Ciprofloxacin Therapy Results in Mitigation of ATP Loss after Irradiation Combined with Wound Trauma: Preservation of Pyruvate Dehydrogenase and Inhibition of Pyruvate Dehydrogenase Kinase 1

Authors: Swift, Joshua M., Smith, Joan T., and Kiang, Juliann G.

Source: Radiation Research, 183(6) : 684-692

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RR13853.1

RADIATION RESEARCH **183**, 684–692 (2015)
0033-7587/15 $15.00

DOI: 10.1667/RR13853.1

Ciprofloxacin Therapy Results in Mitigation of ATP Loss after Irradiation Combined with Wound Trauma: Preservation of Pyruvate Dehydrogenase and Inhibition of Pyruvate Dehydrogenase Kinase 1

Joshua M. Swift,[a,b,c] Joan T. Smith[a] and Juliann G. Kiang[a,c,d,1]

[a] *Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20889; and* [b] *Departments of Military and Emergency Medicine;* [c] *Radiation Biology and* [d] *Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814*

Swift, J. M., Smith, J. T. and Kiang, J. G. Ciprofloxacin Therapy Results in Mitigation of ATP Loss after Irradiation Combined with Wound Trauma: Preservation of Pyruvate Dehydrogenase and Inhibition of Pyruvate Dehydrogenase Kinase 1. *Radiat. Res.* 183, 684–692 (2015).

Ionizing radiation exposure combined with wound injury increases animal mortalities than ionizing radiation exposure alone. Ciprofloxacin (CIP) is in the fluroquinolone family of synthetic antibiotic that are available from the strategic national stockpile for emergency use and is known to inhibit bacterial sepsis. The purpose of this study was to evaluate the efficacy of ciprofloxacin as a countermeasure to combined injury mortality and determine the signaling proteins involved in energy machinery. B6D2F1/J female mice were randomly assigned to receive either 9.75 Gy irradiation with Co-60 gamma rays followed by skin wounding (combined injury; CI) or sham procedure (sham). Either ciprofloxacin (90 mg/kg/day) or vehicle (VEH) (water) was administered orally to these mice 2 h after wounding and thereafter daily for 10 days. Determination of tissue adenosine triphosphate (ATP) was conducted, and immunoblotting for signaling proteins involved in ATP machinery was performed. Combined injury resulted in 60% survival after 10 days compared to 100% survival in the sham group. Furthermore, combined injury caused significant reductions of ATP concentrations in ileum, pancreas, brain, spleen, kidney and lung (−25% to −95%) compared to the sham group. Ciprofloxacin administration after combined injury resulted in 100% survival and inhibited reductions in ileum and kidney ATP production. Ileum protein levels of heat-shock protein 70 kDa (HSP-70, a chaperone protein involved in ATP synthesis) and pyruvate dehydrogenase (PDH, an enzyme complex crucial to conversion of pyruvate to acetyl CoA for entrance into TCA cycle) were significantly lower in the CI group (vs. sham group). Using immunoprecipitation and immunoblotting, HSP-70-PDH complex was found to be present in the ileum tissue of CI mice treated with ciprofloxacin. Furthermore, phosphorylation of serine residues of PDH resulting in inactivating PDH enzymatic activity, which occurred after combined injury, was inhibited with ciprofloxacin treatment, thus enabling PDH to increase ATP production. Increased ileum levels of pyruvate dehydrogenase kinase 1 protein (PDK1, an enzyme responsible for PDH phosphorylation) after combined injury were also prevented by ciprofloxacin treatment. Taken together, these data suggest that ciprofloxacin oral administration after combined injury had a role in sustained ileum ATP levels, and may have acted through preservation of PDH by HSP-70 and inhibition of PDK1. These molecular changes in the ileum are simply one of a host of mechanisms working in concert with one another by which ciprofloxacin treatment mitigates body weight loss and drastically enhances subsequent survival after combined injury. To this end, our findings indicate that oral treatment of ciprofloxacin is a valuable therapeutic treatment after irradiation with combined injury and warrants further analyses to elucidate the precise mechanisms involved.

INTRODUCTION

Casualties after a nuclear power plant accident, detonation of a radiologic dispersal device or nuclear attack are likely to suffer from a number of injuries, as a result of both radiation exposure and trauma due to blast and thermal effects [i.e., combined injuries (CI)]. These complex injuries lead to enhanced mortality compared to radiation injury alone, although taken individually, neither trauma injury nor radiation exposure may be lethal (*1, 2*). Furthermore, after a nuclear detonation a large percentage of casualties have sustained injuries resulting from more than just exposure to ionizing radiation. In Hiroshima and Nagasaki, for example, 60–70% of radiation victims sustained additional traumatic injury, primarily in the form of burns, mechanical wounding and hemorrhage (*3, 4*). Therefore, a comprehensive understanding of the mechanisms involved in combined injuries and the development of appropriate countermeasures to mitigate or inhibit those effects is an essential undertaking.

In previous studies of mouse models, we reported that wound trauma induces lethality after nonlethal radiation exposure (*1, 5*). Furthermore, combined injury exacerbates radiation-induced mortality, potentially due in part to

[1] Address for correspondence: Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Building 46, Bethesda, MD 20889-5603; e-mail: juliann.kiang@usuhs.edu.

systemic bacterial infection from the gastrointestinal tract and skin wound (*5*). Within the ileum, combined injury reduces villus height and width and decreases tunica muscularis thickness, resulting in severe gastrointestinal injury and bacteria translocation into the bloodstream (*1, 5, 6*). Furthermore, combined injury induces apoptosis of villous epithelial cells and autophagy of crypt cells (*6, 7*).

The synthetic antibiotic agent, ciprofloxacin, is an FDA-approved fluoroquinolone widely used as an antimicrobial. Ciprofloxacin is included in the Strategic National Stockpile, which is maintained by the United States Department of Health and Human Services, to control bacterial infection during a national emergency such as a nuclear detonation or other radiological incident. In addition to the antimicrobial activity, ciprofloxacin exerts immunomodulatory effects in rodents and humans (*8, 9*), improving a wide spectrum of conditions including inflammatory bowel disease (*10, 11*), rheumatoid arthritis (*12, 13*), chemotherapy-induced neutropenia (*14, 15*) and radiation-induced apoptosis (*16*). These favorable improvements are irrelevant to its antimicrobial activity, but rather are attributable to the stimulation of hematopoiesis (*17, 18*) and reduction of inflammation (*9*). In addition, ciprofloxacin administered after whole-body irradiation reduces systemic bacterial infection, making it an attractive countermeasure for radiation combined injury (*6, 19*). However, the effectiveness of ciprofloxacin for increasing survival, as well as the molecular mechanisms by which ciprofloxacin functions in the ileum have yet to be determined.

Adenosine triphosphate (ATP) production is partly regulated by the expression of pyruvate dehydrogenase (PDH), and its enzymatic activity might be altered by combined injury, an aspect that has not yet been investigated. PDH is the enzyme that converts pyruvate to acetyl CoA, which enters the tricarbonic acid cycle (TCA), subsequently generating significant amounts of ATP (*20*). Reductions in PDH protein and/or its enzymatic activity may result in decreased ATP. Conversely, increased PDH protein or its enzymatic activity could increase ATP levels.

Wang *et al*. (*21*) reported that in Tibet minipigs, exposure to radiation reduced energy metabolism in the small intestine. The ATP reduction in the small intestine 24 h after X irradiation was found to be dose-dependent. Their results suggest that the degree of ATP reduction was a potential indicator of the severity of small intestinal injury. The ATP effect after combined injury had not been investigated before.

The aim of the our study was to determine whether the synthetic antibiotic, ciprofloxacin, effectively inhibited the deleterious effects of radiation combined wound injury on ATP levels in various tissues, PDH and related proteins and increased survival. We hypothesized that wound trauma after exposure to ionizing radiation would reduce mouse survival, deplete tissue ATP and reduce PDH levels in the ileum after 10 days. We further hypothesized that daily ciprofloxacin treatment would increase mouse survival and attenuate the deleterious effects of combined injury trauma on ileum ATP and related proteins.

MATERIALS AND METHODS

Animals

Female B6D2F1/J mice (~25 gm body mass; n = 40) were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed one week to acclimate to their surroundings. Upon completion of the acclimation period, all animals were 12–20 weeks old and were rank ordered by body mass to one of four experimental groups (n = 10/group): sham controls (sham); sham controls administered ciprofloxacin (sham + CIP); radiation plus wounding (combined injury or CI); and CI plus CIP treatment (CI + CIP). Because it has been previously reported that wounding alone and radiation alone are nonlethal by day 10 (*22*), and to minimize the number of animals utilized in the experiment [in accordance with the IACUC (3R's)], we chose to focus on the effects on mice of combined injury with or without ciprofloxacin treatment.

The experiments were conducted at the Armed Forces Radiobiology Research Institute's (AFRRI) animal facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-International (AAALAC-I). All animals were housed in a temperature-controlled (23 ± 2°C) room with a 12:12 light-dark cycle and were provided standard rodent chow (Teklad 8604; Harlan® Laboratories Inc., Indianapolis, IN) and water *ad libitum*. All animals were monitored for health on a daily basis. Animal care and experimental procedures described in this investigation were conducted in accordance with the AFRRI Institutional Animal Care and Use Committee regulations. Euthanasia was performed in accordance with recommendations and guidelines of the American Veterinary Medical Association.

Prior to experiments, hair on the dorsal surface of mice was removed using electric clippers. On the day of the experiments, mice were first irradiated and skin wounding was performed under anesthesia by methoxyflurane inhalation. All mice, including controls, were administered an intraperitoneal injection of 0.5 mL sterile isotonic 0.9% NaCl fluid therapy immediately after combined injury or sham injury to avoid radiation-induced dehydration. After injuries, mice were assigned to clean cages and provided with proper food and acidified water.

On day 10, all mice were euthanized by cervical dislocation under methoxyflurane anesthesia and a small portion of the brain, spleen, kidney, ileum, pancreas and lung was removed from all mice and frozen immediately at −80°C until immunoblotting and ATP assays were performed. Tissues were minced, sonicated for 15 s and subsequently centrifuged at 10,000*g* for 10 min. Supernatant was saved for determination of the total amount of protein in each lysate sample prior to immunoblot analyses. Cellular ATP levels were measured, and proteins and moieties were assessed by immunoprecipitation and immunoblotting between HSP-70 and PDH, and PDH and phosphoserine.

Irradiation

On day 0, all mice were place individually in well ventilated acrylic boxes and administered either 0 (n = 20) or 9.75 Gy (n = 20) whole-body irradiated with ^{60}Co γ photons at ~0.4 Gy/min bilaterally in the AFRRI Cobalt Radiation Facility. The 9.75 Gy dose is ~$LD_{70/30}$ according to previous work (*23*). Dosimetry was performed using the alanine/electron paramagnetic resonance system. Calibration of the dose rate with alanine was traceable to the National Institute of Standards and Technology (NIST) and the National Physical Laboratory (NPL) (United Kingdom). Sham-irradiated mice (0 Gy) were placed in ventilated acrylic boxes, taken to the radiation facility and restrained for a time period similar to irradiated mice.

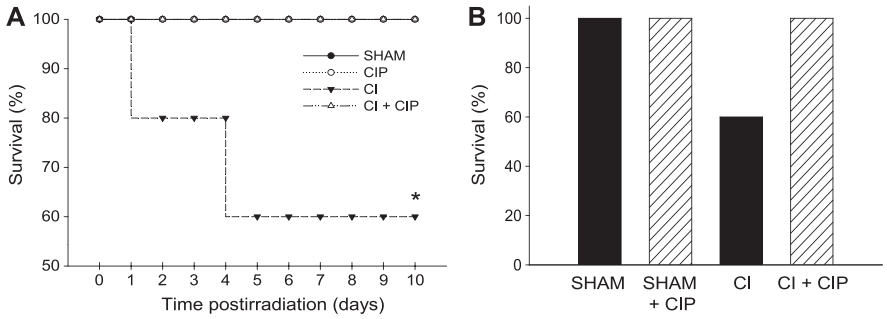

FIG. 1. Effects of daily ciprofloxacin (CIP) treatment on combined injury (CI)-induced mortality in mice. Female B6D2F1/J mice received 0 (SHAM) or 9.75 Gy γ rays (^{60}Co) followed by skin wounding within 1 h (CI; n = 10/group). One hour later, and thereafter daily for 10 days, mice were orally administered ciprofloxacin (CIP; 90 mg/kg/day). The experiment was performed twice. The data presented are from only one experiment. *$P < 0.05$ vs. SHAM, SHAM + CIP and CI + CIP.

Skin Wounding

Within 1 h of radiation exposure, mice were anesthetized under methoxyflurane by inhalation. An experimental wound was administered 19 ± 1.3 mm from the occipital bone and between the scapulae using a stainless steel punch on a Teflon®-covered board cleaned with 70% alcohol before each use. The panniculus carnosus muscle and overlying skin (23.5 ± 1.1 mm long and 14.9 ± 0.7 mm wide) were removed. Sham-wounded mice were treated identically to other groups except without wounding. The wound remained open to mimic the scenario of mass casualty after a nuclear weapon detonation or accidents. Four mice were housed in each cage.

Preparation and Administration of Ciprofloxacin

Veterinary, oral-use ciprofloxacin tablets (500 mg/each) (Dr. Reddy's Laboratories Ltd., Hyderabad, India) were used to prepare fresh solution each week. Tablets were ground, dissolved in sterile water (vehicle) and, after a brief centrifugation, sterile filtered using 0.22 μm cellulose nitrate filter system (Corning Inc., Corning, NY). Each dose of 0.2 mL ciprofloxacin solution delivered 90 mg/kg, based on average mouse body mass. All mice received 0.2 mL of either ciprofloxacin or vehicle via oral route once per day for 10 days, starting within 2 h of combined injury (*23, 24*) and through day 10. The treatment regimen has been justified based on previous studies (*19, 25*) and pharmacokinetics data (*26*) to treat more severe polymicrobial endogenous sepsis in combined injury (*5*). Mice were gently restrained by hand and fed using oral feeding needles attached to 1 mL syringes. Feeding needles were disinfected with 70% ethanol on a gauze sponge between mice in each cage and new needles were used for every cage of mice.

Survival

Survival after combined injury and ciprofloxacin therapy was evaluated with 10 mice per group. The gross appearance, general health and survival of each mouse were followed by visual inspection daily for 10 days in parallel with other assessments.

Tissue ATP Levels

Tissue ATP levels were determined using the ATP Bioluminescence Assay Kit HS II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Luminescence was measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Data were normalized to total protein, and the tissue ATP level is expressed as picomoles (p*M*) per microgram of protein.

Western Blot Analysis

Ileum samples were resolved on SDS-polyacrylamide slab gels (precast 10% gel; Invitrogen™, Carlsbad, CA). Protein was blotted onto a nitrocellulose membrane (type NC, 0.45 μm; Schleicher & Schuell Bioscience GmbH, Dassel, Germany) using a Novex® blotting apparatus and the manufacturer's protocol. The nitrocellulose membrane was blocked by incubation for 90 min at room temperature in phosphate buffered saline (PBS) containing 5% nonfat dried milk. The blot was then incubated overnight with specific antibodies primary antibodies to actin, HSP-70, HSP-90 (Santa Cruz Biotechnology® Inc., Dallas, TX); PDH (BD Transduction Laboratories, San Diego, CA); phosphoserine (Abcam, Cambridge, MA); and pyruvate dehydrogenase kinase 1 (PDK-1; Cell Signaling Technology®, Danvers, MA) in PBS-5% bovine serum albumin (BSA). The blot was washed three times (10 min each) in Tris-buffered saline (TBS), 0.1% Tween® 20 before it was incubated for 60 min at room temperature with a 1,000× dilution of species-specific IgG peroxidase conjugate (Santa Cruz Biotechnology) in 1% gelatin in PBS. The blot was washed six times (5 min each) in TBS, 0.1% Tween 20 before detection of the peroxidase activity using the Enhanced Chemiluminescence Kit (Amersham Life Science Products, GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Immunoprecipitation

Tissue lysates containing 300 μg protein were incubated with the specific antibody (5 μL), chilled on ice for 1 h, mixed with protein A/G agarose beads (50 μL) (Santa Cruz Biotechnology) and incubated overnight on a nutator at 4°C. The immunoprecipitate was collected by centrifugation at 12,500*g* for 10 min and washed twice with 500 μL of stop buffer and once with 500 μl PBS wash buffer. The pellet was resuspended in 50 μL of electrophoresis sample buffer without 2-mercaptoethanol, boiled for 5 min and then centrifuged for 30 s to remove the agarose beads. The supernatant was incubated with 5% 2-mercaptoethanol at 37°C for 1 h. Sample (25 μL) were loaded onto 10% Tris-glycine polyacrylamide precast gels for Western blots.

Statistical Analysis

All data are presented as mean ± standard deviation (SD) and evaluated using the statistical package SPSS (version 15, Chicago, IL). All data were compared using one-way ANOVA with Fisher's LSD post hoc tests when appropriate. For all data, statistical significance was accepted at $P < 0.05$.

RESULTS

Ciprofloxacin Inhibits Combined Injury-Induced Mortality in Mice Exposed to 9.75 Gy Gamma Radiation

Ten days after combined injury (9.75 Gy), only 60% of the vehicle-treated mice survived (Fig. 1A and B). However, 100% survival (n = 10) was observed in the combined injury group administered ciprofloxacin. In addition, there were no deaths in the sham group treated with ciprofloxacin or vehicle. Since $LD_{50/30}$ for combined injury in B6D2F1/J mice is 8.95 Gy, we wanted to analyze combined injury-associated changes in ileum ATP and related proteins on day 10, which would correlate with continued mortality in the combined injury group (*5*).

Treatment with Ciprofloxacin Rescues Combined Injury-Associated Reductions in Tissue ATP Levels

ATP levels in various tissues were measured to determine whether combined injury affects ATP in mouse organs and the effect ciprofloxacin treatment has on these measures (Fig. 2A–G). Combined injury resulted in significantly lower ATP levels in ileum (−82%), pancreas (−93%), brain (−47%), spleen (−95%), kidney (−25%), lung (−51%) and liver (−99.6 %) compared to sham injury. Ciprofloxacin treatment after combined injury inhibited ATP loss in ileum and kidney. Most strikingly, ciprofloxacin treatment in the combined injury group completely prevented reductions in ileum ATP, resulting in fivefold higher ATP levels compared to those of the combined injury mice. Furthermore, the combined injury mice treated with ciprofloxacin showed significantly attenuated reductions in ATP levels in the pancreas (−41%) and spleen (−49%) compared to sham mice. Reductions in brain, lung and liver ATP levels were unaffected by ciprofloxacin treatment after combined injury. It is worth noted that the basal levels of cellular ATP among organs were in sequence from the highest to the lowest: liver > brain > ileum > pancreas > lung > kidney > spleen.

Ciprofloxacin Inhibits Reductions in Ileum HSP-70 and HSP-90 and Attenuates PDH Protein Decreases Associated with Combined Injury

Since the most significant effect of ciprofloxacin treatment after combined injury was on the ileum ATP, compared to the other six organs, we chose this organ to further examine the effects of ciprofloxacin on related proteins. Heat shock proteins 70 and 90 kDa, chaperone proteins involved in ATP synthesis (*23*), were greater in CI + CIP mice compared to CI + VEH mice (+130–181%; Fig. 3A and B). PDH, an enzyme crucial to ATP generation, was lower in CI + VEH mice (−76% vs. sham; Fig 3C). Reductions in PDH were mitigated with ciprofloxacin treatment after combined injury (+114%) compared to the combined injury treated with vehicle.

Daily Administration of Oral Ciprofloxacin after Combined Injury Results in the Formation of HSP-70-PDH Complex

To determine whether the formation of an HSP-70-PDH complex occurred in ileum tissue with ciprofloxacin treatment, immunoprecipitations of lysates with anti-HSP-70 were performed. Precipitates were immunoblotted with anti-PDH antibody and the PDH band was detected in all groups, except for the combined injury group, demonstrating that HSP-70 can form a complex with PDH (Fig. 4). Combined injury significantly reduced HSP-70-PDH complex formation (−69%) compared to sham-treated mice. Ciprofloxacin administration after combined injury inhibited this reduction in ileum tissue, resulting in significantly greater formation of the HSP-70-PDH complex formation (+141% vs. CI). To determine whether HSP-70 also formed complex with phosphorylated PDH, precipitates were immunoblotted with anti-phosphoserine antibody but no visible bands with 70 kDa and 60 kDa, respectively, were detected (Fig. 4A). To determine whether the HSP-70 forming complex with PDH was specific, the precipitate was immunoblotted with anti-PDK1 antibody and no visible band with 60kDa was found (Fig. 4A).

Ciprofloxacin Treatment after Combined Injury Inhibits Phosphorylation of PDH at Serine Residues and Reduces PDK in the Ileum

Phosphorylation of PDH by PDK inhibits ATP synthesis, whereas inhibition of PDK-1 protein by prevention of PDH phosphorylation upregulates synthesis of ATP molecules. To determine whether a PDH-phosphoserine residue formation occurred, ileum tissue lysates were immunoprecipitated with anti-PDH antibody and the precipitates were subsequently immunoblotted with anti-phosphoserine. Combined injury significantly increased ileum phosphoserine-PDH (+43% vs. sham), demonstrating increased phosphorylation of PDH at serine residues (Fig. 5A). Conversely, ciprofloxacin treatment after combined injury inhibited serine phosphorylation of PDH (−69% vs. CI).

Further evidence that combined injury prevents ileum tissue ATP generation was determined from immunoblots of PDK1. Combined injury resulted in a significant elevation in ileum PDK1 activation (+89% vs. sham), which was completely inhibited with ciprofloxacin treatment (Fig. 5B). Taken together, these data demonstrate that combined injury results in ileum phosphorylation of PDH at serine residues by the upregulation of PDK1 activity, which renders PDH inactive and unable to convert pyruvate to acetyl CoA, thus inhibiting ileum ATP molecule generation. Ciprofloxacin treatment prevents these actions, resulting in normal ileum ATP production.

DISCUSSION

We previously reported that hematopoietic death begins to occur approximately 10 days after exposure to ionizing

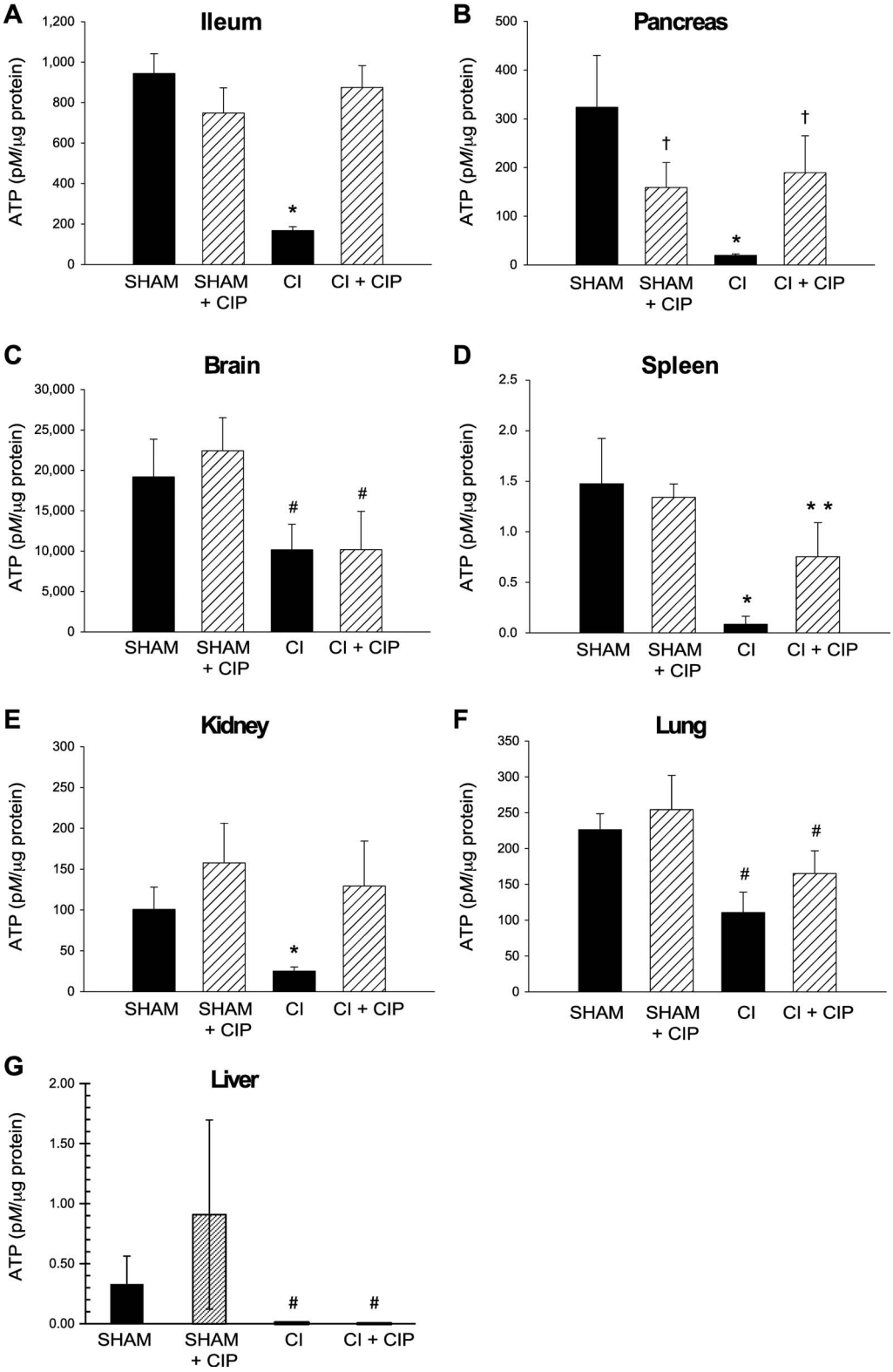

FIG. 2. Effects of daily ciprofloxacin (CIP) treatment on various ATP levels in the tissue 10 days after combined injury (CI) or sham injury (SHAM). Panel A: ileum; panel B: pancreas; panel C: brain; panel D: spleen; panel E: heart; panel F: lung and panel G: liver. $^{*}P < 0.05$ vs. SHAM, SHAM + CIP and CI + CIP. $^{**}P < 0.05$ vs. SHAM, SHAM + CIP, CI. $^{\#}P < 0.05$ vs. SHAM and SHAM + CIP. $^{\dagger}P < 0.05$ vs. SHAM and CI.

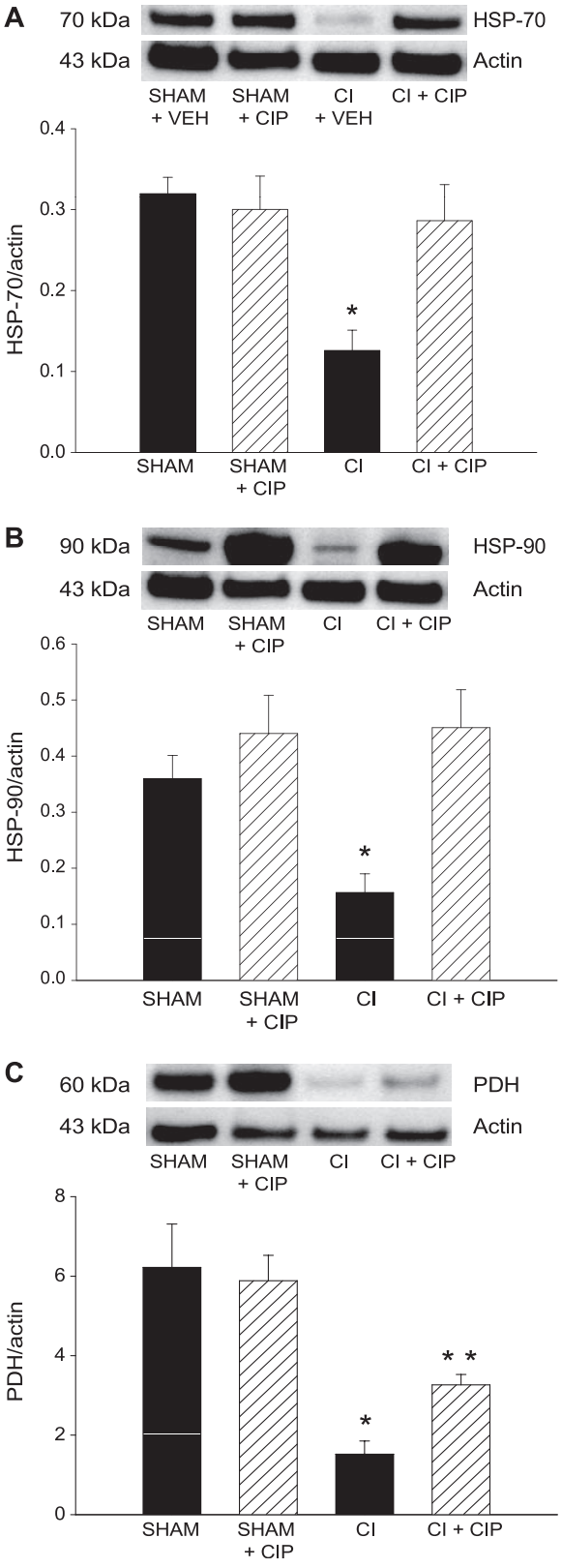

FIG. 3. Effects of daily ciprofloxacin (CIP) treatment on ileum protein expression of heat-shock protein 70 (HSP-70) (panel A), heat-shock protein 90 (HSP-90) (panel B) and pyruvate dehydrogenase (PDH) (panel C) after combined injury (CI) or sham injury (SHAM). $*P < 0.05$ vs. SHAM, SHAM + CIP and CI + CIP. $**P < 0.05$ vs. SHAM, SHAM + CIP and CI.

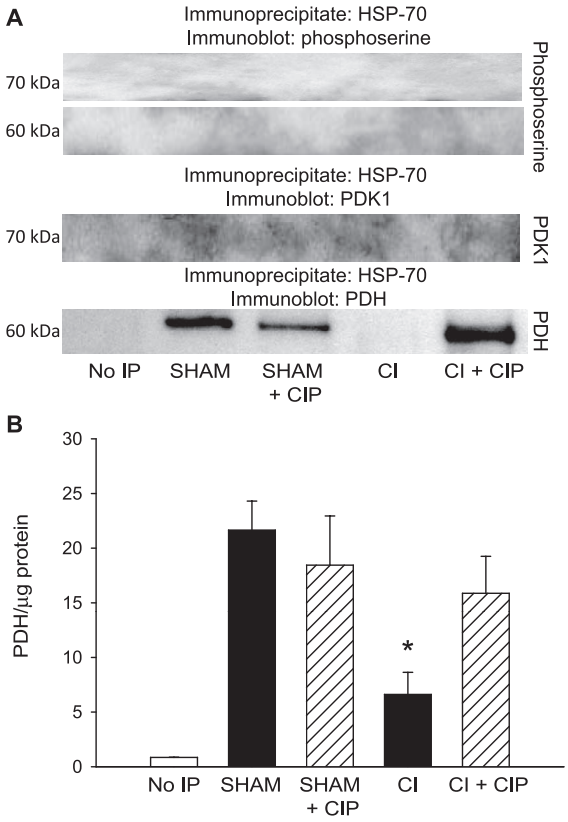

FIG. 4. Effects of daily ciprofloxacin treatment on formation of HSP-70-PDH complex in mouse ileum after combined injury (CI) or sham injury (SHAM). Panel A: Representative gels of HSP-70 phosphorylation, PDH phosphorylation and HSP-70 specificity in HSP-70 immunoprecipitates (IP). No observable HSP-70 phosphorylation, PDH phosphorylation and PDK1 were detected. Panel B: Quantitative bar graph corresponding to representative gels of HSP-70-PDH complex. $*P < 0.05$ vs. SHAM, SHAM + CIP and CI + CIP.

radiation. However, skin wound trauma after irradiation accelerates the onset of radiation-induced mortality by 3–8 days depending on the radiation dose, indicating the presence of the gastrointestinal syndrome (*5, 6*). The time course of reduction in survival is also correlated with body weight loss (*22*), suggesting this may be due to either: 1. reduction of food intake; 2. impairment of GI function; or 3. the combination of these two factors, leading to lack of energy to maintain the body weight. ATP is the main energy form for a variety of cellular processes, including DNA, RNA and protein synthesis, maintenance of the cytoskeleton, signaling, ion transport and repair. Herein, we reported that combined injury significantly reduced cellular ATP contents in ileum along with pancreas, brain, spleen, kidney and lung. Reductions in ATP are known to disintegrate the cell structure and function, leading to necrosis, apoptosis or autophagy (*27–32*). A normal ATP production is maintained by glycolysis and the TCA cycle taking place in mitochondria (*20*). PDH is the critical enzyme for converting pyruvate to acetyl CoA, whereas PDK is a critical enzyme for phosphorylating PDH, resulting in inactivation of PDH. As a result, the homeostasis between

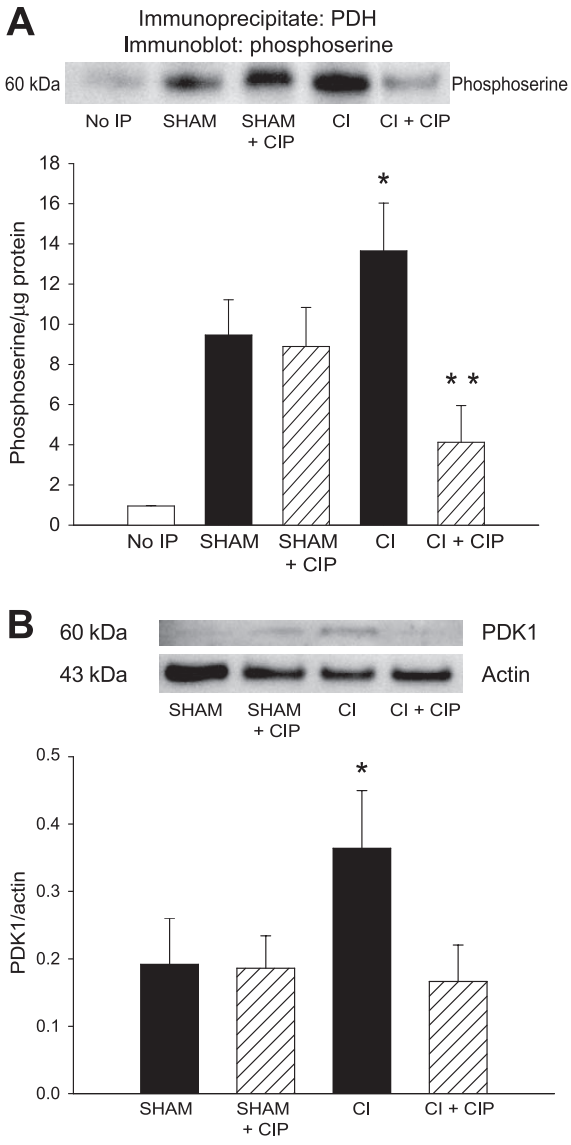

FIG. 5. Effects of daily ciprofloxacin treatment on formation of PDH-phosphoserine complex (panel A) and inhibition of pyruvate dehydrogenase kinase 1 (PDK1) (panel B) in mouse ileum after combined injury (CI + CIP) or sham injury (SHAM + CIP). *$P <$ 0.05 vs. SHAM, SHAM + CIP and CI + CIP. **$P < 0.05$ vs. SHAM, SHAM + CIP and CI.

the enzymatic activities of PDH and PDK remains critical to cell integrity for survival. It appears that combined injury disrupted the metabolic homeostasis, which may in turn have led to diminished ATP production, thus contributing to the combined injury-induced blood cell depletion (*22*).

Combined injury diminished the amount of PDH complex but increased PDK, a symptom similar to that found in PDH deficiency disease (*33*), which is a genetic disorder. The possibility of combined injury-induced DNA damage on the PDH gene cannot be ruled out, since combined injury has previously demonstrated the ability to increase DNA strand breaks (*16*). It is interesting to note combined injury-induced reduction in PDH is in agreement with combined injury-induced reduction in red blood cells (*23*).

Ciprofloxacin, an FDA-approved drug for clinical use, a widely prescribed antimicrobial agent and a known topoisomerase-II inhibitor, is very safe, even in high concentrations (*34*). In our previous work, we observed that after combined injury, treatment with ciprofloxacin significantly improved 30 day survival, attenuated DNA strand breaks and blood cell depletion (*23*), modulated cytokine profiles in serum and mitigated bone marrow damage and small intestinal injury in mice, in addition to demonstrating an ability to eliminate Gram-negative bacteria (*6, 24*). Moreover, ciprofloxacin treatment of human peripheral blood mononuclear cells provided protection from radiation by increasing Bcl-2 (anti-apoptotic) protein and decreasing p53 (apoptotic) protein (*16*), indicating that ciprofloxacin is capable of maintaining the homeostasis of programmed cell death, in addition to its known antimicrobial property. In this study, we found that ciprofloxacin treatment correlated with reduced PDK protein levels and increased PDH. The latter is consistent with the observation of ciprofloxacin mitigating the combined injury-induced red blood cell reduction (*23*). In addition, our data demonstrated that PDH conjugated with HSP-70, suggesting that HSP-70 may have played a role in the prevention of PDK phosphorylation of PDH. This observation was consistent with our previous work in which HSP-70 conjugates with iNOS in geldanamycin-treated hemorrhagic mice to prevent apoptosis (*35*) and with PDH in the same mice to prolong PDH activity (*28*). It was documented that cells overexpressing HSP-70 protein contained a high ATP level and PDH activity and vice versa (*28*), which further supports the theory that HSP-70 may regulate PDH activity and ATP production.

In addition to PDK phosphorylating PDH, PDK is known to increase γ-H2AX formation in response to DNA damage through Akt/GSK 3β activation. Inhibition of this protein led to decreases in γ-H2AX formation (*36*). This observation was in agreement with the *in vivo* experiments on peripheral blood cells of animals treated with ciprofloxacin (*23*) and *ex vivo* experiments of human peripheral blood cells (*16*). Determining activation of Akt signaling and other mechanisms in the small intestine of combined injury mice treated with ciprofloxacin are necessary to further determine whether this pathway is involved.

Ciprofloxacin treatment correlated with improved cellular ATP amounts in ileum, pancreas, spleen and kidney, but not brain, lungs and liver. It is possible that brain ATP levels were not affected by ciprofloxacin treatment after combined injury because of the inability of ciprofloxacin to penetrate the blood-brain barrier (*37*). Orally administered ciprofloxacin may have been degraded by the liver before it had a chance to recover ATP levels after combined injury. Although our data provide the crucial evidence as to the effects of ciprofloxacin on survival and the potential for ATP preservation to be one factor for survival, further metabolic analyses of relevant intracellular and molecular pathways are necessary for conclusive arguments to be

made. Furthermore, time-course investigations involving ciprofloxacin and combined injury are necessary to fully comprehend degradation of ciprofloxacin and alterations in ATP production in these tissues.

To capture the notion of ciprofloxacin correlations with changes in ATP production in ileum of combined injury mice, ciprofloxacin administration may increase complex formation of PDH and HSP-70. We speculated that this may make PDH unavailable for phosphorylation by PDK1, thus preserving ATP production. Furthermore, ciprofloxacin administration may decrease PDK1 to maintain the ATP production after combined injury. PDH is a highly regulated enzyme, and factors other than PDK1, such as levels of glucose and its metabolites regulating PDH levels (*20*), should not be excluded.

We have previously reported that ciprofloxacin treatment eliminates Gram-negative bacteria in the blood of combined injury mice, in which Gram-positive bacteria were still present (*6*), yet still significantly enhances survival after combined injury. Thus, the early death of combined injury mice prior to day 10 (Fig. 1) could be attributed to the presence of Gram-negative bacteria in blood. It is possible that the antimicrobial capability of ciprofloxacin is related to the preservation of ATP production and survival. After day 10, ciprofloxacin appears to promote erythropoiesis in spleen via increasing IL-3 concentrations (*24*), as confirmed by the observation of ciprofloxacin mitigating combined injury-induced reductions in red blood cells, hemoglobin and hematocrit, but not reductions in white blood cells (*23*). In addition, ciprofloxacin also effectively reduces cytokine/chemokine concentrations in blood and DNA strand breaks in bone marrow cells of combined injury mice (*23*).

Most strikingly, while ciprofloxacin therapy is specific to the survival improvement after combined injury, a broad-spectrum of antibiotics like levofloxacin or amoxicillin are ineffective in improving survival after combined injury.[2] Further definitive results involving topical gentamicin application on wounds along with oral levofloxacin administration, two broad-spectrum antibiotics, demonstrate the inability of this class of drugs to positively affect survival after combined injury in a manner as robust as ciprofloxacin (*38*). These data, in combination with our current and previously published results, support the hypothesis that the effect of ciprofloxacin treatment after combined injury is not simply protection from sepsis, and that it does involve a number of complementary mechanisms, all leading to improved health and survival (*6, 23, 24*).

In summary, ciprofloxacin significantly improved survival after combined injury and resulted in increases in cellular ATP levels in small intestine that might be partly correlated with increases in PDH protein and decreases in PDK protein and changes in HSP-70 regulatory activity. Although more research is necessary to definitively determine the effect that ciprofloxacin has on metabolic outcomes and the correlation of these to enhanced survival after combined injury, these data provide crucial evidence to the growing understanding of the complementary physiological mechanisms involved.

[2] Elliott TB, Bolduc DL, Ledney GD, Kiang JG, Fatanmi OO, Wise S, et al. Combined immunomodulator and antimicrobial therapy eliminates polymicrobial sepsis and modulates cytokine production in mice exposed to radiation and combined injury. Accepted for publication by International Journal of Radiation Biology (May 20, 2015).

ACKNOWLEDGMENTS

The authors gratefully acknowledge Neil G. Agravante, HM1, USN, David Bolduc, Ph.D., Risaku Fukumoto, Ph.D., True M. Burns, Thomas B. Elliott, Ph.D. and G. David Ledney, Ph.D. at AFRRI for their technical assistance and Donald A. McClain, MD/Ph.D. at the University of Utah School of Medicine for his insightful discussion. Furthermore, the authors thank the AFRRI Radiation Science Department staff for their dosimetry support and the AFRRI Veterinary Services Department veterinarians and staff for their support with animal care. This work was funded by NIH/NIAID grant no. R33-AI080553 (JGK). The views, opinions and findings contained herein are those of the authors and do not necessarily reflect official policy or positions of the Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, the National Institutes of Health or the United States Government.

Received: July 3, 2014; accepted: March 30, 2015; published online: May 26, 2015

REFERENCES

1. Ledney GD, Elliott TB. Combined injury: factors with potential to impact radiation dose assessments. Health Phys 2010; 98:145–52.
2. Wintz H. Forecare and aftercare in Roentgen ray therapy. Radiology 1923; 1:74–81.
3. Iijima S. Pathology of atomic bomb casualties. Acta Pathol Jpn 1982; 32 Suppl 2:237–70.
4. Kishi HS. Effects of the ''special bomb'': recollections of a neurosurgeon in Hiroshima, August 8–15, 1945. Neurosurgery 2000; 47:441–5; discussion 45–6.
5. Kiang JG, Jiao W, Cary LH, Mog SR, Elliott TB, Pellmar TC, et al. Wound trauma increases radiation-induced mortality by activation of iNOS pathway and elevation of cytokine concentrations and bacterial infection. Radiat Res 2010; 173:319–32.
6. Fukumoto R, Cary LH, Gorbunov NV, Lombardini ED, Elliott TB, Kiang JG. Ciprofloxacin modulates cytokine/chemokine profile in serum, improves bone marrow repopulation, and limits apoptosis and autophagy in ileum after whole body ionizing irradiation combined with skin-wound trauma. PloS One 2013; 8:e58389.
7. Gorbunov NV, Kiang JG. Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol 2009; 219:242–52.
8. Dalhoff A. Immunomodulatory activities of fluoroquinolones. Infection 2005; 33 Suppl 2:55–70.
9. Dalhoff A, Shalit I. Immunomodulatory effects of quinolones. Lancet Infect Dis 2003; 3:359–71.
10. Lahat G, Halperin D, Barazovsky E, Shalit I, Rabau M, Klausner J, et al. Immunomodulatory effects of ciprofloxacin in TNBS-induced colitis in mice. Inflamm Bowel Dis 2007; 13:557–65.
11. Thia KT, Mahadevan U, Feagan BG, Wong C, Cockeram A, Bitton A, et al. Ciprofloxacin or metronidazole for the treatment of perianal fistulas in patients with Crohn's disease: a randomized, double-blind, placebo-controlled pilot study. Inflamm Bowel Dis 2009; 15:17–24.
12. Breban M, Fournier C, Gougerot-Pocidalo MA, Muffat-Joly M,

Pocidalo JJ. Protective effects of ciprofloxacin against type II collagen induced arthritis in rats. J Rheumatol 1992; 19:216–22.

13. Lewis AJ, Keft AF. A review on the strategies for the development and application of new anti-arthritic agents. Immunopharmacol Immunotoxicol 1995; 17:607–63.
14. Freifeld A, Marchigiani D, Walsh T, Chanock S, Lewis L, Hiemenz J, et al. A double-blind comparison of empirical oral and intravenous antibiotic therapy for low-risk febrile patients with neutropenia during cancer chemotherapy. N Engl J Med 1999; 341:305–11.
15. Kloskowski T, Gurtowska N, Nowak M, Joachimiak R, Bajek A, Olkowska J, et al. The influence of ciprofloxacin on viability of A549, HepG2, A375.S2, B16 and C6 cell lines in vitro. Acta Pol Pharm 2011; 68:859–65.
16. Kiang JG, Garrison BR, Smith JT, Fukumoto R. Ciprofloxacin as a potential radio-sensitizer to tumor cells and a radio-protectant for normal cells: differential effects on gamma-H2AX formation, p53 phosphorylation, Bcl-2 production, and cell death. Mol Cell Biochem 2014; 393:133–43.
17. Kletter Y, Riklis I, Shalit I, Fabian I. Enhanced repopulation of murine hematopoietic organs in sublethally irradiated mice after treatment with ciprofloxacin. Blood 1991; 78:1685–91.
18. Shalit I, Kletter Y, Weiss K, Gruss T, Fabian I. Enhanced hematopoiesis in sublethally irradiated mice treated with various quinolones. Eur J Haematol 1997; 58:92–8.
19. Brook I, Elliott TB, Ledney GD. Quinolone therapy of Klebsiella pneumoniae sepsis following irradiation: comparison of pefloxacin, ciprofloxacin, and ofloxacin. Radiat Res 1990; 122:215–7.
20. Stryer L. Biochemistry. New York: W.H. Freeman; 1980.
21. Wang YJ, Liu W, Chen C, Yan LM, Song J, Guo KY, et al. Irradiation induced injury reduces energy metabolism in small intestine of Tibet minipigs. PloS one 2013; 8:e58970.
22. Kiang JG, Garrison BR, Burns TM, Zhai M, Dews IC, Ney PH, et al. Wound trauma alters ionizing radiation dose assessment. Cell Biosci 2012; 2:20.
23. Kiang JG, Fukumoto R. Ciprofloxacin increases survival after ionizing irradiation combined injury: gamma-H2AX formation, cytokine/chemokine, and red blood cells. Health Phys 2014; 106:720–6.
24. Fukumoto R, Burns TM, Kiang JG. Ciprofloxacin enhances stress erythropoiesis in spleen and increases survival after whole-body irradiation combined with skin-wound trauma. PloS One 2014; 9:e90448.
25. Brook I, Elliott TB. Quinolone therapy in the prevention of mortality after irradiation. Radiat Res 1991; 128:100–3.
26. Canton R, Morosini MI. Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol Rev 2011; 35:977–91.
27. Comelli M, Di Pancrazio F, Mavelli I. Apoptosis is induced by decline of mitochondrial ATP synthesis in erythroleukemia cells. Free Radic Biol Med 2003; 34:1190–9.
28. Kiang JG, Bowman PD, Lu X, Li Y, Ding XZ, Zhao B, et al. Geldanamycin prevents hemorrhage-induced ATP loss by overexpressing inducible HSP70 and activating pyruvate dehydrogenase. Am J Physiol Gastrointest Liver Physiol 2006; 291:G117–27.
29. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997; 185:1481–6.
30. Lemasters JJ. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am J Physiol 1999; 276:G1–6.
31. Lieberthal W, Menza SA, Levine JS. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. Am J Physiol 1998; 274:F315–27.
32. Wiegele G, Brandis M, Zimmerhackl LB. Apoptosis and necrosis during ischaemia in renal tubular cells (LLC-PK1 and MDCK). Nephrol Dial Transplant 1998; 13:1158–67.
33. Ferriero R, Manco G, Lamantea E, Nusco E, Ferrante MI, Sordino P, et al. Phenylbutyrate therapy for pyruvate dehydrogenase complex deficiency and lactic acidosis. Sci Transl Med 2013; 5:175ra31.
34. Sorgel F, Naber KG, Kinzig M, Mahr G, Muth P. Comparative pharmacokinetics of ciprofloxacin and temafloxacin in humans: a review. Am J Med 1991; 91:51S–66S.
35. Kiang JG, Bowman PD, Wu BW, Hampton N, Kiang AG, Zhao B, et al. Geldanamycin treatment inhibits hemorrhage-induced increases in KLF6 and iNOS expression in unresuscitated mouse organs: role of inducible HSP70. J Appl Physiol (1985) 2004; 97:564–9.
36. An J, Huang YC, Xu QZ, Zhou LJ, Shang ZF, Huang B, et al. DNA-PKcs plays a dominant role in the regulation of H2AX phosphorylation in response to DNA damage and cell cycle progression. BMC Mol Biol 2010; 11:18.
37. Liu L, Venkatraman SS, Yang YY, Guo K, Lu J, He B, et al. Polymeric micelles anchored with TAT for delivery of antibiotics across the blood-brain barrier. Biopolymers 2008; 90:617–23.
38. Kiang JG, Zhai M, Liao PJ, Bolduc DL, Elliott TB, Gorbunov NV. Pegylated G-CSF inhibits blood cell depletion, increases platelets, blocks splenomegaly, and improves survival after whole-body ionizing irradiation but not after irradiation combined with burn. Oxid Med Cell Longev 2014; 2014:481392.