An amino acid mixture mitigates radiation-induced gastrointestinal toxicity.
AN AMINO ACID MIXTURE MITIGATES RADIATION-INDUCED GASTROINTESTINAL TOXICITY

Liangjie Yin, Pooja Vijaygopal, Rejeesh Menon, Lauren A. Vaught, Mei Zhang, Lurong Zhang, Paul Okunieff, and Sadasivan Vidyasagar*

Abstract—Electrolyte and nutrient absorption occur in villous epithelial cells. Radiation often results in reduced electrolyte and nutrient absorption, which leads to gastrointestinal toxicity. Therefore, the authors studied: (1) radiation-induced changes in glucose and amino acid absorption across ileal tissues and (2) the effect of amino acid mixtures on absorptive capacity. NIH Swiss mice were irradiated (0, 1, 3, 5, or 7 Gy) using a $^{137}$Cs source at 0.9 Gy min$^{-1}$. Transepithelial short circuit current (Isc), dilution potential, and iso- tope flux determinations were made in Ussing chamber studies and correlated to plasma endotoxin and IL-1β levels. Amino acids that increased electrolyte absorption and improved mucosal barrier functions were used to create a mitigating amino acid mixture (MAAM). The MAAM was given to mice via gastric gavage; thereafter, body weight and survival were recorded. A significant decrease in basal and glucose-stimulated sodium absorption occurred after 0, 1, 3, 5, and 7 Gy irradiation. Ussing chamber studies showed that paracellular permeability increased following irradiation and that the addition of glucose resulted in a further increase in permeability. Following irradiation, certain amino acids manifested decreased absorption, whereas others were associated with increased absorption. Lysine, aspartic acid, glycine, isoleucine, threonine, tyrosine, valine, tryptophan, and serine decreased plasma endotoxins were selected for the MAAM. Mice treated with the MAAM showed increased electrolyte absorption and decreased paracellular permeability, IL-1β levels, and plasma endotoxin levels. Mice treated with MAAM also had increased weight gain and better survival following irradiation. The MAAM has immediate potential for use in mitigating radiation-induced acute gastrointestinal syndrome.

Key words: $^{137}$Cs; gamma radiation; radiation effects; radiation therapy

INTRODUCTION

ACUTE GASTROINTESTINAL (GI) syndrome is a component of acute radiation syndrome. Gastrointestinal cells exhibit substantial functional damage in the first few days following high-dose irradiation, which is followed by bone marrow syndrome a month later. The severity of GI syndrome correlates with the radiation dose. Radiation injury leads to dose-dependent nausea, vomiting, diarrhea, and dehydration, which are the major contributors to morbidity and mortality (Donaldson 1977; Beer et al. 1985; Bloch 1990; Yeoh et al. 1993).

Intestinal epithelial cells are responsible for the absorption of electrolytes, water, and nutrients and constitute a functional barrier. The small intestine is presented with approximately 8 L of fluid from endogenous secretions and daily fluid intake, of which approximately 98% is absorbed by an efficient electrolyte transport machinery. Intestinal epithelial cells are renewed every 3–5 d at the tip of the villi to maintain functional integrity. Radiation damage selectively targets rapidly dividing intestinal cells located in the crypt, leading to villous atrophy and breakdown in the mucosal barrier. Most individuals exposed to radiation, especially to the abdomen, pelvis, or rectum, develop acute enteritis, which becomes clinically evident during the first weeks after irradiation and continues for up to 8 wk after completion of the therapy or the last radiation exposure. Independent studies have shown that early supportive care limits chronic radiation enteritis (Shadad et al. 2013).

There is a spatial segregation of ion-transport processes with absorption of Na$^+$ (via Na-H exchange) and Cl$^-$ (Cl$^-$-HCO$_3^-$ exchange) occurring in the villous epithelial cells and secretory processes occurring in crypt cells (Welsh et al. 1982; Traber et al. 1992; Minhas and Field 1994). Previous studies from this laboratory have shown that radiation results in a dose-dependent increase in electrogenic chloride secretion (Zhang et al. 2011). Very little is known about the effect of radiation on the sodium absorptive processes, including basal electroneutral Na$^+$-H$^+$ exchange (NHE3), glucose-stimulated sodium absorption...
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MATERIALS AND METHODS

Animal preparation

Eight-week-old, male, NIH Swiss mice were fed a normal diet and housed at four mice per cage. Animals were irradiated using a Gammacell 40 EXACT Low-Dose Research Irradiator (Best Theratronics, Ottawa, Ontario, Canada) housing two 137Cs sources in parallel and opposed geometry to deliver isotropic radiation with a dose uniformity within ±3%. All mice were subjected to total body irradiation (TBI). Five mice were secured in a plastic jig in the center of the irradiation chamber and were simultaneously irradiated at a dose rate of 0.9 Gy min−1 to achieve a single fraction. The mice were euthanized on day 6 when peak anion secretion occurred, and the toxicity measured was predominantly due to acute GI syndrome.

Bioelectric measurements and flux studies

Stripped ileal sheets were mounted between two halves of an Ussing chamber with 0.3 cm2 of exposed surface area (P2304, Physiologic Instruments, San Diego, CA). The Ringer solution containing (mmol L−1) Na+ 140, Cl− 119.8, K+ 5.2, HPO4− 2− 2.4, H2PO4− 0.4, Mg2+ 1.2, Ca2+ 1.2, and HCO3− 25 was bubbled with 95% O2 and 5% CO2 bilaterally and was maintained at 37°C. After the tissues were allowed to stabilize for 45 min, the basal Isc was determined by using 10−4 M amiloride (HMA), a selective inhibitor of NHE3 (Masereel et al. 1998), followed by glucose-stimulated sodium absorption and amino acid-coupled sodium absorption.

Although the transport mechanisms and their properties for amino acids in the intestines are generally well documented under normal conditions (Broer 2008), they have been poorly characterized in irradiated tissue. It is unclear which nutrients are most beneficial to the enterocytes, with glutamine being the only nutrient with documented therapeutic effects (Klimberg et al. 1990; Ersin et al. 2000). Therefore, the objective was to characterize the transport of various nutrients, especially amino acids, which could be used as an energy source by enterocytes in irradiated tissue. Specifically, the authors determined that identifying which nutrients are absorbed by irradiated intestinal epithelia might permit for the design of an orally administered nutritional supplement that could offset some of the adverse side effects of radiation therapy and promoting healing of the intestinal mucosa. By extension, this would lead to more effective therapy and an increased survival rate.

This present study describes radiation dose-dependent alterations in electrolyte and nutrient absorption and defects in the mucosal barrier. After all 20 amino acids were studied, lysine, aspartic acid, glycine, isoleucine, threonine, tyrosine, valine, tryptophan, and serine were selected to create a mitigating amino acid mixture (MAAM) based on their ability to increase electrolyte absorption and improve mucosal barrier functions. Survival studies using this MAAM were associated with increased survival, despite high-dose irradiation. These studies have immense potential to be translated into clinical use for the treatment of acute and chronic radiation enteritis.

Dilution potential measurement

The dilution potential is based on the principle that a leaky membrane will allow easy diffusion across the membrane, thereby leading to the loss of electrochemical potential and a relative permeability of Cl− and Na+ (PCl/PNa)
closer to 1 (De Benedetto et al. 2011). Permeability of sodium (PNa) and chloride (PCL) were measured by the dilution potential technique, modified from the methods of Kahle et al. (2004) and Hou et al. (2005). The ion permeability ratio (PNa/PCL) was calculated from the dilution potential by using the Goldman-Hodgkin-Katz equation (Salas and Lopez 1982).

**Saturable kinetics of glucose-stimulated increase in the $I_{sc}$**

Saturation kinetics were studied after increasing concentrations of glucose (up to 8 mM) were added to the lumen side in the presence of 140 mM of NaCl and recording the changes in the $I_{sc}$. Tissues were allowed to equilibrate for 10 min after each addition. The peak current for each glucose concentration was used for fitting the data. The nonlinear curve fit with the Michaelis-Menten model for enzyme kinetics was used to calculate the $K_m$ and $V_{max}$.

**Brush border membrane vesicles (BBMV) preparation**

The mucosa was scraped for BBMV preparation and for immunohistochemistry specimens. BBMV were prepared using modified methods from Stieger et al. (1986) and Binder et al. (1986) and stored in a buffer containing a protease inhibitor mixture (10 mM of iodoactamide, 1 mM of phenylmethylsulphonyl fluoride, and 2 μg mL$^{-1}$ of leupeptin) with a pH of 7.4. Protein concentrations were determined in samples using the Bradford assay.

**Immunohistochemistry**

Immunohistochemistry was performed using methods described previously (Zhang et al. 2007, 2011). Ileal tissues were fixed in formalin (10%) for 20 h at room temperature and then embedded in paraffin. Tissue sections (4 μm) were obtained using a microtome (Leica RM2245, Leica Microsystems, Inc., IL, USA) and then fixed onto glass slides. Primary antibody (NHE3) diluted in TBS (1:500) with 1% BSA was incubated overnight at 4°C. Thereafter, the slides were incubated with fluorophore-conjugated secondary antibody (Alexa Fluor 647 Goat Anti-Rabbit IgG, A-21245, Life Technologies Corp., Carlsbad, CA, USA). The slides were then imaged under confocal laser scanning microscopy (Fluoview 1000 IX81 microscope, Olympus, Tokyo, Japan) using a 633-nm laser.

**Western blot analysis**

Western blot analysis was performed using BBMV or ileal lysate prepared from mice irradiated with different doses. Thirty (7–10 in the case of Panel B) micrograms of ileal protein were resolved by electrophoresis through SDS-7.5% polyacrylamide gels, as described by Ranganathan et al. (2011). Blots were reacted subsequently with rabbit anti-NHE3 antibody (SC-28757, Santa Cruz Biotech, CA, USA) at a 1:500-1,000-fold dilution, followed by HRP-conjugated anti-rabbit IgG secondary antibody (BioRad, CA) at 1:3,000 dilution in Tris-Buffered Saline and Tween 20 (TBST). Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce ECL western blotting substrate, Thermo Scientific Waltham, MA, USA) followed by autoradiography for HRP. β-actin was used as a sample loading control.

**Amino acid mixtures and treatment**

Amino acids that retained or increased absorption along with decreased paracellular permeability after irradiation were used to make the MAAM (Table 1), whereas the amino acids that did not show increased absorption or a decrease in conductance were used to make the non-mitigating amino acid mixture (NAAM) (Table 2a and 2b). The mice were subjected to sub-lethal radiation doses for GI death (10% above the LD50 for bone marrow) and were treated with either MAAM or NAAM gastric gavage (150 μL), once 24 h after irradiation and then once daily for 14 d. Body weight was recorded daily before irradiation and gastric gavage.

**Statistics**

Results are presented as mean ± SEM. Statistical analyses were performed with paired and unpaired t-tests and Bonferroni’s 1-way analysis of variance post hoc test. $p < 0.05$ was considered significant.

**RESULTS**

**Effect of radiation on sodium absorption**

Unidirectional and net flux for Na was determined using $^{22}$Na. Tissues from irradiated mice were studied on day 6 after irradiation (0, 1, 3, 5, or 7 Gy). Sodium-22 flux showed a radiation dose-dependent decrease in net sodium absorption. Ileal tissues mounted in the Ussing chamber exhibited basal, NHE3-mediated (the chief sodium absorptive mechanism in the small intestine) (Hoogerwerf et al. 1996; Maher et al. 1997; Gawenis et al. 2002; Lucioni et al. 2002) sodium absorption of 2.8 ± 0.4 μeq h$^{-1}$ cm$^{-2}$ ($n = 10$). Ileal tissues from mice irradiated with 1 Gy resulted in a 0.6 ± 0.1-fold significant decrease in net sodium absorption when compared to basal sodium absorption (2.8 ± 0.4 μeq h$^{-1}$ cm$^{-2}$ versus 1.7 ± 0.3 μeq h$^{-1}$ cm$^{-2}$; $n = 10$, $p < 0.04$) (Fig. 1). Net sodium absorption measured for mice irradiated with 3 Gy was 0.3 ± 0.1-fold lower than that for 1 Gy (1.7 ± 0.3 μeq h$^{-1}$ cm$^{-2}$ versus 0.5 ± 0.2 μeq h$^{-1}$ cm$^{-2}$; $n = 10$, $p < 0.003$). Further increases in radiation dose did not result in significant decreases in net sodium absorption (Fig. 1).

**Effect of radiation on glucose-stimulated sodium absorption**

Ileal tissues mounted in an Ussing chamber exhibited basal sodium absorption. Glucose (8 mM) added to the
luminal side of the ileal tissues led to a significant increase in sodium absorption across all radiation groups studied, except in 5 Gy and 7 Gy (Fig. 1), with a 2.3 ± 0.5-fold increase at 0 Gy and 3.0 ± 0.6-fold increase at 1 Gy. Net glucose-stimulated sodium absorption decreased with increasing radiation dose (n = 10, p < 0.03); however, significant decreases were not seen in ileal tissues from mice irradiated with 0 Gy or 1 Gy (Fig. 1).

To study the effect of radiation on glucose saturation kinetics, increasing concentrations of glucose (up to 8 mM) were added to the luminal side in the presence of 140 mM of NaCl. Changes in the $I_{sc}$ were recorded. In 0 Gy mice, the glucose revealed saturable kinetics with a $K_m$ of 0.4 ± 0.1 mM. With increasing radiation doses, the ileal tissues exhibited increases in $K_m$ and decreases in $V_{max}$ (Fig. 2a). The maximal increase in $K_m$ was seen at 3 Gy as compared to 0 Gy tissues (4.6 ± 0.6-fold).

### Effect of glucose on paracellular permeability in irradiated mouse small intestines

Conductance ($G$), a measure of the paracellular permeability, was recorded using a computer-controlled voltage/current clamp device (VCC MC-8, Physiologic Instruments), as previously described (Zhang et al. 2007, 2011), and expressed as mS cm$^{-2}$. Radiation resulted in significant increases ($n = 20, p < 0.001$) in conductance in all groups except 1 Gy and 7 Gy. Tissues irradiated with 1 Gy showed a significant decrease in conductance when compared to 0 Gy (n = 20, p < 0.001), whereas 7 Gy tissues did not exhibit a decrease that was significantly different from

### Table 1. Selection criteria for the amino acid included in the radiation mitigation drink.$^{ab}$

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>System</th>
<th>Transport after IR</th>
<th>Conductance after IR</th>
<th>$K_m$ (Before)</th>
<th>$K_m$ (after IR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Essential amino acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Arginine</td>
<td>$y^+, y^L, B^0, B^0$</td>
<td>↑↑</td>
<td>↑↑</td>
<td>0.2 ± 0.1</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>2 Histidine</td>
<td>A, N, L, $B^0, y^L$</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>2.8 ± 0.7</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>3 Methionine</td>
<td>A, $B^0, L, y^L$</td>
<td>↓↓</td>
<td>↑</td>
<td>1.7 ± 0.2</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>4* Threonine</td>
<td>ASC</td>
<td>↑↑</td>
<td>↓↓</td>
<td>—</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>5* Valine</td>
<td>$B^0, L$</td>
<td>↑↑↑</td>
<td>↓↓↓</td>
<td>2.9 ± 1.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>6* Isoleucine</td>
<td>L, $B^0, y^L$</td>
<td>↑↑↑</td>
<td>↓</td>
<td>1.7 ± 0.8</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>7* Lysine</td>
<td>$B^0, y^L, y^L, y^L$</td>
<td>↑↑↑</td>
<td>↓</td>
<td>1.1 ± 0.4</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>8 Phenylalanine</td>
<td>L, T</td>
<td>↓</td>
<td>↓</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9* Tryptophan</td>
<td>L, T</td>
<td>↑↑</td>
<td>↓↓↓</td>
<td>—</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>10 Leucine</td>
<td>$y^L, B^0, B^0$</td>
<td>↑</td>
<td>↑↑</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td><strong>Nonessential amino acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Alanine</td>
<td>A, ASC, asc, PAT, $y^L$</td>
<td>↓↓</td>
<td>↓</td>
<td>2.3 ± 0.7</td>
<td>—</td>
</tr>
<tr>
<td>12 Asparagine</td>
<td>A, N</td>
<td>↑↑</td>
<td>↑</td>
<td>—</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>13* Aspartic acid</td>
<td>X-AG</td>
<td>↑↑↑</td>
<td>↓↓↓</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>14 Cysteine</td>
<td>A, ASC, $y^L$</td>
<td>↑↑↑</td>
<td>↓↑</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15 Glutamine</td>
<td>A, ASC, N, $y^L$</td>
<td>↑↑</td>
<td>↑↑</td>
<td>0.2 ± 0.1</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>16 Glutamic acid</td>
<td>X-AG, $B^0$</td>
<td>↓↓↓</td>
<td>↑↑</td>
<td>0.6 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>17* Glycine</td>
<td>A, asc, Gly</td>
<td>↑↑↑</td>
<td>↓↓↓</td>
<td>1.6 ± 0.6</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>18 Proline</td>
<td>A, $B^0$, IMINO, PAT</td>
<td>↑↑</td>
<td>↑↑</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>19* Serine</td>
<td>A, ASC, N</td>
<td>↑</td>
<td>↓↓↓</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>20* Tyrosine</td>
<td>L, T</td>
<td>↑↑</td>
<td>↓↓↓</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$Na$^+$-dependent amino acid transport.

$^b$Small amino acids such as Ala, Ser, Gln are preferred substrates.

ASC Transports Ala, Ser, and Cys.

B0 Broad specificity for most neutral amino acids.

N Prefers His, Gln, Asn.

Na$^+$-independent amino acid transport.

L Transports neutral amino acids.

$y^+$ Specific for cationic amino acids.

$y^L$ In addition to transporting cationic amino acids, it interacts with neutral amino acids in the presence of Na.

$b0,+$ Transport cationic amino acids, binds to neutral amino acids in the absence of Na.

$^c$‘—’ denotes an instance in which the $K_m$ could not be measured. It is either because of a small amino-stimulated increase in current ($I_{sc}$) or because the current never saturated with increasing concentration of the respective amino acids.

$^d$‘↑’ represents an increase and ‘↓’ represents a decrease.

$^e$‘*’ represents the amino acid selected for making the drink.
that of 0 Gy. However, 7 Gy irradiated tissues exhibited a 0.8 ± 0.03-fold decrease when compared to 5 Gy tissues ($n = 20$, $p < 0.001$) (Fig. 2b). The addition of glucose to the luminal side resulted in 1.1 ± 0.04, 2.1 ± 0.1, 1.2 ± 0.1, 1.2 ± 0.1, and 1.1 ± 0.1-fold increases in conductance at 0, 1, 3, 5, and 7 Gy, respectively (Fig. 2b).

**Effect of radiation on plasma endotoxin and IL-1β**

Plasma samples collected 14 d after irradiation were used to measure plasma endotoxin and plasma IL-1β levels. Endotoxin showed 1.6 ± 0.4, 2.1 ± 0.1, 1.2 ± 0.1, 1.2 ± 0.1, and 1.1 ± 0.1-fold increases in conductance at 0, 1, 3, 5, and 7 Gy, respectively (Fig. 2b).

**Effect of radiation on amino acid transport**

Functional studies showed that glucose increased conductance in irradiated tissues (Fig. 2b) and that its absorption via the sodium-coupled glucose transporter (SGLT1) was decreased following irradiation. Therefore, similar experiments were undertaken using each of the 20 amino acids to determine its sodium absorptive ability and its effect on paracellular permeability in the Ussing chamber. As sodium-coupled

### Table 2a. Amino acid composition of the Mitigating Amino Acids Mixture (MAAM).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CAS No.</th>
<th>Molecular weight</th>
<th>mM</th>
<th>Grams/liter</th>
<th>mOsm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>657-27-2</td>
<td>182.65</td>
<td>4</td>
<td>0.7306</td>
<td>4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>56-84-8</td>
<td>133.1</td>
<td>8</td>
<td>1.0648</td>
<td>8</td>
</tr>
<tr>
<td>Glycine</td>
<td>56-40-6</td>
<td>75.07</td>
<td>8</td>
<td>0.60056</td>
<td>8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>73-32-5</td>
<td>131.17</td>
<td>8</td>
<td>1.04936</td>
<td>8</td>
</tr>
<tr>
<td>Threonine</td>
<td>72-19-5</td>
<td>119.12</td>
<td>8</td>
<td>0.95296</td>
<td>8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>60-18-4</td>
<td>181.2</td>
<td>1.2</td>
<td>0.21744</td>
<td>1.2</td>
</tr>
<tr>
<td>Valine</td>
<td>72-18-4</td>
<td>117.15</td>
<td>10</td>
<td>1.1715</td>
<td>10</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>73-22-3</td>
<td>204.23</td>
<td>8</td>
<td>1.63384</td>
<td>8</td>
</tr>
<tr>
<td>Serine</td>
<td>56-45-1</td>
<td>105.1</td>
<td>10</td>
<td>1.051</td>
<td>10</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>7647-14-5</td>
<td>58.44</td>
<td>72</td>
<td>4.20768</td>
<td>144</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>144-55-8</td>
<td>84.01</td>
<td>4.37</td>
<td>0.3671237</td>
<td>8.74</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>7786-30-3</td>
<td>95.22</td>
<td>1.2</td>
<td>0.114264</td>
<td>3.6</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>10035-04-8</td>
<td>147.03</td>
<td>1.2</td>
<td>0.176436</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Total osmolarity 225.14 mOsm, pH 4.2

### Table 2b. Amino acid composition of the Non-mitigating Amino Acids Mixture (NAAM).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CAS No.</th>
<th>Molecular weight</th>
<th>mM</th>
<th>Grams/liter</th>
<th>mOsm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>74-79-3</td>
<td>174.2</td>
<td>6</td>
<td>1.045</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>71-00-1</td>
<td>155.16</td>
<td>6</td>
<td>0.931</td>
<td>6</td>
</tr>
<tr>
<td>Methionine</td>
<td>63-68-3</td>
<td>149.21</td>
<td>6</td>
<td>0.895</td>
<td>6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>63-91-2</td>
<td>165.19</td>
<td>6</td>
<td>0.991</td>
<td>6</td>
</tr>
<tr>
<td>Leucine</td>
<td>61-90-5</td>
<td>131.17</td>
<td>6</td>
<td>0.787</td>
<td>6</td>
</tr>
<tr>
<td>Alanine</td>
<td>56-41-7</td>
<td>89.09</td>
<td>6</td>
<td>0.535</td>
<td>6</td>
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<tr>
<td>Asparagine</td>
<td>70-47-3</td>
<td>132.12</td>
<td>6</td>
<td>0.793</td>
<td>6</td>
</tr>
<tr>
<td>Cysteine</td>
<td>52-90-4</td>
<td>121.16</td>
<td>6</td>
<td>0.727</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>56-86-0</td>
<td>147.13</td>
<td>6</td>
<td>0.883</td>
<td>6</td>
</tr>
<tr>
<td>Proline</td>
<td>147-85-3</td>
<td>115.13</td>
<td>6</td>
<td>0.691</td>
<td>6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>56-85-9</td>
<td>146.15</td>
<td>6</td>
<td>0.877</td>
<td>6</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>7647-14-5</td>
<td>58.44</td>
<td>72</td>
<td>4.20768</td>
<td>144</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>144-55-8</td>
<td>84.01</td>
<td>4.37</td>
<td>0.3671237</td>
<td>8.74</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>7786-30-3</td>
<td>95.22</td>
<td>1.2</td>
<td>0.114264</td>
<td>3.6</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>10035-04-8</td>
<td>147.03</td>
<td>1.2</td>
<td>0.176436</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Total osmolarity 225.94 mOsm, pH 4.2
amino acid transport results in an increase in the $I_{sc}$, this was used as an index for transport capacity before and after irradiation. Similarly, an increase in conductance, which is a measure of increased paracellular permeability, suggests a breach in the mucosal barrier mechanism. Sixteen amino acids showed increased transport after irradiation, while nine amino acids showed concomitant decreases in paracellular permeability (Table 1). Fig. 4a and 4b represent an amino acid that decreased and increased sodium-absorptive current after irradiation. Amino acids that retained or increased absorption along with decreased paracellular permeability after irradiation were included in the MAAM (Table 2a).

**MAAM decreased the radiation-induced increase in paracellular permeability**

Ussing chamber studies with conductance measurements and dilution potential were used to determine the extent of the loss of epithelial barrier functions. Irradiated mice (5 Gy) MAAM-treated and sham-treated (saline) mice were euthanized on day 14, and tissues were mounted in the Ussing chamber for measuring the dilution potential. Ileal tissues from sham-treated 5 Gy irradiated mice showed significantly increased paracellular permeability when compared to tissues from MAAM-treated 5 Gy and 0 Gy mice ($p < 0.001; n = 6$) (Fig. 5).

**MAAM restored paracellular permeability**

Mice were euthanized at the end of 14 d of treatment with MAAM or saline, and intestinal tissues were used for determining the ion permeability ratio, a measure of paracellular permeability. Tissues irradiated with 5 Gy showed a significant improvement in $\text{PCl/PNa}$ ratio when compared to the 5 Gy saline-treated group ($0.36 \pm 0.01$ vs. $0.82 \pm 0.04$) (Fig. 5). Plasma collected at the time of sacrifice was used for measuring plasma endotoxin and IL-1β levels.

MAAM mitigated radiation-induced increases in plasma endotoxin (Fig. 3a) and IL-1β levels (Fig. 3b). A relative fold reduction in plasma endotoxin levels was observed in MAAM-treated mice (0.7 ± 0.2, 0.6 ± 0.1, 0.5 ± 0.1, 0.5 ± 0.1 in 1, 3, 5, and 7 Gy, respectively). Similarly, a relative fold reduction of 0.3 ± 0.1, 0.3 ± 0.1, 0.3 ± 0.0, and 0.7 ± 0.1 in plasma IL-1β was observed in MAAM-treated 1, 3, 5, and 7 Gy mice, respectively. These results suggest that radiation-induced changes in plasma endotoxin and IL-1β correlated to the changes in paracellular permeability measured using transepithelial electrical resistance and dilution potential.

**Western blot analysis**

Western blot analysis was done using BBMV and whole cell fractions prepared from mucosal scrapings from small intestines. Western blot analysis showed radiation dose-dependent decreases in the protein levels of NHE3. At radiation doses of 1, 3, 5, and 7 Gy, there were 1.6, 1.4, 2.1, and 19-fold decreases in the NHE3 protein level, respectively. Western blot studies on irradiated mouse intestines correlated well with the radiation dose-dependent
decrease in net sodium absorption (Fig. 1). Mucosal scrapings from mice irradiated with a sub-lethal dose (8 Gy) and treated with the MAAM showed a 1.9-fold increase in NHE3 protein levels on day 14 compared with low levels seen in sham-treated irradiated mice (Fig. 6).

**Immunostaining showed changes in NHE3 expression following irradiation**

Immunostaining using a mouse-specific NHE3 antibody demonstrated maximal staining on the villous tips in normal mice (Fig. 6c). Minimal NHE3 expression was seen along the brush border membrane of the villous in 8 Gy irradiated mice (Fig. 6d). Tissue sections from mice treated with MAAM showed NHE3 expression along the brush border membrane of the villous tips (Fig. 6e). These findings correlated well with the protein levels detected using Western blot analysis and $^{22}$Na flux studies in an Ussing chamber.

**MAAM decreased weight loss and increased survival in irradiated mice**

The MAAM was given via gastric gavage 24 h after 8 Gy irradiation. Body weight was recorded at the time of gavage. Sixty-four percent of mice survived with relative body weights at 24 d of ~5% less than the initial weight (Fig. 7). Mice exposed to 8 Gy and treated either with saline or NAAM (negative control) gavage experienced 100% lethality at 17 and 11 d, respectively. In both cases, the body weights showed a steep decline starting approximately 4 d before death of >20% of the mice (Fig. 7a and b). These data suggest that MAAM may enhance electrolyte and nutrient absorption in irradiated mice and be useful as a mitigating therapy to treat acute radiation-induced GI injury.

**DISCUSSION**

Currently, the treatment of acute radiation enteritis is mostly based on symptomatology (Berger et al. 2006), including antidiarrheals to prevent fluid loss, opioids to relieve stomach and rectal pain, and glucose-containing electrolyte solution to correct dehydration or fluid loss. Steroids are also given to relieve acute inflammation due to irradiation. In severe cases of radiation exposure, patients are given parenteral feeding to correct malabsorption of nutrients and electrolytes. It has been shown that it is very difficult to wean patients once they are subjected
to total or partial parenteral nutrition. Beyond the everyday clinical situation, in the case of a potential mass-casualty nuclear event, it is difficult to ascertain an individual’s radiation exposure dose in the absence of reliable onsite dosimetry and to get medical and paramedical help within the first 24 h. Moreover, any agent used to mitigate GI radiotoxicity under such circumstances would be subjected to harsh environmental conditions and would need a long shelf life. Thus, there is a need for an orally administered CONOPS-friendly agent that is effective across various doses and schedules and has a shelf life of at least 12 mo to overcome radiation-induced GI toxicity.

Amino acids are generally considered to be the primary energy source of enterocytes. Various studies have shown that under normal conditions, glutamine, glutamate, and aspartate serve as the primary energy sources, with their oxidation accounting for 80% of the total O2 consumption in enterocytes, with 85% of the total absorbed quantity being metabolized (Okine et al. 1995; Alpers 2000). Other studies indicate that lysine, leucine, phenylalanine, and threonine are also important energy sources, with 30–50% of the absorbed quantity metabolized (Wu 1998; Tome and Bos 2007). In addition, there is evidence that intestinal epithelia become atrophic when deprived of glutamine in cases where total parenteral feeding is used (Alpers 2006). Therefore, it is logical that amino acids would be major components in any oral nutritional supplement that is given to patients undergoing radiation therapy.

In order to encourage the healing of intestinal epithelia and mitigate the effects of radiation damage, an orally administered nutritional supplement should be used

Fig. 5. Effect of radiation and MAAM treatment on dilution potential. Irradiated (5 Gy) mice treated with MAAM for 14 d showed a significant increase in ion permeability ratio compared to tissues from saline-treated 5 Gy irradiated mice ($p < 0.001$). The values are from $n = 6$ tissues.

Fig. 6. NHE3 protein levels and expression pattern following irradiation. (a) Western blot analysis for NHE3 protein levels on tissue extract exposed to different radiation doses. (b) Western blot analysis showing NHE3 protein levels in tissues from irradiated and nonirradiated mice treated with saline or MAAM. For Western blot analysis, the immunoreactive bands were visualized by enhanced chemiluminescence, $\beta$-actin was used as the loading control. (c) Immunohistochemistry for NHE3 expression pattern in nonirradiated tissues. NHE3 expression is seen along the brush border membrane. (d) NHE3 expression in 8 Gy irradiated tissues. Minimal expression is seen along the brush border membrane (white arrow). (e) Immunohistochemistry showing NHE3 expression pattern in 8 Gy irradiated tissues treated with the MAAM. NHE3 expression is seen along the brush border membrane region (white arrow).
to improve the nutrition of patients since enteral feeding seems to provide inherent benefits (Bounous et al. 1975). This component of radiation therapy would likely take the form of an ingestible liquid cocktail of nutrients, all of which are known to be absorbed through irradiated intestinal epithelia and some of which are thought to be an energy source for enterocytes. By providing the enterocytes in damaged epithelia with a constant supply of energy that can be readily absorbed and used, it may be possible to speed up intestinal healing from radiation damage and mitigate its adverse side effects. Survival studies conducted in rat models have shown that irradiated specimens fed a diet enriched in arginine and glutamine (two nutrients thought to be major energy sources for enterocytes) are more resistant to bacterial translocation, exhibit faster intestinal healing, and have increased survival (Klimberg et al. 1990; Ersin et al. 2000). However, there are conflicting reports on the beneficial effects of glutamine. In randomized, controlled trials to determine the efficacy of glutamine in the prevention of acute radiation enteritis, Vidal-Casariego et al. (2013) found that glutamine did not prevent the development of enteritis during radiotherapy. Currently, the main barrier to the design and implementation of such a treatment is the lack of knowledge concerning the transport capabilities of the intestines suffering from radiation damage.

In this study, nine amino acids were selected based on their ability to retain or increase coupled sodium transport and decrease paracellular permeability after irradiation. Most of the absorptive processes are located in the fully differentiated and mature villous tip, with villous atrophy following radiation exposure; these transport machineries are affected, which leads to malabsorption. The presence of unabsorbed or poorly absorbed nutrients and electrolytes alone can produce osmotic diarrhea. The diminished absorptive function can exacerbate the effects of radiation damage and mitigate the tissue’s ability to heal. The reduced regeneration of intestinal epithelia leads to an extended period of malabsorption, causing the patient to become increasingly malnourished and making them more susceptible to infections in the major organs due to bacterial translocation through the damaged intestine.

Glucose saturation kinetics showed a radiation dose-dependent increase in $K_m$, indicating decreased affinity for the glucose transporter. A decrease in the $V_{max}$ suggested a decrease in the number of transporters for glucose transport and corresponded to the villous atrophy that occurs with radiation exposure (Marteau et al. 1997; Berger et al. 2006). The rate at which the functioning of the transporters responsible for glucose-stimulated sodium absorption (SGLT1) and electroneutral sodium absorption (NHE3) decrease after irradiation were not the same, and the ratios were not specific to glucose-mediated transport; therefore, a fold increase in sodium absorption was not used to interpret the data.

Glucose was also shown to increase paracellular permeability across all irradiation groups. Similar changes in paracellular permeability due to glucose have been noted in previous studies (Ballard et al. 1995; Turner et al. 2000). An increase in paracellular permeability permits increased translocation of the luminal contents, such as bacteria and partially digested food substances, into the systemic compartment, thereby leading to various degrees of endotoxemia and an increase in proinflammatory cytokines (Arrieta et al. 2006; Tugendreich et al. 2006). The nine amino acids selected were well absorbed and decreased paracellular permeability after irradiation.

The selected amino acids were dissolved in an electrolyte solution at the optimum pH and osmolarity, which were determined in a series of experiments (method not

Fig. 7. MAAM improved mouse survival and improved body weight following irradiation. (a) Residual weight of irradiated mice that received saline (control), MAAM, or NAAM. Mice treated with NAAM showed > 20% weight loss in less than 10 d, whereas control animals reached > 20% weight loss by the 15th day. Mice treated with the MAAM showed steady weight gain beyond 18 days (data not shown). (b) Mice treated with MAAM showed > 66% increase in survival beyond 25 days. Mice receiving the NAAM died earlier than animals receiving the saline controls. Values are from $n = 25$ animals/group; the error bars indicate SEM.
shown in this manuscript). The resulting MAAM was tested for radiation mitigation through measurements of the overall survival, body weight, plasma endotoxin and IL-1β levels, NHE3 protein expression, and protein levels.

These studies indicate that this MAAM can be used to mitigate acute radiation enteritis when given orally within the first 24 h after radiation exposure. The agent is safe at radiation doses below the GI syndrome level (“worried well”) and in all patients, including men, women, children, and pregnant women. The MAAM may also be beneficial in bone marrow ARS, which can occur at radiation doses below that which causes GI ARS. Also, the MAAM may be used in combination with other radiation mitigation agents and should not reduce the benefits of other therapies for combined diseases. However, the dosage and treatment time windows may need to be optimized for humans.

All of the ingredients used in the formulation of the MAAM are listed in the “Generally Recognized as Safe” list of chemicals and substances added to food; thus, the formulation is exempt from the usual Federal Food, Drug, and Cosmetic Act. Consequently, this MAAM has immediate potential to be used in patients with acute radiation-induced GI toxicity, under a medical food category for rapid rehydration and radiotoxicity mitigation.

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