A prospective observational study of the effect of critical illness on ultrastructural and microscopic morphology of duodenal mucosa

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The gastrointestinal tract epithelium acts as a barrier against luminal organisms and antigens. It has been suggested that, during critical illness, paracellular defects in intestinal barrier function allow bacteria, other micro-organisms and antigens to translocate across the gut epithelium, predisposing patients to sepsis, organ failure and death.\(^1\,^2\)

The integrity of intestinal tight junctions is considered pivotal to barrier function. In animal models, it has been observed that various triggers for critical illness, such as infection and trauma, are associated with structural and functional tight junction abnormalities, which facilitate paracellular translocation of organisms and antigens.\(^3\,^5\) Other ultrastructural abnormalities of intestinal epithelium have been found, by the use of electron microscopy, in animal models of critical illness. These include less dense microvilli, mitochondrial abnormalities and swollen and degenerated vascular endothelial cells.\(^6\,^7\)

It is generally assumed, based on these animal data, that critical illness causes leaky tight junctions, which affect intestinal barrier function and contribute to adverse outcomes in this group of patients. However, the intestinal ultrastructure of critically ill patients has never been assessed using electron microscopy. Our primary aim was to describe tight junction morphology of the duodenum in critically ill patients, compared with a control group of otherwise healthy participants who were having endoscopies as outpatients. Our secondary aims were to describe microvillus length and density, vascular endothelium morphology and mitochondrial density and morphology, examined with electron microscopy, and morphology examined with light microscopy.

**Results:** We observed no abnormalities of tight junction ultrastructure in either group. There was a tendency towards shorter microvilli in the critically ill group: mean length in critically ill patients, 1.17 μm (interquartile range [IQR], 1.05–1.60 μm) v mean length in control patients, 1.58 μm (IQR, 1.30–1.72 μm); \(P = 0.07\). There was a tendency towards less dense microvilli in the critically ill group: mean density in critically ill patients, 7.29 microvilli/μm (IQR, 6.83–8.05 microvilli/μm) v mean density in control patients, 8.32 microvilli/μm (IQR, 7.34–9.11 microvilli/μm); \(P = 0.07\). Vascular endothelium appeared normal in all critically ill patients and abnormal in one control participant. Abnormal mitochondrial morphology was noted in one critically ill patient and one control participant, and no differences were seen in mitochondrial density. Using light microscopy, we saw more apoptotic cells in the critically ill patients (\(P = 0.018\)), but villus height, crypt depth and lymphocyte density were normal.

**Conclusions:** We did not detect any morphological abnormalities of duodenal tight junctions in critically ill patients. Our results should be interpreted with caution because of the small sample population, but our observations challenge the concept that paracellular translocation facilitates secondary sepsis.

**ABSTRACT**

**Objective:** Disturbed intestinal barrier function due to ‘leaky’ tight junctions may cause secondary sepsis via paracellular translocation across the gut wall. Our objective was to describe the effects of critical illness on duodenal morphology and ultrastructure.

**Design, setting and participants:** Prospective observational study of 12 mechanically ventilated critically ill patients in an intensive care unit and 15 control participants in an outpatient endoscopy suite.

**Intervention:** We took six endoscopic biopsy samples of the duodenum from each participant for analysis by electron and light microscopy.

**Main outcome measures:** Our primary outcome was tight junction morphology, examined with electron microscopy. Secondary outcomes were microvillus length and density, vascular endothelium morphology and mitochondrial density and morphology, examined with electron microscopy, and morphology examined with light microscopy.

**Results:** We observed no abnormalities of tight junction ultrastructure in either group. There was a tendency towards shorter microvilli in the critically ill group: mean length in critically ill patients, 1.17 μm (interquartile range [IQR], 1.05–1.60 μm) v mean length in control patients, 1.58 μm (IQR, 1.30–1.72 μm); \(P = 0.07\). There was a tendency towards less dense microvilli in the critically ill group: mean density in critically ill patients, 7.29 microvilli/μm (IQR, 6.83–8.05 microvilli/μm) v mean density in control patients, 8.32 microvilli/μm (IQR, 7.34–9.11 microvilli/μm); \(P = 0.07\). Vascular endothelium appeared normal in all critically ill patients and abnormal in one control participant. Abnormal mitochondrial morphology was noted in one critically ill patient and one control participant, and no differences were seen in mitochondrial density. Using light microscopy, we saw more apoptotic cells in the critically ill patients (\(P = 0.018\)), but villus height, crypt depth and lymphocyte density were normal.

**Conclusions:** We did not detect any morphological abnormalities of duodenal tight junctions in critically ill patients. Our results should be interpreted with caution because of the small sample population, but our observations challenge the concept that paracellular translocation facilitates secondary sepsis.
**Methods**

**Critically ill patients**

We studied mechanically ventilated critically ill patients in an intensive care unit who were suitable to receive enteral nutrition. Patients were excluded if they were younger than 18 years, were pregnant, had a contraindication to endoscopy, had a history of small intestinal surgery (excluding appendectomy), had a history of small intestinal abnormality, had active gastrointestinal bleeding, had an international normalised ratio > 1.5, had a platelet count < 50 x 10^9/L, were receiving drugs known to alter platelet aggregation or thrombus formation, or had religious beliefs that prevented administration of blood products. Patients were fasted for at least 6 hours before endoscopy.

**Control participants**

To provide a comparison group, we asked people who were already booked for an outpatient endoscopy at the Royal Adelaide Hospital Department of Gastroenterology to participate. The same exclusion criteria applied to these control participants.

**Duodenal biopsies**

We obtained duodenal biopsy samples from 12 mechanically ventilated critically ill patients and from 15 control volunteers. A total of six biopsy samples from the second part of the duodenum were obtained from each participant: three samples were preserved in glutaraldehyde for electron microscopy and three were preserved in formalin for light microscopy.

**Intestinal ultrastructure visualised with electron microscopy**

Our primary outcome was assessment of tight junction morphology, which was categorised as either normal or abnormal. Our secondary outcomes were microvillus length and density, vascular endothelium morphology and mitochondrial morphology and density. All qualitative outcomes were initially reviewed by two independent investigators (V L and A D), coded, and analysed in a blinded fashion by a pathologist with expertise in electron microscopy of the gastrointestinal tract (A B). To perform quantitative analysis, we used the image-processing software Fiji (http://fiji.sc/Fiji).

Because of the absence of published data, we first developed our own pragmatic criteria to quantify microvillus length, microvillus density and mitochondrial density.

- To quantify microvillus length, we identified three consecutive epithelial cells that appeared to be representative of all epithelial cells, and measured the length of 10 representative microvilli from each epithelial cell to calculate the mean microvillus length per participant.

- To quantify mitochondrial density, we counted the number of normal mitochondria in the area of the cell above the nucleus, divided by area, and repeated this across three representative epithelial cells to calculate the mean mitochondrial density per participant. Mitochondria were only counted if they were > 0.5 μm in length, and we defined normal mitochondria as having an intact border, an oval shape with length greater than width, and intact cristae or granules.

**Intestinal structure visualised with light microscopy**

All duodenal biopsy samples were processed for paraffin section examination and stained using the haematoxylin and eosin (H&E) method. Slides were examined by two pathologists (N D and A S) in a blinded fashion, using an Olympus BX51 microscope.

Each slide was photographed at a magnification of × 400 using an Olympus DP27 digital microscope camera with Olympus Stream imaging software. We performed histological morphometric analysis, including villus height and crypt depth, which were measured on three consecutive well preserved and anatomically oriented villi and crypts, using a Polyline measuring tool. We measured villus height from the tip of the villus to its base, and crypt depth from the base of the villus to the muscularis mucosae, and calculated the mean for each variable.

We performed lymphocyte counts on three consecutive well preserved and anatomically well oriented villi. We counted lymphocytes at × 400 magnification, and recorded the number of lymphocytes per 100 enterocytes. We then obtained the mean for each biopsy sample for measured villi.

We used light microscopy of H&E-stained slides to detect apoptosis. The criteria for apoptosis included cell shrinkage with small vacuoles containing karyorrhectic nuclear debris and surrounded by free space. We examined three consecutive preserved and anatomically well oriented villi, counted the total number of apoptotic cells for each biopsy sample, and calculated the mean.

**Clinical sepsis outcomes**

For clinical significance, we also recorded whether the critically ill patients developed features of the systemic inflammatory response syndrome (SIRS) or sepsis after the biopsy. We used standard definitions of sepsis and recorded results for all specimens that were obtained and analysed for clinical reasons. We categorised all positive cultures as those for which the organism cultured was potentially due to a gastrointestinal organism (ie, gram-negative rods and...
Candida species). We reported all such positive cultures along with the site of the culture, because an individual patient could provide more than one organism and/or site. Gastric volumes were aspirated every 6 hours, and we defined intolerance to enteral nutrition as an aspirate greater than 250 mL in the preceding 48 hours.10

Our study was conducted in accordance with the National Health and Medical Research Council National statement on ethical conduct in human research and local legal requirements for the conduct of research involving unconscious persons, with the protocol approved by the Royal Adelaide Hospital Human Research Ethics Committee. Written informed consent was obtained from the next of kin of critically ill patients before the study. Patients having outpatient endoscopy and participating as the control group provided written informed consent before endoscopy.

Statistical analysis
As there were no previous data in humans and data from animal models showed that the phenomenon of intestinal barrier dysfunction occurred frequently, we did not perform an a priori power calculation. Rather, we anticipated that a small group of critically ill patients (n = 12) would be enough to identify the marked ultrastructural abnormalities that we anticipated we would observe.

We present data as frequencies for categorical variables, and as medians and interquartile ranges (IQRs) for continuous variables. As categorical observations were infrequent, we did not conduct inferential analyses for these data. We compared continuous variables (length and density) between groups using the Mann–Whitney U test. A two-sided P < 0.05 was considered statistically significant. We conducted all analyses using SPSS, version 22.0 (SPSS Inc).

Results
Characteristics and outcomes of patients
The median age of the 12 critically ill patients was 51 years (IQR, 32–69 years); all were men and they were admitted for a variety of reasons (sepsis, 5; pancreatitis, 3; trauma, 3; subarachnoid haemorrhage, 1), with a median Acute Physiology and Chronic Health Evaluation II score on admission of 19 (IQR, 15–22). Patients underwent endoscopy at a median of Day 9 (IQR, Day 4 to Day 13) of their ICU admission and, at the time, only nine were receiving enteral nutrition (three were receiving parenteral nutrition), with four patients tolerant and five patients intolerant of enteral feeding.

All participants tolerated endoscopy and biopsy with no adverse events. Patients remained in the ICU for a median of 13 days (IQR, 9–14 days) after biopsy. All patients had features that met the SIRS criteria on at least 1 day. Eight patients had an episode of sepsis in the ICU after biopsy and, for seven patients, an organism was cultured that was potentially a gastrointestinal pathogen (n = 2, Enterococcus, Klebsiella and Candida; and n = 1, Pseudomonas, Enterobacter and Citrobacter) with positive cultures grown from urine, sputum and blood (n = 4, 2 and 2, respectively). Five patients developed severe sepsis.

The median durations of ICU and hospital admission were 21 days (IQR, 15–34 days) and 45 days (IQR, 32–56 days), respectively. One patient died in hospital.

Characteristics of control participants
The median age of the 15 control participants was 52 years (IQR, 41–64 years); seven (47%) were men, and the participants were having an elective outpatient endoscopy for a variety of reasons (epigastric discomfort, 6; gastroesophageal reflux symptoms, 2; iron deficiency anaemia, 2; post-hiatus hernia repair follow-up, 2; dysphagia, 2; and non-cardiac chest pain, 1).

Figure 1.
Electron microscopy
We observed no abnormalities of tight junction morphology in patient and control groups (Figures 1A and 1B). The differences between groups in point estimates for microvillus length and density did not reach statistical significance, but suggested that critical illness was associated with shorter and less dense microvilli (median microvillus length: critically ill patients, 1.17 μm [IQR, 1.05–1.60 μm] v control participants, 1.58 μm [IQR, 1.30–1.72 μm]; P = 0.07; median microvillus density: critically ill patients, 7.29 microvilli/μm [IQR, 6.83–8.05 microvilli/μm] v control participants, 8.23 microvilli/μm [IQR, 7.34–9.11 microvilli/μm]; P = 0.07).

Vascular endothelial morphology appeared normal in all critically ill patients but was abnormal in one control participant (Figure 2). Mitochondrial morphology was abnormal in one patient and one control participant. Median mitochondrial density (Figure 3) was similar between the two groups (critically ill patients, 0.26 mitochondria per μm² [IQR, 0.21–0.39 mitochondria per μm²] v control participants, 0.36 [IQR, 0.23–0.48 mitochondria per μm²]; P = 0.26).

Light microscopy
We observed between zero and four apoptotic cells per villus, with statistically significantly more apoptotic cells in the critically ill patients (median, critically ill patients, 0.3 apoptotic cells/villus [IQR, 0.0–1.0 apoptotic cells/villus] v control participants, 0.0 apoptotic cells/villus [IQR, 0.0–0.3 apoptotic cells/villus]; P = 0.018). There were no statistically significant differences between the groups in median villus height (critically ill patients, 399 μm [IQR, 301–534 μm] v control participants, 422 μm [IQR, 370–524 μm]; P = 0.54), median crypt depth (critically ill patients, 221 μm [IQR, 165–247 μm] v control participants, 184 μm [IQR, 151–208 μm]; P = 0.28) and median lymphocyte (lc) density (critically ill patients, 8.5 lc/100 enterocytes [IQR, 2.5–13.7 lc/100 enterocytes] v control participants, 11.3 lc/100 enterocytes [IQR, 10.3–27.7 lc/100 enterocytes]; P = 0.11).

Discussion
We evaluated duodenal ultrastructure in critically ill patients and compared these features to those in a group of outpatients undergoing endoscopy for a variety of symptoms. To our surprise, we did not observe any effect of critical illness on our primary outcome of interest, tight junction ultrastructure. There was a tendency for shorter and less dense microvilli in the critically ill patients, which did not reach statistical significance, but there was no effect of critical illness on vascular endothelial morphology, mitochondrial morphology or mitochondrial density. Using light microscopy, we observed an increase in the number of apoptotic epithelial cells in the critically ill patients but no difference in villus height, crypt depth or lymphocyte density.

Comparison with other data
Our ultrastructural findings do not support previous animal data, which had reported marked intestinal tight junction structural changes during critical illness.3,5,11-13 Our light microscopic findings confirm previous studies in critically ill patients that reported intestinal epithelial cell apoptosis,14,15 but we did not observe previously reported intestinal mucosal atrophy.16

Our study is the first to evaluate intestinal ultrastructure in critically ill humans using electron microscopy, but previous investigators have used light microscopy to evaluate intestinal epithelium. In 15 critically ill patients, Hernandez and colleagues observed a marked reduction in villus height and crypt depth when compared with healthy participants;16 observations that we did not replicate. However, these data were obtained in the 1990s and all patients were fasted for at least 4 days, which does not reflect current clinical practice.17 Prolonged enteral nutrient deprivation has been reported to cause similar histological changes in animals18,19 and...
humans. There has also been considerable advancement in care of the critically ill since that study was done, with changes that may have an effect on gastrointestinal epithelium. For example, mechanical ventilation using large tidal volumes has been reported to cause damage to intestinal epithelium in rabbits, which suggests that non-gastrointestinal interventions may be deleterious to the gut. Therefore, we believe our observations more closely reflect modern intensive care practice.

Hotchkiss and colleagues were able to obtain full thickness tissue from the large intestine of 10 patients who underwent emergency laparotomy after a motor vehicle accident or gunshot injury. They compared that tissue with large intestinal tissue from six patients who had had an elective laparotomy and resection to treat large intestinal malignancy. Using light microscopy and immunohistochemical staining for examination, they reported extensive epithelial apoptosis. We also observed more apoptotic cells in the epithelium in critically ill patients. Hotchkiss and colleagues obtained tissue from all patients soon after injury, and also obtained tissue from one patient during surgery 4 days later. They reported that features of apoptosis were not observed in that patient at the second operation, which suggests that structural changes of intestinal mucosa are focal rather than a generalised phenomenon, or that changes are reversible with the care that can be provided in modern ICUs. Our observations with electron and light microscopy support that hypothesis.

Strengths

The key strength of our study is that we studied a group of critically ill patients who were likely to display ultrastructural abnormalities for several reasons: all patients were mechanically ventilated, had been admitted for several days before endoscopy, and remained in the ICU for several days after endoscopy. In addition, three of our patients (25%) were deprived of enteral nutrition before their biopsy, and nutrient deprivation is associated with structural changes. Finally, several of our patients developed sepsis after their biopsy, which suggests that if leaky tight junctions are a true and frequent phenomenon in the critically ill, we should have observed these even in our relatively small group. Another strength of our study is that we were able to obtain intestinal tissue using endoscopy. This method overcomes the inherent limitations of analysing post-mortem tissue and the pathological changes associated with a disease process necessitating laparotomy.

Limitations

There are several limitations to our study, apart from those inherent to our observational design. First, based on the marked differences observed in intestinal ultrastructure of animals, and the considerable technical challenges of obtaining intestinal tissue in patients, we only studied a relatively small number of patients who were critically ill from a variety of causes. There was therefore considerable risk of a type II error, and we may have observed leaky tight junctions if we had studied a larger number of patients, patients earlier in their ICU admission, or patients from groups with specific disorders. However, because we failed to observe any patients with distorted intestinal tight junction architecture, it appears that this phenomenon is less frequent in unselected critically ill patients, particularly those who remain in the ICU for several days, than is suggested by animal models.

Second, the lack of previous data meant that there were no validated criteria for defining intestinal ultrastructural abnormalities and we had to develop our own pragmatic criteria. However, for the reasons listed above, we expected to see stark ultrastructural changes and were of the opinion that pragmatic criteria would be sufficient.

Third, because of the considerable logistical challenges of obtaining tissue, we did not obtain additional tissue at multiple time points or from the distal small and/or large intestine, so we caution against extrapolating our observations to more distal tissue. However, previous investigators have reported that both the small and large intestine are similarly affected by critical illness, and animal studies have reported that bacterial translocation occurs in the duodenum as well as in more distal segments of the small intestine.

Fourth, our control group, having been referred for diagnostic endoscopy, cannot be assumed to represent true health. Finally, we wish to emphasise that we only evaluated structure, and that function may be affected even if tissue appears structurally normal.

Clinical implications

The gut is presumed to be a source of ongoing or secondary sepsis in critically ill patients. Interventions aimed at attenuating this phenomenon are currently under investigation, including selective digestive tract decontamination, use of probiotics and avoidance of prophylactic acid suppressive therapy. If our data are reproducible, and the paracellular intestinal barrier is more intact than previously thought, we would consider the mechanism of any benefit from such interventions to be mediated through the transcellular pathways, intestinal immune function or non-gastrointestinal mechanisms. Bacterial translocation across the gut epithelium may not be as prominent a mechanism in critically ill humans as it is in animals.

Future directions

Although it is thought-provoking, we recognise that our data require validation with a larger sample. Ideally, future
studies should include a surrogate marker of permeability, such as a dual-sugar test,\textsuperscript{38,39} to assess function, and include immunohistochemical data to further investigate lymphocyte and epithelial cell apoptosis.\textsuperscript{14,15,34,35} Attempts to evaluate the ultrastructure of tissue distal to the duodenum appear to be warranted before conclusions about more distal intestinal tight junctions can be made.

Conclusions

Within the limitations of a small sample population, we observed no ultrastructural evidence to support the concept that abnormalities of tight junctions facilitate intestinal translocation via paracellular pathways during critical illness.

Author contributions

Victor Liew was responsible for acquisition and interpretation of data and drafting the manuscript. Marianne Chapman, Nam Nguyen, Caroline Cousins, Mark Plummer, Lee-anne Chapple, Yasmine Ali Abdelhamid, Nicholas Manton, Adam Swalling, Peter Sutton-Smith and Alastair Burt contributed to the acquisition and interpretation of data and critical revision of the manuscript for important intellectual content. Adam Deane was responsible for the study conception and design, interpretation of data and drafting the manuscript. Adam Deane is guarantor of this work so had full access to all the data in the study and takes responsibility for the integrity of these data and the accuracy of the analysis.

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Competing interests

None declared.

Previous presentation

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