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Epidermal growth factor improves intestinal integrity and survival in murine sepsis following chronic alcohol ingestion

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Abstract

Epidermal growth factor (EGF) is a cytoprotective protein that improves survival in preclinical models of sepsis through its beneficial effects on intestinal integrity. Alcohol use disorder worsens intestinal integrity and is associated with increased morbidity and mortality in critical illness. We sought to determine whether chronic alcohol ingestion alters the host response to systemic administration of EGF in sepsis. Six week old FVB/N mice were randomized to receive 20% alcohol or water for 12 weeks. All mice then underwent cecal ligation and puncture (CLP) to induce polymicrobial sepsis. Mice were then randomized to receive either intraperitoneal injection of EGF (150 µg/kg/day) or normal saline. Water-fed mice given EGF mice had decreased seven-day mortality compared to water-fed mice (18% vs. 55%). Alcohol-fed mice given EGF also had decreased seven day mortality compared to alcohol-fed mice (48% vs. 79%). Notably, while systemic EGF improved absolute survival to a similar degree in both water-fed and alcohol-fed mice, mortality was significantly higher in alcohol+EGF mice compared to water+EGF mice. Compared to water-fed septic mice, alcohol-fed septic mice had worsened intestinal integrity with intestinal hyperpermeability, increased intestinal epithelial apoptosis, decreased proliferation and shorter villus length. Systemic administration of EGF to septic alcohol-fed mice decreased intestinal permeability compared to septic alcohol-fed mice given vehicle, with increased levels of the tight junction mediators claudin-5 and JAM-A. Systemic administration of EGF to septic alcohol-fed mice also decreased intestinal apoptosis with an improvement in the Bax/Bcl-2 ratio. EGF also improved both crypt proliferation and villus length in septic alcohol-fed mice. EGF

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administration resulted in lower levels of both pro- and anti-inflammatory cytokines MCP-1, TNF and IL-10 in alcohol-fed mice. EGF is therefore effective at improving both intestinal integrity and mortality following sepsis in mice with chronic alcohol ingestion. However, the efficacy of EGF in sepsis is blunted in the setting of chronic alcohol ingestion, as intestinal integrity and mortality in alcohol-fed mice given EGF improves animals to levels seen in water-fed mice given vehicle but does not approach levels seen in water-fed mice given EGF.

Keywords

Sepsis; alcohol; EGF; cecal ligation and puncture; apoptosis; permeability; proliferation; cytokine

INTRODUCTION

Between 230,000 and 370,000 patients die of sepsis annually each year in the United States (1). The incidence of sepsis is rising (2), and despite intellectual advances in both defining and managing sepsis (3–5), mortality and long-term disability from this devastating syndrome remain unacceptably high. In addition, despite significant improvements in the understanding of the molecular underpinnings of sepsis (6), advances at the bench have not translated into advances at the bedside with well over 100 negative clinical trials and no approved therapies for sepsis beyond rapid administration of antibiotics and fluid resuscitation.

Although sepsis is commonly referred to as a single entity, both host and pathogen factors can significantly impact outcome. Chronic co-morbidities are associated with a significantly worse outcome from sepsis (7). Notably, not all co-morbidities are of equal importance as some play a disproportionate role (8;9). A commonly seen co-morbidity in hospitalized patients is alcohol use disorder. Alcohol use disorder affects nearly 33 million people in the United States (10), and deaths from alcohol recently reached a 35-year high (11). Notably, an estimated 20–40% of patients admitted to the hospital has alcohol use disorder, and up to one third of patients admitted to the intensive care unit (ICU) carry this diagnosis (12;13). Importantly, patients with alcohol use disorder have more frequent admissions to the ICU, longer ICU stays, and an increased risk of death (14).

Epidermal growth factor (EGF) is a cytoprotective, trophic peptide that has been shown to improve survival following sepsis and other forms of critical illness (15–18). EGF acts via its receptor EGF-R on multiple cell types. However, the survival advantage conferred by EGF in sepsis is mediated, at least in part, through the intestinal epithelium (16). The gut is frequently referred to as “the motor” of the systemic inflammatory response syndrome, and sepsis diminishes intestinal epithelial integrity, with increases in permeability and apoptosis, combined with decreases in proliferation and villus length (19–21). In addition to improving intestinal integrity, overexpression of EGF solely in villus enterocytes of transgenic mice improves survival following sepsis, demonstrating that the intestine is sufficient and potentially necessary to mediate the beneficial effects of EGF in sepsis (16).

While there are multiple reasons why preclinical trials of sepsis have failed to translate into therapeutic gain at the bedside, one potential reason is that research using animal models is

performed in young mice without comorbidities, which is not reflective of the patient population that develops sepsis. Notably, multiple agents that are effective in preclinical trials in young mice without comorbidities lose their effectiveness or become harmful in the setting of aging or pre-existing comorbidities (22). To determine if alcohol use disorder impacts the efficacy of EGF in sepsis, we randomized mice to drink water or to a model of chronic alcohol ingestion that is associated with increased mortality and intestinal injury following sepsis (23). All mice were then made septic and mice were again randomized to receive either EGF or vehicle. This study design allowed us to determine a) the impact of chronic alcohol ingestion in sepsis and b) whether EGF has the same benefits in the setting of chronic alcohol ingestion as it does in animals without pre-existing comorbidities.

MATERIALS AND METHODS

Animals

Six week old male FVB/N mice were purchased from a commercial vendor (Charles River Laboratories, Wilmington, MA). After a one week acclimatization period, animals were randomized to receive either alcohol or water (details below) and were then watched for an additional twelve weeks before being subjected to cecal ligation and puncture (CLP, also detailed below). Following CLP, mice were followed for 1–7 days depending on whether they were used for non-survival or survival experiments. Thus, animals were 19–20 weeks old at time of sacrifice. Experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine (Protocol DAR-2002473-090316BN). All animals were housed in an approved university animal facility and were given free access to food and either water or alcohol throughout. Prior to CLP, all animals were given a single dose of buprenex (0.1mg/kg, McKesson Medical, San Francisco, CA) in an attempt to minimize animal suffering. Buprenex was re-dosed post-operatively as deemed appropriate by the staff of the Division of Animal Resources at Emory University. Animals were sacrificed 24 hours following CLP for non-survival studies by exsanguination under deep isoflurane anesthesia. A different cohort of mice were followed for 7 days post-CLP for survival. All animals were checked twice daily to determine if they were moribund, and if they were, they were sacrificed using humane endpoints. Moribund animals were identified as follows: a) surgical complications unresponsive to immediate intervention (wound dehiscence, bleeding), b) medical conditions unresponsive to treatment including self-mutilation, severe respiratory distress, icterus, or intractable diarrhea, or c) clinical or behavioral signs unresponsive to appropriate intervention persisting for 1 day including significant inactivity, labored breathing, sunken eyes, hunched posture, piloerection/matted fur, one or more unresolving skin ulcers, and abnormal vocalization when handled. Animals in mortality studies that survived 7 days following CLP were sacrificed using asphyxiation by CO₂.

Chronic alcohol ingestion model

Mice were randomized to receive either alcohol or water in a 1:1 ratio. Mice in the alcohol group were acclimated to alcohol by increasing the concentration from 0% to 20% (volume/volume) over a course of two weeks (5% v/v for 4 days, 10% v/v for 4 days, 15% v/v for 4

days) and then received 20% alcohol concentration for an additional ten weeks for a total of 12 weeks (31). At the conclusion of 12 weeks of either drinking alcohol or water, mice had similar body weights and renal function (data not shown), gut integrity and liver histology (23). Following CLP, all mice received water regardless of whether they had been randomized to the alcohol or water groups for the previous 12 weeks. The rationale behind this is to mimic the clinical scenario in which all septic patients are given hydration in the ICU but are not given alcohol in the hospital even if they have a chronic history of alcohol use.

Sepsis model

CLP was performed according to the method of Baker et al. (32). Under isoflurane anesthesia, a small midline abdominal incision was made. The cecum was ligated just distal to the ileocecal valve by a technique that did not result in intestinal obstruction, punctured twice with a 25 gauge needle and then gently squeezed to extrude a small amount of stool. The cecum was then replaced into the abdomen, which was closed in layers. To mimic the clinical scenario where septic patients receive fluid resuscitation and antibiotics, all animals received these treatments following CLP. Specifically, all mice received a subcutaneous injection of 1 ml of 0.9% saline post-operatively to account for insensible fluid losses that occurred during surgery. Animals that were sacrificed 24 hours after CLP received two doses of ceftriaxone 50mg/kg (Sigma-Aldrich, St. Louis, MO) and metronidazole 30 mg/kg (Apotex Corp, Weston, FL) given subcutaneously at 3 and 15 hours post-operatively. Animals followed for survival received antibiotics every 12 hours for a total of 48 hours.

Systemic EGF administration

Both water-fed and alcohol-fed animals were then randomized to receive either EGF or saline vehicle. EGF (Harlan Bioproducts, Indianapolis, IN) was given in a post-treatment fashion following CLP via intraperitoneal injections of 75 µg/kg administered twice a day (total dose of 150 µg/kg/day). EGF injections were continued twice a day until animals were sacrificed. This dosage and timing was chosen based on published work giving EGF following sepsis (15). This resulted in four groups hereafter referred to as: a) water (mice that drank water prior to sepsis and received vehicle after CLP), b) alcohol (mice that drank alcohol for 12 weeks prior to sepsis and received vehicle after CLP), c) water+EGF (mice that drank water prior to sepsis and received EGF after CLP), and d) alcohol+EGF (mice that drank alcohol prior to sepsis and received EGF after CLP).

Intestinal permeability

Mice were gavaged with 0.5 ml of fluorescein isothiocyanate conjugated-dextran (FD-4, 22mg/ml, molecular mass 4.4 kD, Sigma-Aldrich) five hours prior to sacrifice (33–35). At time of sacrifice, blood was collected and centrifuged at 10,000 rpm at 4°C for 10 min. Plasma (50 µl) was then diluted with an equal amount of sterile phosphate-buffered saline (pH 7.4), and the concentration of FD-4 was determined using fluorospectrometry (Synergy HT, BioTek, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm with serially diluted samples as standards. All samples and standards were run in duplicate.

Intestinal protein expression analysis

At the time of sacrifice, segments of jejunum were snap-frozen in liquid nitrogen and stored at -80°C . These were subsequently weighed and added to 5x volume-to-weight ice-cold lysis buffer (50mM Tris HCl; 10mM EDTA; 100mM NaCl; 0.5% Triton X-100) containing the commercially available protease inhibitor mix Complete Mini, EDTA-free (Roche, Indianapolis, IN) for homogenization. After 30 minutes on ice, homogenates were centrifuged at $10,600 \times g$ for 10 minutes at 4°C . Sample total protein concentration was determined on the supernatant using the Pierce 660nm protein assay (Thermo Scientific, Rockford, IL). For protein expression analysis, 40 μg of protein was added to an equal volume of Laemlli buffer and heated for 5 minutes at 95°C . Protein was separated by the SDS-PAGE method on 4–20% gradient stain-free gels (BioRad, Hercules, CA) at 120V for ~50 minutes. The stain-free gel and proteins were activated for 5 minutes and the protein was transferred to polyvinylidene difluoride membranes using a semi-wet method via Transblot Turbo (BioRad) at 25V for 10 minutes. Membranes were blocked in 5% non-fat milk in TBS with 0.1% Tween-20 for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with rabbit anti-claudin 1 (1 $\mu\text{g}/\text{mL}$, Invitrogen, Camarillo, CA), rabbit anti-claudin 2 (1:1000, Abcam, Cambridge, MA), rabbit anti-claudin 3 (1 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-claudin 4 (1 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-claudin 5 (1 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-claudin 8 (1 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-occludin (3 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-JAM-A (1 $\mu\text{g}/\text{mL}$, Invitrogen), rabbit anti-Bax (1:1000, Cell Signaling Technologies, Danvers, MA) or rabbit anti-Bcl-2 (1:1000, Cell Signaling Technologies). After membranes were washed, they were incubated at room temperature for 1 hour in anti-rabbit secondary antibody linked to horseradish peroxidase (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with a chemiluminescent system (GE Healthcare, Buckinghamshire, UK) and visualized with a charged coupled device (ChemiDoc Touch, BioRad). Resulting bands were analyzed using intensity quantification software (ImageLab 5.2, BioRad). Linear dynamic detection range with stain-free technology was used for lane protein normalization and comparisons (24). Data is presented as relative protein expression compared to water mice.

Intestinal epithelial apoptosis

Apoptotic cells in the crypts were quantified by two complementary methods: morphologic analysis via H&E-staining and functional analysis via active caspase-3 staining (25). For both methods, apoptotic cells were quantified in 100 contiguous well-oriented crypt-villus units per animal by an examiner blinded to sample identity. Apoptotic cells were identified on H&E-stained jejunal sections by characteristic nuclear condensation and fragmentation. For active caspase-3 staining, jejunal sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 minutes. Slides were then immersed in Antigen Decloaker (Biocare Medical, Concord, CA) and heated in a pressure cooker to facilitate antigen retrieval for 45 minutes. Following this, sections were blocked with 20% goat serum (Vector Laboratories, Burlingame, CA), and incubated with rabbit polyclonal anti-active caspase-3 (1:100; Cell Signaling Technology) overnight at 4°C . Sections were then incubated with goat anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 30 minutes at room temperature, followed by Vectastain Elite ABC reagent (Vector

Laboratories) for an additional 30 minutes and developed with diaminobenzidine followed by hematoxylin counterstaining.

Intestinal proliferation

Ninety minutes prior to sacrifice, mice received an intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU, 5 mg/mL diluted in 0.9% saline; Sigma) to label crypt cells in S-phase. Intestinal sections were subsequently deparaffinized, rehydrated, incubated in hydrogen peroxide, immersed in Antigen Decloaker and heated in a pressure cooker. Sections were then blocked for 30 min with Protein Block (Dako, Carpinteria, CA) and incubated with rat monoclonal anti-BrdU (1:500; Accurate Chemical & Scientific, Westbury, NY) overnight at 4° C. After being incubated at room temperature with goat anti-rat secondary antibody (1:500; Accurate Chemical & Scientific) for 30 min, sections were incubated with streptavidin-horseradish peroxidase (1:500; Dako) for 30 min and developed with diaminobenzidine, followed by counterstaining with hematoxylin. Jejunal S-phase crypt cells were quantified in 100 contiguous well-oriented crypt-villus units by an examiner blinded to sample identity.

Intestinal villus length

Villus length was measured on H&E-stained sections as the distance in μm from the crypt neck to the villus tip in 12 well-oriented jejunal villi per animal using Image J software (National Institutes of Health, Bethesda, MD) by an examiner blinded to sample identity.

Serum cytokines

Blood taken at the time of sacrifice was centrifuged at $10,600 \times g$ for 10 minutes. Cytokines concentrations were determined on the resultant serum using a multi-plex cytokine assay kit (Bio-Rad) according to manufacturer protocol. All samples were run in duplicate.

Lung wet to dry weight and histopathology

The left lung from each mouse was harvested and immediately weighed to determine a "wet" weight. After drying in an oven at 115 °C for 24 hours, the tissue was re-weighed to establish a dry weight, and a ratio of weight to dry weight was calculated. A separate cohort of animals had lung tissue sectioned and stained using H&E. Histopathologic assessment of lung injury was performed by an examiner blinded to sample identity.

Statistics

Data were analyzed using the statistical software program Prism 6.0 (GraphPad, San Diego, CA) and are presented as mean $\pm$ SEM. Data sets were evaluated for a Gaussian distribution using the D'Agostino-Pearson omnibus normality test. Multiple group comparisons were done with one-way analysis of variance followed by the Tukey post-test for data that was normally distributed. If data sets were not normally distributed, comparisons were done with the Kruskal-Wallis non-parametric one-way analysis of variance followed by the Dunn's post-test. Survival was analyzed via the Log-rank test. A p-value of <0.05 was considered to be statistically significant in all studies.

RESULTS

Effect of EGF on mortality from sepsis with and without chronic alcohol ingestion

Initial comparisons were made between animals with chronic alcohol ingestion for 12 weeks prior to the onset of sepsis and mice that drank water during the same time period prior to the onset of sepsis. Mice were then randomized to receive EGF or vehicle following CLP. Water+EGF mice had a lower seven-day mortality following CLP than water mice (18% vs 55%, Fig. 1). Alcohol+EGF mice had a lower seven-day mortality following CLP than alcohol mice (48% vs 79%). Notably while EGF improved mortality in alcohol mice to a level similar to water mice, mortality was significantly higher in alcohol+EGF mice compared to water+EGF mice.

Effect of alcohol and EGF on intestinal permeability and tight junction protein expression following sepsis

Subsequent mechanistic experiments were performed only on water, alcohol, and alcohol +EGF mice. The rationale behind this experimental design was that this allowed an assessment of the impact of alcohol in sepsis (water vs. alcohol) and the impact of EGF in sepsis superimposed on chronic alcohol ingestion (alcohol vs. alcohol+EGF). We did not include water+EGF mice in subsequent experiments in an effort to minimize the number of animals used since we have already published extensively comparing septic water vs. water +EGF mice (15–17).

Alcohol mice had higher serum levels of FD-4 five hours after oral gavage compared to water mice (Fig. 2A). Serum levels of FD4 were decreased in alcohol+EGF mice compared to alcohol mice, bringing levels of intestinal permeability to similar levels seen in water mice. To determine potential mechanisms through which EGF improved intestinal permeability, western blots were performed on intestinal tight junction proteins. Claudin 5 expression decreased significantly in alcohol mice compared to water mice (Fig. 2B). However, claudin 5 levels increased in alcohol+EGF mice compared to alcohol mice, similar to levels seen in water mice. Alcohol had no impact on JAM-A expression, as levels were similar in water and alcohol mice (Fig. 2C). However, EGF administration led to increased levels of JAM-A, as protein levels were higher in alcohol+EGF mice than alcohol mice. Expression of claudin 1, 2, 3, 4, 8, and occludin were comparable in water, alcohol and alcohol+EGF mice (data not shown).

Effect of alcohol and EGF on intestinal epithelial apoptosis following sepsis

Intestinal epithelial apoptosis was higher in alcohol mice than water mice whether assayed by H&E (Fig. 3A, B) or active caspase 3 (Fig. 3C, D). In contrast, EGF administration decreased gut apoptosis, as alcohol+EGF mice had lower levels of intestinal cell death compared to alcohol mice. To understand potential mechanisms responsible for alterations in intestinal apoptosis with both alcohol and EGF, western blot analysis of jejunal protein expression was performed for the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2. Bax levels were unaffected by either alcohol or EGF as levels were similar in water, alcohol and alcohol+EGF mice (Fig. 4A). In contrast, Bcl-2 expression was decreased 2.5-fold in alcohol mice compared to water mice (Fig. 4B) while EGF administration increased

Bcl-2 levels in alcohol+EGF mice. The ratio of Bax/Bcl-2 is often used as a rheostat for cellular survival (26). The Bax/Bcl-2 ratio was increased in alcohol mice compared to water mice (Fig. 4C) while EGF administration decreased this ratio in alcohol+EGF mice.

Effect of alcohol and EGF on villus length and crypt proliferation following sepsis

Villus length was decreased in alcohol mice compared to water mice (Fig. 5). EGF administration increased villus length in alcohol+EGF mice. Crypt proliferation was similarly decreased in alcohol mice compared to water mice (Fig. 6). EGF administration increased levels of crypt proliferation, as alcohol+EGF mice had higher levels of S-phase cells than alcohol mice; however, EGF was unable to restore crypt proliferation in alcohol+EGF levels to levels seen in water mice.

Effect of alcohol and EGF on circulating cytokines following sepsis

Both the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine MCP-1 were markedly increased in alcohol mice compared to water mice (Fig. 7A, B, Table 1). EGF administration decreased levels of both of these cytokines in alcohol+EGF mice. The pro-inflammatory cytokine TNF was not affected by alcohol (Fig. 7C, Table 1). However, administration of EGF lowered TNF as cytokine levels were lower in alcohol+EGF mice than alcohol mice. Neither alcohol nor EGF impacted levels of IL-1 β , IL-2, IL-6 or IFN- γ (Table 1).

DISCUSSION

EGF improved survival when administered following CLP to mice with chronic alcohol ingestion prior to the onset of sepsis. This survival benefit was associated with an improvement in multiple facets of intestinal integrity including permeability, apoptosis, villus length and proliferation. Further, EGF decreased some (but not all) pro- and anti-inflammatory cytokines without impacting lung injury in septic mice following chronic alcohol ingestion. The survival benefit conferred by EGF in sepsis was similar in both alcohol and water mice in terms of absolute percentage (31% and 37% respectively). However, mortality was significantly higher in alcohol+EGF mice compared to water+EGF mice demonstrating that the efficacy of EGF in sepsis is blunted in the setting of this pre-existing comorbidity, since it cannot fully overcome the detrimental effects of chronic alcohol ingestion.

The results presented herein examine two complementary comparisons – the impact of EGF in the setting of chronic alcohol ingestion prior to sepsis (alcohol vs. alcohol+EGF) and the impact of alcohol alone prior to sepsis (water vs. alcohol). While it is not possible for ICU providers to alter a patient's pre-existing alcohol use disorder, the experimental design helps to clarify what impact this co-morbidity could potentially have in critical illness. In order to put the results into context, it is important to emphasize first that the control group in all experiments (water mice) already had significant abnormalities in every variable examined compared to unmanipulated hosts. Water mice were animals that had free access to drinking water both before and after CLP, and as such, are representative of 18 week old inbred septic mice. Multiple previous studies have shown that sepsis induces abnormalities in multiple

facets of intestinal integrity (permeability, apoptosis, proliferation, villus length) and cytokine levels (19;21), although the presence of lung injury in CLP is more controversial (27).

EGF administration following CLP in mice with chronic alcohol ingestion markedly improved intestinal integrity. This is consistent with prior studies in mice that drank water prior to sepsis that subsequently received systemic EGF (15). Tight junction proteins are known to be affected by a) sepsis, b) alcohol, and c) EGF. CLP, in isolation, increases intestinal permeability. This is associated with increases in jejunal claudin 2 and JAM-A as well as decreases in claudin 5 and occludin in FVB/N mice (the same strain used in this study) (28). In contrast, localization (but not levels) of claudins 1, 3, 4, 5 and 8 are all altered in colonic tight junctions following CLP in C57Bl/6 mice (29). Next, ZO-1 and occludin are decreased in septic alcohol mice compared to septic water mice (23). Additionally, intestine-specific overexpression of EGF normalizes sepsis-induced increases in claudin 2 (16). In this study, EGF decreased intestinal permeability in alcohol mice and this was associated with an increase in the tight junction proteins claudin 5 and JAM-A. Previous studies have shown that JAM-A deficient mice have an increase in intestinal barrier dysfunction. Thus, the increase in intestinal JAM-A expression in alcohol+EGF mice may confer a protective effect and improve intestinal barrier function. Of note, the mechanisms through which EGF improves sepsis-induced hyperpermeability are different in alcohol mice compared to water mice given the same agent at the same dose. Together, these findings suggest that control of intestinal permeability via tight junction alterations is highly complex and differentially regulated in sepsis, alcohol and via EGF since even the same drug (EGF) can have differing effects on the tight junction depending on baseline comorbidities.

EGF also decreased sepsis-induced intestinal epithelial apoptosis in alcohol mice. This was associated with an increase in Bcl-2 expression without a change in Bax expression, leading to a decrease in the Bax/Bcl-2 ratio. The decrease in sepsis-induced intestinal apoptosis is consistent with previous studies giving EGF – either systemically or in an intestine-specific manner – to water mice subjected to either CLP or *Pseudomonas aeruginosa* pneumonia (15–17). EGF signaling has previously been shown to prevent apoptosis by altering the balance of Bcl-2 family members in the mitochondrial pathway of cell death. However, EGF has also been shown to alter extrinsic death receptor pathways when given to water-fed septic mice, suggesting that the control of apoptosis is also regulated via a complicated collection of signaling outputs (15).

EGF-R is expressed on multiple cell types, and we therefore made a pre-hoc determination to study whether there were effects of EGF outside of the intestine. EGF decreased systemic levels of IL-10, MCP-1 and TNF in alcohol mice. Of note, transactivation of EGF-R protects intestinal epithelial cells from TNF-induced apoptosis, suggesting a potential mechanistic link between systemic cytokines and gut apoptosis (30;31). Both alcohol and sepsis independently increase serum cytokines, although both alcoholic and non-alcoholic septic patients have similar inflammatory responses (32). The impact of EGF found herein contrasts with water-fed mice subjected to *Pseudomonas aeruginosa* pneumonia-induced sepsis, where EGF did not impact serum cytokines (17). This also contrasts with water-fed mice with intestinal overexpression of EGF subjected to CLP, which similarly did not induce

any change in systemic cytokines. However, our results are consistent with findings that EGF decreases hepatic levels of TNF and IL-10 in unmanipulated animals following chronic alcohol ingestion (33).

We also examined potential extra-intestinal effects of EGF in lung tissue and did not see any differences in pulmonary edema or injury with either alcohol or following EGF administration (data not shown). The rationale for examining lungs was that EGF improves endotoxin-induced lung injury in rabbits (34). Of note, the degree of lung injury induced by sepsis in mice is controversial, with a recent study demonstrating that CLP does not cause lung damage (27). Additionally, although alcohol increases susceptibility of the lungs to secondary insults such as ARDS (35), alcohol does not impact lung wet to dry ratio or pulmonary histology when superimposed on sepsis, although it does increase pulmonary MPO levels and BAL cytokines (23).

This study has a number of limitations. The decision to initiate EGF immediately following the onset of sepsis models the ideal clinical scenario where sepsis is identified and very rapidly treated; however, these conditions are almost impossible to replicate in patients where delays in presentation and delays in management are common. Initiating EGF at a later timepoint might have offered additional insights. Additionally, animals were only sacrificed at a single timepoint (24 hours). It is likely that a timecourse where samples were taken at multiple different timepoints following the onset of sepsis would have yielded additional mechanistic insights. Next, only male animals were used. While this experimental design was common when the experiments were performed (2012–2015), the NIH has subsequently released policies related to rigor and reproducibility which emphasize that both genders should be used, and the use of a single gender limits the generalizability of our results (36). In addition, outside of survival experiments, we chose not to include a water +EGF group. This decision was made to minimize the number of animals used in experiments since we have already published extensively on water+EGF in sepsis. To verify that published results are reproducible, it would have been reasonable to repeat these experiments here although this point requires balancing a desire to reduce animal use with reproducibility. It should be noted that the survival curve comparing water to water+EGF in sepsis (Figure 1) reproduces published work comparing these two groups (15) and the decision to not include water+EGF mice in mechanistic studies was taken only when we demonstrated that at least one major element of the response to EGF in sepsis was reproducible. Finally, while intestine-specific effects have been demonstrated to be sufficient for EGF to confer a survival advantage in mice that drink water prior to CLP, it is likely that systemic EGF had additional extra-intestinal effects not examined in this study in mice with chronic alcohol ingestion.

Despite these limitations, this study demonstrates that systemic administration of EGF improves survival in septic mice with a history of chronic alcohol ingestion. However, EGF is unable to improve survival to the same level as when EGF is given to septic mice without a history of drinking alcohol, implying that both EGF and chronic alcohol ingestion play a role in mediating mortality. This suggests that EGF may represent a potential novel therapeutic agent in the treatment of sepsis, but comorbid conditions may play a role in impacting both its efficacy as well as its mechanism of action.

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FIG. 1. Survival in septic water, water+EGF, alcohol, alcohol+EGF mice

EGF improved survival in mice that drank water prior to CLP ($n=11-17$, $p=0.03$). EGF also improved survival in animals with chronic alcohol ingestion prior to CLP ($n=24-27$, $p=0.04$). However, survival in mice given EGF following chronic alcohol ingestion did not reach levels seen in mice given EGF that drank water prior to CLP. Survival was similar between water mice (no EGF) and alcohol+EGF mice ($p=0.77$).

FIG. 2. Intestinal permeability and tight junction expression in septic water, alcohol, and alcohol +EGF mice

Intestinal permeability was higher in alcohol mice than water mice (A, $p=0.01$). EGF decreased permeability as FD-4 concentration in the bloodstream was lower in alcohol+EGF mice compared to alcohol mice ($n=7-10$, $p=0.01$). Protein levels of claudin-5 were lower in alcohol mice than water mice (B, $p=0.04$). EGF increased levels of claudin-5 in alcohol mice ($n=10-11$, $p=0.03$ alcohol vs. alcohol+EGF). Representative blot showing relative expression of claudin 5 is shown. JAM-A levels were similar between water and alcohol mice (C, $p>0.99$). However, EGF increased levels of JAM-A in alcohol mice ($n=8-9$, $p=0.01$, alcohol vs. alcohol+EGF). Representative blot showing relative expression of JAM-A is shown.

FIG. 3. Intestinal epithelial apoptosis in septic water, alcohol, and alcohol+EGF mice

Apoptosis was higher in alcohol mice than water mice, when measured by both H&E (A, $p=0.02$) and active caspase 3 (C, $p=0.002$). EGF decreased intestinal apoptosis as numbers of apoptotic cells/100 crypts were lower in alcohol+EGF mice compared to alcohol mice $n=7-8$ H&E, $p=0.04$, $n=6$ active caspase-3, $p=0.02$). Representative histomicrographs demonstrate apoptotic cells measured by morphologic criteria in H&E-stained sections (B) or functional criteria (active caspase-3-stained apoptotic cells stain brown, D). Arrows point to apoptotic cells. Magnification 400 $\times$.

FIG. 4. Apoptotic mediators in septic water, alcohol, and alcohol+EGF mice

No statistically significant differences were seen in the pro-apoptotic mediator Bax in jejunal sections of mice regardless of whether they had chronic alcohol ingestion prior to the onset of sepsis or received EGF after the onset of sepsis (A, $n=9-11$, $p>0.05$ for all comparisons). Representative blot showing relative expression of Bax is shown. In contrast, protein levels of the anti-apoptotic protein BCL-2 were lower in alcohol mice than water mice (B, $p=0.04$). EGF increased levels of Bcl-2 in alcohol mice ($n=8-12$, $p=0.03$ alcohol vs. alcohol+EGF). Representative blot showing relative expression of Bcl-2 is shown. The ratio of Bax/Bcl-2, a commonly used rheostat of cell survival was higher in alcohol mice than water mice (C, $p=0.02$) whereas EGF decreased this ratio in alcohol mice ($p=0.02$). Note: the Bax-Bcl-2 ratio calculated only on blots that were stained for both Bax and Bcl-2 on same animals ($n=4$ water, $n=10$ alcohol, $n=10$ alcohol+EGF).

FIG. 5. Villus length in septic water, alcohol, and alcohol+EGF mice

Villus length was decreased in alcohol mice compared to water mice ($p=0.009$). EGF increased villus length in alcohol mice ($p=0.02$, $n=6-7$).

FIG. 6. Intestinal proliferation in septic water, alcohol, and alcohol+EGF mice

Proliferation was decreased in alcohol mice compared to water mice (A, $p < 0.0001$). EGF increased crypt proliferation in alcohol mice ($n=6$, $p=0.0005$). Representative micrographs of jejunal crypts show S-phase cells labelled with BrdU staining brown (B, magnification 400 $\times$).

FIG. 7. Serum cytokines in septic water, alcohol, and alcohol+EGF mice

The anti-inflammatory cytokine IL-10 was higher in alcohol mice than water mice (A, $p < 0.0001$). EGF decreased IL-10 in alcohol mice ($n=8-9$, $p=0.0001$). A similar trend was seen for the pro-inflammatory cytokine MCP-1. MCP-1 levels were higher in alcohol mice than water mice (B, $p=0.0006$) while EGF decreased MCP-1 in alcohol mice ($n=6-8$, $p=0.003$). In contrast, TNF levels were similar in alcohol mice and water mice (C, $p=0.91$). However, EGF decreased TNF in alcohol mice ($n=9/\text{group}$, $p=0.047$).

Table 1

Serum cytokines 24 hours after CLP

Serum (pg/mL)	Water	Alcohol	Alcohol+EGF	Water vs. Alcohol (p value)	Alcohol vs. Alcohol+EGF (p value)
IL-1β	2333 $\pm$ 227	1608 $\pm$ 309	1374 $\pm$ 451	>0.05	>0.05
IL-2	183 $\pm$ 23	186 $\pm$ 12	132 $\pm$ 25	>0.05	>0.05
IL-6	1905 $\pm$ 267	2045 $\pm$ 277	1492 $\pm$ 135	>0.05	>0.05
IL-10	2022 $\pm$ 369	34363 $\pm$ 7349	5548 $\pm$ 1555	<0.0001	0.0001
MCP-1	24340 $\pm$ 4949	221164 $\pm$ 45768	56304 $\pm$ 9374	0.0006	0.0042
IFN-γ	2 $\pm$ 1	4 $\pm$ 1	3 $\pm$ 1	>0.05	>0.05
TNF	1510 $\pm$ 146	1439 $\pm$ 101	999 $\pm$ 118	>0.05	0.047