than that observed in humans. Similarities between NHPs and humans may be attributed to physiological similarities between the two species.

Radiation-induced damage is generally proportional to the cell division rate. Cells with a high division rate such as the hematopoietic lineages (28) and the intestinal crypts (29) are more radiosensitive than cells in areas with low rates of cell division such as the central nervous system, skeletal muscle or the bones (30, 31). While crypt cell regeneration, cell apoptosis and mucosal surface measurements are the most commonly quantified parameters post-radiation exposure (26), these morphologic/morphometric endpoints require tissue sampling and use of invasive methodologies. However, as a plasma biomarker, citrulline allows for the conduct of repeated measurements (in time course studies) from the same individual animal in the larger species, thus reducing the number of animals required as per NC3R (the replacement, refinement and reduction in the use of animals in research and testing). The effects of radiation on intestinal function have been correlated with impaired absorptive capacity due to epithelial cell loss (32-35) and citrulline can be used as a surrogate biomarker to quantify the intestinal epithelial cell mass (12, 16, 26). Plasma citrulline levels decreased rapidly following partial irradiation in mice reaching their lowest concentrations between on Day 3.5. The time course of plasma citrulline changes noted in mice was comparable to the kinetic profile characterized by others in this same species (26). In partially irradiated minipigs, where shielding accounted for 50-55% of bone marrow, the nadir citrulline levels were noted later with increasing radiation dose levels (i.e., Day 5 at 8 Gy and Day 7 at 12 Gy and 16 Gy). The NHPs in this study presented a slower kinetic of post-radiation citrulline reduction when compared to mice, reaching nadir values by Day 7 at radiation dose levels higher than 9 Gy. Lower radiation dose levels were associated with an earlier nadir (Day 5 at 6.72 Gy). As would be anticipated, allometric differences are widely reported between species for physiological parameters when determined at baseline (36); however, differences are also observed during the conduct of drug development studies (37) and

during the conduct of radiation biology studies (38) with smaller species presenting faster kinetic profiles typically proportional to body surface area. In this study, citrulline recovery was more rapid with lower radiation doses in all species; however, the recovery was incomplete with TBI at 9.2-13 Gy in NHPs. Partial recovery of citrulline levels to pre-radiation levels could be attributed, albeit to a moderate degree, to the short duration of monitoring (i.e., only 10 days for NHPs) in the current study. In the minipigs, citrulline levels appeared to rebound beyond basal levels, although given the low sample size (n=2, 1 beyond Day 35 at 12 and 16 Gy respectively) it is difficult to ascertain the cause of the excessive rebound. Plasma citrulline levels could potentially be used as a biomarker for biodosimetry that can be quantitatively related to the magnitude of the radiation dose received as shown by decreased citrulline levels with increasing dose of radiation in our study.

Citrullinemia is modulated by clinical factors such as renal and liver function, metabolic stress and inflammation (8). In ARS animal models, opportunistic bacterial infections, which are often associated with inflammation, could represent a potential confounding factor with regard to the interpretation of citrulline values; however, the timing of development of neutropenia (which typically coincides with the onset of opportunistic infections) occurs much later than the citrulline nadirs observed in our studies. The present study showed that experimental conditions such as feeding and anesthesia can modulate basal citrulline levels in NHPs. The plasma citrulline concentrations that were determined in these studies were slightly, but statistically significantly, decreased (-12.2%) in the postprandial state in NHPs which is comparable to observations reported in humans (6). However this change may not be clinically relevant against the backdrop of the extent of the radiation effect. As a consequence, a decreased appetite (which is a common occurrence with radiation exposure) needs to be monitored accurately for

interpretation of citrulline levels in GI-ARS studies. In this study we also demonstrate that a standard anesthetic regimen in NHPs (i.e., ketamine and acepromazine) could also potentially modulate citrulline levels in NHPs. To our knowledge, this is the first time that an anesthetic regimen consisting of ketamine and acepromazine has been reported to decrease plasma citrulline concentrations. Anesthesia may be required in studies conducted with large NHPs and our results suggest that standardization of the interval between anesthesia and the blood collection time points for citrulline monitoring should be considered in the study design as it could help reduce potential variability within and between study groups. The exact mechanisms by which anesthesia impacts citrulline levels or whether all anesthetics could have a similar effect remains unknown; however, in our studies the effect was noted as early as eight minutes after induction of anesthesia by a ketamine/acepromazine regimen.

The data reported herein suggest that dose-dependent changes in citrulline plasma concentrations occur after radiation exposure in different animal species used in non-clinical models of GI-ARS. However, when conducting such studies it should be noted that citrulline levels can also be modulated by other factors such as feeding regimens and use of anesthetic agents.

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CONFLICTS OF INTEREST

None of the authors have any conflicts of interest, other than their employment in commercial pharmaceutical companies or contract research organizations.

FIGURE LEGENDS

Figure 1. Plasma citrulline levels in different species. Basal plasma citrulline levels in rhesus NHPs (n=209), C57BL/6 mice (n=20) and Gottingen minipigs (n=81). Data are presented as mean \pm SEM.

Figure 2. Plasma citrulline levels post-irradiation. Plasma citrulline levels decreased after irradiation with partial bone marrow shielding in C57BL/6 mice (A) and Gottingen minipigs (B). Plasma citrulline levels are also shown after total body irradiation in rhesus NHPs (C). The line (- ----) represents the average basal citrulline levels determined prior to study conduct and across all available animals. Values are presented as mean \pm SEM. Individual sample sizes in the radiation dose groups ranged from 4 to 8 for mice, 1 to 20 for minipigs and 2 to 20 for NHPs, not accounting for any mortality on-study.

Figure 3. Plasma citrulline levels decrease in a dose-dependent manner. Plasma citrulline levels following irradiation in C57BL/6 mice at Days 3.5 and 7 (A) and Gottingen minipigs at Day 5 (B). Plasma citrulline levels also decrease as shown after total body irradiation in rhesus NHPs at Days 3, 5 and 7 (C). Values are presented as mean \pm SEM.

Figure 4. Effect of feeding on plasma citrulline levels in rhesus NHPs. Plasma citrulline levels were measured in rhesus NHPs either when fasted (n=88) or in a postprandial (n=47) state. Data are presented as mean \pm SEM. * $P < 0.05$ when compared to the fasted animals.

Figure 5. Effect of ketamine/acepromazine anesthesia on plasma citrulline levels in rhesus NHPs. Plasma citrulline levels were measured in rhesus NHPs with (n=16) or without (n=16) the use of a ketamine/acepromazine anesthetic regimen. ‘Anesthesia’ represents pooled data collected within three hours of the anesthetic regimen. ‘No anesthesia’ represents pooled data collected in excess of three hours after the anesthetic regimen or from animals which had not been previously anesthetized. Data are presented as mean \pm SEM. $**P < 0.01$ when compared to unanesthetized animals.

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Table 1: Summary of Animal Demographics for Citrulline Analysis

Species	N	Age Range Pre-irradiation	Gender	Radiation Dose Range (Gy)	Radiation Dose Rate (cGy/min)	Shielding Conditions
Mouse (C57BL/6)	20	9 weeks	Males	13-17	0.60	Left leg distal to mid-femur
Minipig (Göttingen)	81	5-8 months	Male (N= 38)and Female (N=43)	8-16	0.50	Head, thorax and pelvic limb
Non-human primate (rhesus macaques)	209	2.5-6 years	Male (N=153) and Female (N=56)	6.72-13	0.60-0.80	None