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The Effects of Alcohol Intoxication and Burn Injury on the Expression of Claudins and Mucins in the Small and Large Intestines

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Abstract

Alcohol intoxication at the time of burn injury exacerbates post-burn pathogenesis. Recent findings suggest gut barrier integrity is compromised after combined alcohol and burn insult, which could contribute to these complications. Tight junction proteins and mucins play critical roles in keeping the gut barrier intact. Therefore, the goal of this study was to examine the effects of alcohol and burn injury on claudin and mucin expression in the intestines. We also evaluated if the combined insult differentially influences their expression in the small and large intestines. Male C57BL/6 mice were given a single dose of 2.9g/kg ethanol prior to a ~12.5% body area burn. One and three days following injury, we profiled expression of several tight junction proteins, mucin, and bacterial 16S rRNA genes in small and large intestine using qPCR. We observed >50% decrease in claudin-4 and claudin-8 genes in both ileal and colonic epithelial cells one day after injury. Claudin-2 was significantly upregulated, and occludin was down-regulated in small intestine one day following injury. Mucin-3 expression was substantially elevated (>50%) in small intestine, whereas mucin-2, and mucin-4 were considerably diminished in the colon (>50%)

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Conflicts of Interest:

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one day following injury. Most parameters were normalized to sham levels on day three, except for mucin-3 and claudin-8, which remained decreased in large intestine. Neither alcohol nor burn alone resulted in changes in junction or mucin gene expression compared to shams. This was accompanied with increases in the family of Gram-negative bacteria, Enterobacteriaceae, in both small and large intestine one day following injury. These findings suggest that alcohol and burn injury disrupts normal gut microbiota and alters tight junction and mucin expression in the small and large intestines.

Keywords

Ethanol; Trauma; Inflammation; Tight Junctions; Mucus; Gut Barrier

Introduction

Alcohol (ethanol) abuse remains a serious problem worldwide. A study from the World Health Organization reported that alcohol attributed deaths exceeded three million in 2012(1). One of the leading consequences of excessive intoxication is the increased risk factor it poses for the occurrence of a traumatic injury(2). According to the American Burn Association, burn injury alone leads to 40,000 patient hospitalizations annually in the United States(3). Confounding data have shown that approximately 50% of hospitalized burn patients have detectable blood alcohol levels at the time of their admission (2, 4). A host of studies have shown the combination of alcohol and burn injury results in worsened patient prognosis including increased susceptibility to infection, sepsis, multiple organ failure, and death (4, 5). The intestines are the major reservoir of bacteria within the host, and disruptions of the intestinal barrier could exacerbate any of these sequelae(5, 6).

Our lab has previously demonstrated increases in intestinal permeability following combined alcohol and burn injury in mice and rats (7–9). Gut leakiness following the combined insult is paired with significant increases in pro-inflammatory mediators including IL-6, IL-18, and MCP-1(9–11). In addition, bacterial translocation to extra-intestinal sites such as the mesenteric lymph nodes has been observed as early as 24 hours post-injury(7). Others have demonstrated gut leakiness and immune dysregulation following burn injury, which may perpetuate the ability of bacteria and other pathogens to extravasate from the intestines following traumatic injury (12, 13) further demonstrating the clear necessity for gut barrier maintenance.

In healthy individuals, proper intestinal function is accomplished through the formation and maintenance of a selective barrier formed by tight junctions which allow the intestinal wall to retain commensal microbes to the lumen, while allowing the passage of nutrients out of the gut. Previous studies from our laboratory have demonstrated significant reductions in the expression and phosphorylation status of occludin and claudin-1 in the ileum of rats following alcohol and burn injury(14). Studies from other laboratories have shown significant disruptions in occludin and ZO-1 protein expression in mouse ileum following the combined injury (15, 16). However, studies in knockout mice have suggested that occludin may not be essential for the maintenance of epithelial barrier integrity(17), and that

claudin-1, -3, -4, -5, and -8 are most involved in strengthening the intestinal barrier (18, 19). Of these, claudin-4 and -8 are highly expressed in the ileum and colon of mice, and dysregulation of either could contribute to the leakiness observed following injury(18).

In addition to changes in tight junction expression, the mucus layer serves to protect barrier integrity by limiting the amount of interaction between luminal contents and intestinal epithelial cells. The mucus layer becomes more prominent moving distally down the intestinal tract, likely due to the correlating increase of commensal bacteria. In murine small and large intestine, the most prevalent mucin is mucin-2, and aberrant mucin-2 profiles have been observed in diseased states such as inflammatory bowel disease(20). In addition, a reduction in mucin-2 has also been correlated with increased ability of pathogens to cross the intestinal barrier (21). As such, alterations in mucin expression as a result of intestinal inflammation are correlated with the severity of pathogenesis and serve as an excellent marker to assess intestinal damage and leakiness. Changes in junction and mucin proteins may contribute to gut leakiness following alcohol and burn injury, which can result in multiple organ failure.

Therefore, we profiled mucus and tight junction gene expression in mouse ileum and colon epithelial cells one and three days following alcohol and burn injury. We hypothesized that both mucin and tight junction expression would be decreased in both the small and large intestine one day following alcohol and burn injury. Our results demonstrate that both ileal and colonic epithelial cells have decreased tight junction and mucin gene expression, and that goblet cell presence and mucin glycoprotein expression is largely reduced in colonic tissue one day following injury. These changes correlate with increases in the Gram-negative family, Enterobacteriaceae, in both the small and large intestine feces one day following the combined injury. Taken together, our results indicate that alcohol and burn injury affects the small and large intestines differently, and this may be significant in the development of gut barrier leakiness following alcohol and burn injury.

Materials and Methods

Animals

C57BL/6 male mice 10–12 weeks old (23–25g body weight) were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA.

Murine Model of Binge Alcohol and Burn Injury

For our animal experiments, we used the well-established mouse model of alcohol and burn injury we have described previously (10). Mice were randomly assigned into one of the four experimental groups: sham injury + vehicle (water), sham injury + ethanol, burn injury + vehicle, or burn injury + ethanol. On the day of injury, mice were gavaged with 400µl of 25% ethanol in water (2.9 g/kg). Animals in the vehicle groups were gavaged with 400µl of water. Four hours following gavage, mice were anesthetized by intraperitoneal injection with a ketamine hydrochloride/xylazine cocktail (~80 mg/kg and 1.2 mg/kg, respectively). The dorsal surfaces of mice were shaved, and animals were placed in a prefabricated

template exposing approximately 12.5% total body surface area calculated using Meeh's formula as described by Walker and Mason(22). Animals in burn groups were immersed in 85–87°C water bath for ~7 seconds to induce a full-thickness scald burn injury. Sham animals were placed in a 37°C water bath for 7 seconds. Immediately following burn or sham injury, mice were gently dried and given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages and allowed food and water *ad libitum*. All animal experiments were conducted in accordance with the guidelines set forth in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at Loyola University Health Sciences Division.

Intestinal Epithelial Cell Isolation

One or three days following the injury procedure, mice were humanely euthanized. Isolation of intestinal epithelial cells was performed as described previously by Weigmann *et al.*(23). Small and large intestines were removed from the peritoneal cavity. For small intestine studies, the distal 10cm of the small intestine was separated from the remainder of the small intestine for analysis. The entirety of the colon was harvested for analysis. The tissues were cut longitudinally and placed in ice cold PBS + 1% penicillin/streptomycin (pen/strep) cocktail. Following two washes in PBS + pen/strep, tissues were placed in a digestion solution containing 5% heat-inactivated fetal bovine serum (FBS), 1% HEPES, 1% pen/strep, 0.5% gentamicin, 5mM EDTA, and 1mM dithiothreitol (D.T.T.) in Hank's Balanced Salt Solution (HBSS) at 37°C. Tissues were placed in a 37°C incubator and shaken on a rotator at 250rpm for 20 minutes. Tissues were vortexed to separate the epithelial cells from the tissue and passed through a 100µm filter. Cells were counted on a hemocytometer to determine epithelial cell purity (90%). Intestinal epithelial cells were then processed for downstream applications.

Epithelial Cell RNA Isolation and cDNA synthesis

RNA isolation was performed using a RNeasy Mini Kit (Qiagen, Valencia, CA) as we have described previously(7). Genomic DNA was removed by DNase digestion using an RNase-free-DNase Set (Qiagen). Concentration of isolated RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Bannockburn, IL). Only samples with a 260/280 ratio of 2.0 were used for cDNA synthesis. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) and reactions were run on a Veriti 96-well Fast Thermocycler (Life Technologies) per the manufacturer's instructions.

Real-Time PCR

Expression of occludin, claudin-2, claudin-4, claudin-8, E-cadherin, mucin-1, mucin-2, mucin-3, and mucin-4 mRNA levels were analyzed by qPCR using TaqMan primer probes and TaqMan Fast Advanced Master Mix (Life Technologies). Target gene Ct cycle values were normalized to housekeeping control beta actin Ct values. Data were calculated using the Ct method, and all groups were expressed relative to the sham + vehicle group(24).

Immunohistology

Sections of the distal ileum (1cm) and proximal colon (1cm) were fixed in Carnoy's Solution. Tissue sections were prepared by the Loyola University Health and Sciences Division Processing Core. Briefly, tissues were cleared in 100% ethanol and embedded in paraffin as described previously(25). 5µm sections were cut, stained with alcian blue to stain for mucin expression in the ileum and colon, and cover slipped for analysis. All images were taken on a Zeiss Axiovert 200m microscope and processed using Adobe Photoshop CS3.

Isolation of Stool Genomic DNA

At the time of euthanasia, stool samples from the distal 5 cm of the ileum and the complete colon were sterilely collected. Wet weight of feces was obtained followed by isolation of fecal genomic DNA using a QIAmp DNA Stool Isoaltion Kit (Qiagen) per the manufacturer's instructions, and included incubation at 95°C to improve bacterial cell lysis. Genomic DNA purity and concentration was obtained using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

16s rRNA Bacterial Quantitative real-time PCR

Real-time PCR analysis using specific primer sets targeting small subunit (SSU) 16s rRNA of total bacteria and Enterobacteriaceae were used to quantify the fecal microbiome as described previously (26). Primer sequences were: 340F (ACTCCTACGGGAGGCAGCAGT) and 514R (ATTACCGCGGCTGCTGGC) for total bacteria, and 515F (GTGCCAGCAGCCGCGGTAA) 826R (GCCTCAAGGGCACAACCTCCAAG) for Enterobacteriaceae (Invitrogen). Standard curves for PCR quantification were generated using 10-fold serial dilutions of bacterial SSU ribosomal copy number from *Blautia producta* (Prevot) Liu et al. (*Ruminococcus productus*) genomic DNA (ATCC, Manassas, VA) for total bacteria, and *Escherichia coli* genomic DNA (ATCC) for Enterobacteriaceae. Reactions were run using 1X iTaq Universal SYBR Green Supermix (Bio-Rad) with a total of 300nM of forward and reverse primers per reaction. Reactions were run as follows: 95°C for 3 min, 40 cycles of 95°C for 15s, followed by data collection at 63°C (Bacteria) or 67°C (Enterobacteriaceae) for 1 min. Reactions were performed on a Step One Plus Real-Time PCR instrument (Applied Biosystems). Because changes in total bacteria 16S rRNA were observed in ethanol + burn samples, Enterobacteriaceae was expressed at the relative copy number per gram of feces. All groups are expressed as fold-increase in copy number relative to the sham + vehicle treated group.

Statistics

The data, wherever applicable, are presented as means + SEM and were analyzed using analysis of variance (ANOVA) with Tukey's post-hoc test or Student's t test (GraphPad Prism6). A p-value of <0.05 was considered statistically significant.

Results

Alterations of small intestine claudins following alcohol and burn injury

Previous data from our laboratory have demonstrated alterations in both the expression and phosphorylation status of occludin and claudin-1 in the ileum of rats one day following alcohol and burn injury(14). However, many different claudins are expressed within murine intestine, and may play a significant role in maintaining the gut barrier following alcohol and burn injury. To further expand the underlying mechanisms of gut leakiness following the combined insult, we performed gene expression analysis of several key tight junction markers including claudin-2, claudin-4, and claudin-8. Real-time PCR data obtained from isolated intestinal epithelial cells from the distal ileum of mice demonstrate significant reductions in occludin (60% reduction), and claudin-8 (76% reduction) one day following alcohol and burn injury compared to sham animals (Fig. 1A). Claudin-4 levels were significantly decreased following alcohol and burn injury when analyzed by student's t test ($p=0.0001$). Claudin-2, a leaky claudin that is permissive to water and ions, was significantly elevated (80%) relative to sham mice (Fig. 1A). All mRNA levels returned to sham levels three days following injury, indicating that gene expression in the small intestine is able to recover following injury (Fig. 1B).

Alterations of large intestine claudins following alcohol and burn injury

In spite of the large number of studies examining the effects of alcohol, trauma, or disease states on tight junctions in the colon, no studies to our knowledge have examined the effects of combined alcohol and burn injury in the context of the large intestine. As in the ileum, mRNA expression from isolated colonic epithelial cells was quantified by qPCR on day one and day three post-injury (Fig. 2). Similar decreases in claudin-4 (46% reduction) and claudin-8 (57% reduction) were observed in colonic epithelial cells one day following the combined injury (Fig. 2A). A trend of a decrease in occludin ($p = .07$ by student's t test) was also observed, but no significant changes in claudin-2 gene expression were seen in the colon. Similar to the results observed in the ileum, all gene expression returns to sham levels three days following injury with the exception of claudin-8 (41% reduction, Fig. 2B).

No significant changes in intestinal adherens genes following alcohol and burn injury

To determine if adherens junctions were altered following combined alcohol and burn injury, E-cadherin gene expression was analyzed in ileal and colonic epithelial cells as above. One day following the combined insult, a trend of a decrease in E-cadherin gene expression ($p = .0519$ by student's t test) was observed in colon epithelial cells (Fig. 3B). However, E-cadherin expression was unchanged in ileal epithelial cells one day after alcohol and burn injury (Fig. 3A). Additionally, no changes were observed in cadherin-17 (data not shown).

Alterations in intestinal mucin expression following alcohol and burn injury

In addition to tight junction complexes between intestinal epithelial cells, the mucus layer serves as a buffer between the epithelial cells and the luminal microbiome. Thus, we sought to profile key mucin gene expression from isolated distal small intestine and colon epithelial

cells following alcohol and burn injury. Data in Figure 4A demonstrate significant elevations in mucin-3 (76%) are observed in the distal ileum one day following the combined insult. However, mucin-2 levels were significantly decreased (20%) compared to sham + vehicle animals by student's t test ($p = 0.02$). A trending decrease in mucin-4 (44%, $p = .06$ by student's t test) was also observed in the small intestine one day following injury. Similarly to tight junction gene expression, mucin mRNA expression in ileum of all groups is similar to the sham vehicle group three days after injury with the exception of mucin-1 (Fig. 4B). The most notable differences in mucin gene expression were observed in colonic epithelial cells one day following injury (Fig. 5A). Both mucin-2, the most highly expressed mucin in mouse intestine, as well as mucin-4 were significantly reduced 51% and 54%, respectively. Three days following injury, mucin gene expression returns to levels similar to sham vehicle in all groups, except in the case of mucin-1 ($p = .03$, student's t test) and mucin-3 (Fig. 5B).

Changes in large intestine mucin expression following alcohol and burn injury

To validate the changes in mucin gene expression observed by qPCR, we performed alcian blue histological stains on both distal small intestine and colon tissues. Alcian blue stains acidic mucins, which are ubiquitously expressed in both murine small and large intestines. Representative images from all groups are shown (Fig. 6). Data demonstrate slightly diminished goblet cell presence in ileum of alcohol + burn mice. However, no demonstrable changes were observed in luminal mucin expression one day after injury (Fig. 6A). However, substantial reductions of mucin expression and goblet cell number were observed in the colon of five out of eight animals one day after receiving combined alcohol and burn injury compared to sham animals (Fig. 6B).

Increase in Enterobacteriaceae in small and large intestine feces following alcohol and burn injury—Feces were isolated from distal ileum and colon one day following injury. DNA was isolated and both total bacteria and Enterobacteriaceae family populations were analyzed by quantitative real-time PCR (Fig. 7). As compared to shams, a 13.6-fold increase was observed in total bacteria in the distal ileum feces one day following injury (Fig. 7A). No changes were observed in the total bacteria populations in the colon (Fig. 7B). However, several fold increases in the Gram-negative family Enterobacteriaceae was observed in both the small (184-fold) and large (75-fold) intestines one day following alcohol and burn injury (Fig. 7C, D). While the ethanol alone did not appear to affect the bacterial populations in small and large intestines, burn injury alone caused a ~30-fold increase in Enterobacteriaceae in the large intestine (Fig. 7D). While these increases were not found to be statistically significant due to high variability, changes in the microbiome are clearly visible following ethanol and burn injury.

Discussion

In the current study, we observed significant decreases in claudin-4 and claudin-8 gene expression in both the small and large intestines one day following alcohol and burn injury. Additionally, claudin-2 was significantly up-regulated in small intestine epithelial cells one day following injury. Levels of all claudin genes were restored in both the small intestine

and colon three days post-injury, with the exception of decreased claudin-8 expression in the colon. Finally, we observed a several fold increase in Enterobacteriaceae in both small and large intestines one day following injury. While we observe a restoration of most of the tight junction and mucin genes by three days following injury, it is plausible that bacteria and other pathogens are able to quickly invade even in very acute lapses in intestinal barrier integrity following injury. Our lab has generated data to support this where we have observed bacteria in the mesenteric lymph nodes of alcohol burn injured mice one day following injury(7). We further found that only mucin-2 was statistically reduced in ileal epithelial cells. In contrast, mucin-2 and -4 gene expression were significantly decreased in the colon one day following injury. Considerably reduced alcian blue staining in the colon one day following injury supported these observations. These results were not surprising considering the high level of mucin expression in the colon of healthy animals.

Few studies in the literature have examined the *in vivo* effects of alcohol or burn injury alone on the intestinal barrier (5, 13, 27, 28). Ma *et al.* have shown in cultured Caco-2 cells that even low concentrations of alcohol (10%) can induce rapid disruptions of tight junction complexes including on ZO-1, actin, and myosin filaments, which was correlated with significantly increased myosin light-chain kinase (MLCK) activity(29). MLCK activation results in the phosphorylation of myosin light chain (MLC), causing cytoskeletal shifting, and disruption of epithelial cell junctions. Supporting *in vivo* studies in an alcohol burn injury model performed by Zahs *et al.* have also shown increased MLCK activity in the ileum of mice after alcohol and burn injury, which was rescued by using the membrane permeant inhibitor of MCLK (PIK)(15). In addition, our laboratory has shown substantial decreases in phosphorylation of occludin and claudin-1 in rodent ileum following alcohol and burn injury(14). Our current findings not only add supporting data to these previous studies, but expand the analysis from the ileum to the colon. Furthermore, we profile several additional tight junction genes that may be significant to maintaining barrier integrity in addition to the ones already investigated(14–16). The variance in regulation of gene expression observed between the small and large intestine highlights the significance of different junction proteins and mucins that may contribute to the increase in leakiness in these two segments of the intestine. Many claudins are expressed throughout the intestines, but most are only expressed within specific regions. While claudin-4 is highly expressed in both the small and large intestines, claudin-8 protein is only observed in the ileum and colon of rodents(18). It is plausible that these regional variations in tight junction protein expression play a significant role in gut leakiness following injury. In addition, enzyme activity modulating cytoskeletal rearrangements certainly plays a significant role in barrier integrity following alcohol and burn injury, which may also have feedback to limiting tight junction gene expression in epithelial cells following a traumatic insult.

To our knowledge, very few studies have examined mucin expression in the intestines following alcohol exposure. Hartmann *et al.* examined the extent of alcoholic liver damage and found that Muc2^{-/-} were protected from intestinal bacterial overgrowth and dysbiosis following chronic alcohol feeding(30). They found that antimicrobial peptides were significantly elevated in Muc2^{-/-} mice, which likely account for the decreased bacterial translocation observed. These studies suggest that it takes very high concentrations or

chronic exposure of alcohol to perturb the mucus layer of the intestines. It is known that mucin levels increase in the intestine of chronic alcoholic feeding in rodents(31), however, in our model of a single binge of alcohol it is unknown what role mucin would play in the intestines following the combined injury. In the current study, we observed significant reductions in mucin gene expression in both the small and large intestines one day following this combined insult. Such decreases in mucin expression could potentially contribute to increased translocation of bacteria and other pathogens as mucin is known to contain bacteriophages and IgA antibodies to prevent such occurrences (32).

While the mechanism underlying alterations in tight junction proteins and mucin expression remains to be explored, our findings suggest a role for IL-22 in disrupted barrier function after alcohol and burn injury. IL-22 has been shown to play a critical role in the maintenance of intestine homeostasis, and is known to modulate intestinal epithelial cell proliferation and apoptosis, and promote mucin and antimicrobial peptide secretion (33). Previous work from our laboratory has shown that IL-22 levels in small intestine are significantly reduced following combined injury, and this is paired with increased intestinal permeability(7). In addition, we observe large increases in the amount of IL-18 present in the intestines following alcohol and burn injury, which may also contribute to altered tight junction proteins and increases in gut leakiness (10, 14).

A resounding observation from these results is the lack of significant changes in tight junction and mucin expression following burn injury alone. Other models of burn injury without alcohol intoxication demonstrated significant changes in the intestinal barrier tight junctions and mucin expression(27). These models of burn injury are much more extensive (20–60% total body surface area) than the burn injury we administer in our combined alcohol and burn model. We believe that the extent of our burn injury alone (~12.5% total body surface area) is not sufficient to induce intestinal damage similar to that observed in larger burn area models. Thus, our results highlight that alcohol intoxication at the time of burn injury exacerbates post-burn pathogenesis, even in small surface area burns. Our data further suggest that both ileum and colon respond differently to the combined insult of alcohol and burn injury, and that these changes are visible as early as one day following the combined injury. Although, the mechanism underlying the differential response remains to be established, previous studies suggest that the intestines are highly dynamic, and the microenvironments of the small and large intestines vary drastically. As such, intestinal homeostasis is differentially regulated in the small and large intestines to account for the different environments that exist in each. In a recent study, we observed that large intestine exhibits a higher inflammatory response to a combined insult of alcohol and burn injury compared to the small intestine, which supports this notion (11).

Finally, our study demonstrates changes in the local microbiome may be one likely cause of the barrier disruption in the intestines following the combined injury, as we observed significant differences in the bacterial populations between the ileum and colon. Our findings show large increases in both total bacteria and Enterobacteriaceae populations in the ileum one day following injury, whereas only Enterobacteriaceae increased in the colon. The underlying cause for the observed differences in bacterial populations and how this affects the barrier in small and large intestines following injury remains to be established,

and is one limitation of our study. However, previous studies have demonstrated a role of the microbiome in intestinal barrier maintenance, including tight junction and mucin expression (34, 35), and this will be the focus of future studies.

Taken together, our findings presented in this manuscript highlight the differential regulation that occurs in both tight junction and mucin genes in the small and large intestines following alcohol and burn injury. Regardless of the underlying mechanism, disruption in barrier integrity can allow gut bacteria to gain access to extra intestine sites. While the intestinal barrier is restored three days following injury, these bacteria have already infiltrated and may contribute to infection, sepsis, and multiple organ failure observed following injury. These findings suggest that although acute administration of a single dose of alcohol alone is not likely to cause infection and/or sepsis in healthy individuals, the presence of alcohol in the context of a second insult such as burn injury becomes a serious confounding factor that significantly impacts the recovery of the host. Therefore presence of alcohol at the time of injury should be considered as one factor in the overall outcome of burn patients.

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Figure 1. Tight junction mRNA profiles in small intestine ileum epithelial cells one and three days following alcohol and burn injury

RNA was isolated from small intestine epithelial cells and gene expression was examined using qPCR to examine expression of several tight junction genes. **A.)** Significant reductions in occludin, claudin-4, and claudin-8 were observed one day following combined alcohol and burn injury. Claudin-2 was expressed at higher levels than in sham vehicle controls. **B.)** All tight junction mRNA levels were normalized three days post-injury (n = 3–8, *p < 0.05, **p < 0.01 by ANOVA, #p < 0.05 by student's t test compared to sham + vehicle).

Figure 2. Tight junction mRNA profiles in large intestine epithelial cells one and three days following alcohol and burn injury

RNA was isolated from large intestine epithelial cells and gene expression was examined using qPCR to examine expression of several tight junction genes. **A.)** Significant reductions in claudin-4, and claudin-8 were observed one day following combined alcohol and burn injury. **B.)** All tight junction mRNA levels were normalized three days post-injury with the exception of decreased claudin-8 expression ($n = 3-8$, $*p < 0.05$, $**p < 0.01$ by ANOVA compared to sham + vehicle).

Figure 3. Adherens mRNA profiles in small and large intestine epithelial cells one day following alcohol and burn injury

RNA was isolated from small and large intestine epithelial cells and gene expression was examined using qPCR to examine expression of E-cadherin. **A.)** No observed changes in E-cadherin expression was observed between any groups in ileum epithelial cells. **B.)** A trend of a decrease ($p = .0519$ by student's t test) was observed between sham + vehicle and alcohol + burn large intestinal epithelial cells one day following the combined insult ($n = 5-9$ animals/group).

Figure 4. Small intestine ileum epithelial cell mucin profiles mostly unaffected by combined alcohol and burn injury

RNA was isolated from small intestine epithelial cells and gene expression was examined using qPCR to examine expression of several mucin genes. **A.)** mRNA levels of mucin-3 were observed to be significantly elevated, while mucin-2 levels were diminished one day following the combined insult. **B.)** All mucin mRNA levels returned to sham levels three days post-injury except for mucin-1 ($n = 3-8$, **** $p < 0.0001$ by ANOVA, # $p < 0.05$ by student's t test compared to sham + vehicle).

Figure 5. Significant reductions in large intestine epithelial cell mucin profiles following combined alcohol and burn injury

RNA was isolated from large intestine epithelial cells and gene expression was examined using qPCR to examine expression of several mucin genes. **A.)** Large decreases in both mucin-2 and mucin-4 were observed in the large intestine one day post-injury. **B.)** Mucin gene levels were largely restored by three days following combined alcohol and burn injury. However, significant decreases were observed in mucin-3 analyzed by ANOVA, and also in mucin-1 analyzed by student's t test ($n = 3-8$, $*p < 0.05$, $****p < 0.0001$ by ANOVA, $\#p < 0.05$ by student's t test compared to sham + vehicle).

Figure 6. Significant reduction in large intestine mucus protein expression following combined alcohol and burn injury

Alcian blue staining one day following alcohol and burn injury was performed on **A.)** distal ileum and **B.)** proximal colon sections. Five images of five separate microscopic fields were taken at 200X total magnification for each animal. Substantial reductions in the amount of mucin glycoprotein expression and goblet cell presence was observed in five out of eight mice receiving alcohol and burn injury in the large intestine compared to shams ($n = 5-8$).

Figure 7. Increase in Enterobacteriaceae in small and large intestine feces one day following ethanol and burn injury

Changes in total bacteria and Enterobacteriaceae populations within the small and large intestine feces one day following injury were analyzed using domain (total bacteria) and family (Enterobacteriaceae) specific primers by qRT-PCR. Data are expressed as the copy number of 16S rRNA per gram of feces relative to the sham + vehicle group. Large increases in total bacterial were observed in the **A.)** ileum, but not in the **B.)** colon. Enterobacteriaceae was highly increased in both the **C.)** small and **D.)** large intestine feces one day following injury (n = 5–8).