Intestinal Structure and Injury of Extremely Premature Lambs During Artificial Placenta Support

ABSTRACT

Background: There are many causes of intestinal complication in premature infants, ranging from structural issues to dysbiosis. This study aims to investigate the effect of an Artificial Placenta (AP) utilizing extracorporeal membrane oxygenation (ECMO) on intestinal structure and injury.

Methods: Small intestine from 3 groups of premature lambs (n=5 from each) was analyzed. These groups included: lambs of 118-121 days estimated gestational age (EGA) delivered via caesarian section and placed on the AP (AP group); lambs of 115-121 days EGA and 125-131 days EGA delivered via caesarian section and immediately sacrificed (early tissue control, ETC group; late tissue control, LTC group, respectively). Post necropsy, jejunal samples were formalin fixed and stained for analysis with H&E, Ki-67, and lysozyme. From H&E slides, crypt depth (CD), villus height (VH), and CD:VH ratio were analyzed. From Ki-67 slides, cell proliferation was analyzed, and from lysozyme slides paneth cell counts were taken to determine small intestine response to injury. In addition, injury scores evaluating epithelial injury, congestion, villus atrophy, and inflammation were determined.

Results: CD, VH, and CD:VH ratio were similar between the three groups ($p>0.05$). AP lambs demonstrated more enterocyte proliferation (95.7 ± 21.8) than ETC lambs (49.4 ± 23.4; $p=0.003$) and LTC lambs (66.1+11.8; $p=0.04$), and more Paneth cells (81.7 ± 17.5) than ETC lambs (41.6 ± 7.0; $p=0.0005$) and LTC lambs (40.7 ± 8.2, $p=0.0004$). There was more epithelial injury and congestion seen in AP lambs compared to ETC and LTC lambs, however the results were not significant ($p=0.05$; $p=0.07$, respectively). No villus atrophy or inflammation was present in any group.

Conclusion: Results support that the AP preserves small intestine architecture and promotes cellular turnover. Small intestine injury on the AP was minimal. Future studies will focus on obtaining a profile of intestinal bacteria to investigate differences in microbiota composition between different AP lambs and AP and term lambs.

INTRODUCTION

Immune system of the gastrointestinal tract

It is easy to consider the immune system and bacteria as separate, but in actuality, a huge proportion of the immune system is located in the gastrointestinal (GI) tract. The GI tract is where bacteria and the immune system meet. Defense of the GI system includes the adaptive and innate immune systems as well as the protective benefits from the gut microbiota.

Induction of the gut adaptive immune response involves the usual activation of B and T cells by antigen presenting cells and the resulting effector functions of these adaptive cells. Secretory IgA (sIgA) antibodies are the major humoral defense factor of gut immunity. These antibodies serve as a main line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms. This is done through immune exclusion, promoting the clearance of antigens and pathogens from the intestinal lumen by blocking their access to gut epithelial receptors, entrapping them in mucous, and facilitating their removal by peristaltic and mucocilliary means[1]. Meanwhile, regulatory T cells have also been shown to play an important role in the cellular defense and response to intestinal antigens[2].

Induction of the gut innate defenses starts with physical and chemical barriers and extends to secreted antimicrobial peptides (AMPs). Physical barriers include the epithelial cell layer and actions of gut motility, and chemical barriers include mucous, bile, gastric acid, and pancreatic enzymes[3]. AMPs mostly come into play once a pathogen has breached these barriers

and invaded the mucosal membrane. When this happens, the intestinal cells recognize pathogen associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs)[4] such as Toll-like receptors (TLRs); when a PAMP binds a PRR, AMPs including cathelicidins, defensins, and lysozymes are secreted[5].

Defensins are small, cationic, antimicrobial peptides. They are found in high concentrations in the granules of phagocytes as well as in Paneth cells, specialized innate immune cells of the small intestine. Defensins have the ability to kill and inactivate a wide range of microbial pathogens including gram-negative and gram-positive bacteria, fungi, protozoa, and viruses. Their cationic and amphipathic nature allows them to easily cross negatively charged microbial membranes. Cathelicidins are a class of AMPs that have varying bactericidal potential. They are synthesized as larger precursor molecules with a variant C-terminal antimicrobial functional domain. The heterogeneity of the C-terminal is what allows for the wide range of cathelicidin activity, and there is a wide overlap in activity within the families of cathelicidins. Cathelicidin families can also be chemotactic for innate immune cells such as monocytes and neutrophils. Lysozyme is an extremely potent AMP. Lysozyme expression has been noted in a variety of cell and tissue types, including in the paneth cells of the small intestinal crypts. In the small intestine, lysozyme is found to be secreted into the crypts; it works to decrease the integrity of pathogens' cell walls, thus causing death of the pathogen[5].

Paneth Cells

As seen above, paneth cells are an important component of the GI innate immune response. They are located in the small intestine crypts of Lieberkühn. Their apical granules point towards the intestinal lumen, and synthesize and secrete AMPs and various proteins. They

are key in modulating and mediating host-microbe interactions, including the homeostatic balance with the colonizing microbiota and the innate immune protection from pathogens. They also secrete factors that have been known to help sustain and modulate the progenitor cells and epithelial stem cells of the crypts and play a role in revitalizing the small intestine epithelium.

Paneth cell dysfunction can contribute to the pathogenesis of inflammatory intestinal diseases[6]. A study by Nita H Salzman demonstrated that paneth cell defensins are actually able to regulate the composition of the intestinal bacterial microbiome. She noted defensin-dependent reciprocal shifts in the dominant bacterial species of the small intestine without changes in overall bacterial numbers. This further supports the importance of paneth cells and their secretory components in maintaining intestinal homeostasis[7].

Host microbiota function in defense

Beyond the immune system, immunity is also provided by the host microbiota. The microbiota is the name given to the complex and dynamic population of microorganisms that inhabit the gut. Soon after birth, organisms establish their own unique gut microbiota determined by their maternal flora as well as genetic and epigenetic factors[3]. Beyond these initial factors, diet is also main driver in shaping the microbiota of the gut across an organism's lifetime. The microbiota has co-evolved with their host to form an intricate and mutually beneficial relationship, so much so that these mutualistic intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting the gut by outcompeting pathogens. In addition, the microbiota also offers benefits to the host through physiological functions such as strengthening gut integrity, shaping the intestinal epithelium, and harvesting energy. In fact, altered composition of gut bacteria (dysbiosis) has been associated with the pathogenesis of many inflammatory diseases and infections[8]. This is why so many antibiotics

have gastrointestinal complications as side effects; they drastically alter the gut microbiota. The intestinal microbiota is extremely important for normal gastrointestinal development, protection, and function.

Necrotizing enterocolitis

When any aspect of gut immunity is compromised, aberrant colonization by pathogenic bacteria (i.e. infection) can lead to devastating effects. One such infection, which targets premature infants, is necrotizing enterocolitis (NEC). Just hearing this term strikes fear in the hearts of preemie parents, as NEC is one of the most catastrophic comorbidities associated with prematurity. It is a devastating gastrointestinal disease that is associated with severe sepsis, intestinal perforation, and significant morbidity and mortality[9]. Though the pathogenesis is still not fully understood and is believed to be multifactorial, dysbiosis by way of disruption or delay of acquisition of commensal microbiota has been implicated as a key risk factor in the pathogenesis of NEC. Conversely a beneficial complement of commensal intestinal microbiota has been found to protect the neonatal gut from inflammation and injury. Nowadays, interventions aimed at providing or restoring a healthy microbiota, such as probiotic therapy, are extremely promising treatments to prevent NEC. Shifting the balance of intestinal bacterial from pathogenic to protective can protect the gut from the inflammation and injury characteristic of NEC. Studying and better understanding NEC is incredibly important as 20-30% of infants who develop NEC will succumb to it[10]. The biggest risk factor for NEC is prematurity and an immature gastrointestinal tract; the disease disproportionately affects premature and extremely low birth weight (ELBW) infants.

Prematurity

Every year an estimated 15 million babies, around 1 in every 10, are born prematurely (less than 37 weeks gestational age) in the US. Being born prematurely usually comes with a host of complications that follow the child for their entire life, if they survive infancy; preterm birth complications are the leading cause of death for children under 5 years of age, and accounted for nearly 1 million deaths in 2015[11]. Among the preterm babies delivered, those born at less than 28 weeks, the micro-preemies, face the highest rates of mortality and complications.

Most of these babies are born at ELBW (weighing less than 2 pounds, 3 ounces) and almost all require treatment with surfactant, oxygen, and mechanical ventilation to help them breathe. In addition to their lungs and gastrointestinal tract, other organs and systems are underdeveloped, leading multiple complications outside of simply breathing and NEC. ELBW newborns can face neurological complications that extend from an immature renal system due to sodium retention, as their immature kidneys' cannot concentrate urine well. They are also especially susceptible to retinopathy of prematurity (ROP), a disease brought on by high oxygen levels that can cause retinal detachment and blindness if not treated. In addition, they are unable regulate their own temperature, and spend weeks in temperature controlled incubators to combat hypothermia[12].

For babies born under 24 weeks, the prognosis is most grim. Outcomes research puts survival for babies born at 24 weeks at 56%, 23 weeks at a mere 26%, and 22 weeks at only 5%[13]. Furthermore, most micro-preemies face one if not many of the above mentioned health complications because of how underdeveloped their organs systems are and more often than not, these complications contribute to, if not directly cause, death. For those babies lucky enough to survive their premature birth, most of these complications do not simply disappear once they

leave the NICU, and often other complications such as cerebral palsy, emotional and behavioral complications, and neurological delays can only be uncovered as the child grows and misses developmental milestones.

Due to severely underdeveloped lungs, premature babies are often left with bronchopulmonary dysplasia (BPD), a chronic lung disease that causes abnormal lung growth and inflammation. For some children, over time the lungs can heal some, but most face asthma-like symptoms throughout their lives. Babies who survive NEC in the hospital oftentimes have to undergo surgery to remove the infected parts of their intestines, and as a result of this shortening of their GI tract, can face issues with proper nutrient absorption from the food they eat. Furthermore, scarring from the NEC can lead to intestinal blockages and associated pain. Many babies who develop ROP, too, must undergo surgery in a timely fashion to correct the problem or face lifetime blindness. Still, even with proper medical treatment and surgical intervention, many ROP babies face vision problems as they age[14,15]. Overall, preterm birth accounts for the leading cause of death for children under the age of 5[11].

Mechanical ventilation and associated complications

The current standard of care for premature babies with severe respiratory distress syndrome (SRDS) is intubation followed by invasive mechanical ventilation. This allows for premature babies who cannot sufficiently support themselves breathing on their own to still receive oxygen and discard carbon dioxide[16]. In regular breathing, the inhalation process is active. The diaphragm contracts, moving downwards and increasing the space in the chest cavity. This allows for the lungs to expand with air. The exhalation process is passive. The diaphragm relaxes and moves upwards; this tightening of space forces air out of the lungs[17]. Invasive

ventilation works by way of positive pressure. Similar to the active inhalation process, it blows air, with or without extra oxygen depending on the baby's needs, into the airways and lungs. However, on a ventilator, the exhalation process is also active, as the ventilator takes over this part of breathing as well[18]. Since the introduction of invasive mechanical ventilation as standard of care for premature babies, mortality from SRDS has decreased from 268 in every 100,000 births in 1971 to 14.7 in every 100,000 births in 2008[19].

However, while invasive mechanical ventilation has saved the lives of countless newborns with SRDS, it comes with many complications of it's own. Mechanically, the small size of the patients results in challenges with high respiratory rate, rapidly changing lung compliance, highly compliant chest wall, short inspiratory time, and small tidal volumes. In addition, as literature has shown associated tracheal injury using cuffed endotracheal (ET) tubes[20], premature infants are intubated with uncuffed ET tubes, and are thus victim of air leaks. These air leaks coupled with incredibly small tidal volumes (sometimes as low as 2-3 mL) makes detection of flow and accurate measurement of both inspiratory and expiratory tidal volume extremely complex[16].

Besides mechanical difficulties, this kind of positive pressure ventilation can actually contribute to the health complications premature infants face, such as BPD[21]. This disorder is primarily seen in ELBW infants and micro preemies requiring ventilator support and supplemental oxygen, as oftentimes, their frail and immature lungs simply cannot handle the positive pressure force of the ventilator. These patients experience decreased alveolarization of pulmonary tissue and small airway diseases, as well as inflammation and fibrosis, at a reported rate reported between 30 and 40%[22].

Other complications of mechanical ventilation include intraventricular hemorrhage (IVH), again, due to the positive pressure being forced into the baby's system, and NEC. In fact, a study done by Carter and Holditch-Davis showed length of mechanical ventilation as a primary predictor of NEC in preterm infants. Infants who required mechanical ventilation during the neonatal period were 13 times more likely to develop NEC[23], and as the number of days an infant spent on a mechanical ventilator increased, so did their risk of developing NEC[24].

The Artificial Placenta

Currently, mechanical ventilation can be seen as the lesser of two evils, preferable to death by SRDS but not the solution. What premature infants really need is some sort of technology that can simulate the intra-uterine environment, thus allowing for continuation of critical organ growth and development despite a newborn's early arrival. The artificial placenta (AP) is exactly this kind of technology. The AP uses extracorporeal membrane oxygenation (ECMO) to provide the premature baby with adequate oxygenation and carbon dioxide removal without their underdeveloped bodies needing to do any of the hard work. An ECMO circuit consists of cannulas placed into major blood vessels (an artery and vein for AV-ECMO or two veins for VV-ECMO) that are connected via silicon tubing to a pump and oxygenator. Based on how the circuit is hooked up, ECMO can act as either a patient's lungs (in the case of VV ECMO) or their heart and lungs (AV-ECMO). When a patient is on ECMO, blood drains out of their body and is pumped through an oxygenator. Within the fibers of the oxygenator, oxygen is diffused into the blood and carbon dioxide out of the blood. This blood is then circulated back into the body[25]. This is life saving technology.

The AP functions by utilizing VV-ECMO while still maintaining fetal circulation[26,27]. Fetal circulation differs from typical circulation in that in-utero, the placenta does the work the developing lungs are supposed to do. Furthermore, fetuses have three shunts, the ductus venosus, ductus arteriosus, and foramen ovale, which direct blood around the lungs and liver. In a healthy newborn, fetal circulation ceases when the baby is born and takes their first breaths of air. However, for a premature newborn on the AP, the end goal is to "trick" the baby into thinking they are still in-utero, so prostaglandins are given intravenously (IV) and the oxygen saturation of the blood is left low to keep the ductus arteriosus patent. The ductus arteriosus connects the pulmonary artery to the descending aorta and allows blood from the right ventricle to bypass the lungs, allowing them to develop without risk of congestion from too much blood flow[28].

The goal of the AP is to be a passive environment that provides extremely premature newborns with the support they need to grow and develop as they would in-utero. In addition, as the AP doesn't require invasive mechanical ventilation or any sort of positive pressure, associated risks such as BPD and IVH become non-issues. The hope is that this device will also lead to a decrease in cases of NEC. The AP is supposed to both decrease morbidity and mortality associated with preterm birth and increase quality of life for premature newborns. This being said, investigation into the device's impact on all organs must be done to make sure this is actually the case. This investigation will start with the small intestine.

This study aimed to understand small intestine growth and injury of extremely premature lambs on the AP in order to better comprehend why the premature gut is so much more susceptible to NEC. This analysis provides a structural understanding by specifically evaluating mucosal architecture, cellular proliferation, injury, and response to injury. We hypothesized that small intestine architecture would be preserved during AP support and injury and response

would be comparable to tissue controls. The hope is that a better understanding of structural development on the AP will lead to future microbiological studies of the lambs' small intestine to understand the effects of the AP on microbiota development. Together, this structural investigation and microbiological survey should provide a better understanding of NEC susceptibility in premature infants.

METHODS[29]

The experimental procedure was performed in an ovine model following protocol approval by the University of Michigan Institutional Animal Care and Use Committee (IACUC) (protocol 00007211). All sheep used for the experiment were treated in compliance with the Guide for Care and Use of Laboratory Animals, 8th edition.

Lambs used for the experiment were divided into three groups: Artificial Placenta (AP) Group, Early Tissue Control (ETC), and Late Tissue Control (LTC). Age and weight of each lamb were recorded.

Experimental Groups

AP Group

Premature lambs of 116-121 days EGA (term = 145; n =5) were delivered via C-Section. 10-14Fr cannulas (Terumo: Ann Arbor, MI) were placed in the jugular vein (drainage) and umbilical vein (reinfusion). The circuit was completed with ¼" tubing (Tygon: Lima, OH), a collapsible-tubing roller pump (MC3: Ann Arbor, MI), and oxygenator/heat exchanger (either Medos HiLite, Xenios: Heilbronn, Germany or Capiox Baby Rx, Terumo, Ann Arbor, MI; **Figure 1**). Veno-venal extracorporeal life support (VV-ECLS) was initiated and the lambs were

monitored closely. A 5 Fr arterial line (Covidien-Medtronic: Minneapolis, MN) was placed into the umbilical artery for hemodynamic monitoring and arterial blood gas (ABG) blood draws. The second umbilical vein was cannulated with a 5 Fr triple lumen venous line (Covidien-Medtronic: Minneapolis, MN) for intravenous fluid, TPN, heparin sulfate (SAGENT, Schaumburg, IL) (100 U/hr, titrated to a goal activated clotting time (ACT) of 200-250 seconds), and Prostaglandin E_1 (Pfizer, New York, NY) (0.2mcg/kg/min) infusion to maintain ductal patency. The lambs were intubated and lungs were filled with fluid (amniotic fluid, Ringer's Lactate, or perfluorodecalin [Origen: Austin, TX]).

All lambs were supported on TPN infused via the umbilical vein. The TPN (ExactMix, Baxter Healthcare Corporation, Englewood, CO. Baxter International Inc. Supplied by the University of Michigan HomeMed- Home Infusion Pharmacy) was made with a standard composition including: Amino Acids (15%) (16 GM); Dextrose (45 GM); IntraLipds (80 ML); and electrolytes (Sodium Phosphate (5.4 MM); Potassium Chloride (8.1 MEQ); Magnesium Sulfate (1.6 MEQ); and Calcium Gluconate (1 MEQ)) in 400 mL volume administered at a rate of 5 mL/kg/hr. All AP lambs remained nil per os (NPO) during support. Hemodynamics, urine output, and bowel movements were monitored. All lambs were given prophylactic intravenous antibiotics (piperacillin-tazobactam [Hospira Inc., Lake Forest, IL]) and antifungals (fluconazole [SAGENT, Schaumburg, IL]) to prevent infection. Solumedrol (Pfizer, New York, NY) 0.63 mg/kg was given every 12 hours to prevent hypocortisolemia. Diazepam (Hospira Inc., Lake Forest, IL) 2.5mg and Buprenorphine (Parr Inc., Spring Valley, NJ) 0.3mg were used sparingly for pain or agitation. In cases of volume-resistant hypotension, vasopressors (norepinephrine (Claris LifeSciences Inc., North Brunswick, NJ), epinephrine (Hospira Inc., Lake Forest, IL), or

dopamine (Baxter, Deerfield, IL)) were used to maintain a (mean arterial pressure) MAP >40 mmHg. AP support was continued for 7-10 days, and then the animals were euthanized.

Tissue Control Groups

Early and Late Tissue Controls (ETC; n=5 and LTC; n=5) were delivered at 115-121 and 125-131 days respectively. They were immediately sacrificed.

Necropsy, Tissue Preparation, and Histological Analysis

After sacrifice, the small intestine was removed en bloc and formalin-fixed. Standardized 2 cm longitudinal sections of jejunum were taken from 6 cm distal to the duodeno-jejunal junction to be used for mucosal measurements and staining. Sections from proximal, mid, and distal jejunum, terminal ileum, and cecum were also harvested for injury scoring. All samples were sectioned 3-5 μm thick. Slides were stained with Hematoxylin and Eosin (H&E), Ki-67, and Lysozyme, then digitized and reviewed via ImageScope (Leica Biosystems Imaging, Inc 2016, USA). Cell counts and measurements were summed for each sample then averaged for each group. Injury scores were marked as present or absent in the 5 anatomic locations for each animal. All measurements were done in a blinded fashion.

Histological Measurements

H&E Stain to Evaluate GI Mucosa Crypt Depth and Villus Height

Slides were stained with H&E (Fischer Scientific, Pittsburgh, PA). After review of each slide using ImageScope, Crypt depth (CD), villus height (VH), and CD:VH ratio (12/slide) were measured in micrometers and averaged. Measurements of CD and VH were only taken for those

in which the plane of sectioning ran vertically from the tip of the villus to the base of the adjacent crypt. VH was measured from tip of villus to the crypt mouth, and CD was measured from crypt mouth. For each slide, 12 of the most representative villi were used for measurement and the corresponding 12 crypts.

Ki67Antibody Stain to Measure GI Epithelial Cell Proliferation

Slides stained with Ki67 antibody (Abcam, Cambridge, MA) were used to evaluate GI enterocyte proliferation. 50 crypts from 5 different places of 10 consecutive crypts were reviewed per slide, and all the cells in the crypts stained dark brown with Ki67 were counted per slide and averaged. Measurements were taken and compared between groups.

GI Mucosal Injury Severity Scores:

H&E-stained slides were reviewed and scored by experienced pediatric pathologist, Dr. Raja Rabah, who was blinded to the groups. Intestinal injury scores were measured using presence of inflammation, epithelial injury, congestion, hemorrhage, and villus atrophy. Presence was measured as 1 and absence as 0. Since there was absence of inflammation, villus atrophy, and only minor hemorrhage among the groups, only epithelial injury and congestion were compared between groups.

Paneth Cells Stained with Lysozyme Antibody to Measure Immune Response

Proximal jejunal sections were stained with Lysozyme antibody (Invitrogen, IL, USA). Images with clearly defined Paneth cells within the crypts were included in the analysis. Paneth

cell counts were averaged per slide. Those values were then averaged per animal in each group and compared between groups.

Statistical Analysis

The Graphpad Prism version 7.0 statistical software (GraphPad Software Inc, 2017, USA) and ANOVA with post-hoc Tukey's Test was used to compare groups with Ki-67 stain, Lysozyme stain, and CD, VH, and CD:VH Ratio. IBM Corp. Released 2013. IBM SPSS Statistics, Version 22.0 (Armonk, NY), Fischer Exact Test was used to compare presence of injury score for each anatomic GI location for epithelial injury and congestion. $P<0.05$ defined statistical significance.

RESULTS[29]

GI Mucosal Development in Premature Lambs (H&E Stain)

The objective of staining the GI slides with H&E was to evaluate GI mucosal development. There was no significant difference between CD in the small intestines of ETC (68.5±7.3) and LTC (77.2±8.2) with p=0.18. Although the CD in AP small intestine (77.5±5.9) was not statistically different than ETC (p=0.16) and LTC (p=0.99), there was an increasing trend of AP CD similar to that of the LTC group. VH of the AP Group (315.6±70.4) was comparable to ETC (314.9±7.3; p=0.99) and LTC (297.9±32.6; p=0.88), without differences compared to ETC and LTC VH (p=0.89). Similarities were also found between the AP CD:VH Ratio (4.1±0.9) compared to ETC (4.7±1.4; p=0.58) and LTC (3.9±0.6; p=0.96). No differences were found between CD:VH ratio of ETC and LTC (p=0.42) (**Figure 2**).

GI Proliferation in Premature Lambs (Ki67 Stain)

The objective of staining the GI slides with Ki-67 was to evaluate enterocyte proliferation in the GI tract. Significantly more enterocyte proliferation was found in the AP group (95.7±21.8) when compared to ETC (49.4±23.4; p=0.003) and LTC (66.1+11.8; p=0.04). There were no significant differences between both tissue control groups (p=0.35) (**Figure 3**).

GI Paneth Cell Count in Premature Lambs (Lysozyme Stain)

The objective of staining the GI slides with lysozyme was to obtain paneth cell counts in order to determine intestinal response to injury. There were significantly more positively stained Paneth cells in the AP Group (81.7±17.5) when compared to both ETC (41.6±7.0; p=0.0005) and LTC (40.7±8.2; p=0.0004). The number of lysozyme stained Paneth cells were similar between the ETC (41.6±7.0) and the LTC (40.7±8.2), p=0.99.

GI Injury Severity in Premature Lambs (H&E Stain)

The objective of analyzing GI injury was to determine injury severity between the AP, ETC, and LTC lambs. There was no evidence of inflammation or villus atrophy in any of the three groups (AP, ETC, LTC). One AP animal exhibited presence of hemorrhage in the mid jejunum and colon, but otherwise there was no hemorrhage found in any other animals or anatomic locations. Epithelial injury was present in ETC and AP groups, while congestion was present in LTC and AP groups. The distal jejunum showed more epithelial injury and congestion in the AP group, however these numbers were not statistically significant. There was, however, a trend towards significance (p=0.05; p=0.07, respectively). There was otherwise no statistical difference between groups (**Table 1)**.

DISCUSSION

This study aimed to investigate small intestine growth and development as well as intestinal injury and response to injury. The data showed a protective effect from the AP on intestinal growth and development, as lambs supported on the AP maintained intestinal structure and integrity. This was evidenced by CD, VH, and CD:VH ratio. Overall, the small intestine of AP supported lambs showed minimal evidence of injury. AP lambs also showed an appropriate and adequate response to injury evidenced by increased cell proliferation and paneth cell counts.

CD, VH, and CD:VH ratio are literature-supported measures of intestinal growth and development. As a fetus develops in utero, villus length and crypt depth increase, finally reaching maturity around term. Part of this increase is thought to be due to increase in cell proliferation and slower rates of cell shedding when compared to proliferation[30]. The data from this study showed no significant difference in CD between the AP, ETC, and LTC groups, supporting that small intestine structure is preserved in lambs on the AP. Additionally, though the numbers did not reflect true significance, CD of AP lambs increased towards CD of LTC lambs, providing evidence that the AP might actually aid intestinal growth and development. VH and CD:VH ratios of AP lambs were similar to both ETC and LTC lambs. Furthermore, the increase in cell proliferation and CD in AP supported lambs is consistent with the in utero increase in CD and VH, further supporting the AP's clinical relevance as a simulation of the intra-uterine environment.

Apart from being evidence of CD and VH increase with intra-uterine development of the fetus, increase in cell proliferation is also seen as a response to injury[31]. Paneth cells, too have been known to be markers of both intestinal development[32] and an injury/inflammation

response[33]. Paneth cells first appear in the first trimester of pregnancy, mature by the age of viability (22-24 weeks), and then increase in number by full term gestation[32]. Thus, they increase and mature as the fetus develops. It is these mature paneth cells that then work as markers of inflammation and injury. Upon invasion of the intestines by pathogenic bacteria and fungi, paneth cells release AMPs, lysozyme, and growth factors into the lumen of the intestines in an attempt to protect and preserve the intestinal epithelia[33]. In pathogenic intestinal infections such as NEC, after the acute phase infection, studies have shown that paneth cell numbers decrease[34]. However, after recovery from the inflammation, paneth cells are upregulated[35]. Thus, an increase in paneth cells is evidence of proper response to intestinal injury. The increased cell proliferation and increased paneth cell counts seen in the AP supported lambs provide evidence of both intestinal development and an appropriately protective response to injury.

Consistent with the increased proliferation and paneth cell counts, the small intestine tissue of AP supported lambs did show minor evidence of injury. This injury can be classified as minor because despite the injury, intestinal structure was preserved, as evidenced by CD and VH measurements. To determine small intestine injury, this study investigated inflammation, villus atrophy, hemorrhage, congestion, and epithelial injury, paralleling the variables of epithelial cell inflammation, edema, necrosis, and hemorrhage used in established studies[36]. Regardless of group, none of the lambs' intestines showed evidence of inflammation or villus atrophy. There was minimal hemorrhage noted in the mid-jejunum and colon of one AP supported lamb; all other lambs from all groups showed no hemorrhage anywhere in the small intestine. There was, however, noted congestion and epithelial injury in the distal jejunum in AP supported lambs; this might suggest that this area of the intestine is more susceptible to injury, however further studies will need to be performed to make any definitive conclusions.

Small intestine injury in AP supported lambs can arise from a variety of factors including the administration of vasopressor medications, hypoperfusion, and lack of enteral feeds.

Two of the 5 AP lambs were supported on vasopressor medications for anywhere from 3-6 days. Vasopressor medications can increase peripheral vascular resistance. As a result, they can cause intestinal hypoperfusion, as they are oftentimes associated with a decrease in regional blood flow, which can also lead decreased splanchnic blood flow. Impaired blood flow to splanchnic organs including the small intestine can cause injury such as intestinal necrosis, renal insufficiency, and gastric ulceration[37]. Furthermore, the tissue damage that results from this reduced perfusion of the small intestine can cause damage to and disruption of the mucosal barrier, thus allowing bacteria in the GI tract access to systemic circulation[38]. This can result in severe infection leading to systemic sepsis[39]. Though no such evidence was seen in this study, this is something to be considered with the clinical translation of the AP, especially given the increased susceptibility of premature infants to infection.

Hypoperfusion of the intestines could also be due to the actual AP device. The AP utilizes ECLS technology, wherein the blood is oxygenated outside of the body. Normally, under rest conditions, the intestines receive about 20% of cardiac output and anywhere from 20-35% of systemic oxygen delivery. However, blood flow and oxygen supply to the GI tract is reduced in critical conditions to maintain perfusion to the brain and heart muscle[39]. When the blood pressure of a patient on ECLS drops, similar effects occur, and many of the AP supported lambs showed intermittently low MAPs as low as mid 20s (target range of MAP in this study was 40-50).

Most notably, however, is the fact that all the AP lambs were maintained NPO, getting their nutrients solely from TPN. In utero, the human fetus is constantly inhaling and swallowing amniotic fluid, infesting anywhere from 750-1000 mL daily. Meanwhile, a sheep fetus ingests

anywhere from 100-1000 mL daily[40]. Established studies have shown that swallowing this fluid can have a protective effect on the intestines, and in fact contributes to increased mucosal development. Conversely, a lack of fluid leads to decreased birth weight and abnormal intestinal structure, including decreased intestinal mucosal thickening, and decreased VH and villus cell density[41]. This just goes to show that liquid enteral nutrition provides a positive effect to developing intestines. Further supporting this point is the fact that premature human infants in the NICU are given minimal amounts of breast milk enterally in an effort to prime their GI tracts. Studies have shown that premature human infants provided with minimal enteral nutrition have lower rates of NEC than those maintained NPO[42]. This could be due to the fact that the microbiome is vital in protecting the intestines and enteral nutrition contributes to timely acquisition of commensal microbiota. Thus, a lack of enteral feeding can contribute to a dysbiosis that can increase NEC susceptibility[43].

There are potential limitations to this study. First and foremost, the AP lambs were only supported on the device for 7 days. It might take more than 7 days for changes to occur to the bowel mucosa. This is could be another explanation for why CD, VH, and CD:VH ratios were similar between the AP, ETC, and LTC groups. Furthermore, the administration of vasopressor medications was not consistent across all the lambs in the AP group. Earlier experiments had a lower threshold for starting these medications, but currently, these medications are avoided unless absolutely necessary. Thus it is possible that future AP experiments (on the current vasopressor protocol) will show less bowel injury. Lastly, this study only focused on changes in bowel structure as cause for injury even though the importance of the microbiota in maintaining a healthy GI tract and providing protection against injury and inflammation is very well known[8].

Nevertheless, overall it seems as though the AP is doing what it is intended to do. The data from this study supports that premature lambs on the AP show intestinal structure and development similar to the in-utero structure and development of the tissue control lambs and that AP lambs and show similar responses to inflammation and injury as term infants. However, despite this, a number of the AP supported lambs have succumbed to GI related complications including suspected NEC. This study showed that these complications aren't likely due to structural or developmental issues, thus future studies on AP lambs will focus on investigating the gut microbiome. Comparisons can be done between AP lambs showing evidence of GI compromise or that died of supposed NEC and those that were electively sacrificed.

During necropsy of all AP and tissue control lambs, small sections of the small intestine were obtained and frozen for future analysis. I propose a slow thawing of this tissue, followed by homogenization and PCR to replicate the 16S rRNA gene. By doing this, only the bacterial cells will be amplified. The PCR product can then be sent off for next generation sequencing. This will allow for identification of the bacterial species that colonized the small intestines of the AP lambs. These bacterial profiles can be compared between lambs electively sacrificed and those suspected of having GI complications. Furthermore, a study can be done comparing these microbiological profiles from those obtained from stool samples from term lambs. NEC disproportionately affects premature infants, so maybe this can elucidate whether there are differences in the microbiota of premature vs. term infants. If so, these profiles could provide valuable information about bacteria associated with NEC.

CONCLUSION

This study supports the hypothesis that intestinal architecture would be preserved during AP support and that injury and injury response of AP supported lambs would be comparable to tissue controls. The AP can provide revolutionary support to extremely premature newborns, allowing them to continue normal organ growth and development even after birth in a simulated intra-uterine environment. This device will be promoted both as a way to decrease morbidity and mortality and to increase quality of life for extremely premature newborns. As a result, thorough investigation of its effects on the various organ systems needs to be done. The analysis of the effect of the AP on intestinal growth and development has been, so far, positive.

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Figures[29]:

Figure 1. AP Diagram

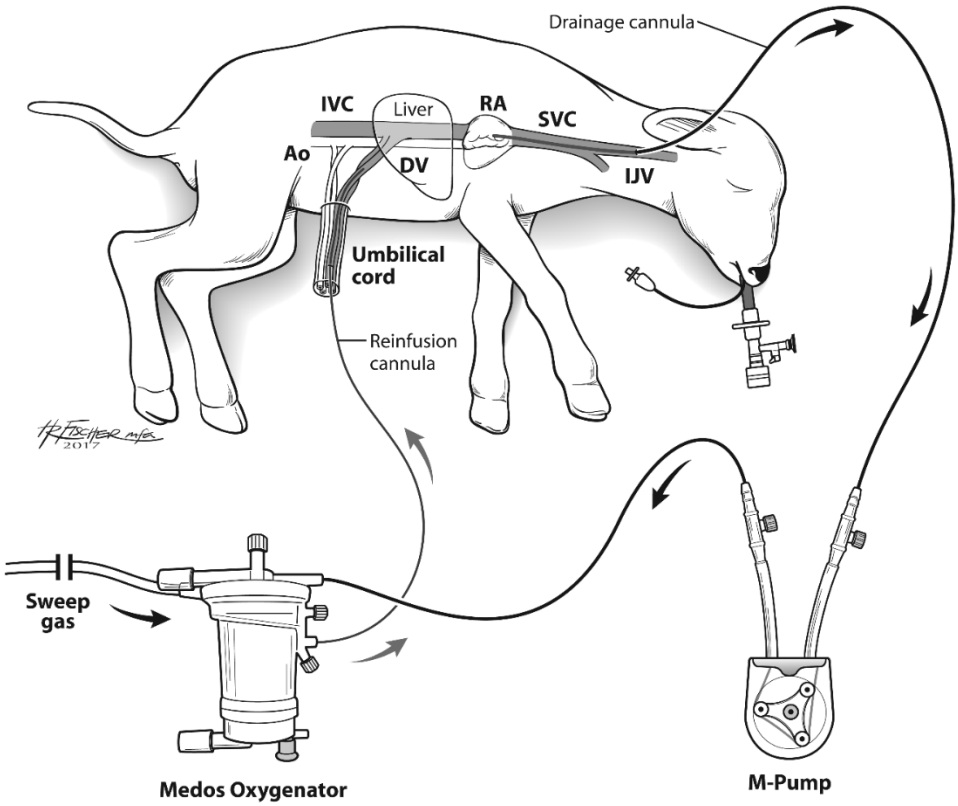

Fig. 1[29]**.** Schematic of the artificial placenta (AP) circuit in a premature lamb model demonstrating the cannulated lamb for VV ELCS, collapsible-tubing roller pump (M-Pump), and oxygenator/heat exchanger. Ao: Aorta; IVC: Inferior Vena Cava; DV: Ductus Venosus; RA: Right Atrium; SVC: Superior Vena Cava; IJV: Internal Jugular Vein. Thanks to Dr. Alvaro Rojas-Peña for the providing this schematic design.

Figure 2. GI Structure: Crypt Depth and Villus Height

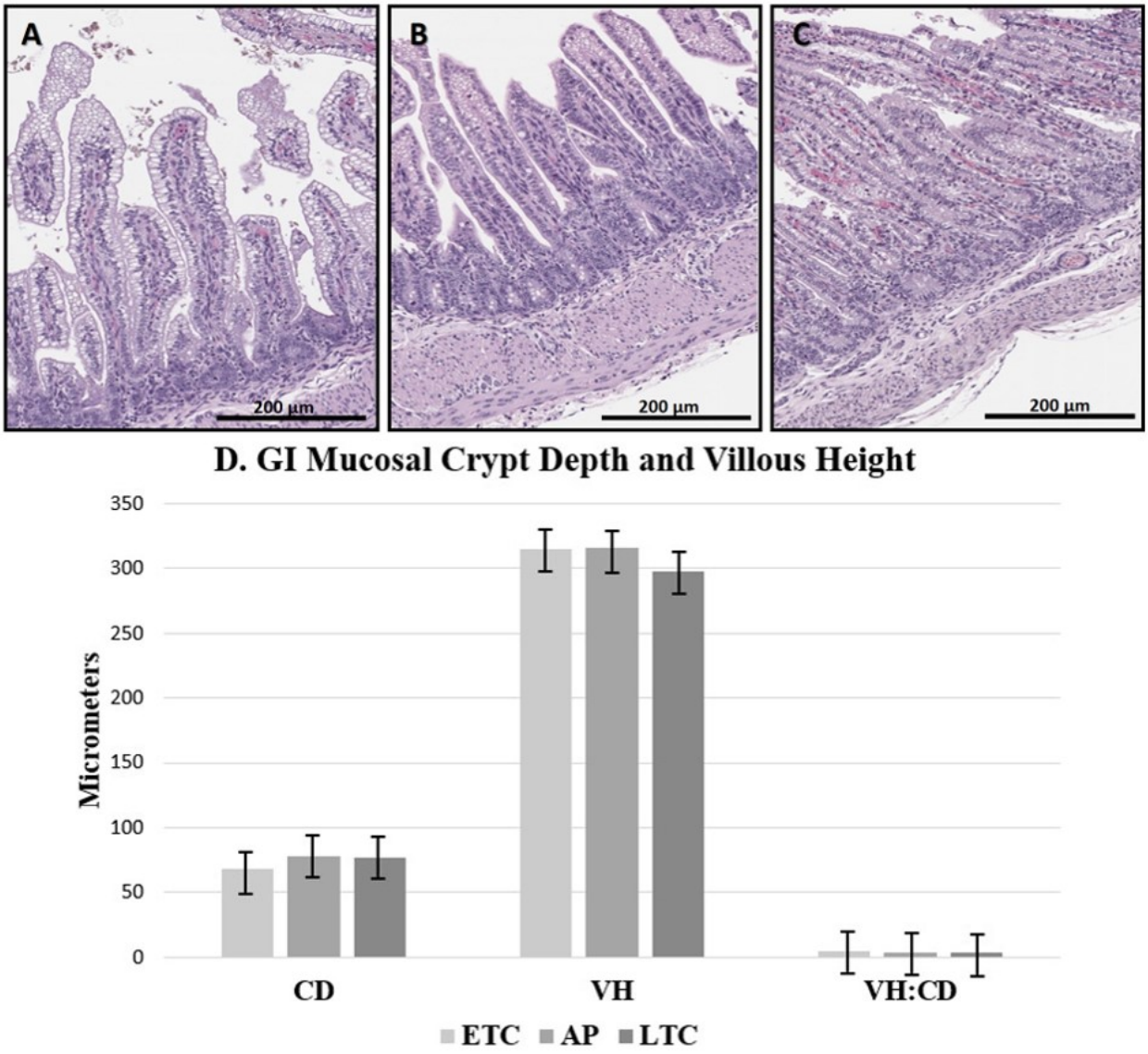

Fig. 2[29]**.** Representative histological images and graph comparing GI morphology of all groups. **(A)** ETC [H&E Stain]; **(B)** AP [H&E Stain]; **(C)** LTC [H&E Stain]; H&E, 200x magnification; **(D)** Graph demonstrating no significant difference when comparing Crypt Depth (CD), Villus Height (VH), or CD:VH Ratio of all groups. ETC: Early Tissue Control; AP: Artificial Placenta; LTC: Late Tissue Control. P-values calculated by ANOVA. * Indicates significance $p<0.05$.

Thanks to Dr. Jennifer McLeod for assistance in making the graph schematic (D).

Figure 3. GI Enterocyte Proliferation

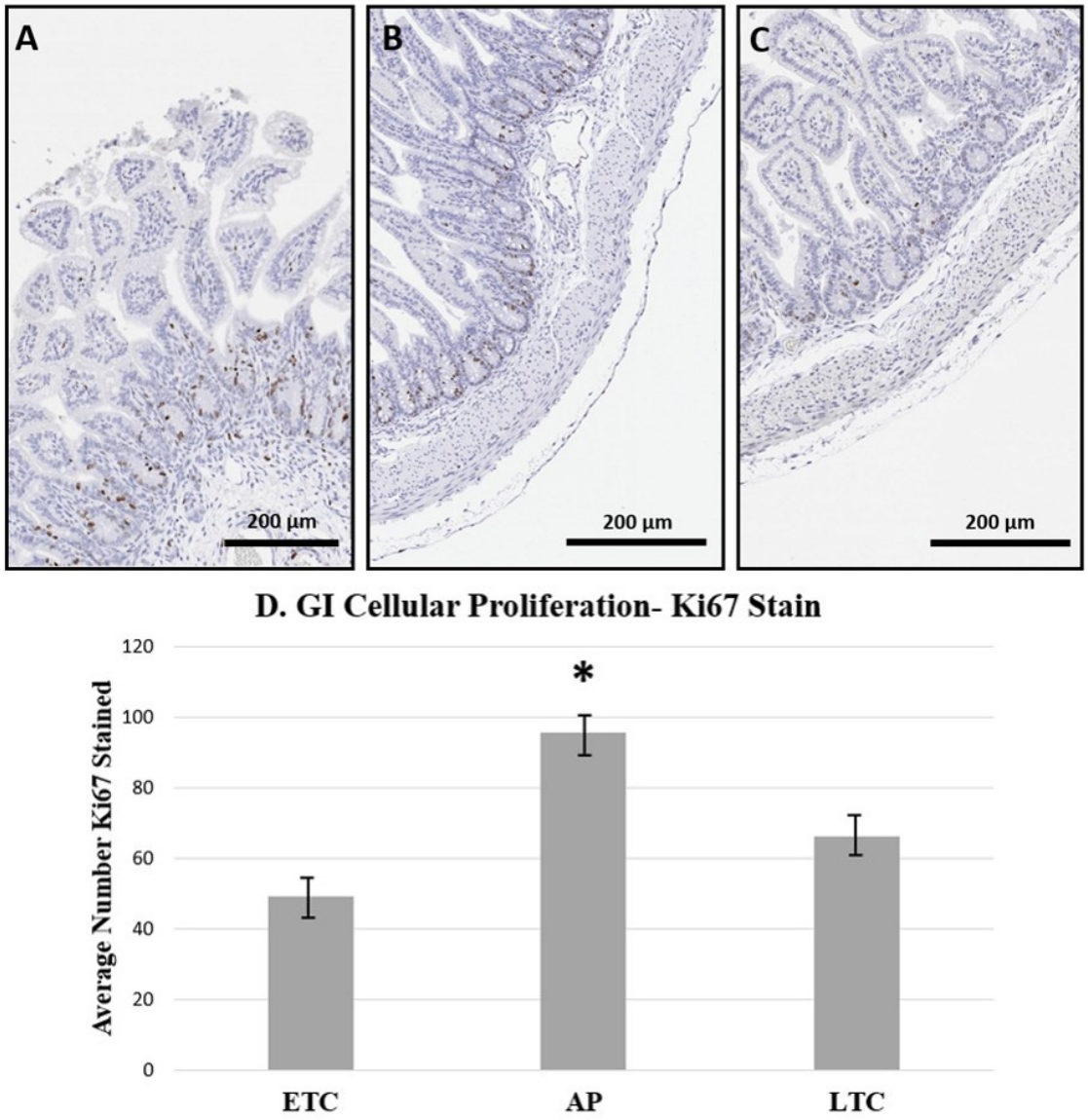

Fig. 3[29]**.** Representative histological images and graph comparing enterocyte proliferation of all groups. **(A)** ETC [Ki-67 Stain]; **(B)** AP [Ki-67 Stain]; **(C)** LTC [Ki-67 Stain]; Ki-67, 200x magnification; **(D)** Graph demonstrating significantly increased proliferation in enterocytes of the AP group compared to both ETC and LTC groups. ETC: Early Tissue Control; AP: Artificial Placenta; LTC: Late Tissue Control. P-values calculated by ANOVA. * Indicates significance $p<0.05$. Thanks to Dr. Jennifer McLeod for assistance in making the graph schematic (D).

Table 1. Presence of GI Mucosal Injury

		AP (n=5)	ETC (n=5)	LTC (n=5)	p-value
Prox Jej	***Epithelial Injury***	**2**	**1**	**0**	**0.73**
	Congestion	**2**	**0**	**1**	**0.73**
Mid Jej	***Epithelial Injury***	**3**	**1**	**0**	**0.23**
	Congestion	**3**	**0**	**1**	**0.23**
Distal Jej	***Epithelial Injury***	**4**	**1**	**0**	**0.05**
	Congestion	**4**	**0**	**2**	**0.07**
TI	***Epithelial Injury***	**3**	**1**	**0**	**0.23**
	Congestion	**3**	**0**	**2**	**0.25**
Colon	***Epithelial Injury***	**1**	**0**	**0**	**1.0**
	Congestion	**1**	**0**	**0**	**1.0**

Table 1[29]**.** Numbers represent number of samples manifesting injury type within specified region (out of n=5). AP: Artificial Placenta; ETC: Early Tissue Control; LTC: Late Tissue Control; TI: Terminal ileum; Jej: jejunum; Prox: proximal; p-values calculated by Chi-square. * Indicates significance $p<0.05$. Thanks to Dr. Jennifer McLeod for assistance in creating the table schematic.

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