ABSTRACT

MCKINNEY-AGUIRRE, CAROLINE ANN. Advancing Biomimetics to Accelerate Intestinal Epithelial Repair. (Under the direction of Dr. Liara Gonzalez).

The following projects evaluate the potential of biological extracts and a biologically relevant machine system for accelerating intestinal epithelial repair following severe injury. The three primary disease models explored in this work are Necrotizing Enterocolitis (NEC) in neonatal infants, severe colic in horses, and intestinal failure requiring transplantation in adults. All have unacceptably high mortality rates and are impossible to prevent, thus necessitating effective treatments, versus reliance on preventatives. Intestinal epithelial recovery following severe injury requires activation of the intestinal stem cell (ISC) niche. Unfortunately, there are no current clinically employed therapies which directly upregulate ISC-mediated epithelial healing. Most treatments are designed to remove the most severely affected intestine, if possible, and then provide the patient with supportive care until their intestinal epithelium can recover on its own. This delayed timeline, however, contributes to these elevated mortality rates and thus warrants more direct intervention.

Chapter 1 reviews experimental agents evaluated in NEC to highlight promising cellular mechanisms which may contribute to the development of novel therapeutics. This review emphasizes the need for studies to be done with therapeutic agents for NEC as over two-thirds of those in literature only evaluate agents prophylactically. Many of the reports analyzed in this review used murine models of disease. While these models are very valuable, they have inherent limitations in translatability, especially when modeling severe intestinal disease and recovery, which is reviewed in Chapter 2.

The remaining chapters report findings from projects utilizing large animal models and their primary cell cultures. Chapter 3 addresses the need for novel therapeutics in NEC with an evaluation of a human placental extract (HPE) applied to neonatal porcine models therapeutically and preventatively. Through this work, we demonstrate that HPE can enhance the reparative capacity of the neonatal epithelium in the context of NEC. This is demonstrated through an upregulation of epithelial migration and proliferation, *in vitro*, and reduced tissue injury, weight loss, and increased epithelial crypt proliferation *in vivo.* With encouraging results using HPE, we then created a parallel product from equine placentas (ePE) to evaluate in *in vitro* models of severe colic, which often results in similar epithelial injury to that observed in NEC. This work is

summarized in Chapter 4. Chapter 4 also includes a discussion of the application of extracellular vesicles (EVs), known to be produced by the placenta and a potential subcomponent of ePE, in the same models of intestinal injury in horses. Like findings regarding HPE, ePE and EVs were able to upregulate *in vitro* equine epithelial repair through increasing epithelial migration and, unique to these products, tight junction recovery.

Moving from biological products to biologically relevant systems, Chapter 5 evaluates a novel method of intestinal allograft storage prior to transplantation: Normothermic Machine Perfusion (NMP). The current standard of care for pre-transplantation intestinal storage, Cold Storage (CS), contributes to prolonged ischemia reperfusion injury within the graft and the low survival rates following intestinal transplantation. Previous work in the Gonzalez lab demonstrated that NMP is superior to CS in preserving intestinal allograft epithelial health and proliferative potential. In this chapter, the impact of NMP on allograft inflammation, a significant contributor to graft rejection, is explored. We demonstrate that NMP reduces inflammatory cell populations while increasing certain markers which may be associated with epithelial recovery.

Continued investigations of novel therapeutics are needed to enhance epithelial recovery from severe intestinal injury. Additionally, improved understandings of the mechanisms involved in upregulating repair will expedite these efforts. Future work, as outlined in Chapter 6, will determine the mechanisms contributing to this repair with the ultimate goal of identifying novel therapeutics.

Advancing Biomimetics to Accelerate Intestinal Epithelial Repair.

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina
2024

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DEDICATION

I dedicate this dissertation to my family. Joe, Gabriel, Simon, Oscar, and Luna, thank you for being my inspiration and constant source of love and happiness. I am eternally grateful for you. To my parents, thank you for supporting my passion for learning my entire life. Mom, thank you for encouraging my love for animals and sacrificing so much so I could achieve my goals. Dad, thank you for always being willing to help. Thank you also to the many animals who have cared for me as much as I could ever care for them.

BIOGRAPHY

Caroline Ann McKinney was born on April 29, 1990, in Mount Kisco, New York. She grew up with her younger brother, Connor, dogs, cats, cockatiels, horses, and a pony. Growing up, her family moved from Connecticut to Florida and then settled in a suburb of Chicago. There, Caroline graduated from Neuqua Valley High School in 2008. She went on to receive a Bachelor of Arts degree in Medicine, Health, and Society from Vanderbilt University in Nashville, Tennessee in 2012. It was at Vanderbilt that Caroline met the love of her life, Joseph Aguirre. Following graduation, Caroline relocated to Massachusetts to pursue a dual Doctorate in Veterinary Medicine and master's in public health at the Cummings School of Veterinary Medicine at Tufts University, graduating in 2016. Caroline then returned to Nashville to complete a one year rotating equine internship at Tennessee Equine Hospital in Nashville, where she gained experience in internal medicine, surgery, anesthesia, and field medicine. Intrigued by complex medical cases, she returned to Tufts to pursue a Large Animal Internal Medicine residency, but this time brought her now fiancé, Joe. During residency training, Caroline became especially interested in intestinal disease and equine colitis. While working on projects evaluating fecal microbial transplantation for patients with colitis, she also developed a passion for research and drive to understand the mechanisms of intestinal injury, recovery, and how biologic products can be best leveraged to encourage healing. To pursue her research interest, Caroline moved to North Carolina the fall of 2020, to begin a PhD in Comparative Biomedical Sciences at North Carolina State University (NCSU) under the direction of Dr. Liara Gonzalez. She has been blessed to learn so much through this program and meet many friends and mentors along the way. Caroline has a passion for intestinal physiology, teaching, and internal medicine. She is looking forward to a career combining these interests in veterinary academia.

ACKNOWLEDGMENTS

Firstly, I would like to acknowledge the animals who made these studies possible. Their contributions will alleviate suffering in veterinary and human patients alike and their sacrifice is thoroughly appreciated. There are many people to thank for their help and guidance through this PhD process. Dr. Liara Gonzalez, thank you for welcoming me into the lab and showing me constant support in all my interests and inquiries. Your mentorship, guidance, friendship over the past 4 years has been an invaluable gift. Thank you also to the other members of my committee -- Drs. Casey Theriot, Ben Callahan, John Rawls, and Matt Fisher - for your support and guidance through my development into a PhD. To the Gonzalez lab members past and present – particularly John, Amy, Cecilia, Brittany, Elsa, and Elizabeth – your assistance and encouragement made all this work attainable. I am so grateful for our friendships that extend well beyond the walls of the lab. Sincere thanks to the many friends gained during this phase of my life, you brought me so much joy. To the many inspiring and supportive gastrointestinal researchers who I met at NCSU, Dr. Jody Gookin, Dr. Laurianne Van Landeghem, Dr. Chris Dekaney, thank you for always sharing your wisdom and encouragement. Sincere appreciation to the staff in the Central Procedures Laboratory and Laboratory Animal Resources in taking care of the animals and assistance with our projects. Thank you to Dr. Sam Jones and the NCSU Comparative Medicine Translational Research Training Program (NIH T32) for supporting my training. Finally, thank you to my husband, son, and family for supporting me through this process and in the next steps to come.

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CHAPTER 1

The NEC-t step in Therapeutics: A Review of Experimental Therapeutics for NEC

Introduction

Necrotizing enterocolitis (NEC) is the leading cause of death among neonatal gastrointestinal diseases. As the most common gastrointestinal emergency in premature neonates, it is responsible for up to 10% of all admissions to the neonatal intensive care unit.[1,2] The overall mortality rate for affected neonates is approximately 30% but increases to 50% in those requiring gastrointestinal surgery.[1,3,4] Beyond the devastating individual patient and family burdens, the annual cost of treating NEC exceeds $1 billion in the United States alone.[5] The pathogenesis of NEC is multifactorial and believed to be secondary to intestinal inflammation triggered by a combination of immature gut mucosal immunity, intestinal dysbiosis, and recurrent hypoxic-ischemic events.[6] NEC results in damage to the intestinal epithelium, which is the singular barrier between noxious intestinal contents and the bloodstream. Early identification and intervention are critical to prevent disease progression. Significant effort has been allocated to exploring preventative techniques for NEC; however, even in the presence of these preventatives, disease occurs and requires treatment in the face of already-established injury.[7] Beyond surgical resection of affected intestine, current medical treatment options are designed to dampen intestinal inflammation and are thus largely supportive. Infants who survive NEC often face lifelong implications such as short bowel syndrome, malabsorption, malnutrition, total parenteral nutritional-related complications, intestinal stricture, and neurological impairment.[4,8] Novel therapeutics are needed to directly target and enhance recovery of the intestinal barrier injured in NEC. This review will begin by discussing the mechanisms implicated in NEC development, as they are integral to the design of targeted therapeutics, briefly review currently employed clinical therapeutics, and finally explore experimental agents which have been evaluated for the management of NEC.

Intestinal Epithelial Damage and Mechanisms of NEC

There are a few hypotheses for how epithelial damage occurs in NEC. The leading pathophysiological pathway characterizes NEC as a primary inflammatory disease which, through the accumulation of inflammatory cells and their byproducts within the intestinal mucosa, leads to a reduction in microvascular blood supply and epithelial damage.[9] Another proposed pathophysiology suggests that NEC progresses from mucosal damage followed by bacterial translocation and, finally, an excessive inflammatory response to endotoxin.[10] In either

scenario, mucosal injury ranges from mural edema and apoptosis only affecting the most proximal, luminal-facing mucosal epithelium of the intestinal villi, to the complete destruction of all layers of the intestinal wall resulting in necrosis and perforation.[11,12] Even if epithelial damage may not be the initiator of NEC-mediated injury, it provides a portal of entry for the translocation of bacteria and bacterial byproducts, which beget further inflammation, thus feeding into a viscous inflammatory cycle. There are many mechanisms reported to contribute to the development of NEC. The hallmark elements of these pathways have been simplified and summarized in Figure 1.

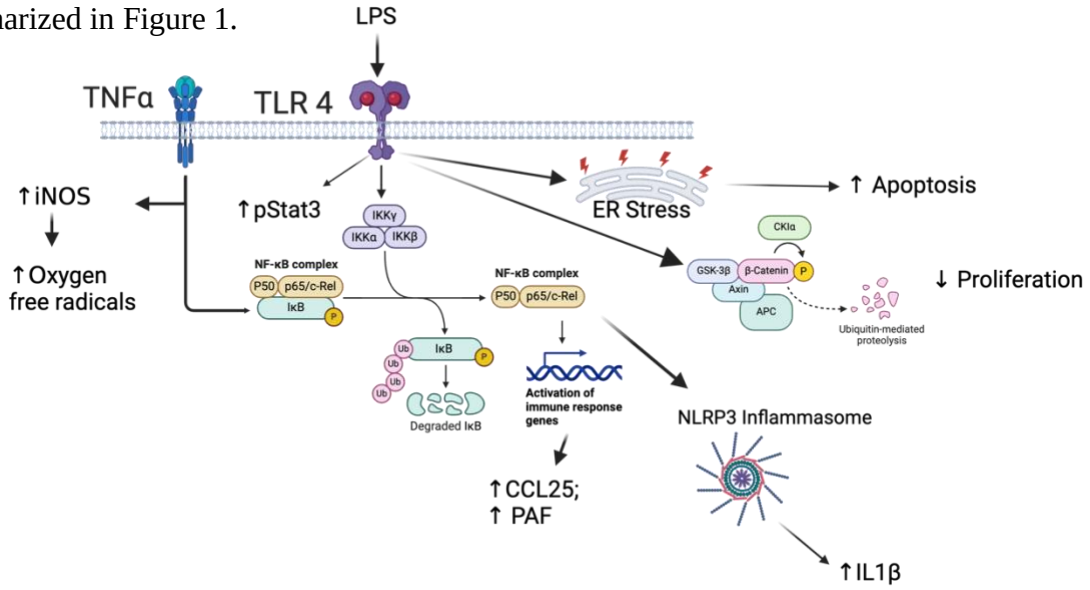

Figure 1. Simplified pathways of NEC-induced intestinal inflammation and injury. This simplified illustration provides a general background for the mechanisms which contribute to epithelial injury in NEC.

Immunologically, the development of NEC is associated with the uncontrolled accumulation of inflammatory mediators, including cytokines, inducible nitric oxide (iNOS), and platelet activating factor (PAF).[12] Some of the inflammatory cytokines elevated in NEC include IL-17, IL-1β, IL-6, IFNγ, TNFα, IL-12, and IL-18.[13] [14,15] Of the inflammatory cytokines elevated in NEC, TNFα has received significant attention as a contributor to damage. TNFα is a proinflammatory cytokine secreted by a variety of immune cells.[16] This cytokine is one of the most powerful inducers of oxygen radicals production by neutrophils.[17] It drives the production of matrix-degrading enzymes in the gut wall, especially metalloproteinase stromelysin–l, through

which it may create extensive tissue injury.[15] TNFα also activates nuclear factor kappa beta (NFκB), which contributes to its role in the NEC inflammatory cascade.

These cytokines are produced locally in intestinal mucosal lymphocytes, which are known to shift from a homeostatic T-regulatory to an inflammatory Th17 phenotype in NEC.[14,18] Toll-like receptor 4 (TLR4) is a known trigger of that phenotypic shift, through its upregulation of phosphorylated STAT3 (pSTAT3). Increased TLR4 expression is also known to produce cognate chemokine ligand 25 (CCL25), a lymphocyte chemoattractant.[14] The role of TLR4 in NEC pathogenesis extends beyond its impact on lymphocyte behavior. Activation of TLR4 upregulates glycogen synthase kinase 3 beta (GSK3β) expression which in turn enhances phosphorylation and degradation of β-catenin, thus reducing enterocyte proliferation critical to the maintenance of the epithelial barrier.[19,20] TLR4 activation in the enteric glia also contributes to the development of NEC. TLR4 leads to glial loss and thus reduction in of an important glial product, brain derived neurotrophic factor (BDNF), which regulates neurotransmission in the intestinal smooth muscle.[21] Impacting yet another element of gastrointestinal homeostasis, TLR4 reduces intestinal microcirculation through the downregulation of endogenous nitrous oxide (eNOS), thus further contributing to NEC-mediated inflammation.[22]

TLR4 activation leads to increased expression of nuclear factor-κB (NFκB), which is another instigator of the inflammatory response seen in NEC. NFκB upregulates many proinflammatory chemokines, leukocyte adhesion molecules, and cytokines which enable inflammatory cells to home to and incite damage in the intestinal mucosa.[23] Constitutively present in an inactive state, bound to the inhibitor of κB (IκBs), TLR4 activation leads to the phosphorylation and degradation of IκB, which then allows NFκB to translocate to the nucleus and upregulate inflammatory mediators.[24] One of the immune processes upregulated by NFκB is the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome. This inflammasome is a cytoplasmic protein complex, which mediates the activation of IL-1β, IL-8, and programmed cell death.[25] Increased activation of NLRP3 has been implicated in the development of NEC.[26]

Increased oxidative damage in the intestine is another pathway associated with NEC damage. Generally, premature infants, who most often develop NEC, have reduced oxidant capacity compared to full-term neonates.[27] While nitric oxide (NO), and its precursor sodium nitrate, are integral to maintaining intestinal blood flow and vascular tone in the infant intestine,

over-expression of NO and its oxidant-product, peroxynitrite, can contribute to epithelial injury.[28,29] In the face of inflammation, and signaling provided through cytokines like TNFα, more of the inducible isoform of nitric oxide synthase (iNOS) is present, which increases the activity of free nitrogen radicals and thus, oxidative damage and intestinal epithelial apoptosis.[29]

Overall, TLR4 appears to be central to the development of NEC. While it may not be the only initiator, this receptor and its downstream sequelae are capable of magnifying inflammation and injury initiated by other mechanisms. This is especially applicable to the preterm neonatal intestine, where TLR4 is over-expressed.[30] Regardless of the role of TLR4, the management of downstream accumulation of lymphocytes and damaging cytokines, oxygen free radical formation, endoplasmic reticulum stress, and inhibition of pathways associated with epithelial proliferation and tight junctions is key to attenuating intestinal inflammation and treating NEC.

Pathways Highlighted in NEC Recovery

Recovery from NEC requires alleviation of inflammation, discontinuation of ongoing damage, and renewal of the damaged intestinal epithelium. Cessation of ongoing damage involves inhibiting the pathways discussed above. Epithelial repair, however, requires unique pathways. The purely regenerative pathways which will be discussed in this review are summarized in Figure 2. Epithelial regeneration necessitates activation of the intestinal stem cell (ISC) niche, which produces epithelial cells to replace those that have been lost in injury.[31,32] Wnt-β catenin signaling is integral to the maintenance of the ISC niche.[32] Li and colleagues demonstrated that, in humans and rodents, Wnt/β-catenin signaling is decreased during NEC, as is ISC activity and intestinal regeneration.[1] Activation of Wnt downregulates GSK3β expression, thus protecting β-catenin from degradation and increasing enterocyte proliferation.[19,20] The downregulation of GSK3β is one of the mechanisms through which breast milk protects against NEC.[33]

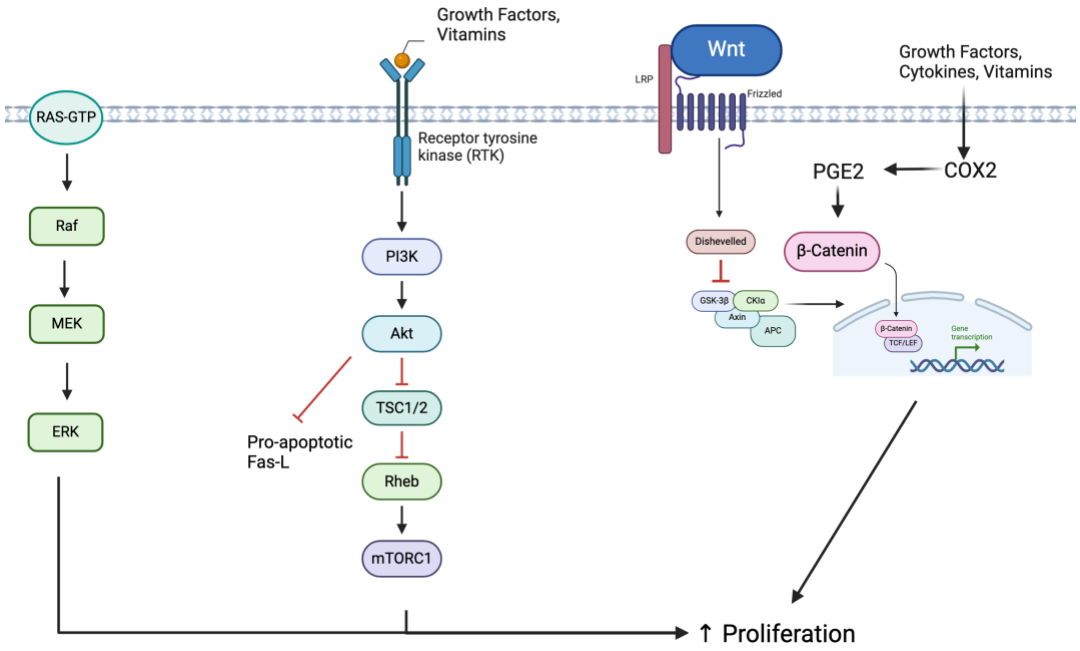

Figure 2. Simplified pathways of NEC recovery. This simplified graphic provides a general background for the pathways involved in epithelial recovery following NEC.

Like Wnt, the epithelial growth factor family (EGF) of ligands is reduced in the saliva and cord blood of premature infants and those who develop NEC.[33-36] EGF signaling is critical for preserving the intestinal epithelial barrier, improving nutrient absorption, and increasing intestinal enzyme activity in addition to accelerating epithelial recovery.[37] This family includes eleven ligands including, as relevant to NEC, EGF, transforming growth factor α and β (TGF-α, β), heparin-binding EGF- like growth factor (HB-EGF), the neuregulins (NRG1-4), and lactadherin.[38] One of the ways in which this family protects the intestinal epithelium by inducing the maturation of hydrolytic enzymes in the brush border and stimulating the proliferation of epithelial cells, in part through supporting Paneth cell populations.[39] EGF regulates the expression of tight junction proteins such as claudin-3 and occludin, in addition to increasing mucus production via intestinal goblet cells.[3,40] The protection afforded by the EGF family comes in part from its upregulation of signaling, including the phosphoinositide 3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) and the RAF-MEK-ERK pathways.[38] This family of growth factors is also able to downregulate the expression of TLR4, as was demonstrated through work in breast milk.[33] Beyond supporting proliferation and epithelial cell maturation, lactadherin (EGF VIII) contributes to the phagocytosis of apoptotic cells, which is

critical to the regular turnover of the intestinal epithelium.[41] HB-EGF also has unique properties of increasing epithelial migration and intestinal micro-vascularity, in part through binding to its specific receptor, N-arginine dibasic convertase (NrdC)[4 4,42]

Cyclooxygenase 2 (COX2) is an enzyme whose expression is induced by growth factors, including vascular endothelial growth factor (VEGF), vitamins, and inflammatory cytokines. It has a variety of roles though that which is most relevant to NEC recovery is its ability to increase cell proliferation and decrease apoptosis.[43] COX2 increases prostaglandin E2 (PGE2), which then increases cyclic adenosine monophosphate, which can, in turn, activate β-catenin and PI3K signaling to increase cell growth and proliferation.[43] The PI3K/AKT and mTOR signaling pathways are crucial for cell growth and survival, especially during stress.[44] These two pathways are so interconnected that they are often considered one. This signaling is often activated by growth factors, such as HB-EGF and hepatocyte growth factor (HGF), but upregulation has also been associated with vitamin administration.[45] Once activated, downstream signaling results in upregulation in cell cycle progression and inactivation of apoptotic factors such as Fas-ligand, resulting in improved cell survival.[44]

Yet another pathway supporting intestinal epithelial proliferation and survival, and which has been associated with the mechanism of agents studied in NEC, is the RAF-MEK-ERK pathway.[46,47] Signal transduction through this pathway is triggered by a variety of ligands including EGFR and vitamin D. These purely regenerative pathways are surely not the only ones involved in intestinal healing from NEC; however, when combined with inhibition of the inflammatory pathways previously mentioned, they provide an overview of the mechanisms involved in NEC recovery.

Clinically Employed Therapeutics

As the inciting causes of NEC are incompletely understood and prevention is impossible, current clinical practices are largely reactionary, aiming to limit the sequelae of NEC through removing intestinal insults and controlling the effects of a damaged epithelial barrier.[4,42] Medical management typically includes orogastric decompression, bowel rest, broad-spectrum antibiotics, and hemodynamic support.[6,48] Affected infants often require 7-10 days of bowel rest with concurrent total parenteral nutrition to provide adequate caloric, hydration, and electrolyte support.[6] Ultimate reintroduction of enteral feeds, preferentially with breast milk, is a gradual

process dependent upon a patient's clinical stability.[6] Broad-spectrum intravenous antibiotics are another pillar of treatment, given the association of atypical intestinal bacterial colonization and reduced intestinal microbiota diversity with NEC.[49,50] These medications are also critical to minimizing the inflammation caused by the translocation of bacteria across the injured intestinal epithelial barrier into the bloodstream.[51,52] The combination of antimicrobial medications typically includes ampicillin, gentamicin, and, if perforation has occurred, metronidazole, though a variety of different antibiotics have been employed based on culture and sensitivity information.[6] The duration of antibiotic treatment is titrated to the severity of the infant's disease. In addition to antibiotics, medications to manage coagulation may also be required as NEC can result in clotting disturbances secondary to insults like ongoing ischemia in the intestine, overall inflammation, and imbalances in pro- and anti-clotting factors secondary to these elements of disease.[6] These may include treatment with platelets, packed red cells, fresh frozen plasma, or cryoprecipitate.[53] Similarly, iatrogenic blood loss from frequent clinical sampling may result in anemia requiring packed red cell transfusion.[54] Infants are often also treated with intravenous fluids or total parenteral nutrition to ensure adequate hydration. While these medical treatments provide overall systemic support, none of them directly enhance intestinal healing.

Additionally, even when initiated in early-stage NEC, medical management is unable to prevent over 30% of patients from developing segmental bowel necrosis, which requires surgical resection.[55] Neonates requiring surgical intervention face significantly higher mortality rates of up to 50%.[3] Despite medical or surgical intervention, these infants remain at risk for serious complications such as sepsis, multiorgan compromise, and shock.[56,57] Additionally, 6-10% of infants are at risk for recurrence and long-term gastrointestinal dysfunction.[58,59] The gravest complication following a NEC episode is short bowel syndrome secondary to significant intestinal resection and intestinal failure, which are associated with early mortality.[6] Clearly, additional therapeutic agents are needed to improve the overall outcomes of neonates facing NEC. One of the significant gaps in current therapeutic approaches is targeted treatment tools to specifically address and reverse the damage to the intestinal epithelium. Novel therapeutics are needed to directly accelerate epithelial recovery and eliminate this nidus of inflammation and point of translocation for luminal bacteria into the bloodstream. Researchers have been working for decades to identify potential therapeutic agents for NEC. This review will discuss experimental agents evaluated for their therapeutic versus preventative potential and the

proposed mechanisms to emphasize pathways that may hold promise for future NEC therapeutics.

Experimental NEC Therapeutics

Review of Experimental NEC modeling

A variety of animal models have been employed to study NEC and to test potential therapeutic agents.[60] Murine models are the most commonly utilized, although the piglet model is often used due to its more translatable similarities to human neonates.[1,8,61] Key differences in model species, however, change the necessary NEC induction protocols and can influence a study's conclusions. For example, NEC is sufficiently induced in piglets that are delivered preterm by cesarean section and fed formula, whereas, in murine models, those two elements must be combined with hypoxia, and often hypothermia, lipopolysaccharide administration, and oral gavage of NEC-associated bacteria.[55,61,62] Furthermore, it is important to clearly distinguish between studies whose models replicate treatment versus prevention in order to best evaluate the efficacy of the novel agent being tested. Most of the research regarding NEC interventions, to date, has revolved around preventative approaches. Of the 89 studies evaluated for this review, only 20 analyzed therapeutic approaches. This review will focus on NEC treatment and defines a treatment as an agent administered after every element of the NEC induction protocol was initiated, compared to a preventative, which is given at any time prior (i.e. preterm delivery and formula feeding versus only preterm delivery). Such approaches are designated as treatment or therapeutic compared to those used prior to NEC induction, for which descriptors like pretreatment, preventative, or prophylactic are used. A supplemental table (Table S1) is provided with details regarding the NEC induction model, timing of the experimental agent, and general outcomes of the model. Therapeutics evaluated range from biologic products, including placental derivatives and breast milk, to synthetic small molecule inhibitors. While the object of this review is to evaluate treatments initiated after disease onset, the potential application of preventative mechanisms to post-injury repair necessitates the inclusion of many of these interventions as well. Where recent and comprehensive reviews are available, these have been cited to allow emphasis on protocols which have not yet been so thoroughly discussed.

Placental Derivatives

As NEC is often associated with pre-term delivery and thus premature removal of the infant from the uterine environment, derivatives from perinatal membranes and fluids that facilitate *in utero* maturation are attractive candidates for NEC therapeutics. These derivatives include amniotic fluid, perinatal stem cells, perinatal-derived extracellular vesicles, and synthetic products designed to parallel these elements. Thus far, many of these agents have only been evaluated prophylactically, though some treatment explorations have been completed.

Amniotic Fluid:

Amniotic fluid (AF) provides numerous nutritive resources to the developing intestine *in utero,* including taurine, arginine, and glutamine.[63,64] Beyond providing nutrition to these epithelial cells, AF encourages growth and maturation through the activity of factors such as EGF, TGFα, TGFβ-1, insulin-like growth factors 1 and 2 (IGF-1,2), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), granulocyte colony-stimulating factor (GCSF), and erythropoietin (EPO).[11,63,64] AF also has a multitude of antimicrobial factors, including alpha-defensins and lactoferrin, and, with its high concentration of IL-10, is overall anti-inflammatory.[63,64]

Amniotic fluid has been evaluated as a preventative treatment for NEC in several studies.[11,64,65] When given prior to NEC induction, AF reduced disease severity through decreasing intestinal inflammatory genes and TNFα production by dendritic cells, while increasing body weight gain.[64] This evaluation stressed AF's ability to downregulate TNFα, a critical regulator of the inflammatory response and apoptosis, and its capacity to reduce the bacterial load in the intestine.[64] Amniotic fluid, however, did not show consistent therapeutic potential when given at the same time as the last element of NEC induction: enteral feeding introduction.[64] In another model of NEC prevention, AF reduced TLR4-mediated signaling and gut inflammation in a mouse model of NEC-like intestinal injury.[64,65] One mechanism through which AF inhibits TLR4 activity is via its enriched concentration of EGF.[65] An additional evaluation of the prophylactic capacity of AF in rat pups suggested that HGF, the most abundant growth factor in rat AF, plays a critical role in protecting against experimental NEC through interacting with its dedicated receptor, *c-met,* to increase activity through PI3K signaling.[66] HGF also has the capacity to transactivate receptors in the EGF family (EGFR: ErbB2, ErbB3), which parallels the NEC-protecting impact of EGF.[65,66]

These preventative assessments evaluated complete AF fluid, including its many, variable subcomponents. As identified by de Kroon and colleagues in their comprehensive review, the processing of amniotic fluid, and its subparts, significantly influences the capacity of this biologic.[11] Simulated amniotic fluid (SAF) is a promising new approach to providing the trophic and nutritional benefits of amniotic fluid in a highly controlled format. Like AF, most evaluations of SAF, including clinical trials with premature infants, have explored the ability of SAF to prevent, versus treat, NEC.[67] One therapeutic evaluation of the administration of SAF in 30 neonates diagnosed with mild NEC found that SAF was well-tolerated with no newly developed gastrointestinal signs such as diarrhea, bloody stools, or abdominal distension.[39] Because of the relatively small number of infants enrolled, and other variables in their care, no conclusions could be made about the impact of SAF on overall case outcome. While SAF provides opportunities for personalization of the trophic elements administered and avoids potential ethical dilemmas of amniotic fluid collection, it may inadvertently lack critical elements of complex amniotic fluid.[11] Additional efforts are needed to explore and optimize the ingredients in SAF to create the greatest benefits for infants with NEC.

Within the greater milieu of amniotic fluid, perinatal stem cells have been proposed to significantly contribute to its beneficial impacts.[8,68] Perinatal stem cells include amniotic fluid stem cells (AFSC), amniotic membrane stem cells, placental stem cells (PSC), those derived from the umbilical cord, and hematopoietic stem cells (HSC) derived from cord blood (Figure 1).[69,70] These stem cells reduce inflammation and upregulate regenerative pathways to repair epithelial damage.[55] The application of perinatal stem cell therapy to NEC has been recently and thoroughly reviewed elsewhere.[48,68,70,71] Many studies have confirmed the prophylactic potential of perinatal stem cells though significantly fewer have investigated their impact after NEC-mediated inflammation is underway.[48,72,73] Preventative perinatal stem cells were able to reduce the incidence of NEC and, when compared, do so, as well as bone marrow-derived mesenchymal stem cells (MSC), though AFSC were suggested to be the most practical and clinically translatable.[71,73]

AFSC demonstrate pluripotency and rapid growth capacity, potentially exceeding that seen in MSC.[8,72,74] Application of AFSC, selected by stem cell selector c-kit (CD117) positivity, improved intestinal function, morphology, inflammation, and clinical status, including fluid retention, and ultimately survival when therapeutically applied during experimental NEC.[8,72,75]

Other investigations have found similar benefits when AFSC were given preventatively.[8] In both scenarios, the retention of AFSC in sites of intestinal injury is variable, thus many studies suggest a paracrine mechanism of action.[8,72] Both approaches identified upregulation of the Wnt-β catenin pathway following the application of AFSC in NEC models.[8,72] Wnt-β catenin is integral to the maintenance of the ISC niche. In therapeutic assessments, AFSC increased the expression of genes related to Wnt-associated ISC function (AXIN, APC, CTNNA1).[72] Prophylactic administration resulted in the upregulation of ISC markers such as leucine-rich G protein coupled receptor 5 (Lgr5) and proliferative markers such as *PCNA*.[8] In their therapeutic assessments of AFSC, Zani and colleagues also identified increased expression of genes associated with cell migration (chemokine stromal cell-derived factor-1, CXCL12), growth factors associated with maintaining epithelial integrity and reducing mucosal injury (IGF-1, FGF-1,3,4, and receptor-1 (FGFR-1)) and activation of the EGFR following AFSC treatment.[72] Additionally, when challenged with LPS, AFSC increased expression of important growth factors like Vascular Endothelial Growth Factor α (VEGFα), FGF-2, TGFß-1, TGFα, and platelet-derived growth factor (PDGF)β, compared to MSC.[72] These growth factors can induce COX2 expression from stromal cells, which is known to increase epithelial proliferation while diminishing epithelial apoptosis and inflammation to increase healing.[72]

In addition to providing COX2-related ISC support, preventative studies have also suggested that AFSC are able to reduce epithelial endoplasmic reticulum stress associated-apoptosis and tight junction disruption through activation of protective stress pathways.[76] Combined with other evaluations, these findings suggest that AFSC counteract NEC-mediated inflammation through preserving ISC-mediated proliferation via COX2 and EGFR-associated Wnt-β catenin signaling while upregulating tight junction recovery and preventing apoptosis through activation of the ER stress pathway.[72,76,8]

Compared to AFSC, placental stem cells (PSC) are more accessible and collected from placental tissue, which is otherwise regarded as medical waste. PSC are known to have immunomodulatory potential and to express epithelial regeneration and recovery factors in a variety of injury models.[55,77] In work by Weis and colleagues, human placental stem cells (hPSC) improved villi/crypt morphology, re-established essential epithelial cell populations, including those in the crypt, and reduced damage in the ileum.[55] Interestingly, these histological improvements did not translate to benefits in clinical sickness scores, body weight, or

inflammation. The impacts of hPSC on the ISC niche, and increased expression of β-catenin^{+} cells suggested their ability to support Wnt/β-catenin signaling.[55] hPSC are also known to express EGFR and its ligand, NRG1, which protects Paneth cells in their critical role of supporting the ISC niche.[77] Like AFSC, hPSC were able to produce positive effects via paracrine signaling.[55] Overall, hPSC share therapeutic mechanisms with AF and AFSC, including EGFR-activation and upregulation of Wnt/β-catenin.

Yet another source for fetal-associated stem cells is the umbilical cord. Umbilical cord-derived stem cells (UC-MSC) were therapeutically applied in one case of an early term (37 week) infant who developed NEC following recurrent episodes of supraventricular tachycardia. UC-MSCs reportedly resulted in improved mesenteric blood flow and facilitated the return to enteral feeding with ultimate ostomy removal 46 days following surgery.[78] As a clinical case report, mechanistic inquiries were not possible, however, overall recovery suggests a healthy intestinal epithelial barrier, which requires a functioning ISC niche and thus Wnt/β-catenin-related signaling.

Stem Cell-Produced Mediators

AFSC produce mediators independently capable of accelerating recovery from NEC. Prophylactic administration of human AFSC-derived conditioned media (hAFSC-CM) resulted in similar protections afforded by AFSC: increased survival, epithelial proliferation, stem cell activity, and angiogenesis, including VEGF expression, with decreased intestinal epithelial damage, apoptosis, intestinal inflammation.[79] After identifying the benefits of hAFSC-CM, the same group evaluated the preventative potential of hAFSC extracellular vesicles (EV), specifically, and found that these EVs reduced NEC incidence, intestinal injury, and intestinal inflammation while increasing stem cell expression and proliferation.[80] Extracellular vesicles contain pro-proliferative, intestinal stem cell niche supporting growth factor, Wnt.[8] These reports suggest the importance of upregulating the ISC niche in preventing NEC and provide elements capable of providing that enhanced activity, including Wnt activation and increased expression of VEGF.

Exosomes are small extracellular vesicles measuring approximately 100 nm in size, containing RNA, miRNA, DNA, and protein. When given preventatively, exosomes produced by AFSC reduced NEC-mediated injury, likely through an anti-inflammatory response, though no

specific mechanistic exploration was undertaken.[5] In a separate experiment, therapeutic AF-derived exosomes reduced NEC injury and inflammation, while increasing ISC populations.[81] When separately applied to intestinal epithelial cells in culture, these exosomes increased epithelial migration following scratch wounding, suggesting a general process contributing to their therapeutic effect.[81] Which nucleic acids or proteins within exosomes may be conferring the observed protection remains to be elucidated.[5,82] Additionally, in order to determine their true therapeutic value for NEC, evaluations of their application after complete disease induction are needed.[5,82]

The secretome of amnion-derived cells, such as ST266, has demonstrated therapeutic potential in mice and preventative capacity piglets.[61] The beneficial impact of ST266 was associated with decreased expression of TLR4 in the intestinal epithelium independent of EGF.[61] RNA sequencing of the intestinal epithelium of mice treated with ST266 identified upregulation of genes associated with energy metabolism, intestinal immunity, gut differentiation, and remodeling.[61] Additional work investigating its therapeutic value in models beyond mice is needed to confirm this potential application.

Non-Perinatal-Derived Mesenchymal Stem Cells and their Products

Placental and amniotic-derived stem cells, as described above, are both types of extraembryonic, reproductive membrane-associated MSC.[48] MSC can also be derived from adult bone marrow and adipocyte tissues. Generally, MSCs are considered pro-proliferative and anti-inflammatory in the context of the injured intestinal epithelium. Human bone marrow-derived human MSC (BM-MSC) rescued clinical sickness scores, reduced microscopic intestinal damage, and improved weight gain when given intraperitoneally in rat pups following complete NEC induction. Unlike AFSC, BM-MSC homed to the injured intestine in this study.[83] When used to treat fetal intestinal epithelial cells in vitro after hypoxic injury, BM-MSC and BM-MSC-conditioned media improved cell viability through decreasing apoptosis via Fas-mediated mechanisms.[84] These therapeutic studies, however, did not specifically explore mechanisms through which MSC may have impacted Fas-signaling.

MSCs are generally known to secrete a wide variety of anti-inflammatory cytokines and proliferation-inducing growth factors, many of which have been identified in other therapeutics. These elements may be delivered by exosomes. BM-MSC-produced exosomes have prevented

NEC-mediated injury and did so as well as BM-MSC alone, suggesting they may be the primary effector of BM-MSC-related benefits.[82] Additionally, when assessed using *in vitro* scratch assays, BM-MSC-exosomes accelerated epithelial wound healing, though this *in vitro* model is the only therapeutic application that has been explored to date.[82] Beneficial cytokines and growth factors known to be enriched by BM-MSC include IL-6, FGF, VEGF, HGF, and IGF-1, which may enhance intestinal epithelial repair through an NF$\kappa\beta$-related mechanism.[83-85]

Cytokines

As a necro-inflammatory disease, NEC is associated with excessive pro-inflammatory signaling associated with a shift in mucosal lymphocytes to an inflammatory Th17-associated phenotype.[13] Th17 T cells produce IL-17 as their effector cytokine, which has been shown to induce a loss of tight junctions, increase crypt apoptosis, and reduce enteroid proliferation in both *in vivo* and *in vitro* models.[14] Conversely, preventative administration of an IL-17 receptor antibody restores tight junctions, reduces crypt apoptosis, and supports crypt proliferation, culminating in a reduction of NEC severity.[14] The Th17 phenotypic shift can be induced by increased expression of pSTAT3 in the immature intestinal epithelium, which is associated with TLR4 expression known to be increased in NEC. As such, prophylactic inhibition of STAT3 in neonatal mice led to a reduction in Th17 cells, an increase in T-regulatory populations, and attenuation of NEC-mediated inflammation.[14] Increased CCL25, also enhanced by TLR4, contributes to inflammation by attracting lymphocytes to the intestinal epithelium. Preventative inhibition of CCL25 with lentiviral particles prevented lymphocyte infiltration and thus reduced NEC severity.[14] Together, this work suggests that inhibition of TLR4-mediated T-cell phenotypic shifts and infiltration into the intestinal mucosa prevents NEC, though additional work is needed to evaluate its therapeutic potential.

Elevation of TNFα, an inflammatory cytokine that induces apoptosis and activates NFκB, is often identified in NEC.[16] Etanercept, a recombinant dimer of human TNFα receptor protein and thus antagonist, reduced the severity of NEC, levels of TNFα, IL1-ß, and oxidative damage when administered prophylactically.[16] TNFα antibodies have similarly shown protective effects, with reduced ileal injury, inflammatory cytokines (TNFα, IL-18), and paracellular permeability.[15,86] Pentoxifylline, another anti-TNFα agent, has also been evaluated in NEC but has shown mixed success in two different administration approaches, prophylactic versus

therapeutic, in animal models of NEC.[17,27] In the treatment study, pentoxifylline reduced the incidence and severity of NEC in a complex neonatal rat model of NEC. A specific mechanism of these improvements was not investigated, though pentoxifylline's ability to inhibit TNFα and platelet-activating factor (PAF), and thus free radical accumulation and leukocyte activations, was presumed to play a role.[17] In contrast, when given prophylactically in a neonatal rabbit model of NEC, pentoxifylline had no impact on tissue injury or evidence of oxidative damage.[27] Though assessments thus far are mostly preventative, and inconsistent, direct TNFα inhibition may be a valuable mechanism to evaluate for NEC therapeutics.

Considering the pro-inflammatory environment of the intestine during NEC, anti-inflammatory antibodies are an intuitive target for NEC researchers. IL-37 is an anti-inflammatory cytokine that functions as an inherent suppressor of innate immune and inflammatory responses. Many NEC-associated pro-inflammatory signals, including IL-1ß, TNFα, IL-18, IFNɣ, and TLR4, trigger IL-37 expression, which then can provide negative feedback on accumulating inflammation caused by these factors.[87] When given preventatively, IL-37 reduced overall mortality, tissue injury, TLR4-activation, and preserved the alpha diversity of the intestinal microbiota.[88] Another anti-inflammatory cytokine with established roles in promoting intestinal epithelial integrity and modulating mucosal immune responses that has been evaluated in NEC is IL-10.[89] In studies by Emami and colleagues, prophylactic IL-10 reduced intestinal epithelial apoptosis, increased epithelial tight junction integrity, and decreased iNOS in a murine model of NEC.[89] These effects of IL-10 suggest the promotion of a healthier intestinal epithelial barrier that contributed to the overall improved survival in pre-treated groups.[89] For both IL-10 and IL-37, confirmation of their efficacy in therapeutic models is necessary to observe their utility as a potential therapy to treat NEC.

Production of TGF-ß, another cytokine with anti-inflammatory properties, is decreased in the intestine in the setting of NEC.[90] This cytokine plays a role in regulating cell growth, differentiation, and adhesion.[91] TGF-ß enacts its anti-inflammatory effects by inhibiting NF-κB activation.[92] In a preventative approach, TGF-ß reduced NEC incidence, inflammatory cytokine accumulation, and NF-κB signaling, through the canonical Smad4 pathway.[90] Another anti-inflammatory cytokine known to be diminished in the premature intestine is IL-22.[93] IL-22 is a critical modulator of intestinal microbial communities in addition to increasing intestinal epithelial proliferation and reducing apoptosis in adult animals.[94] When administered

prophylactically, IL-22 upregulated antimicrobial peptides, reduced inflammation, and enhanced epithelial regeneration, likely through the proliferation of transit amplifying cells above the ISC niche.[93] In sum, anti-inflammatory cytokines are able to prevent NEC through reducing the accumulation of inflammatory cells and oxidative damage in the intestinal mucosa, preserving epithelial tight junctions, and increasing epithelial proliferation. Additional work is needed to evaluate the therapeutic potential of these cytokines in NEC.

Dietary Agents:

Dietary agents have also shown promise in preventing the development of NEC. Retinoic acid, derived from Vitamin A, reduces inflammatory Th17 populations, increases beneficial T-regulatory cells, and promotes epithelial growth.[14] This is particularly relevant to NEC since vitamin A levels are reduced in affected patients.[95] Pretreatment with vitamin A and retinoic acid reduced inflammation and increased tight junction proteins while supporting beneficial *Bacteroides* populations in the gut microbiome.[95] All-trans retinoic acid (ATRA), a metabolite of Vitamin A, prevented NEC via supporting regulatory T cells and reducing pro-inflammatory Th17 cells through the inhibition of STAT3 signaling.[14] Niño and colleagues followed this initial work with demonstrations that ATRA's lymphocyte restoration protected ISCs depleted in NEC.[96] As suggested by its influence on lymphocytes, ATRA appears to act downstream of TLR4. Treatment with a pro-vitamin A compound, ß-carotene, in an intestinal epithelial cell line (IEC-6) exposed to lipopolysaccharide (LPS) to induce NEC-like inflammation, reduced cellular apoptosis and autophagy through the PI3K/AKT/mTOR signaling cascade.[45]

Like vitamin A, vitamin D levels are also reduced in preterm infants who develop NEC, and administration has been shown to be protective.[97] In a murine NEC model, preventative vitamin D reduced intestinal tissue damage, inflammatory cytokine mRNA expression, and markers of oxidative stress and apoptosis and was suspected to do so through the ERK signaling pathway.[47] Similar results were observed in a second prophylactic study, where Vitamin D improved overall survival, attenuated intestinal damage and epithelial apoptosis, suppressed inflammation, and protected tight junction protein expression, while downregulating TLR4 expression.[98] In a different, vitamin D-related preventative mechanism, a flavonoid derivative Astragaloside IV (AS-IV), which is a frequently-used herbal prescription in Eastern medicine, reduced ileal damage, inflammation, and oxidative stress through upregulating vitamin D3-

upregulated protein 1/NFκB signaling in a rat NEC model.[99] Vitamin E, known to function as an intracellular antioxidant and reduce free radical-mediated oxidation of polyunsaturated fatty acids in cell membranes has been explored for its therapeutic potential in treating NEC. Vitamin E treatment reduced epithelial damage and oxidative damage in a neonatal rat model of NEC.[100] The specific mechanism through which vitamin E may achieved that recovery, however, was not explored.

Expanding beyond vitamin-derivatives, polyunsaturated fatty acids (PUFA), known to reduce inflammation, have been interrogated for their potential impact on NEC as well. Most exploration has been purely preventative and thus will be briefly reviewed here. An early, randomized, controlled clinical trial demonstrated that pre-supplementation of PUFA, specifically egg phospholipids, reduced NEC incidence in preterm neonates.[101] In a neonatal rat model of NEC, pups treated with formula containing two PUFA, arachidonic acid (AA), and docosahexaenoic acid (DHA), reduced overall mortality, endotoxemia, and inflammatory factors such as PAF, though had no impact on intestinal epithelial apoptosis or iNOS expression.[102] Working to understand this mechanism further in the same model, this group found that PUFA supplementation suppressed TLR4 expression, as a mechanism for preventing NEC.[103] Additional studies with PUFA in treatment scenarios during NEC is needed.

The use of pre-digested fat (PDF) has also shown promise in preventing NEC. Unlike other fats, PDF does not require lipase metabolism, which is known to be lacking in the intestinal mucosa of premature infants.[104] PDFs are typically delivered as a formula supplement and, when given preventatively, decreased the severity of intestinal injury induced by NEC through reducing inflammatory cytokines and reactive oxygen species in the intestine, though the exact mechanism remains unknown.[104,105] A source of omega-3 fatty acids, fish oil, has also been explored for its NEC-modulating properties. Prophylactic fish oil was found to decrease intestinal PAF and inflammatory leukotriene concentrations and overall intestinal necrosis in NEC.[106] When added to the paternal diet prior to conception, fish oil reduced the risk of offspring's development of NEC through supporting the overall diversity of the neonatal mouse intestinal microbiota, though further mechanistic interactions are yet to be determined.[107] Though additional work is needed to explore their therapeutic potential, the possibility that dietary modifiers may help prevent NEC, and do so through modulating the TLR4, PI3K/AKT/mTOR, ERK, and ultimately NFκB signaling is very exciting. A review of the ideal parenteral nutrition

for neonates with NEC before and after surgical intervention has recently been published, and readers are recommended to refer to this for additional dietary modification interests.[108]

Anti-inflammatory and Antioxidant Medications:

Oxidative stress is a well-accepted contributor to the pathogenesis of NEC. Many of the interventions to date that have aimed to reduce oxidative damage and inflammation have been limited to prevention, rather than treatment. However, several studies have evaluated the therapeutic potential of this approach. Melatonin, a neurohormone produced both in the brain and intestinal tract, is a potent antioxidant.[109] When applied as a treatment for NEC, melatonin reduced tissue injury, inflammatory cytokines, and elevated antioxidant enzyme activity.[109] Additional studies exploring the preventative capacity of melatonin found similar results and identified downregulation of TLR4/NFκB and elements of the inflammasome, including NOD-like receptor family pyrin domain containing 3 (NLRP3) as a potential protective mechanism.[110] Treatment with another antioxidant, medical ozone, known to modulate phagocytic activity of peritoneal macrophages, was also able to reduce the severity of experimental NEC tissue injury, indicators of oxidative damage, and inflammatory cytokines while increasing endogenous antioxidants.[111]

Inducible nitric oxide synthase (iNOS) contributes to oxidative damage through the activity of free nitrogen radicals, which induce intestinal epithelial apoptosis. Resveratrol is a polyphenol compound recognized for its ability to modulate enzymes like iNOS and its general antioxidant and scavenging properties.[12] When given preventatively, resveratrol reduced tissue injury and decreased the presence of oxidative damage in NEC.[12] A potential mechanism for this oxidative relief, resveratrol is known to modulate COX2 expression, a pathway through which AFSC also improves recovery from NEC, though COX2 was not evaluated in this specific study. In contrast to iNOS, superoxide dismutase (SOD), can reduce free radicals. Historically, therapeutic intravenous administration of SOD reduced NEC severity and eicosanoid release in a surgical model of NEC.[112] The benefits of controlling oxidative radical accumulation, both preventatively and therapeutically, support further exploration of antioxidants for the treatment of NEC.

Nicotinamide adenine dinucleotide (NAD+) is an important regulator of oxidative stress. NAD+ concentrations upregulates Sirtuin 1 (SIRT1) activity, which is a key modifier of

inflammation.[113] SIRT1 can prevent inflammation in vascular endothelial cells through its modification of eNOS acetylation.[114] The precursor for NAD+, nicotinamide riboside (NR), is naturally found in breast milk.[115] When given preventatively, NR improves overall survival via NAD+/SIRT1 signaling that reduces NEC severity, including epithelial damage, oxidative damage, inflammation, and improves intestinal microcirculatory perfusion.[115] Another antioxidant medication that has been evaluated for the prevention of NEC is Dexpanthenol (Dxp). Dxp is an analog of pantothenic acid (PA) and is oxidized to PA in tissues. PA is known to support antioxidant systems in cells, including glutathione, glutathione peroxidase (GPx), catalase (CAT), and SOD, through increasing the level of glutathione, coenzyme-A, and ATP within cells.[116] Dxp pre-treatment reduced tissue injury, oxidative stress indices, and inflammatory cytokines while increasing tissue antioxidants in NEC modeling.[116]

Hydrogen sulfide is an antioxidant gastrotransmitter that has been explored recently for its therapeutic potential in inflammatory, cardiovascular, and neurodevelopmental diseases.[48] This endogenously-produced gas plays a role in the differentiation and proliferation of MSCs and appears to require hydrogen sulfide to promote mesenteric vasodilation.[117,118] When given preventatively, GYY4137, a hydrogen sulfide donor, was shown to improve mesenteric perfusion, intestinal histological score, clinical status, and overall weight gain in NEC.[119] Potential mechanisms for these improvements were associated with increased anti-inflammatory cytokines such as IL-6 and increased VEGF measured in the lung and liver. Overall, antioxidant agents appear to prevent, and occasionally treat, NEC through reducing oxidative damage, the TLR4/NFκB pathway, NLRP3 inflammasome activations, and inflammatory cytokines while also supporting mucosal microvascular perfusion.

Small Molecule Inhibitors:

Small molecule medications are those with low molecular weights that allow easy entry into cells. As different molecular pathways are identified as contributors to NEC, these have become targets for small molecule intervention. Thus far, these small molecules have been predominantly evaluated for their preventative, rather than therapeutic, impact on NEC. Pathways that have been targeted to date include TLR4 and NLRP3 inflammasome activation.

With the importance of TLR4 in the development of NEC, small molecules capable of inhibiting this receptor are a natural target for investigators. One compounds identified for its potential TLR4 inhibitors include C34 ($C_{17}H_{27}NO_9$), a 2-acetamidopyranoside, and its Beta-anomeric cyclohexyl analog, C35.[120] C34 prevented systemic inflammation in a mouse model of NEC. Although the precise mechanism was not illuminated *in* vivo, *in vitro* studies demonstrated that the substance inhibited TLR4 signaling in enterocytes and macrophages.[120] The ability of this inhibitor to treat NEC remains to be seen. However, in diseased explants collected from infants undergoing surgical resection for NEC, C34 treatment reduced TNFα signaling and dampened iNOS expression suggesting this compound may have therapeutic potential as well.[120] When given preventatively, TLR4-eliminating adenoviral vectors were also able to preserve β-catenin signaling by blocking TLR4 in NEC.[19] Similarly, prophylactic treatment of cells with small interfering RNA (siRNA) targeting Focal Adhesion Kinase (FAK), through which TLR4 slows epithelial migration, was able to reverse the restitution-inhibition induced by NEC.[121] To address TLR4's modulation of enteric glia, researchers identified a small molecule, J11, able to induce glia's BDNF release, which TLR4 otherwise obstructs.[21] Pretreatment of neonatal mice with J11 resulted in the reduction of NEC severity, intestinal epithelial inflammation, and small intestinal motility.[21] Together, these small molecules, acting in several different branches of downstream TLR4 signaling, support the preventative, and potentially therapeutic, capacity of small molecule TLR4 modulation in NEC.

Transient receptor potential melastatin 7 (TRPM7) is a membrane protein involved in the development of inflammation and, specifically, LPS-induced responses.[122] TRPM7 has also been shown to interact with NLRP3 inflammasome activation, which is known to play a role in NEC.[26] Pre-treatment with a small molecule inhibitor of TRPM7, NS8593, resulted in improved body weight and reduced tissue injury and inflammation.[26] Additional evaluations are necessary to determine if TRPM7 inhibition is able to accelerate recovery from NEC, though these initial results support the benefit of reducing excessive inflammatory responses in models of NEC.

Modifications of the Intestinal Microbiota:

Though the exact pathogenesis of NEC remains undetermined, there is convincing evidence that derangements in the intestinal microbiota play a significant role in the development

of the disease. Specifically, infants who develop NEC tend to have decreased resident Firmicutes and increased Proteobacteria in the stool prior to NEC onset.[123] As such, approaches to modify preterm intestinal tract bacterial populations have been eagerly pursued by NEC researchers though largely for the prevention, rather than treatment, of NEC. These approaches, including the use of probiotics, prebiotics, and synbiotics, have been recently and thoroughly reviewed elsewhere.[124] As such, this review will only provide a brief discussion of these tools with an emphasis on those evaluated therapeutically.

Prebiotics:

Prebiotics are beneficial substrates selectively used by host microorganisms to support the growth of beneficial bacteria like Bifidobacteria and Lactobacilli.[124] Prebiotic undigestible sugars such as inulin, galacto-oligosaccharides, and fructo-oligosacharides, provide fermentable substrate which supports the growth and fitness of these desirable bacteria and may also have immunological impacts.[125-127] Many of these prebiotics are also found in human milk, thus significant overlaps exist in the benefits of these agents. In preterm infants, prebiotic oligosaccharides beneficially alter the microbiota.[128,129] A meta-analysis of randomized controlled trials of prophylactic prebiotics alone found a significant reduction in the incidence of sepsis and mortality, though no significant differences between prebiotic or control when comparing NEC morbidity.[130] To our knowledge, no purely therapeutic evaluations of prebiotics in NEC have been completed.

Probiotics:

Probiotics are living, nonpathogenic, anaerobic bacteria. These bacteria have potential applications in NEC through producing beneficial bioactive compounds and competing with pathogenic bacteria, thus preventing their attachment and reducing the probability of their colonization and translocation into the blood supply, leading to sepsis.[131,132] A number of trials have evaluated the preventative potential of a variety probiotics. These showed a significant reduction in severe NEC and mortality following enteral probiotic treatment.[133] Because of the extensive analysis already completed, probiotics will only be briefly reviewed here.

Bifidobacterium and Lactobacillus species are the most common commercially available probiotic strains studied.[134] Members of both species, *Bifidobacter lactis* and *Lactobacillus rhamnosus G*, reduce tight junction permeability in neonatal humans and mice, respectively.[135,136] Bifidobacteria, known to dominate the term infant intestinal microbiota and with inherent anti-inflammatory properties, have significant potential for the prevention of NEC.[137] In a cell line LPS-challenge model, pretreatment with several strains of Bifidobacteria were able to inhibit LPS-induced NFκB activation, while two strains, *B. bifidum S17 and B. longum NCC2705*, were capable of reducing inflammatory cytokine production. Other work demonstrated that they may compete with Clostridial species which are known to dominate the intestine of preterm infants in neonatal intensive care units.[138] Preventative treatment with *Lactobacillus reuteri* strains, specifically DSM 17938, also reduced the incidence and severity of NEC.[139] This probiotic resulted in reduced inflammatory cytokine signaling and upregulation of anti-inflammatory IL-10. Additionally, preventative *L. reuteri* administration resulted in a reduction in TLR4 expression and LPS-induced phosphorylated IκB activity in vitro and in vivo, with neonatal rats showing improved survival, decreased incidence and severity of NEC and improved inflammation.[139] These assessments support the ability of *Bifidobacterium and Lactobacillus* to reduce NEC-induced inflammation when given prior to disease onset. Because of concerns for the immaturity of epithelial barriers and immune system, probiotic administration must be carefully considered to ensure administered bacteria are not contributing to the development of disease, as has been reported.[140,141] With additional clinical trials and increased regulation, probiotics may become a regularly employed NEC preventative and, potentially, with additional exploration, considered in therapeutic situations as well.

Synbiotics:

Administration of synbiotics, or combinations of prebiotics and probiotics, and their impact on NEC is still in early stages of evaluation. Evaluations of the incidence of NEC following the use of preventative synbiotics have yielded mixed results. Some studies have demonstrated reductions in NEC incidence following supplementation with complex combinations of synbiotics including probiotics such as *L. acidophilus, B. bifidum, S. thermophiles, B. longum,* with fructooligosaccharides (FOS) or *Bifidobacterium* plus inulin, but not after inulin alone.[142] Other work, however, using *Lactobacillus* +/- *Bifidobacterium* with

FOS did not report benefits in preterm infants who received these synbiotics prior to any indication of NEC.[138] Beyond uncertain efficacy, the potential mechanism through which synbiotics take preventative effect, and their potential to have a therapeutic role, have yet to be determined.

Short chain fatty acids:

Short chain fatty acids (SCFA) are produced by symbiotic intestinal bacteria from indigestible carbohydrates. Apart from serving as a nutritive source, SCFA also inhibit intestinal inflammation.[143,144] The most abundant SCFAs in the intestinal tract include butyrate, acetate, and propionate, which, in neonates, are primarily the byproducts of fiber and human milk oligosaccharides created by Bacteroidetes and Firmicutes.[145] The levels of these SCFAs are reduced in infants with NEC.[144] When administered preventatively, oral butyrate inhibited NEC-associated apoptosis, cytokine expression, and histological injury.[143,146] This was achieved through repressing SIRT1-mediated deacetylation of Notch intracellular domain (NICD). This upregulated Notch activity then downregulated TLR4 signaling through the increased activity of TLR-inhibitors like single immunoglobulin interleukin-1-related receptor (SIGIRR), toll-interacting protein (TOLLIP), and A20.[143] This study demonstrates that SCFA may be yet another preventative method to suppress TLR4 activity and thus NEC development, though therapeutic potential remains to be seen.

Fecal Microbial Transplantation (FMT):

Fecal microbial transplantation (FMT), or the administration of fecal material derived from healthy donors, may be considered akin to synbiotic administration. Preventatively, enema-FMT reduced NEC severity scores, oxidative stress, TLR-4 signaling, intestinal apoptosis, and bacterial translocation while recovering luminal populations of Lactobacillus and Bifidobacterium in an experimental model of NEC.[147] These improvements were hypothesized to be secondary to FMT's ability to dampen oxidative stress.[147] Prado and colleagues evaluated both the preventative and therapeutic potential of orogastric FMT.[148] For both interventional approaches, FMT reduced inflammation, oxidative damage, and the extent of intestinal injury caused by NEC and was suspected to occur secondary to FMT's ability to enhance microbiota

maturation.[148] In a therapeutic-only assessment, FMT administered via enema, not orogastric intubation, reduced NEC incidence, attenuated inflammatory cytokines, and increased the integrity of tight junctions. Interestingly, FMT administration did not significantly change the proportion of Lactobacillus or Proteobacteria compared to untreated NEC groups, though microbiome evaluation was limited to the level of phyla.[149] The inferiority of orogastric FMT in this study, which in fact led to increased inflammatory cytokine expression, was also found in another FMT study using preterm piglets, though without other elements of NEC modeling, and was suspected to be secondary to the overload of bacteria and metabolites in the proximal gut.[149,150] In contrast, another therapeutic study using a rabbit model of NEC found that FMT delivered via orogastric route, and especially when given in combination with sulperazone, resulted in reduced NEC severity scoring, improved survival, and recovery of intestinal microbiota diversity and transcriptomic activity.[151] This potentially occurred through a combination of inhibiting pathogenic bacteria, by sulperazone, and direct replacement with beneficial bacteria by FMT, though the mechanism was not explicitly explored.[151]

When compared to Probiotic Consortium Transplantation (PCT), including *Lactobacillus reuterii* and *Bifidobacterium longum*, FMT was able to more effectively prevent oxidative damage, through the activity of vasodilatory eNOS, though both treatments significantly improved tissue damage.[152] Preventative evaluations in neonatal piglet models of NEC have demonstrated donor-dependent impacts on FMT efficacy, with some donations providing more significant protection, including reduced NEC damage and increased weight gain, suspected to be secondary to overall improved gut microbiota diversity and specifically increased rescue of Lactobacilli.[153] Because of concerns about transplanting potentially pathogenic bacteria or toxins during FMT, Prado and colleagues compared pretreatment with sterilized FMT, via centrifugation, filtration, and ultraviolet radiation, to fresh FMT. This group found sterilized FMT to be equally effective to fresh FMT in reducing tissue damage, inflammatory cytokines, oxidative damage, and increasing anti-inflammatory cytokines.[154] With similar motivations, Brunse and co-investigators evaluated the preventative efficacy of fecal filtrate transplantation (FFT), which eliminates bacteria, but not bacteriophages, through additional centrifugation and 0.45 µM polyether sulfone (PES) filtration, compared to unfiltered FMT. Oro-gastric, versus enema-delivered, FFT was demonstrated to be superior to FMT in preventing NEC through reducing tissue injury while increasing enteric viral diversity and Proteobacteria.[155] Overall,

FMT has significant potential as both a preventative and therapeutic tool for NEC through a combination of oxidative damage control, TLR4 signaling inhibition, and repopulating the enteric microbiota.

Breast milk:

Breast milk is the most effective preventative strategy for NEC.[156] As a powerful milieu of biological entities, breast milk has been shown to prevent NEC through a variety of mechanisms, including immunomodulatory and antibacterial properties.[64,157] Breast milk contains many immunomodulatory agents, including human milk oligosaccharides, leukocytes, immunoglobulins, antimicrobial peptides, growth factors, cytokines, lactoferrin, lysozyme, lactadherin, L-glutamine, and nitric oxide.[3] This combination enhances neonatal intestinal barrier function and immunological defense while attenuating inflammatory responses. In addition to regulating the enteric immune response, breast milk is also capable of stimulating gastrointestinal maturation.[3,158] These components have been thoroughly reviewed elsewhere, and thus, only those that have been experimentally interrogated in NEC, and especially therapeutically, will be briefly mentioned here. Additionally, components of breast milk that have been evaluated in other sections of this review (antioxidants, growth factors) were only included in this section if breast milk-specific evaluations of those agents were performed.

<u>Antibodies in Breast Milk:</u>

There are numerous antibodies in breast milk that may contribute to its NEC-preventative capacity. Secretory immunoglobulin A (sIgA) is the most abundant antibody in human milk.[3] sIgA inhibits pathogen attachments to mucosal surfaces and neutralizes microbial toxins, binding potentially pathogenic bacteria like Enterobacteriaceae, which have been shown to be associated with the development of NEC.[134] In addition to preventing pathogenic activity, sIgA also encourages commensal microbiota colonization and reduces the expression of pattern recognition receptors and inflammation.[159] In a neonatal mouse model of NEC, pups of IgA-deficient mothers were predisposed to NEC despite receiving typically protective breast milk, whereas those receiving sIgA-rich milk had reduced incidence and severity of NEC, characterized by improved histological scores, survival, and body weight.[160] This effect was suspected to be

secondary to sIgA's ability to bind to, and thus limit the activity of enteric Enterobacteriaceae, though the specific mechanism of this binding is unknown. IgG and IgM are also present in human milk and have immune-surveillance activities, but are of reduced presence and thus have not been extensively evaluated.[3] Future evaluations of sIgA in a therapeutic context is required to determine if this antibody may be beneficial in the face of established NEC.

Antimicrobial properties of Breast Milk:

In addition to sIgA, breast milk contains another powerful microbiota manipulator: lactoferrin. Lactoferrin is a broad-spectrum antimicrobial glycoprotein found in colostrum and milk, which can modulate the innate and adaptive immune system.[161] This protein may mediate its NEC-protective capacity through direct interaction with epithelial cells lining the intestine, the microbes that inhabit the intestinal lumen, and the immune cells that regulate host responses.[162] Lactoferrin reduces pathogenic bacterial growth by controlling iron availability, destabilizing microbial membranes, and binding host-microbe receptors.[161,163] Lactoferricin, the proteolytic byproduct lactoferrin in acidic conditions, can neutralize endotoxin and is thus also antibacterial. Lactoferricin's impact extends beyond bacteria as it also provides antiviral properties and can prevent the secretion of cytokines, including IL-1β, IL6, IL8, and TNF-α.[164,165] Specific to epithelial recovery, lactoferrin can also upregulate intestinal epithelial cell proliferation, critical for replenishing enterocytes lost in NEC-induced injury.[166] Thus far, lactoferrin has only been evaluated preventatively, and its efficacy remains controversial. In two different systematic reviews of enterally administered lactoferrin in preterm infants, lactoferrin reduced the incidence of NEC and late-onset sepsis, though the quality of the evidence was deemed low.[162,167] A large randomized controlled trial investigating oral supplementation with bovine lactoferrin, however, found that it did not impact the incidence of NEC, sepsis, or mortality.[168] Given the nature of these studies, a specific mechanism of action could not be suggested.

Growth Factors in Breast Milk:

Breast milk is also rich in many growth factors likely contributing to its ability to prevent NEC. Several notable constituents of breast milk are members of the EGF family. In fact, maternal milk and colostrum are the predominant postnatal sources of intestinal EGF.[3]As introduced previously, this growth factor family is critical for maintaining epithelial homeostasis. Pretreatment with breast milk resulted in a reduction of NEC incidence and severity, characterized by reduced indicators of oxidative damage and epithelial apoptosis with enhanced proliferation, in a mouse model of NEC.[33] In this study, breast milk appeared to provide protection via EGF and downregulation of TLR4 signaling.[33] Another growth factor, Lactadherin (EGF VIII), is found in the fat globules of milk and is known to contribute to the phagocytosis of apoptotic cells, which is critical to the regular turnover of the intestinal epithelium, in addition to epithelial cell migration.[41] Pretreatment with lactadherin reduced NEC incidence and severity, including weight loss, histological injury, and tight junction permeability.[169] In fact, lactadherin was noted to encourage the reorganization of tight junction proteins to promote a stronger gut epithelial barrier.[169] However, the mechanism behind such reorganization was not thoroughly explored. When EGF was applied to fetal human primary intestinal epithelial cell lines (H4) preventatively at concentrations found in human breast milk, it reduced the cellular release of the pro-inflammatory cytokine, IL-8, in response to IL-1β or TNFα stimulation.[158] Another member of the EGF family, HB-EGF is also found in breast milk. As previously introduced, HB-EGF has a reparative, regenerative role in the intestinal epithelium which it exacts through upregulating mucin secretion and reducing epithelial permeability.[36,170]

Human Milk Oligosaccharides:

Beyond growth factors, oligosaccharides, which serve as metabolic substrates and prebiotics, are also plentiful in breast milk.[134] Human milk oligosaccharides reduce adhesion of leukocytes to endothelial cells and platelet adhesion to leukocytes, alter the composition of the microbiome, and reduce adherence of certain microbes to the intestinal epithelium surface.[171-174] This pathogen limitation occurs, in part, through the ability of HMOs to bind and thus prevent their interaction with intestinal epithelial cells.[175] HMOs, when combined with a glycoconjugate, also contribute to the production of human milk glycans (HMGs) which further support

Bifidobacterial growth, thought to be protective in NEC.[176] Beyond microbial modifications, these oligosaccharides have been shown to promote epithelial cell differentiation.[177]

More than 100 unique human milk oligosaccharides (HMOs) have been identified, with varying concentrations and compositional changes with the lactation stage.[178] Of these many HMOs, 2'-fucosyllactose (HMO-2'FL) is the most abundant.[179] In a neonatal mouse model of NEC, prophylactic HMO-2'FL reduced the severity of NEC, including improved NEC severity, reduction in proinflammatory markers, and increase in mesenteric circulation.[62] The mechanism mediating the proective effects of HMO-2'FL was thought to occur through the upregulation of eNOS.[62] Disialyllacto-N-tetraose (DSLNT) is another HMO that, when given preventatively, also reduced the incidence and severity of NEC, though the mechanism was not explored.[62,180] In contrast, in a neonatal piglet model of NEC, a complex mixture of 4 to 25 synthetic HMOs, including DSLNT and 2'FL, had no impact on NEC incidence or severity.[181] Overall, the preventative efficacy of HMOs remains uncertain, and their therapeutic application remain unexplored. However, emphasis on their potential ability to reduce NEC incidence and severity through the upregulation of eNOS, and thus protection of intestinal perfusion, may prove useful for considering pathways of interest for other therapeutics.

Amino Acids in Breast Milk:

Glutamine and glutamic acid are abundant free amino acids in human milk known, generally, to increase cell proliferation and differentiation, reduce apoptosis, and decrease epithelial permeability via tight junction expression.[182,183] In a neonatal rat model of NEC, preventative glutamine reduced the severity of NEC characterized by decreased mucosal injury, inflammation, and apoptosis.[184] Ultimately, these changes were hypothesized to be secondary to glutamine-associated TLR4 reduction.[184] When orally given to premature neonates, glutamine reduced the incidence of NEC and septicemia and was associated with reduced intestinal permeability.[185] In these studies, preventative parenteral supplementation of glutamine in preterm infants also decreased the incidence of NEC.[186] However, a Cochrane review, which included 12 clinical trials and over 2800 premature infants, did not find a decreased incidence of NEC.[187]

Breast Milk-derived Immunomodulators:

Leukocytes within breast milk are able to survive digestion and translocate into the infant's blood, lymph nodes, spleen, and liver.[176,188,189] Cytokines produced by these lymphocytes and contained within breast milk, such as IL-1, IL-2, IL-6, IL-8, IL-10, IFNɣ, and TNFα, are systemically absorbed and provide passive protection and immune modulation through a mixture of pro- and anti-inflammatory activity. Many of these cytokines have been evaluated only in their trends associated with NEC development rather than as specific preventatives or therapeutics. The preventative capacity of IL-10, at concentrations parallel to those found in breast milk, however, has been evaluated and found to reduce IL-8 secretion following IL-1β or TNFα stimulation in an *in vitro* model of NEC.[158] In a follow-up *in vitro* model of NEC, another group found that breast milk is able to suppress the transcription of NFκB triggered by IL-1β, providing mechanistic context for the impacts of breast milk-associated IL-10.[190] Overall, additional efforts to profile the preventative and therapeutic benefits of the specific immunomodulatory components of breast milk in NEC are needed.

Exosomes:

Exosomes are yet another biologically active agent within breast milk. Breast milk is known to be rich in exosomes with greater concentrations in earlier-produced milk.[191] The phospholipid membrane of exosomes protects their protein, microRNA, and mRNA contents and allows them to reach the intestinal epithelium intact.[192] Treatment with breast milk exosomes reduced NEC histological injury and inflammation while supporting the ISC niche in a neonatal mouse model of NEC.[81] These mechanisms were also observed in a separate experiment using *in vitro* intestinal epithelial cells (IEC-18), without additional injury or damage, where viability, proliferation, and stem cell activity were all increased.[192] In support of clinical applicability, human milk-derived extracellular vesicles (HMEV), the broader category of nanoparticles that includes exosomes, are able to survive neonatal digestion. Additionally, neonatal human enteroids are capable of absorbing HMEV, suggesting a direct mechanism for these agents to impact the neonatal intestine.[193] Additional work exploring exosomes and HMEV in complex

models of NEC is ongoing, with expectations that this exosomes may provide both preventative and therapeutic options for NEC.

Growth Factors:

Growth factors are found in many biological products and have already been introduced in this review. This section is dedicated to specific applications of individual growth factors beyond what has been introduced in discussions of the more complex agents. As is true for the rest of the review, greater attention was given to growth factors which have been evaluated in therapeutic, versus preventative, NEC modeling.

Wnt:

Intestinal stem cell-mediated epithelial recovery, which is required following NEC, depends upon Wnt/β-catenin signaling. Exogenous Wnt (3a and 7b) supplementation to enteroids led to improved epithelial survival and growth in human and mouse NEC-derived organoids and *in vitro* NEC modeling.[1,8] Further, therapeutic administration of exogenous Wnt7b, found to be significantly reduced in NEC-afflicted intestinal epithelium, improved the regenerative phenotype of organoids isolated from humans and mice with NEC.[1] This study also explored the preventative impact of intraperitoneal Wnt7b in NEC where it increased survival, proliferation, and stem cell populations, while decreasing tissue damage and inflammatory cytokines.[1] Apart from this work, Wnt7b has not been thoroughly evaluated in the intestine, though it has roles in regeneration in the epithelium of other organs.[1] In the skin, it activates epithelial recovery through prostaglandin E2.[194] Given these considerations and *in vitro* therapeutic potential, controlled Wnt supplementation may hold promise as a therapeutic approach for NEC.

EGF Family:

With the importance of EGF in preserving and recovering the intestinal epithelial barrier, it has been an attractive target for NEC interventions. As discussed earlier, EGF has been identified and implicated as a potential mechanism of action in amniotic fluid, amniotic fluid stem cells, and human placental stem cells. Investigating direct therapeutic EGF administration, a double-blind, randomized controlled study evaluating continuous intravenous infusion of EGF

in eight human neonates with severe NEC showed improved rectal crypt proliferation, though ultimately had no impact on clinical outcome.[195] Rectal samples were employed in this study versus the more clinically relevant ileum, as they were less invasive to obtain. Because of the clinical nature of this work, more mechanistic exploration was not feasible. Other studies, however, have demonstrated that EGFR can induce COX2 activity. Interestingly, COX2 and EGFR are mutually supportive in that COX2 products like prostaglandin E2 (PGE2) activate EGFR while EGFR induces COX2 expression in enterocytes.[196] As discussed previously, COX2 is able to increase epithelial proliferation while reducing epithelial apoptosis, inflammation, and oxidative damage, thus increasing healing in the context of NEC.

Preventative HB-EGF attenuates NEC with reduced histological injury[4] and intestinal permeability, leading to reduced disease incidence and prolonged survival time.[9,42] When given prior to NEC induction with MSCs, HB-EGF and MSC were found to work synergistically to improve survival and reduce the incidence of NEC.[197] This was suspected to occur through the ability of HB-EGF to encourage the migration and proliferation of epithelial cells, decrease oxygen-free radical formation, and improve nutritional transport and intestinal enzyme activity.[4,9,197] Enhanced restitution has been credited to the ability of HB-EGF to activate the PI3K/Akt and MEK/REK pathways.[197] However, when administered therapeutically in a NEC model, HB-EGF did not have any significant effect.[4] Moreover, excess EGF receptor activation has been linked to several cancers, including head and neck cancers, raising concerns about long-term sequelae after treatment.

Conclusion

With the rapid onset of NEC, prevention is not always possible and thus, there is an urgent need for expanded therapeutic options. However, of the 89 studies exploring novel agents and NEC which were reviewed for mechanistic insights (Table S1) , only 20 analyzed agents utilized therapeutically in complex models of NEC. While the purely therapeutic options for NEC remain limited, several promising therapeutic agents have been identified such as TLR4 modifiers, antioxidants, fecal microbial transplantation, mesenchymal stem cells, and amniotic and placental-derived biologics that include stem cells and secreted factors. Generally, these agents reduce tissue injury and inflammation while improving intestinal microbial diversity and, ultimately, survival. Pathways that have been associated with recovery from NEC, with

recognized overlap between them, include downregulating TLR4 and NFκB signaling, their associated downstream sequelae, and reducing oxidative damage and endoplasmic reticulum stress. Meanwhile, upregulation of the Wnt/β-catenin, PI3K/Akt/mTOR, and the ERK pathway can contribute to intestinal healing, largely through increased epithelial proliferation. A simplified summary graphic highlights the different agents, for which publications identified a mechanism of action, and their application to these pathways (Figure 3). It is our hope that, with continued dedication to pre-clinical studies and subsequent clinical trials, additional therapeutics to help address the devastation caused by NEC will arrive at the bedside soon.

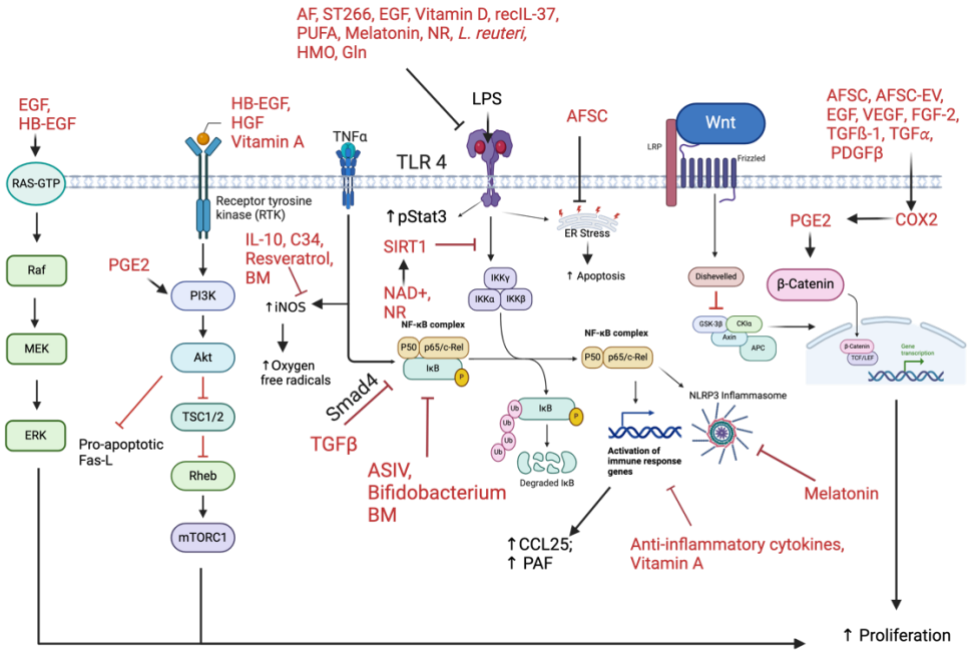

Figure 3. NEC interventions and pathways. This illustration demonstrates the signaling pathways and therapeutic targets related to interventions explored in the publications reviewed, in relation to intestinal injury and repair seen in NEC.

Table S1: Experimental Agents Evaluated as NEC Treatments

	Agent	Author	Year	Model and NEC-induction protocol	Proposed Mechanism:
1	AFSC	Zani	2014	Neonatal rat: formula, hypoxia, oral LPS	Increased COX-2 expressing stromal cells surrounding the intestinal crypt, increased proliferation and migration of epithelial cells, decreased apoptosis. Reduced intestinal lipid peroxidation and neutrophil sequestration
2	AFSC	Zani	2014	Neonatal rat: formula, hypoxia, oral LPS	Reduction in intestinal inflammation and damage via COX2 stromal cell activation
3	SAF	Lima-Rogel	2003	Neonatal infants	rhG-CSF, rhEPO, and electrolyte pre-supplementation enhanced maturation of the intestinal epithelial brush border.
4	Human placental stem cells (hPSC)	Weis	2020	Neonatal rat: formula, hypoxia, oral LPS	Reduced epithelial apoptosis and increased proliferation, replenishment of ISC and Paneth cells.
5	Amnion-derived multipotent cell secretome ST266	Sodhi	2022	Neonatal mouse: formula with NEC fecal bacterial stock, hypoxia	Reduced histological damage, reduces inflammation, and inhibits TLR4 activation
6	Human Bone Marrrow-Derived Mesenchymal Stem Cells (hBM-MSC)	Tayman	2011	Neonatal rat: formula, hypoxia, hyperthermia, hyperoxia for 3 days	Increased weight gain, clinical sickness score, and histological evaluation.
7	hBM-MSC Conditioned Media*	Weil	2009	Human fetal intestinal epithelial cells (FIE) exposed to hypoxia	Reduced apoptosis and increased proliferation, IL-6, HGF and VEGF.
8	β-Carotene*	Xu	2022	IEC-6 cells with LPS treatment	Improved cellular viability, reduced apoptosis, upregulated Akt/mTOR pathway

Table S1 (continued)

9	Vitamin E	Okur	1995	Neonatal rat: singular hypoxia exposure followed by reperfusion	Reduced histological and oxidative damage.
10	Melatonin	Guven	2011	Neonatal rat: formula, hypoxia, hypothermia, hyperoxia	Reduced weight loss, clinical sickness score, tissue damage, inflammatory tissue metabolites, and inflammatory cytokines; Increased anti-inflammatory metabolites (SOD, GPx).
11	Medical ozone	Guven	2009	Neonatal rat: formula, hypoxia, hypothermia, hyperoxia	Reduced weight loss mortality, histological damage, indicators of oxidative damage, and inflammatory cytokines; increased tissue antioxidants
12	Superoxide dismutase (SOD)	Miller	1988	Weanling rabbit: Surgical exteriorization of intestine followed by luminal injection of acidified protein and calcium	Reduced oxidative damage and intestinal injury.
13	Pentoxifylline	Travadi	2006	Neonatal rat: premature delivery, formula, hypoxia, hypothermia	Reduced incidence and severity of histological injury.
14	C34	Neal	2013	NEC-affected intestinal explants from neonatal infants	Reduced TNFα signaling, when given alone or prior to LPS stimulation, and dampened iNOS expression following LPS stimulation.

Table S1 (continued)

15	FMT	Prado	2019	Neonatal rat: formula, hypoxia, hypothermia and oral LPS	Reduced inflammatory cytokines, oxidative stress, and tissue damage
16	FMT	Liu	2020	Neonatal mice: formula with LPS, hypoxia,	FMT-enema improved histological injury, reduced inflammatory cytokines, tight-junction integrity
17	FMT + sulperazone	Lin	2021	Neonatal rabbits: formula, hypothermia, hypoxia	Prolonged survival, reduced histological damage, improved intestinal microbiome diversity and transcriptomic activity.
18	FMT	Hui	2022	Neonatal piglets: premature delivery, formula	Reduced tissue damage and improved intestinal microbiota diversity.
19	Wnt7b*	Li	2019	Organoids from NEC-affected human and murine tissue	In vitro: Improved proliferation and increased intestinal stem cell expression
20	Recombinant human epidermal growth factor 1-48	Sullivan	2007	Neonatal infants	Increased rectal crypt bifurcation and mucosal thickness.
21	HB-EGF, EGF	Radulescu	2009	Neonatal rats: premature delivery, formula, enteral LPS, hypoxia, hypothermia	HB-EGF + EGF reduced the incidence and severity of NEC, intestinal permeability, increased survival time HB-EGF administered 4H after complete NEC protocol initiation reduced NEC incidence and severity, later time points had not effect.
22	Breast milk and amniotic fluid-derived exosomes	Hu	2023	Neonatal mice: formula, hypoxia, hypothermia	Reduced NEC injury and inflammatory cytokines; increased ISC presence

*Asterisk indicates a study only employing cell models of injury

Table S2: Experimental Agents Evaluated as NEC Preventatives

	Agent	Author	Year	Model and NEC-induction protocol	Proposed Mechanism:
1	Amniotic Fluid (AF)	Siggers	2013	Neonatal piglet: premature delivery, formula	Downregulation of TNFa, IL-1a, IL6, NOS; decreased bacterial abundance in colon
2	AF	Good	2012	Neonatal mouse: formula, hypoxia, intraperitoneal (IP) LPS	EGF within AF inhibited TLR4 signaling via peroxisome proliferator-activated receptor
3	AF	Jain	2013	Neonatal rat: premature delivery, formula, hypoxia, hypothermia	HGF within AF increased cell proliferation, migration, and cell survival via increased PI3K signaling
4	Synthetic AF (SAF)	El-Ganzoury	2014	Neonatal infants	rhG-CSF and/or rhEPO prevented villous atrophy.
5	Extracellular vesicles (EV)	McCulloch	2018	Neonatal rat: premature delivery, formula+/- LPS, hypoxia, hypothermia	AFSC- reduced NEC injury, likely through an anti-inflammatory mechanism.
6	Perinatal Stem Cells	McCulloch	2017	Neonatal rat: premature delivery, formula +/- LPS, hypoxia, hypothermia	Decreased the incidence and severity of NEC, mechanism unclear though suggested a paracrine, exosome-related mechanism
7	Amniotic Fluid Stem Cells (AFSC) and AFSC-derived EV	Li	2020	Neonatal rat: formula, hypoxia, oral LPS	AFSC and AFSC-EV increased ISC proliferation

Table S2 (continued).

8	AFSC	Li	2021	Neonatal mouse: formula, hypoxia, oral LPS	Reduction in ER stress and preservation of epithelial tight junctions through the upregulation of *ATF6*, *IRE1α*, *PERK*, and their downstream effectors ATF*4 and CHOP.*
9	Conditioned-media derived from human amniotic fluid stem cells (hAFSC-CM)	O'Connell	2021	Neonatal mice: formula, hypoxia, oral LPS	Reduced intestinal epithelial apoptosis and neutrophil infiltration while increasing intestinal epithelial stem cell activity and VEGF levels.
10	Extracellular vesicles derived from hAFSC-CM	O'Connell	2021	Neonatal mice: formula, hypoxia, oral LPS	Reduced tissue damage, decreased expression of inflammatory markers, and increased intestinal epithelial stem cell activity.
11	Stem cell-derived exosomes (SC-Ex) including AFSC-Ex	McCulloh	2018	Neonatal rat: formula, hypoxia, hypothermia, LPS administration	Reduced histological injury and overall incidence of NEC.
12	Bone marrow-derived mesenchymal stem cell (BM-MSC) produced Exosomes (BM-MSC-Ex)	Rager	2016	Neonatal rat: formula, hypoxia, hypothermia	In vivo, reduced NEC-incidence and tissue damage, including intestinal permeability. In vitro, increased epithelial restitution.

Table S2 (continued).

13	Th17 polarization inhibitors: Anti-IL17 receptor antibody, All-trans retinoic acid (ATRA), WP1066: STAT3 Inhibitor CCL25 lentiviral inhibitor	Egan	2015	Neonatal mouse: formula, hypoxia, enteric bacterial administration	Anti–IL-17R strengthened tight junctions, increased proliferation, and decreased apoptosis. WP1066 (STAT3 inhibitor): Reduced NEC severity, oxidative damage (3-nitrotyrosine) and inflammatory cytokine (Th17 and IL-17) levels. ATRA: Reduced NEC severity, oxidative damage (3-nitrotyrosine), and inflammatory cytokine (Th17 and IL-17) levels.
14	Recombinant IL-37 (recIL-37)	Cho	2020	Neonatal mouse: formula, hypoxia, hypothermia	Reduced tissue injury, clinical sickness score, and inflammation, TLR4-expression while improving intestinal microbiota alpha diversity.

Table S2 (continued).

15	IL-10	Emami	2012	Neonatal mouse: formula, hypoxia, hypothermia	Increased survival, alleviated mucosal injury, decreased mucosal oxidative damage (iNOS).
16	TGFβ1	Shiou	2013	Neonatal rat: premature delivery, enteric bacterial colonization, formula, hypoxia	Reduced NEC severity and incidence, inhibited NFκB signaling, and increased Smad4 pathways.
17	Recombinamt IL-22 (rIL-22)	Mihi	2021	Neonatal mouse: formula with LPS and NEC-associated bacteria, hypoxia	Reduced NC severity, including inflammation, increased transit amplifying cell proliferation, and improved tight junction organization
18	Vitamin A	Xiao	2018	Neonatal mouse: formula, hypoxia, hypothermia	Reduced inflammatory cytokines, increased tight junction integrity, maintained more diverse intestinal microbiota
19	All-trans retinoic acid (ATRA)	Niño	2017	Neonatal mouse: formula with NEC-associated bacteria feeding, hypoxia	Reduced intestinal injury severity, decreased intestinal inflammation, and protected the ISC niche

Table S2 (continued).

20	Vitamin D	Lyu	2020	Neonatal mouse: formula, hypoxia, hypothermia	Reduced intestinal tissue damage, inflammatory cytokine expression, apoptosis, and membrane damage. Increased proliferation, anti-oxidative capacity, and increased ERK pathway activity.
21	Vitamin D	Shi	2018	Neonatal rat: formula, hypoxia, hypothermia	Increased survival and tight junction protein integrity, reduced histological damage, intestinal epithelial apoptosis, inflammation, and TLR4 expression.
22	Astragaloside IV (AS-IV)	Cai	2016	Neonatal rat: hypoxia, hypothermia	Reduced histological damage, increased antioxidant tissue levels, reduced oxidative damage, reduced inflammatory cytokines, reduced NFkB signaling via preventing phosphorylation of IkB.

Table S2 (continued).

23	Polyunsaturated fatty acids (PUFA) – egg phospholipids (EP)	Carlson	1998	Neonatal infant	Reduced NEC incidence in supplemented neonates
24	PUFA – arachadonic acid (AA), docosahexaenoic acid (DHA)	Caplan	2001	Neonatal rat: Premature delivery, hypoxia, hypothermia, formula	Reduced NEC incidence, endotoxemia, and platelet activating factor.
25	PUFA – AA, DHA, EP	Lu	2007	Neonatal rat: Premature delivery, hypoxia, hypothermia, formula	Reduced NEC incidence, TLR4 expression in the small intestine, platelet activating factor (PAF) expression
26	Pre-digested fat (PDF)	Sodhi	2018	Neonatal mouse: formula with NEC-bacteria, hypoxia	Reduced weight loss, mucosal injury, and inflammatory cytokines, and reactive oxygen species
27	Fish Oil	Akisu	1998	Young mouse: singular hypoxia exposure followed by reperfusion	Prevented histological damage, and PAF and leukotriene B4 (LTB4) accumulation
28	Fish Oil	Rumph	2022	Neonatal mouse: formula	Increased intestinal microbiota diversity in neonates and reduced NEC incidence.
29	Melatonin	Xiong	2022	Neonatal mouse: formula with LPS, hypoxia	Increased survival, body weight, and antioxidative markers; Reduced tissue injury, inflammatory cytokines, indicators of oxidative damage. Decreased NLRP3, TLR4, NF-κB, and Caspase- 1.
30	Resveratrol	Ergün	2007	Neonatal rat: formula, hypoxia	Reduced tissue damage, reduced oxidative damage (iNOS)

Table S2 (continued).

31	Etanercept	Yurttutan	2014	Neonatal rat: hypoxia, hypothermia, hyperoxia	Reduced tissue damage, inflammatory cytokines, and oxidative damage-associated enzymes.
32	anti-TNF-antibody (anti-TNF-α)	Halpern	2006	Neonatal rat: premature delivery, formula, hypoxia, hypothermia	Reduced incidence and severity of NEC, including apoptosis, and tissue permeability, inflammatory cytokines
33	anti-TNF-α	Seitz	2005	Neonatal rat: formula, hypoxia, hypothermia	Reduced incidence and severity of NEC, including apoptosis, tissue permeability, and inflammation
34	Pentoxifylline	Erdener	2004	Neonatal rabbit: hypoxia	No significant impact ontissue damage or evidence of oxidative damage
35	Nicotinamide riboside (NR)	Zhang	2022	Neonatal mouse: formula, hypothermia, hypoxia	Increased survival, endothelial cell proliferation, and SIRT1 activity. NR reduced NEC incidence, histological damage, evidence of oxidative damage, inflammation (TLR4)
36	Dexpanthenol (Dxp)	Karadag	2015	Neonatal rat: formula, hypothermia, hypoxia, hyperoxia	Decreased intestinal damage (preserved Goblet cells), reduced oxidative stress and inflammatory cytokines
37	Hydrogen sulfide donor agents	Drucker	2018	Neonatal mouse: hypoxia, formula with LPS, hypothermia	Reduced intestinal injury, increased weight-gain, and improved clinical scores, intestinal perfusion, and improved lung and liver inflammation (IL6, VEGF)

Table S2 (continued).

38	C34	Neal	2013	Neonatal mouse: formula, hypoxia NEC-affected intestinal explants from neonatal infants	Reduced tissue injury and inflammation (iNOS) in mice. Reduced TNFα signaling, when given alone or prior to LPS stimulation, and dampened iNOS expression following LPS stimulation.
39	TLR4 AAV	Sodhi	2010	Neonatal mice: formula, hypoxia	Increased enterocyte proliferation
40	FAK siRNA	Leaphart	2007	IEC6 Cells exposed to LPS or ATRA, then scratch-wounded	Increased in vitro epithelial migration
41	J11 (BDNF)	Kovler	2021	Neonatal mice: formula with NEC-associated bacteria, hypoxia NEC-affected intestinal explants from neonatal infants	Reduced NEC mucosal injury, inflammation, and improved small intestinal motility Reduced LPS-TLR4 induced TNFa expression from diseased explants
42	NS8593 (TRMP7 inhibitor)	Li	2022	Neonatal rats: formula with LPS, hypothermia, hypoxia	Increased body weight, survival; reduced tissue injury and inflammation
43	Bifidobacterium	Riedel	2006	HT-29 Cells: LPS and TNFa exposure	Inhibited LPS-induced NFKb activation response; *B. bifidum S17 and B. longum NCC2705* reduced LPS-induced inflammatory cytokines, cyclooxygenase 2 (Cox-2), and intercellular adhesion molecule 1 (ICAM-1)).
44	*Lactobacillus reuteri* (*L. reuteri*)	Liu	2012	Neonatal rats: formula, hypoxia	Improved survival, decreased incidence and severity of NEC, improved inflammation (reduced TLR4; increased IL10); decreased NFκB signaling

Table S2 (continued).

45	*Bifidobacterium lactis*, inulin, or *Bifidobacterium lactis* + inulin	Dilli	2015	Neonatal infants: <32 weeks, weight <1500 g, transferred to NICU in first week of life	*Bifidobacterium lacti* and *Bifidobacterium lactis* + inulin reduced NEC incidence; All groups reduced NEC mortality and hospital stay
46	Two lactobacillus species plus fructooligosaccharides; several species of lactobacilli and bifidobacteria plus fructooligosaccharides	Underwood	2009	Neonatal infants: <35 weeks, weight 750-2000g, transferred to NICU in first week of life	No differences in weight gain or stool short chain fatty acid content; bifidobacterium supplementation more likely to result in intestinal colonization.
47	Butyrate	Yu	2023	Neonatal mice: formula with LPS, hypoxia	Reduced intestinal apoptosis, cytokine expression, histological injury, NFKB, and IkB; increased NICD, A20, SIGIRR, and TOLLIP
48	Fecal Microbial Transplantation (FMT)	Li	2017	Neonatal mice: formula, hypoxia, hypothermia	Improved survival, intestinal microbiota richness, reduced weight loss, NEC incidence, histological damage, oxidative damage
49	FMT	Prado	2019	Neonatal rat: formula, hypoxia, hypothermia and endotoxin exposures (oral LPS)	Reduced inflammatory cytokines, oxidative stress, and tissue damage
50	FMT and Probiotic Consortium Transplantation (PCT)	Tian	2022	Neonatal mice: formula, hypoxia, hypothermia	FMT and PCT decreased histological damage, increased tissue antioxidant capacity, immune barrier activity, inflammatory cytokines, and intestinal permeability; FMT reduced oxidative damage

Table S2 (continued).

51	FMT	Prado	2022	Neonatal rats: formula, hypoxia, hypothermia, LPS administration	Fresh and sterile FMT reduced tissue damage, increased anti-inflammatory cytokines and decreased proinflammatory cytokines and oxidative damage
52	FMT, Fecal filtrate transplantation (FFT)	Brunse	2022	Neonatal piglets: premature delivery, formula	FFT reduced NEC severity (orogastric and enema) and incidence (orogastric) > FMT; Orogastric FFT increased viral diversity and reduced Proteobacteria
53	Wnt7b	Li	2019	Neonatal mouse: formula, hypoxia, oral LPS	In vivo: Increased survival, proliferation, and stem cell populations; decreased tissue damage and inflammatory cytokines
54	Heparin-binding EGF-like Growth Factor (HB-EGF)	Feng	2006	Neonatal rats: premature delivery, formula, hypoxia, hypothermia, enteral LPS	Reduced the incidence and severity of NEC, intestinal permeability; increased survival time
55	HB-EGF, EGF	Radulescu	2009	Neonatal rats: premature delivery, formula, enteral LPS, hypoxia, hypothermia	HB-EGF and EGF reduced the incidence and severity of NEC, intestinal permeability, increased survival time HB-EGF administered 4H after complete NEC protocol initiation reduced NEC incidence and severity, later time points had not effect.
56	HB-EGF and MSC	Yang	2012	Neonatal rats: premature delivery, formula, hypoxia, hypothermia	HB-EGF and MSC decreased incidence of NEC, intestinal permeability, and improved survival. HB-EGF promoted MSC intestinal engraftment.

Table S2 (continued).

57	Human milk factors: transforming growth factor-β (TGF-β), erythropoietin (Epo), IL-10, or epidermal growth factor (EGF)	Claud	2003	HT29- cl19A, Caco2, fetal human primary intestinal epithelial cell line (H4), and temperature-sensitive conditionally immortalized fetal human intestinal (ts-FHI) cell lines	TGF- β and Epo decreased H4's IL8 secretory response to TNF-α or IL-1β application
58	Secreted Immunoglobulin A (sIgA)	Gopalakrishna	2019	Neonatal mice: hypoxia, *Enterobacter spp.* and *Enterococcus spp.* administration	Reduced NEC incidence with improved histological scores, survival, and body weight
59	Enteral lactoferrin: bovine and recombinant human sources	Pammi	2017	Neonatal infants: preterm	Decreased NEC incidence
60	Lactoferrin or talactoferrin or lactotransferrin or bovine lactoferrin or human lactoferrin or lactoferricin	He	2018	Neonatal infants: preterm	Decreased NEC incidence
61	Bovine lactoferrin	Griffiths	2018	Neonatal infants: preterm	No change in NEC incidence
62	Breast milk (BM)	Good	2015	Neonatal mice: formula with NEC-associated bacteria, hypoxia	Reduced NEC severity, oxidative damage, epithelial apoptosis, and enhanced proliferation.
63	Lactadherin	Shen	2019	Neonatal rats: formula, hypoxia, hypothermia	Reduced NEC incidence and severity including weight loss, histological damage, and tight junction permeability

Table S2 (continued).

64	Human milk oligosaccharide 2′-fucosyllactose (HMO-2'FL)	Good	2016	Neonatal mice: formula with NEC-associated bacteria, hypoxia	Reduced the severity of NEC including a decrease in pro-inflammatory markers, preservation of mucsoal architecture, and increased vasodilatory substances (eNOS)
65	HMO disialyllacto-N-tetraose (DSLNT)	Jantscher-Krenn	2012	Neonatal rats: formula, hypoxia	Improved survival and reduced NEC incidence and severity including decreased pathology scores
66	Human milk oligosaccharide	Rasmussen	2017	Neonatal pigs: preterm delivery, formula	No impact on NEC incidence or severity
67	Glutamine (Gln)	Zhou	2014	Neonatal rats: formula, hypoxia, hypothermia	Reduced mucosal injury, markers of inflammation and apoptosis
68	Gln	Sevastiadou	2010	Neonatal infants: preterm	Reduced incidence of NEC and septicemia.
69	Gln	Bober-Olesińska	2005	Neonatal infants: preterm	Reduced incidence of NEC
70	Breast milk	Minekawa	2004	Caco2 cells with IL1β exposure	Reduced IL-1β and LPS-induced IL-8 expression via downregulation of NFkB

*Asterisk indicates a study only employing cell models of injury

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CHAPTER 2

Large Animal Models Enhance the Study of Crypt-Mediated Epithelial Recovery from Prolonged Intestinal Ischemia Reperfusion Injury

Abstract

Intestinal ischemia and reperfusion injury (IRI) is a deadly and common condition. Death is associated with sepsis due to insufficient epithelial repair, requiring stem cell-driven regeneration, typically beginning 48 hours after injury. Animal models are critical to advancing this field. To effectively study epithelial healing, models must survive clinically relevant intestinal ischemic injury extending to the crypt. Though mouse models are indispensable to intestinal research, their application for studying epithelial repair following severe IRI may be limited. Ischemic injury was induced in mouse and porcine jejunum for up to 3 hours, with up to 72 hours of reperfusion. Histologic damage was scored by Chiu-Park grade and animal survival was assessed. Findings were compared between species. A mouse IRI literature review was performed to evaluate the purported degree of injury, duration of recovery, and reported survival rates. In mice and pigs, 3 hours of ischemia induced severe, reliable injury extending into the crypt. However, at 48 hours, mouse survival was only 23.5% compared to 100% survival in pigs. In literature, ischemia was induced for >1 hour in only 4 of 102 mouse studies and none to 3 hours. Recovery was attempted for 48 hours in only 6 reports. 47 studies reported intestinal crypt injury. Of those that featured histologic intestinal crypt damage, survival rates at 48 hours ranged from 10-50% (median 30%). Mouse models are not ideal to study intestinal stem cell mediated recovery from severe IRI. Alternative large animal models, like pigs, are recommended.

Introduction

Intestinal ischemia and reperfusion injury (IRI) is a critical pathologic component of numerous disease states.(1) IRI induces significant intestinal epithelial barrier compromise and permits bacterial translocation, potentially resulting in sepsis and shock. Acute mesenteric ischemia, for example, has a mortality rate approaching 80%.(2) The inability to prevent these lesions and challenge of early diagnosis contributes to disease severity. As such, patients face significant epithelial loss and rely upon inherent intestinal epithelial healing to prevent damaging sequelae. Though the small intestine is highly susceptible to IRI, crypt-base intestinal stem cells drive impressive epithelial recovery.(3-5) Unfortunately, few studies induce clinically relevant ischemic injury that effectively recapitulates the severe epithelial loss seen in humans. Even

fewer studies use adequate survival periods to examine tissue repair mechanisms. Ultimately, this has likely contributed to limited development of clinically relevant therapeutics.

Animal models have been vital to elucidate mechanisms of ischemic injury and recovery, and rodents are the most employed. However, key anatomic and physiologic intestinal dissimilarities between humans and rodents limits their translational capacity.(1, 6) Specifically, mice have significant differences in intestinal permeability, vascular anatomy, tissue enzymes, and hypoxic response pathways which could impact the induction of and response to IRI.(6) (7-9) Overall, there is a greater anatomic and physiologic correlation between humans and pigs.(7) Thus, selection of the best animal model for the pathophysiology in question is of paramount importance.

Other attributes of experimental design can also limit the utility of IRI models. Most rodent studies, for example, only use short durations of ischemia which do not reflect most human clinical timelines. In humans, less than 30 minutes of ischemia results in minor lifting of the villus tip epithelium from the basement membrane (Gruenhagen space) which can reseal within 60 minutes of reperfusion.(10) Prolonged ischemia, up to 3 hours (hr), is more common in clinical patients and induces crypt-base injury equivalent to Chiu-Park grade 4.(3) Appropriate modeling of both prolonged ischemia (up to 3hr) and sufficient time for cellular proliferation (>12hr reperfusion) is necessary to understand intestinal crypt driven recovery. (11) Specifically, our previous work demonstrated that 48hr is required for early intestinal stem cell regeneration and epithelial recovery following prolonged IRI.(4)

In this report, we examined the utility of intestinal repair experimentation in mouse and porcine models following prolonged ischemia. We focused on severe injury that both parallels clinical cases and impacts progenitor cells within the epithelial crypt base. To evaluate this, we performed experimental ischemia-reperfusion modeling to compare species-specific outcomes and reviewed mouse IRI literature. Through this approach, we highlighted the strengths and limitations of these IRI animal models.

Methods

The Institutional Animal Care and Use Committee (IACUC) of North Carolina State University approved all animal experimentation (mice: IACUC #20-475, n=25, 8-15 weeks old, 20-25g, C57BL6; pigs: IACUC #21-491, n=7, 8-10 weeks old, 15-25 kg, Yorkshire cross). Mice

and pigs of mixed sex were subjected to segmental mesenteric arterial occlusion for 30 minutes – 3hr.(3, 4, 12) Food was withheld for no longer than 18hr in some mice and all pigs (n=15 mice; n=7 pigs).

Mice were anesthetized using 5% vaporized isoflurane in 100% O2 followed by maintenance anesthesia at 1-2% vaporized isoflurane via nosecone. Pigs were anesthetized following induction with xylazine (1.5mg/kg intramuscularly (IM)) and ketamine (11-20mg/kg IM). Orotracheal intubation was performed, and pigs were maintained under general anesthesia with isoflurane (2-5%, in 100% O_2). For mice, anesthetic depth was monitored by withdrawal reflex and respiratory rate whereas pigs were additionally monitored with jaw tone, palpebral response, and parameters including electrocardiography, pulse oximetry, and indirect blood pressure measurement. For both, temperature was monitored and supported using a water-circulating heating mat and heat lamps. Mice were administered 1mL of subcutaneous normothermic saline, which also provided hydration, while pigs received intravenous isotonic fluids (15 ml/kg/hr). Abdomens were aseptically prepared with a surgical scrub (Chlorhexidine ®) and alcohol. Ventral midline skin incision (mice: 3cm; pigs: 12cm) was followed by incision into the linea alba. In pigs, jejunum was identified 40cm oral to the ileocecal junction, whereas in mice small intestine was isolated at the approximate midpoint between stomach and colon. Segments of intact intestine (mice: 2cm; pigs: 10cm) were carefully exteriorized and kept hydrated with saline. Loops were demarcated with luminal clamping (mice: Johns Hopkins Bulldog clamps; pigs: doyen forceps). Ischemia was induced by clamping vascular supply to a designated segment of intestine using Johns Hopkins Bulldog clamps for up 3hr. The intestinal segments were replaced into the abdominal cavity, skin temporarily closed with towel clamps and covered with saline-soaked gauze for the duration of ischemia.

For both species, after ischemia clamps were removed, intestines were replaced in the body cavity, and carboxymethylcellulose added to reduce adhesion risk. In pigs, sterile saline abdominal lavage was also performed. The abdomen was closed in 2 (mice) or 3 (pig) layers and animals were recovered. During recovery, animals were monitored continuously until they regained ability to stand and ambulate appropriately. Prior to ambulation, mice were treated with 0.01 mg/kg buprenorphine subcutaneously for pain. Additional pain medication (buprenorphine subcutaneously: mice 0.01 mg/kg; pigs 0.02–0.05 mg/kg) was administered as needed. Regular monitoring, every 6-12hr, was continued until the recovery end point, up to 72hr, or humane

endpoint. Mice were euthanized via cervical dislocation following anesthesia using isoflurane. Pigs were euthanized with pentobarbital (85-100 mg/kg intravenously) following induction with xylazine and ketamine.

Tissue was collected at the time of euthanasia for histologic analysis of epithelial injury using Chiu-Park grading (Table 1).(13) A survival curve was determined for animals where recovery was planned or attempted.

Table 1: Chiu-Park grading criteria applied to ischemically-injured intestine.

Chiu-Park Score	Description of Tissue Damage
0	Normal mucosal villi
1	Formation of subepithelial Gruenhagen's space at villus apex; capillary congestion
2	Subepithelial space extension with moderate lifting of epithelial layer from the lamina propria
3	Massive epithelial lifting down the sides of the villi; a few villus tips may be denuded
4	Denuded villi exposing lamina propria and dilated capillaries; increased cellularity of lamina propria may be noted
5	Lamina propria is digested and disintegrated; hemorrhage and ulceration are present

To evaluate current practices and durations of ischemia and reperfusion reported in the field, over 100 original research articles utilizing this methodology were reviewed. Articles in PubMed (2001-2024) were identified using Boolean operators (AND, OR) to combine terms such as intestinal, ischemia, reperfusion, mouse. Relevant articles were identified through title and abstract screening, followed by full-text assessment.

Results and Discussion

Results

Mouse and Porcine Ischemia-Reperfusion Experiments:

A time-dependent, progressive loss of epithelium beginning from the villus tip and extending toward the crypt was observed, as previously described in mouse, porcine, and human injury.(1, 3, 12) Crypt damage was observed by 3hr of ischemia, but not prior, in both species (Figure 1).

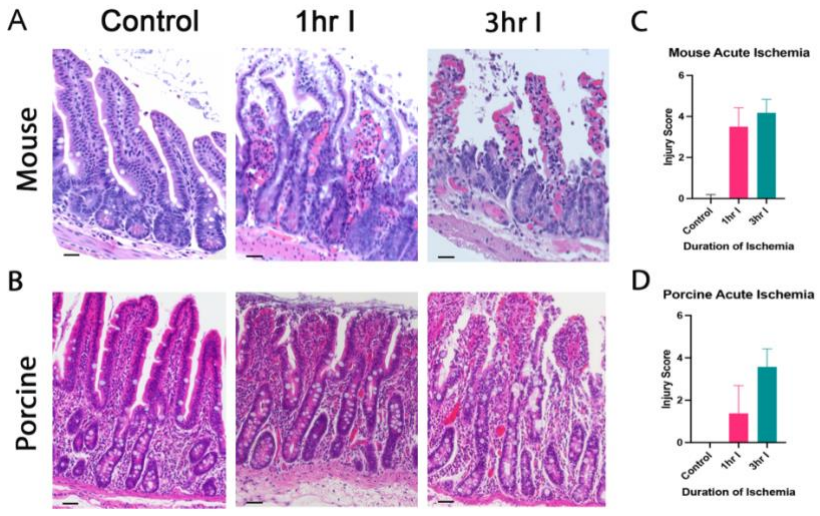

Figure 1: Prolonged ischemia is required to damage the epithelium to the level of the crypt. Representative images of jejunum following no ischemia (control), 1hr of ischemia (1hr I), and 3hr of ischemia (3hr I) in mice (A) and pigs (B). Scale bar = 50 μm, all images taken at 20X. Graphical representation of Chiu-Park Injury scores assigned after injury for mice (C, n= 4) and pigs (D, n= 4-7).

Mouse mortality rate during 3hr of ischemia (3hr I) was 4.2%. However, in the 12hr following, 41.2% of remaining mice were found dead or required emergency euthanasia, increasing to 52.9% at 24hr. By 48hr, 76.5% had died and, by 72hr, mortality increased to 84.6%. In contrast, all pigs who underwent 3hr I survived to 72hr (Figure 2).

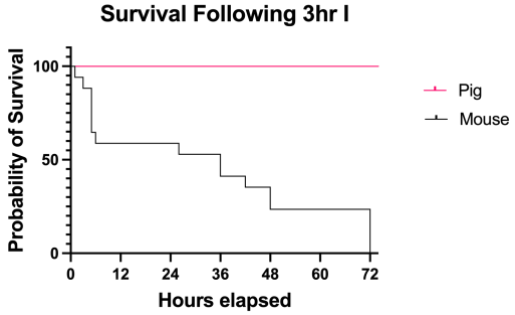

Figure 2: Mouse survival after prolonged ischemia is insufficient to study epithelial recovery. Kaplan-Meier survival prediction comparing mice and pigs following 3hr I.

Furthermore, mice that survived until 48hr following 3hr I showed no histological evidence of recovery-associated crypt depth expansion or re-epithelialization, contrasting such reparative events observed in pigs (Figure 3). In pigs, evidence of progressive repair was observed at 72hr of recovery (Figure 3B). Due to lack of survival, representative mouse imaging

is not available. All mice who underwent only 30 minutes of ischemia survived until 48hr (data not shown).

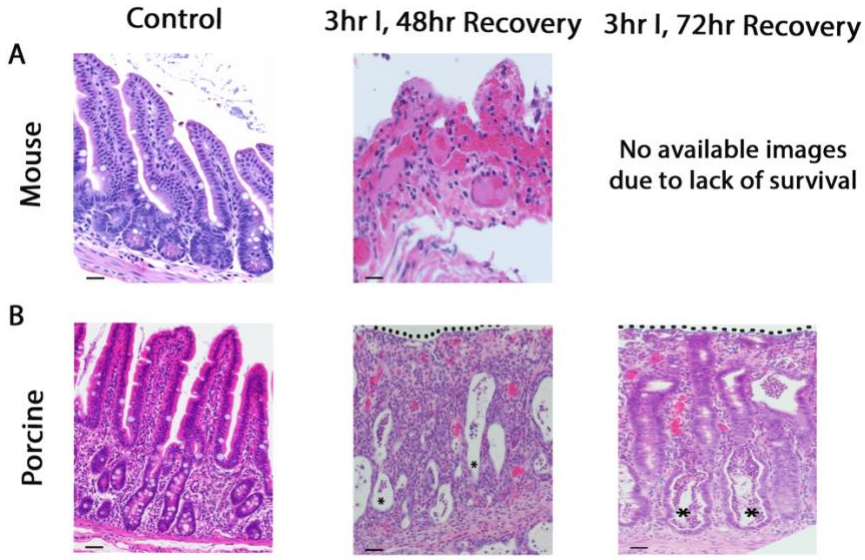

Figure 3: Mouse intestinal epithelium does not show productive recovery following prolonged ischemia. Representative images of jejunum following no ischemia (control), 48hr following 3hr ischemia in mice (A) and porcine tissue (B) (3hr I, 48hr Recovery), and 72hr following 3hr ischemia in porcine (B). Dotted lines indicate re-epithelialization and asterisks indicate crypt regeneration. Scale bar = 50 μm, all images taken at 20X.

Evaluation of Published Mouse Ischemia-Reperfusion Experiments:

One hundred and two articles were reviewed based upon their application of mouse intestinal IRI (Supplementary Table 1). Only 4/102 articles extended ischemia beyond 1hr, and none continued ischemia beyond 90 minutes. All studies employed superior mesenteric artery clamping, compared to the segmental ischemia used here. Durations of ischemia and reperfusion reported by each study are highlighted in the table. Of those reporting injury scores, 47/102 reported damage equivalent to a Chiu-Park score of 4 or higher, consistent with epithelial damage to the level of the intestinal crypt. For those which reported this degree of damage and euthanasia endpoints, 16/47 attempted recovery for 12hr, whereas 15/47 did so for 24hr, and only 6/47 for 48hr, with all others euthanizing mice several hours following ischemia. Within those attempting 12hr of recovery, 8/16 of papers reported survival rates which ranged from 25-100% (mean 72.5%). 7/15 of papers tracked recovery to 24hr which ranged from 0-100% (mean 49.38%), and 4/6 of those recovering for 48hr recorded survival rates of 0-50% (mean 31.0%).

While 47 papers reported injury scores of a Chiu-Park score 4 or higher, our re-evaluation of their representative images found that only 3 reports featured images consistent with this degree of injury. Within these 3 reports, 2 reported survival rates at 12, 24, and 48hr which ranged from 75-100% (mean 100%), 10-75% (mean 60%), and 10-50% (mean 30%) respectively.

Discussion

Mouse models are an invaluable asset to research; however, in severe IRI studies, they have significant limitations. From our experimentation, severe IRI and survival cannot be reliably studied in mice, as compared to pigs. Additionally, we summarized similar limitations in other IRI mouse model papers. Our findings suggest that large animal models, specifically porcine, provide a more reliable and translational framework to study mechanisms of and advance therapies for severe IRI.

From a technical perspective, severe injury (3hr ischemia) can be induced in mice; however, subsequent survival, required to evaluate stem-cell mediated recovery, is less attainable. The challenges of recovering mice beyond severe IRI is evidenced, in part, by the lack of prolonged recovery attempted in the literature, with only 16/47 of papers attempting recovery for 12hr. As El-Assal and colleagues remarked, their initial ischemia modeling "showed that 90 minutes of ischemia resulted in over 70% mortality," which precludes sufficient data collection to draw conclusions.(14) Our attempts to increase survival through segmental vascular occlusion, which reduces the total length of ischemic intestine compared to the more commonly employed superior mesenteric artery occlusion, did not improve mortality rates and instead was associated with intestinal distension. This approach was utilized, in part, to reduce the proportion of experimentally manipulated intestine compared to both the entire intestinal tract and animal size. Given the smaller size of mice, inducing injury in only 2cm of the total small intestine represents a significant proportion of tissue damage and increased risks of mortality.(2, 15) As pigs and humans have more comparable total lengths of small intestine, this factor is less of a confounding variable.(1, 16) The increased length of porcine intestine also allows for the creation of discrete sections of intestine that can be isolated to induce different degrees of injury or introduce local therapeutics, with each animal serving as an internal control.(1, 3, 4, 12)

Animal size also impacts the extent of feasible surgical monitoring and supportive care. Here, both species were managed with constant surveillance during the peri- and immediate

postoperative periods and treated with standard of care following recovery. Because of their smaller size, intravenous fluid resuscitation is nearly impossible in mice and thus only subcutaneous fluids were administered.(17) Additionally, murine endotracheal administration is very challenging and thus nose cone-delivered inhalant anesthesia was used. Both species were treated with the same analgesic agent, buprenorphine, following surgery, though pigs' approved dosing ranges extended higher. Until equipment is available to better facilitate murine endotracheal intubation, blood pressure monitoring, and intravenous fluids and medication administration, these challenges will persist, thus highlighting another benefit to working with larger species. Within the confines of available technologies, efforts were taken to provide comprehensive care and monitoring, however, in the future more standardized protocols may result in improved survival in mice. Beyond procedural difficulties, injury evaluation methodology is also limited. The most employed scoring method, Chiu-Park, is not ideal for evaluating tissue during reperfusion, requiring researchers to develop independent systems making comparisons between studies challenging.(4) The inclusion of additional markers of tissue injury and apoptosis, such as Cleaved Caspase 3 or intestinal fatty acid binding protein, and markers of stem cell-mediated regeneration including sex-determining region Y-box 9 and Ki67 colocalization or olfactomedin 4, would expand future model comparisons.

Mortality, though a simplistic representation of experimental success, was evaluated here as it ultimately determines the degree of recovery which can be studied. It is critical to acknowledge, however, the many biological responses contributing to mortality. These include differences in anatomy, baseline physiology, and inflammatory responses. Direct comparisons to clinically relevant human IRI is complicated by the paucity of human tissue samples with controlled durations of injury and recovery. One group successfully created a model of human small intestine IRI and demonstrated that after brief periods of ischemia (<1hr) epithelial loss is present only in the apical villi, as observed in mouse and pig models (10, 18). To our knowledge, longer durations of human IRI have not been experimentally explored.

In comparison to humans, and pigs, mice have reduced vascular arborization and venules which directly impacts sensitivity to ischemic injury.(19)(20, 21) Rodents also have increased intestinal mucosal oxidase, which contributes to reperfusion injury exceeding that seen in humans with similar degrees of ischemia.(6) Increased xanthine oxidase triggers initial neutrophilic infiltration and tissue inflammation, representing another difference between

humans, pigs, and mice.(1, 16) Beyond intestinal-specific differences, mice have an overall higher metabolic rate compared to pigs, who are much more comparable to humans.(17, 22, 23) Reductions in available oxygen during ischemia may lead to more significant disturbances in energy metabolism which may exacerbate intestinal injury experienced by mice during identical durations of ischemia in pigs and humans.(24) As a further nuance of mouse models, they also have the unique capacity to swiftly and extensively decrease their basal metabolic rate, which further complicates interpretations of IRI responses.(17) Overall, porcine subjects appear to offer a superior option for IRI modeling.

Porcine subjects are not the only large animal which has been evaluated for their utility in IRI modeling. Other species include cats and dogs. Cats, however, like rodents, have increased xanthine oxidase and larger populations of local inflammatory cells, such as neutrophils, which contribute to increased reperfusion injury as compared to humans and pigs.(1, 25, 26) Dogs have similar intestinal anatomy and physiology to humans, however, they are exquisitely sensitive to IRI and experience high associated mortality rates.(27) The use of cats and dogs is also complicated by growing social aversion to companion animal species use in research.(28) Mice, with their easy and inexpensive husbandry and established genetic manipulation tools, will continue to be an indispensable tool for some elements of IRI inquiry, especially that of shorter durations. Specifically, genetic tractability allows for the elimination and/or reduction of cell types in and surrounding the intestinal crypt, which could elucidate the roles of different populations in damage and repair. Additionally, mice will remain a useful early in vivo model for exploring potential IRI dynamics prior to moving to more translatable models like the pig.

Selection of appropriate animal models is necessary to ensure accurate injury representation, translate to species of interest, and reduce and refine research animal use. While mice will remain irreplaceable in the overall study of intestinal epithelial biology, their inability to model prolonged intestinal ischemia and recovery limits their translatability to humans experiencing this very clinically relevant injury. As porcine intestinal anatomy and physiology is inherently more human-like, their size allows multiple replicates and injury grades in one animal, and they survive the injury and recovery consistent with severe IRI in humans, we advocate that they are the ideal model for exploring mechanisms of healing following this injury.

Table S1. Articles evaluating intestinal ischemia and recovery in mouse models.

	Year	Author	Title
1	2024	Huang et al.	E3 ligase TRIM65 alleviates intestinal ischemia/reperfusion injury through inhibition of TOX4-mediated apoptosis
2	2023	Zhang et al.	Organoids transplantation attenuates intestinal ischemia/reperfusion injury in mice through L-Malic acid-mediated M2 macrophage polarization
3	2023	Katada et al.	Role and Potential Mechanism of Heme Oxygenase-1 in Intestinal Ischemia-Reperfusion Injury
4	2023	Chu et al.	Neutrophil extracellular traps aggravate intestinal epithelial necroptosis in ischemia–reperfusion by regulating TLR4/RIPK3/FUNDC1-required mitophagy
5	2023	Tan et al.	Endothelial FOXC1 and FOXC2 promote intestinal regeneration after ischemia-reperfusion injury
6	2023	Liu et al.	Bryostatin-1 attenuates intestinal ischemia/reperfusion-induced intestinal barrier dysfunction, inflammation, and oxidative stress via activation of Nrf2/HO-1 signaling
7	2022	Zhenzhen et al.	miR-146a-5p/TXNIP axis attenuates intestinal ischemia-reperfusion injury by inhibiting autophagy via the PRKAA/mTOR signaling pathway
8	2022	Hu et al.	Lactobacillus murinus alleviate intestinal ischemia/reperfusion injury through promoting the release of interleukin-10 from M2 macrophages via Toll-like receptor 2 signaling
9	2022	Dong et al.	Use of dexmedetomidine to alleviate intestinal ischemia-reperfusion injury via intestinal microbiota modulation in mice
10	2022	Kobritz et al.	H151, a small molecule inhibitor of sting as a novel therapeutic in intestinal ischemia–reperfusion injury
11	2022	Li et al.	Dexmedetomidine Ameliorated Cognitive Dysfunction Induced by Intestinal Ischemia Reperfusion in Mice with Possible Relation to the Anti-inflammatory Effect Through the Locus Coeruleus Norepinephrine System
12	2021	Royster et al.	Therapeutic Potential of B-1a Cells in Intestinal Ischemia-Reperfusion Injury
13	2021	Wu et al.	STING-dependent induction of lipid peroxidation mediates intestinal ischemia-reperfusion injury
14	2021	Funken et al.	Lack of gamma delta T cells ameliorates inflammatory response after acute intestinal ischemia reperfusion in mice
15	2021	Li et al.	MicroRNA-146a overexpression alleviates intestinal ischemia/reperfusion-induced acute lung injury in mice
16	2021	Wu et al.	Complement Initiation Varies by Sex in Intestinal Ischemia Reperfusion Injury

Table S1 (continued).

17	2021	Mi et al.	Remote ischemic post-conditioning alleviates ischemia/reperfusion-induced intestinal injury via the ERK signaling pathway-mediated RAGE/HMGB axis
18	2020	Moore et al.	Sildenafil as a Rescue Agent Following Intestinal Ischemia and Reperfusion Injury
19	2020	Denning et al.	Inhibition of a triggering receptor expressed on myeloid cells-1 (TREM-1) with an extracellular cold-inducible RNA-binding protein (eCIRP)-derived peptide protects mice from intestinal ischemia- reperfusion injury
20	2020	Wu et al.	Eicosanoid production varies by sex in mesenteric ischemia reperfusion injury
21	2020	Zhang et al.	mtDNA-STING pathway promotes necroptosis-dependent enterocyte injury in intestinal ischemia reperfusion
22	2020	Yao et al.	MicroRNA files in the prevention of intestinal ischemia/reperfusion injury by hydrogen rich saline
23	2020	Li et al.	Inhibitor of apoptosis-stimulating protein of p53 inhibits ferroptosis and alleviates intestinal ischemia/reperfusion-induced acute lung injury
24	2020	Li et al.	miRNA-182/Deptor/mTOR axis regulates autophagy to reduce intestinal ischaemia/reperfusion injury
25	2020	Zeng et al.	Aggravated intestinal ischemia-reperfusion injury is associated with activated mitochondrial autophagy in a mouse model of diabetes
26	2020	Chen et al.	Ischemic Postconditioning Alleviates Intestinal Ischemia-Reperfusion Injury by Enhancing Autophagy and Suppressing Oxidative Stress through the Akt/GSK-3β/Nrf2 Pathway in Mice
27	2019	Drucker et al.	Hydrogen Sulfide Donor GYY4137 Acts Through Endothelial Nitric Oxide to Protect Intestine in Murine Models of Necrotizing Enterocolitis and Intestinal Ischemia
28	2019	Yan et al.	The HO-1-expressing bone mesenchymal stem cells protects intestine from ischemia and reperfusion injury
29	2019	Li et al.	Ischemia-induced ACSL4 activation contributes to ferroptosis-mediated tissue injury in intestinal ischemia/reperfusion
30	2019	Dai et al.	MicroRNA-29b-3p reduces intestinal ischaemia/reperfusion injury via targeting of TNF receptor-associated factor 3
31	2019	Levinsky et al.	The olfactomedin-4 positive neutrophil has a role in murine intestinal ischemia/reperfusion injury
32	2018	Jensen et al.	The route and timing of hydrogen sulfide therapy critically impacts intestinal recovery following ischemia and reperfusion injury
33	2018	Karhausen et al.	Ubc9 overexpression and SUMO1 deficiency blunt inflammation after intestinal ischemia/reperfusion

Table S1 (continued).

34	2018	Liu et al.	miR-381-3p knockdown improves intestinal epithelial proliferation and barrier function after intestinal ischemia/reperfusion injury by targeting nurr1
35	2018	Matsuo et al.	Inhibition of ubiquitin-activating enzyme protects against organ injury after intestinal ischemia-reperfusion
36	2018	Kim et al.	Role of iRhom2 in intestinal ischemia-reperfusion-mediated acute lung injury
37	2018	Duranti et al.	Bifidobacterium bifidum PRL2010 alleviates intestinal ischemia/reperfusion injury
38	2017	Takeda et al.	Activation of Erk in ileal epithelial cells engaged in ischemic-injury repair
39	2017	Li et al.	MicroRNA-378 protects against intestinal ischemia/ reperfusion injury via a mechanism involving the inhibition of intestinal mucosal cell apoptosis
40	2017	Zhu et al.	Pretreatment with the ALDH2 agonist Alda-1 reduces intestinal injury induced by ischaemia and reperfusion in mice
41	2017	Ueda et al.	The protective effect of orally administered redox nanoparticle on intestinal ischemia-reperfusion injury in mice
42	2017	Jensen et al.	Hydrogen sulfide improves intestinal recovery following ischemia by endothelial nitric oxide-dependent mechanisms
43	2017	Geha et al.	IL-17A Produced by Innate Lymphoid Cells Is Essential for Intestinal Ischemia-Reperfusion Injury
44	2017	Zhu et al.	Down-regulation of toll-like receptor 4 alleviates intestinal ischemia reperfusion injury and acute lung injury in mice
45	2017	Khadaroo et al.	Metabolomic profiling to characterize acute intestinal ischemia/reperfusion injury
46	2017	Yu et al.	Conventional alpha beta (αβ) T cells do not contribute to acute intestinal ischemia- reperfusion injury in mice
47	2016	Jensen et al.	Harvest tissue source does not alter the protective power of stromal cell therapy after intestinal ischemia and reperfusion injury
48	2016	Liu et al.	MicroRNA-682-mediated downregulation of PTEN in intestinal epithelial cells ameliorates intestinal ischemia–reperfusion injury
49	2016	Daly et al.	Role of matrix metalloproteinase-8 as a mediator of injury in intestinal ischemia and reperfusion
50	2016	Meng et al.	Ischemic post-conditioning attenuates acute lung injury induced by intestinal ischemia–reperfusion in mice: role of Nrf2

Table S1 (continued).

51	2016	Hansen et al.	Sirtuin 1 activator SRT1720 protects against organ injury induced by intestinal ischemia-reperfusion
52	2016	Zhu et al.	Protective effects of fenofibrate against acute lung injury induced by intestinal ischemia/reperfusion in mice
53	2016	Gubernatorova et al.	Murine Model of Intestinal Ischemia-reperfusion Injury
54	2015	Pope et al.	TLR2 Modulates Antibodies Required for Intestinal Ischemia/Reperfusion-Induced Damage and Inflammation
55	2015	Gobbetti et al.	Protective effects of n-6 fatty acids-enriched diet on intestinal ischaemia/ reperfusion injury involve lipoxin A4 and its receptor
56	2015	Peng et al.	Protection by enteral glutamine is mediated by intestinal epithelial cell peroxisome proliferator activated receptor-+ during intestinal ischemia/reperfusion
57	2014	Ma et al.	Modulating the p66shc Signaling Pathway with Protocatechuic Acid Protects the Intestine from Ischemia-Reperfusion Injury and Alleviates Secondary Liver Damage
58	2014	Matsuda et al.	FK866, a Visfatin Inhibitor, Protects Against Acute Lung Injury After Intestinal Ischemia–Reperfusion in Mice via NF-κB Pathway
59	2014	Watanabe et al.	Toll-Like Receptor 2 Mediates Ischemia-Reperfusion Injury of the Small Intestine in Adult Mice
60	2013	Watkins et al.	Synergistic effects of HB-EGF and mesenchymal stem cells in a murine model of intestinal ischemia/reperfusion injury
61	2013	Cho et al.	Remifentanil ameliorates intestinal ischemia-reperfusion injury
62	2013	Matsuo et al.	Cyclic arginine-glycine-aspartate attenuates acute lung injury in mice after intestinal ischemia/ reperfusion
63	2013	Jawa et al.	Mesenteric ischemia-reperfusion injury up-regulates certain CC, CXC, and XC chemokines and results in multi-organ injury in a time-dependent manner
64	2013	Lee et al.	Critical role of interleukin-17A in murine intestinal ischemia-reperfusion injury
65	2013	Wu et al.	The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization
66	2013	Ban et al.	Inhibition of ERK1/2 Worsens Intestinal Ischemia/ Reperfusion Injury
67	2013	Kannan et al.	R-spondin3 prevents mesenteric ischemia/reperfusion-induced tissue damage by tightening endothelium and preventing vascular leakage
68	2013	Dong et al.	Resveratrol ameliorates subacute intestinal ischemia-reperfusion injury
69	2013	Wang et al.	Temporal variations of the ileal microbiota in intestinal ischemia and reperfusion

Table S1 (continued).

70	2012	Gobbetti et al.	Serine Protease Inhibition Reduces Post-Ischemic Granulocyte Recruitment in Mouse Intestine
71	2012	Cai et al.	Keratinocyte growth factor up-regulates Interleukin-7 expression following intestinal ischemia/reperfusion in vitro and in vivo
72	2012	Kim et al.	Isoflurane Post-conditioning Protects Against Intestinal Ischemia-Reperfusion Injury and Multiorgan Dysfunction via Transforming Growth Factor-β1 Generation
73	2012	Wang et al.	SIRT3-mediated deacetylation of PRDX3 alleviates mitochondrial oxidative T damage and apoptosis induced by intestinal ischemia/reperfusion injury
74	2012	Sun et al.	Ginsenoside Rb1 Attenuates Intestinal Ischemia Reperfusion Induced Renal Injury by Activating Nrf2/ARE Pathway
75	2012	Ben et al.	TLR4 Mediates Lung Injury and Inflammation in Intestinal Ischemia-Reperfusion
76	2012	Lapchak et al.	Platelet-Associated CD40/CD154 Mediates Remote Tissue Damage after Mesenteric Ischemia/Reperfusion Injury
77	2012	Watanabe et al.	Activation of the MyD88 signaling pathway inhibits ischemia-reperfusion injury in the small intestine
78	2011	Lapchak et al.	Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury
79	2010	Pope et al.	Complement regulates TLR4-mediated inflammatory responses during intestinal ischemia reperfusion
80	2010	Sparkes et al.	Intestinal lipid alterations occur prior to antibody-induced prostaglandin E2 production in a mouse model of ischemia/reperfusion
81	2010	James et al.	HB-EGF Protects the Lungs after Intestinal Ischemia/Reperfusion Injury
82	2010	Soares et al.	Tumor necrosis factor is not associated with intestinal ischemia/reperfusion induced lung inflammation
83	2010	Victoni et al.	Local and remote tissue injury upon intestinal ischemia and reperfusion depends on the TLR/MyD88 signaling pathway
84	2010	Katada et al.	Carbon Monoxide Liberated from CO-Releasing Molecule (CORM-2) Attenuates Ischemia/Reperfusion (I/R)-Induced Inflammation in the Small Intestine
85	2009	Edgertona et al.	IL-17 producing CD4+ T cells mediate accelerated ischemia/reperfusion-induced injury in autoimmunity-prone mice
86	2009	Souza et al.	The Long Pentraxin PTX3 Is Crucial for Tissue Inflammation after Intestinal Ischemia and Reperfusion in Mice
87	2009	Chen et al.	B cells contribute to ischemia/reperfusion-mediated tissue injury
88	2009	Cui et al.	Milk Fat Globule Epidermal Growth Factor 8 Attenuates Acute Lung Injury in Mice after Intestinal Ischemia and Reperfusion

Table S1 (continued).

89	2008	El-Assal et al.	Heparin-binding epidermal growth factor–like growth factor gene disruption is associated with delayed intestinal restitution, impaired angiogenesis, and poor survival after intestinal ischemia in mice
90	2008	Aprahamian et al.	Toll-like receptor 2 is protective of ischemia–reperfusion-mediated small-bowel injury in a murine model
91	2008	Huang et al.	A Novel Targeted Inhibitor of the Alternative Pathway of Complement and Its Therapeutic Application in Ischemia/Reperfusion Injury
92	2008	Hart et al.	Role of extracellular nucleotide phosphohydrolysis in intestinal ischemia-reperfusion injury
93	2007	Amaral et al.	The Role of Macrophage Migration Inhibitory Factor in the Cascade of Events Leading to Reperfusion-Induced Inflammatory Injury and Lethality
94	2006	Szabó et al.	Gender differences in ischemia-reperfusion-induced microcirculatory and epithelial dysfunctions in the small intestine
95	2006	Cattaruzza et al.	Protective Effect of Proteinase-Activated Receptor 2 Activation on Motility Impairment and Tissue Damage Induced by Intestinal Ischemia/Reperfusion in Rodents
96	2005	Souza et al.	NF-jB plays a major role during the systemic and local acute inflammatory response following intestinal reperfusion injury
97	2005	Vieira et al.	Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury
98	2005	Hart et al.	Gastrointestinal Ischemia-Reperfusion Injury Is Lectin Complement Pathway Dependent without Involving C1q
99	2003	Fleming et al.	C5a causes limited, polymorphonuclear cell-independent, mesenteric ischemia/reperfusion-induced injury
100	2003	Souza et al.	Role of PAF receptors during intestinal ischemia and reperfusion injury. A comparative study between PAF receptor-deficient mice and PAF receptor antagonist treatment
101	2003	Stahl et al.	Role for the Alternative Complement Pathway in Ischemia/Reperfusion Injury
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CHAPTER 3

Human Placental Extract Enhances Neonatal Epithelial Reparative Capacity in Porcine Models

Abstract

The devastation caused by necrotizing enterocolitis (NEC) has continued to claim the lives of infants despite advances in neonatal medicine. To address the acute, and often severe, intestinal epithelial damage caused by NEC, therapeutics are needed that directly target epithelial recovery and cellular regeneration processes. We investigated the capacity of a decellularized human placental extract (HPE) for its ability to prevent and enhance recovery from NEC using *in vitro* and *in vivo* models. Healing responses in primary neonatal porcine ileal epithelial cells were analyzed following hypoxia or scratch-wound injury and HPE application. *In vitro,* HPE treatment accelerated scratch closure and increased proliferating cell number though did not enhance tight junction recovery following hypoxia. *In vivo,* NEC was induced in neonatal piglets through a combination of preterm delivery and formula feeding. Treated piglets received enteral HPE while control were *nil per os* prior to formula initiation. *In vivo,* HPE treatment increased weight gain, decreased macroscopic and histological damage, and increased ileal crypt epithelial cell proliferation. After observation of similar effects using *in vitro* and *in vivo* platforms, transcriptomic analysis of monolayer cultures treated with HPE was undertaken. Increased expression of pathways associated with epithelial wound healing, proliferation, and migration were identified, with key shared genes between those pathways. In sum, these findings suggest that HPE can enhance the reparative capacity of neonatal epithelium in the context of NEC.

Introduction

Despite decades of research and clinical efforts, necrotizing enterocolitis (NEC) remains the most common neonatal gastrointestinal emergency with an unacceptably high mortality rate of over 30% in severely affected infants.[1, 2] [3] The pathogenesis of NEC has not been completely elucidated, but known contributors include prematurity, characterized by both gestational age and low birthweight, antibiotic exposure, intestinal dysbiosis, formula-feeding, and hypoxia.[1, 4] Combinations of these factors manifest as significant inflammation and necrosis of the epithelium predominantly in the ileum and proximal colon which present clinically as abdominal distension, bloody stool, and bile-stained emesis.[5-8] The complex etiology of NEC is matched by a variable onset of symptoms with some cases gradually developing while others have a sudden, dramatic emergence and deterioration.[9] This unpredictable timeline precludes early intervention for all cases and creates a conundrum for clinicians who must decide between prophylactic treatment for those predisposed to developing

NEC or risk initiating therapy after significant damage may already have occurred. Moreover, there are no diagnostic tests approved to determine who will develop the disease.[10] Notwithstanding timing, the therapeutic options for NEC remain overall limited and untargeted. For milder cases, supportive medical therapy consists of feeding cessation, fluid support, gastric decompression, antibiotic therapy, and frequent monitoring.[1] For severely affected infants, especially those with necrotic bowel or intestinal perforation, surgical bowel resection may be necessary.[9] There are no currently employed therapeutic tools to specifically accelerate the recovery of the intestinal epithelium, which is necessary to restore the barrier between intestinal contents and the bloodstream and prevent downstream sequela of NEC, such as sepsis. Epithelial recovery occurs though a process of restitution, comprised of villous contraction, epithelial migration, and tight junction recovery, followed by proliferation, which is driven by intestinal crypt-based stem cells.[11, 12] Thus, potential therapeutics for NEC must be evaluated for their capacity to accelerate those processes.

Though none have been adopted in clinical practice, reproductive membrane-derivatives have shown promise in preventing NEC.[5, 6, 8, 13-20] Many of the therapeutics evaluated thus far are derived from amniotic fluid which is known to be rich in many anti-inflammatory and pro-maturation factors for the intestinal epithelium.[6, 14-25] Amniotic-derived therapies, however, are accompanied by inherent challenges such as limited fluid availability, as only approximately 70 mL are available for collection at cesarean section, and reliance upon advanced culturing techniques to propagate placental stem cells or their byproducts.[6, 26] The placenta, which produces amniotic fluid, however, is generally considered biowaste and available following most deliveries; it is also increasingly recognized as a more ideal source of perinatal stem cells.[8] Thus, we created a proprietary, cell-free human biotherapeutic extract comprised of growth factors, cytokines, and fatty acids isolated from term-pregnancy, post-delivery placentas. Our patented method yields high quality concentrations of the anti-inflammatory, pro-regenerative cytokines that are synthesized, stored, and secreted by the placenta during pregnancy. We evaluated this product using highly translatable neonatal piglet models in a two-pronged approach including established *in vitro* models of epithelial injury in primary cell cultures from neonatal piglet ileum and a validated *in vivo* model of NEC.[27]

In this work, we hypothesized that the application of human placental extract (HPE) would enhance neonatal porcine intestinal epithelial cells' capacity to repair after injury through

the upregulation of epithelial cell migration and proliferation. We evaluated the impact of HPE through a combination of *in vitro* models, where HPE was applied after injury, and *in vivo* models, where HPE was given prior to amplification of a NEC-inducing environment. This combinatorial approach was pursued to provide greater insight into HPE's prophylactic and therapeutic capacity, as the unpredictable progression of NEC often makes treatment timing precarious. We evaluated the efficacy of HPE with markers of *in vitro* barrier recovery including tight junction repair measured with transepithelial electrical resistance (TEER), scratch-wound closure, expression of proliferative biomarkers, and transcriptomic analysis, in addition to *in vivo* outcomes such as weight gain, gross tissue damage, histological injury, and intestinal crypt-based proliferative markers. In support of our hypothesis, HPE increased epithelial cell migration and proliferation *in vitro* and increased weight gain and intestinal crypt epithelial proliferation while decreasing gross and histological damage in *in vivo* models of NEC. Overall, HPE appears to have both prophylactic and therapeutic potential for addressing this devastating disease.

Materials and Methods

Human Placental Extract Production

HPE is manufactured by a proprietary, patented process using donated human placental tissue from term deliveries. Human placental tissue was acquired from FDA registered tissue establishments following Food and Drug Administration 21 Code of Federal Regulations1271 guidance and American Association of Tissue Banking Standards. Placentas were rinsed in 0.9% saline and shipped for processing within 48 hours of birth. Following removal of gross debris, tissues underwent mechanical dissection of the placental disc. Tissue was then rinsed in sterile bioprocessing solutions and homogenized. Blood was removed using red cell lysis buffer with agitation. Following blood removal, a phosphate-buffered saline (PBS) solution containing a proprietary mixture of protease inhibitors, to protect and preserve proteins, was used for multiple rounds of rinsing with centrifugation. The washed tissue was processed to fine particle size to optimize cell lysis. Lysis of the cells within this mixture was accomplished utilizing a specified freeze/thaw procedure. After thawing, up to 10 placental tissues were combined and mixed to reduce individual component variability in the final product. Subsequent and further cell lysis was accomplished by sonication and centrifugation to remove larger extracellular matrix components. Additional processing of the HPE supernatant included lyophilization and terminal sterilization.

For *in vitro* assessments, HPE was reconstituted using dimethyl sulfoxide (DMSO) and frozen at -80C. At the time of application, HPE was thawed at room temperature and then applied, apically, at the listed doses. *In vivo,* HPE was reconstituted in sterile water and delivered by orogastric tube. *In vitro* and *in vivo* doses were analogous, as the intestinal epithelium is ultimately exposed to approximately $1/100^{th}$ of the concentration of treatment given to the animal enterally.[46]

Experimental Animals for Cell Culture

The Institutional Animal Care and Use Committee (IACUC) of North Carolina State University approved all animal experimentation (IACUC #21-491, n = 11). Yorkshire crossbred piglets (< 7 days) of both sexes were used. Piglets were anesthetized with an intramuscular combination of telazol, xylazine, and ketamine (0.03 ml/kg). Once anesthetized, piglets were euthanized with intracardiac pentobarbital (85 – 100 mg/kg). Death was confirmed with the absence of heartbeat and corneal reflex, after which tissues were immediately collected. Ileum was rinsed with 1X PBS and then immediately processed for organoid culture.

Ileal Epithelial Crypt Isolation and Monolayer Culture

The collected ileum was opened longitudinally along the antimesenteric border and then sub-sectioned into 1 cm pieces. Crypt isolation was completed as previously published apart from a shorter duration of incubation (20 minutes) in the first dissociation reagent.[69-72] Crypts were then plated for organoid culture which was performed as previously described.[69-72] Enteroids were grown until reaching an adequate size to provide a sufficient number of cells for monolayer creation.

Enteroids were dissociated from 3D Matrigel matrix (Corning, Durham, NC) using the protocol described by Van der Hee and colleagues with minor deviations.[73] Briefly, ice-cold Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) medium, (ThermoFisher Scientific, Waltham, MA) was applied to Matrigel patties, which were then transferred to 15 mL conical tubes and centrifuged for 5 min at 250xg. The supernatant was removed, and 2.5 mL of TrypLE Express dissociation media (Gibco, ThermoFisher) was added to the pellet, thoroughly mixed with repetitive pipetting, and then allowed to incubate for 10 min at 37C. After incubation, 8mL of 20% fetal bovine serum (FBS) in 80% culture media was added to the vial which was then centrifuged at 600xg for 6 minutes. The supernatant was again removed and the remaining cell pellet resuspended in the necessary volumes of culture media

with 20% FBS (200 uL per monolayer). Cells were quantified using a Cellometer Auto 2000 (Nexcelom, Lawrence, MA) and plated onto 0.5% Matrigel-coated plates at a density of 78,125 – 100,000 cells/cm^2. Monolayers were supplemented with FBS-enriched culture media and growth factor combination as previously published.[69-71] Monolayers were grown until visual confluence (Figure 1A).

Hypoxia Exposure and TEER Measurement

Visually confluent monolayers were assessed for their transepithelial electrical resistance (TEER) using an Endohm-6G dual-electrode chamber and EVOM2 meter (World Precision Instrument, Sarasota FL) and deemed confluent once reaching visual confluency and 1000 Ohms*cm^2. Once confluent, culture media and growth factors were refreshed, and the wells were placed in a Modulator Incubator System (Billups-Rothenberg, inc., Del Mar, CA; 1% O_2, 5% CO_2, balanced with N_2) for 18 hours. Following hypoxia, media and growth factors were again refreshed. Monolayers were treated with media alone (control) or three concentrations of HPE reconstituted in culture media: 0.075, 0.15, or 0.5 mg/mL. Transepithelial electrical resistance was measured every 6 hours for 24 hours following hypoxia with media replacement and treatment at each measurement timepoint (Figure 1A). Rate of TEER change was calculated using each monolayer as its own baseline.

Scratch Assay

Once visually confluent, a 200 uL pipette tip was used to create a longitudinal scratch wound across the center of the well. Following scratch, the wells were washed with media and then fresh media, growth factors, and treatment was applied. Monolayers were treated with media alone (control) or three concentrations of human placental extract reconstituted in culture media: 0.075, 0.15, or 0.5 mg/mL. Scratches were imaged at the same locations along leading edge of the scratch every 6 hours until closure or 24 hours (Figure 1A). To maximize visibility, new media was applied prior to each imaging timepoint with new growth factors and treatment added after imaging. 9 sites along the leading edge of the scratch wound were measured using CellSens at every time and percent closure over time was calculated.

NEC Animal Model

The Institutional Animal Care and Use Committee (IACUC) of Inotiv (Fort Collins, Co) approved the NEC animal model (IACUC #21-28, n = 64). A previously validated piglet model of NEC was used to induce disease in all piglets.[27] As described, piglets were delivered via

caesarean section on day 103-105 of gestation (115 days term), equivalent to a 32-week human pregnancy. In addition to preterm delivery, hyperosmolar formula feedings were also used to induce NEC. Prior to the introduction of formula, and beginning immediately following delivery, piglets received one of three dose concentrations of HPE (240, 480, 960 mg/kg/day) via an orogastric tube or nothing per os (control) and all piglets were maintained on total parenteral nutrition for the first 48 hours of life (Figure 4A). After resuscitation following delivery, piglets were sorted by ascending weight into 3 weight categories containing at max 5 piglets: low (1), middle (2), and high (3). Piglets were then given a random number generated identifier and sorted by ascending random number within the weight category. Each piglet was assigned a group number in a 1:1:2:1 ratio for the treatments including control: 240 mg/kg/day: 480 mg/kg/day: 960 mg/kg/day HPE.

After 48 hours, HPE treatment and intravenous feedings were discontinued, and all piglets were transferred to hyperosmolar oral formula for an additional 48 hours. Animals were then euthanized per IACUC guidelines at 96 hours after the initiation of the study. For data analysis, if any animal within a litter developed NEC, then the entire litter was evaluated with animals grouped as NEC+ or NEC. Piglets receiving all doses of HPE were combined into one treatment group for analysis. Evaluators were blinded to treatment.

Tissue histomorphometric evaluation and immunofluorescent assessment

Following euthanasia, the piglets' abdomens were opened and intestinal sections including duodenum, jejunum, ileum, cecum, colon, rectum, were evaluated by a veterinarian or veterinary pathologist to characterize the extent of damage as indicated by gross evidence of edema, hemorrhage, necrosis, and pneumatosis intestinalis and collected for histologic analyses. Each segment of gastrointestinal tract was given an individual NEC score on a 6-point system as listed: 1: absence of lesions; 2: local hyperemia, inflammation, and edema; 3: hyperemia, extensive edema, and local hemorrhage; 4: extensive hemorrhage; 5: local necrosis and pneumatosis intestinalis; 6: extensive necrosis and pneumatosis intestinalis.[65, 74]

For hematoxylin and eosin staining, gastrointestinal tissues were placed in 10% neutral buffered formalin and transferred to 70% ethanol. For immunohistochemistry, small intestine samples were stored 4% paraformaldehyde (PFA) for 18 hours and then stored in 30% sucrose until placement in optimal cutting solution (OCT) matrix.

Histologically, the intestine was evaluated by a board-certified veterinary pathologist using a four point NEC scoring scheme as follows: 0: normal histological appearance, 1: mild local disruption of villus tips (necrosis and/or ulceration of mucosal epithelium), congestion and/or submucosal edema, may be the solitary finding; 2: mild local villus disruption and separation of the submucosal layer (by edema), with or without congestion; 3: regional villus disruption, blood congestion, and separation of the submucosa and lamina propria (by edema); 4: extensive, complete destruction of the villus architecture, blood congestion, and transmural necrosis. An adapted scoring matrix was applied to the stomach and large intestine, including rectum, also using four grades which was identical to that used above apart from the additions to the following score categories: 1: minimal local hemorrhage, and/or mild or more severe submucosal edema, may be used for any solitary finding without disruption or combination of minimal findings without disruption; 2: mild hemorrhage. A sum of the gross necropsy and computerized histomorphometry NEC scores for all segments were compiled to determine comprehensive overall disease severity. Higher summed scores are indicative of dispersed and more severe damage across the six sections of intestinal tract. Veterinary pathologists determined the definitive NEC status of each piglet. Overall disease severity was stratified by treatment group (control vs HPE) and by NEC status (NEC positive vs NEC negative). Disease severity was assessed by Two-Way ANOVA with a Tukey Post-Test, $p<0.05$ to evaluate the effect of treatment on NEC+ and NEC- animals.

Immunohistochemistry was performed on ileal sections from piglets of litters in whom NEC was successfully induced and scratch injured monolayers. Antibodies against sex-determining region Y-box 9 (SOX9, rabbit, 1:500, Millipore Sigma, Temecula, CA), which is associated with intestinal stem cells, transit amplifying cells, and Paneth cells, and Ki67 (mouse, 1:400, Biocare Medical, Pacheco, CA) a marker of proliferation were applied for overnight incubation at 4C as previously reported.[72] Secondary antibodies were all identically diluted (1:500, Sox9: Alexa 555/Cy3.5; Ki67: Alexa 488/GFP; separately), applied for 45 minutes at room temperature, and counterstained with bisBenzimide H 33258 nuclear stain (DAPI, 1:1,000, Sigma-Aldrich, St. Louis, MO).

Positive cells were counted per well-oriented crypt using Fiji and reported as ratios for the total number of cells present. For scratch wound assay, cultures were incubated for 1 hour with 5-ethynyl-2'-deoxyuridine (EdU Click-iT, Thermo Fisher Scientific, Waltham, MA)

following 12 hours of closure, as previously outlined.[69] Monolayers were then with fixed with 4% PFA for 15 minutes and submerged in PBS until further immunofluorescence analysis for Ki67 expression (Alexa 488/GFP), EdU coloration (Alexa 647/ Cy5) and nuclear staining (DAPI). To quantify the number of actively proliferating cells, a grid was applied to each image and a set area was counted in the same six locations along the leading edge for each scratch. The number of total nuclei ($DAPI^+$) and those cells expressing EdU ($Alexa647^+$), Ki67 ($Alexa488^+$), or both were quantified for each set area.

Progress to Confluence and Bulk RNA-sequencing of neonatal ileal monolayers following treatment with HPE

Neonatal porcine ileal monolayers were derived as summarized above. At the time of plating, wells were either treated with media alone (Control) or HPE (0.075 mg/mL). Monolayers were imaged daily to evaluate progress to confluence with daily replenishment of media, growth factors, and treatment prior to imaging. The percent confluence was recorded at each day and compared between HPE-treated and untreated monolayers (paired T-test or Wilcoxon test).

Once nearly confluent (> 90%), monolayers were harvested for bulk RNA sequencing using the RNeasy ® Plus Mini Kit and protocol for total RNA extraction (Qiagen, Valencia, CA). Baseline yield and quality were determined (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA; 260/280 = 2.09 to 2.12). To further confirm quality, samples were evaluated for RNA Integrity Number (RIN) score (10 for all samples, RNA ScreenTape®, TapeStation Analysis Software 4.1, Agilent Technologies, Santa Clara, CA).

Samples were then submitted for bulk RNA sequencing which was performed through a series of steps including RNA Quantification and Normalization using the DeNovix RNA Fluorescence Assay, mRNA-Seq library preparation (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina), library quantification and normalization (DeNovix DNA Fluorescence Assay), library quality control (Agilent Bioanalyzer HS DNA Kit), and then sequencing (SeqMatic, Novaseq).

Analysis was performed by the Bioinformatics and Analytics Research Collaborative at UNC Chapel Hill using the Nextflow (v23.10.1) nf-core/rnaseq pipeline (v3.14.0) with default settings.[75] Briefly, quality control was performed with FastQC (v0.12.1) and Qualimap (v2.3). Adapters were then trimmed with Trim Galore! (v0.6.7) and reads were aligned to the Sscrofa11

reference genome (Ensembl build 111) with STAR (v2.7.9a).[76] Transcripts were quantified with Salmon (v1.10.1).[77] All processing and analysis were run using Singularity/Apptainer (v1.3.0-1), and the singularity definition file used for analysis is provided in the code repository. Transcript level counts from Salmon were summarized to the gene level in R (v4.3.2) with tximport (v1.32.0).[78] Differential analysis was performed with limma (v3.60.2).[79] Distinct replicates within each piglet were treated as nested replicates, and replicate correlation was modelled with the limma "duplicateCorrelation()" function, and piglet was used as a blocking factor to better control for piglet specific effects. Differentially expressed genes (DEG) with adjusted p-values (Benjamini-Hochberg correction) of <= 0.05 were classified as significantly different. Gene Set Enrichment Analysis (GSEA) was performed with the fgsea package (v1.30.0)[80] using a Biological Process (BP) gene set for *Sus scrofa* that was generated from the Ensembl 111 Sscrofa annotation obtained with biomaRt (v2.60.0).[81] Gene Ontology (GO) offspring terms were mapped and merged for each term with the GO.db package (v3.19.1), such that each term contained all directly annotated genes and genes associated with any offspring terms (see code for details).[82-84] Enrichment of specific BP GO terms within the log_2fold ranked gene list were tested using a manually curated list of pathways (Table 1).

Table 1. BP GO search terms pathways applied to log_2fold ranked gene list.

go_id	go_name
GO:0008283	cell population proliferation
GO:0072089	stem cell proliferation
GO:0050673	epithelial cell proliferation
GO:0010631	epithelial cell migration
GO:0010634	positive regulation of epithelial cell migration
GO:0010633	negative regulation of epithelial cell migration
GO:0007267	cell-cell signaling
GO:0016055	Wnt signaling pathway
GO:0030177	positive regulation of Wnt signaling pathway
GO:0009299	mRNA transcription
GO:0006754	ATP biosynthetic process
GO:0042060	wound healing
GO:0002009	morphogenesis of an epithelium
GO:0048565	digestive tract development
GO:0042113	B cell activation

Acquisition of Images

Cell and monolayer confluence were assessed daily with an inverted, bright field microscope (Zeiss AxioVert A1, White Plains, NY). Images were captured using an inverted fluorescence microscope (Olympus IX83, Tokyo, Japan) fitted with a monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Shizuoka, Japan) and color camera (DP26, Olympus, Tokyo, Japan). Scratches were imaged using the bright field channel whereas immunofluorescence was assessed using appropriate color channels (DAPI, 488, 555, or 647). The objective lenses used were X10, X20, and X40 (numerical apertures of 0.3, 0.45, and 0.6, respectively) (LUC Plan FLN, Olympus, Tokyo, Japan). CellSens imaging acquisition and analysis software was utilized for capturing all images and analyzing scratch wound closure (Evident, Waltham, MA). Fiji was employed for immunofluorescent quantification.[85]

Statistical Analysis

Prism software (GraphPad Software, La Jolla, CA) was employed for statistical analysis. Outliers were removed with the robust regression and outlier removal tool. The normality of raw data was assessed with a Shapiro-Wilk normality test, followed by either a 2-way analysis of variance with Tukey's multiple comparisons, uncorrected Fisher's test, Mann-Whitney U test, or paired T-test. Statistical significance was defined as $P < .05$. All authors had access to all data and have reviewed and approved the final manuscript.

Results

HPE Does Not Accelerate Tight Junction Recovery Following Hypoxic Injury

Though the complete pathophysiology is unknown, hypoxia is a recognized component of NEC.[1, 28, 29] Brief periods of hypoxia weaken the tight junctions between cells without resulting in the complete death or loss of cells and in this way may model very early onset of NEC.[30] The ability of HPE to restore tight junction integrity following hypoxia-induced damage was therefore investigated using monolayer cultures (Figure 1A). HPE did not improve tight junction recovery, measured by TEER, greater than control (culture media), although a mild protective effect was noted at the 0.075mg/ml dose of HPE at 24 hours ($p > 0.999$, Figure 1B). This was the only dose evaluated in this injury context. Since tight junction recovery is only a single component of the complex mechanism of epithelial cellular response to injury, other elements of repair were evaluated.

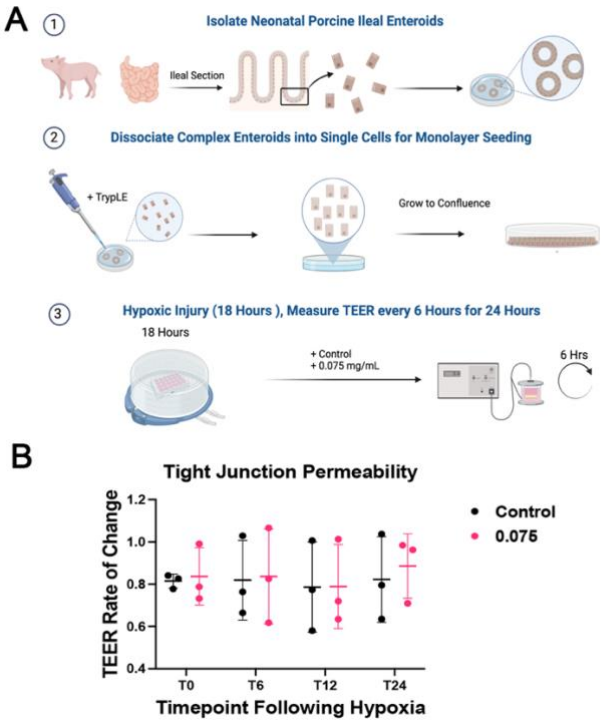

Figure 1: HPE does not significantly increase tight junction recovery following hypoxic injury. A) Summary of Experimental Design. Control treatment consisted of cell culture media only. B) Recovery of tight junction integrity (TEER), represented as a ratio comparing each monolayer's timepoint TEER to its own pre-hypoxia level, was measured with an Endohm-6G dual-electrode chamber and EVOM2 meter every 6H for 24H. n= 3 pigs for both groups, 3 independent experiments. 2way ANOVA followed by Tukey's multiple comparisons. Data presented as mean +/- standard deviation.

HPE Accelerates Neonatal Ileal Restitution

In severe disease, NEC induces significant intestinal epithelial loss which requires cells to restitute to re-establish the epithelial barrier. To evaluate the impact of HPE on epithelial restitution, linear scratch wounds were created in confluent monolayers. Twelve hours post-injury, untreated monolayers achieved a mean closure of 43% +/- 20.78% compared to HPE-treated cultures of 47.875 +/- 6.69% (0.15 mg/mL), 57.91% +/- 9.81% (0.5 mg/mL), and 74.85% +/- 19.23% (0.075 mg/mL) with a statistically significant difference between control and 0.075 mg/mL HPE ($p < 0.0001$) (Figure 2A and 2B). After 24H, all HPE-treated monolayers were fully closed and achieved 23.2% greater closure than untreated wounds (100% +/- 0% treated vs 76.8% +/- 12.91% control; $p< 0.001$) (Figure 2C).

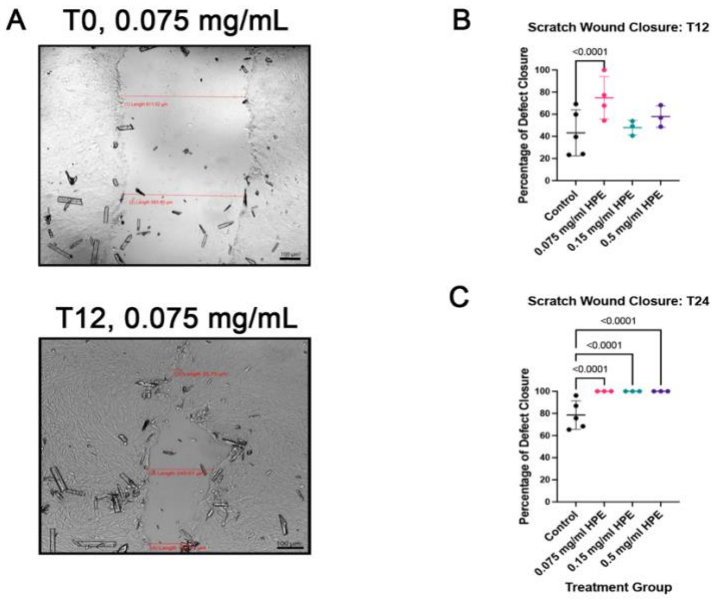

Figure 2: HPE accelerates epithelial restitution *in vitro*. Following scratch wound injury, control cell culture media, 0.075 mg/mL HPE, 0.15 mg/mL HPE, or 0.5 mg/mL HPE was applied to scratch wounds. Images were captured using an inverted microscope (Olympus IX83, Tokyo, Japan) fitted with a monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Shizuoka, Japan) at 10X (Scale bar = 100μm) with CellSens Software. The same 9 sites along the leading edge of the scratch wound were measured using CellSens every 6H until closure. A) Representative images from a 0.075 mg/mL HPE-treated scratch immediately post-scratch (T0) and 12H following (T12). B + C) Comparison of rate of scratch closure between treatments at T12 (B) and T24 (C). n= 3-5 pigs for all groups; 5 independent experiments. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons tests. Data presented as mean +/- standard deviation.

HPE Increases Proliferation at leading edge of Neonatal Ileum Scratch Wounds

As 0.075 mg/mL HPE was the most effective dose for accelerating closure, it was then applied to separate scratch wounds to evaluate the impact of HPE on cellular proliferation (Figure 3A and B). Twelve-hours post-injury, scratch wounds treated with 0.075 mg/mL HPE had significantly expanded proportions of proliferative cells along scratch edges identified with both EdU (EdU/DAPI: 0.066 +/- 0.07 HPE vs 0.0163 +/- 0.025 control, $p = 0.0409$) (Figure 3C) and colocalized Ki67 and EdU ($Ki67^{+}EdU^{+}$/DAPI: 0.029 +/- 0.031 HPE vs 0.005 +/- 0.011 control, $p = 0.006$) (Figure 3D), whereas Ki67 alone was not significantly different (Ki67/DAPI: 0.113 +/- 0.123 HPE vs 0.057 +/- 0.056 control, $p = 0.27$).

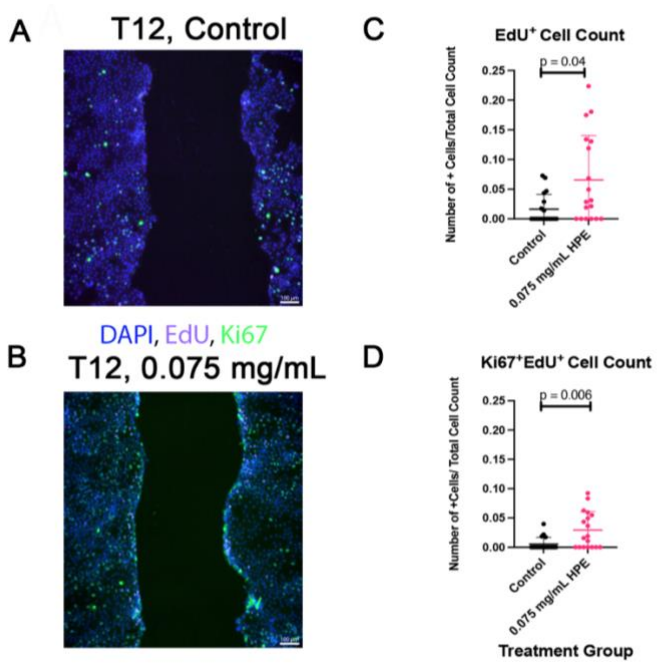

Figure 3: HPE increases proliferation at the leading edge of scratch wounds. Twelve hours following scratch wound and treatment with either 0.075 mg/mL HPE or control media, scratch wounds were EdU-pulsed and fixed. Scratch wounds were processed for DAPI (blue), Ki67 (green), and iFluor-647(purple) to denote EdU^+ cells. Images were captured using an inverted fluorescence microscope (Olympus IX83, Tokyo, Japan) fitted with a monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Shizuoka, Japan) at 10X (Scale bar = 100μm) with CellSens Software. Image quantification and analysis was performed with Fiji. A +B) Representative images for an untreated/control and a 0.075 mg/mL HPE-treated scratch are shown. Cells were counted in consistent areas along the leading edges of scratch wounds and the number of positive cells for C) EdU alone or D) EdU/Ki67 were quantified per total number of cells (DAPI) in a set area. n= 3 pigs for all groups; 3 independent experiments. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons tests. Data presented as mean +/- standard deviation.

HPE Increases Overall Health in Neonatal Porcine Model of NEC

All piglets who received HPE, of any dose and regardless of NEC status, gained significantly more weight than untreated piglets on each day of the study following enrollment (Day 0): Day 1: 0.98 +/- 0.009kg HPE vs 0.935 +/- 0.005kg control p = 0.0024; Day 2: 1.044 +/- 0.014kg HPE vs 0.956 +/- 0.006kg control, p < 0.001; Day 3: 1.08 +- 0.016kg HPE vs 1.036 +/- 0.014kg control, p = 0.0039; Day 4: 1.073 +/- 0.019kg HPE vs 1.016 +/- 0.013kg control, p = 0.0052 (Figure 4B). Representative gross and histological images demonstrate the reduced damage in piglets who received HPE (Figure 4C and D). Additionally, significantly fewer sections of the treated piglets' intestines (comparing duodenum, jejunum, ileum, cecum,

ascending, and descending colon) were characterized as showing evidence of NEC (2 +/- 0.76 HPE vs 4 +/- 1.67 control, p = 0.025) (Figure 4E). Overall disease severity scores were significantly lower in NEC+ piglets who had been treated with HPE (12.08 +/- 5.2 HPE vs 15.43 +/- 8.82 control, p = 0.0132) (Figure 4F).

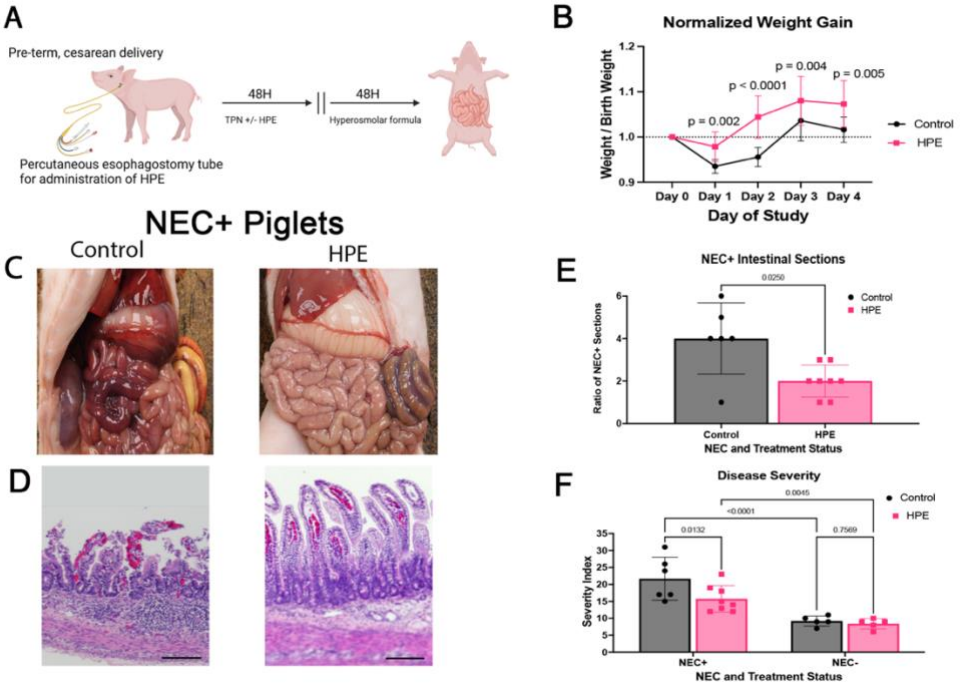

Figure 4: HPE increases weight gain and decreases overall disease severity in piglet model of NEC. A) *In vivo* experimental design. Piglets were given one of three doses of HPE or were nil per os (Control). B) Comparison of bodyweight, compared to birthweight, throughout the course of the study. C) Representative images of gross intestinal tissue damage and D) histological ileal damage in treated compared to untreated piglets with NEC. Images were captured using an inverted microscope (Olympus IX83, Tokyo, Japan) fitted with a monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Shizuoka, Japan) at 10X (Scale bar = 100μm) with CellSens Software. E) Quantification of total sections of intestine with evidence of NEC F) Comparison of combined gross and histological damage scores. n = 24 piglets, 13 received HPE and 11 were control, 2 independent experiments. Statistical significance was determined by 2way ANOVA followed by uncorrected Fisher's test (B +F) and Mann-Whitney U test (E). Data presented as mean +/- standard deviation.

HPE Increases Stem-Cell Associated Proliferation in Treated Piglets

As HPE treatment increased proliferation of porcine neonatal ileal epithelial cells *in vitro,* the ileum of piglets were evaluated for the expression of Ki67, to identify cell proliferation, and Sox9, to localize proliferation to progenitor cells of the crypt. The expression of these markers was compared between piglets using treatment and disease status (Figure 5 A and B). The

number of cells expressing Ki67 were increased in HPE-treated compared to untreated piglets (10.31 +/- 0.75 HPE vs 9.39 +/- 0.45 control) but values did not reach significance between piglets based on treatment or disease status (p = 0.157, Figure 5C). Sox9 was also increased in piglets who received treatment (8.01 +/- 1.83 HPE vs 6.46 +/- 0.71 control, p= 0.995) but was only significantly elevated when comparing the piglets who did not develop NEC (9.23 +/- 4.81 HPE vs 5.59 +/- 2.69 control; p = 0.0141, Figure 5C). Similarly, the number of $Ki67^{+}SOX9^{+}$ cells were overall increased in the piglets who had received HPE versus those who had not (4.86 +/- 1.63 HPE vs 3.49 +/- 0.52 control, p = 0.986) but was only significantly different within the cohort of piglets who did not develop clinical disease (5.11 +/- 2.32 HPE vs 3.52 +/- 2.13 control; p = 0.0133, Figure 5C.)

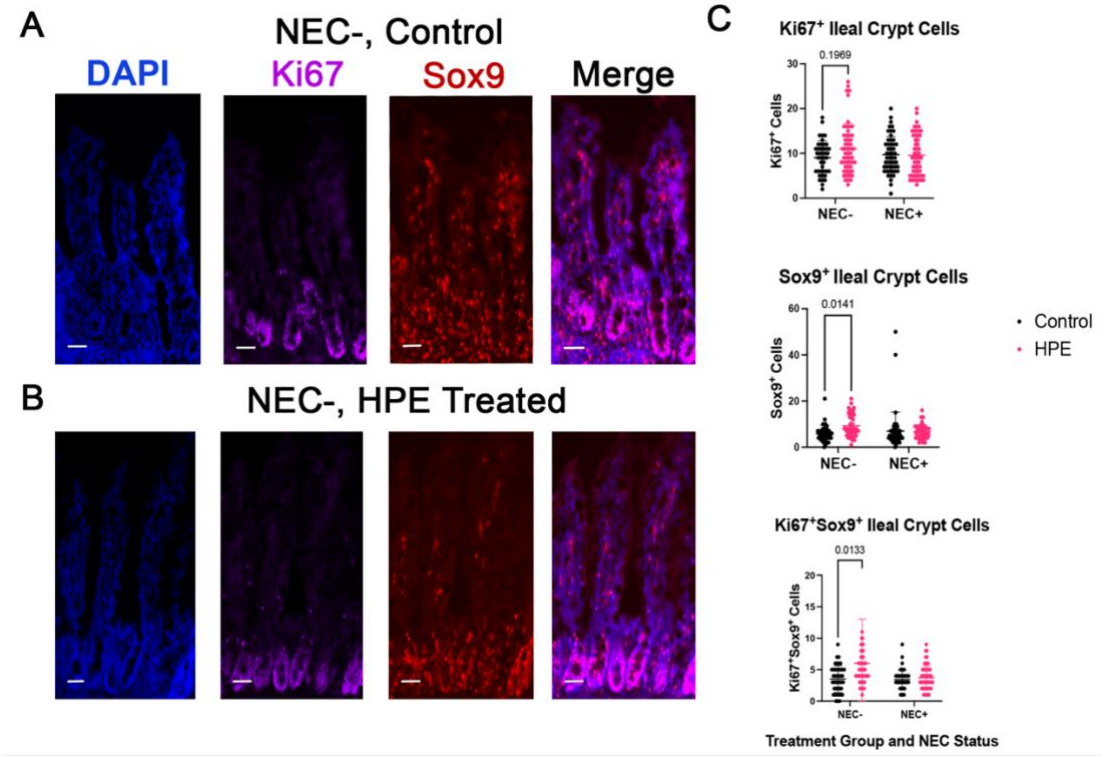

Figure 5: HPE increases stem-cell associated proliferation in treated piglets. A+B) Representative images of ileal tissue from piglets who did not develop NEC without (A, Control) or with HPE treatment (B) (Ki67: purple; Sox9: red; DAPI: blue). Scale bar = 50 μm. Images taken at 20X with CellSens Software. Image quantification and analysis was performed with Fiji. C) Quantification of the total number of positive cells for individual marker and colocalized expression was performed for 10 well oriented crypts per pig. n = 9-10 pigs per group, 2 independent experiments. 2way ANOVA with Tukey multiple comparisons. Data presented as mean +/- standard deviation.

HPE Increases Progress to Confluence and RNA signaling associated with Epithelial Migration and Proliferation

Comparisons of neonatal ileal monolayers treated with HPE during their development to confluence revealed that HPE increased confluency after 3 days of treatment (60% +/- 10.8% HPE vs 19.6% +/- 4.6% control, p = 0.0037). Two or 4 days of treatment were not significantly different (50.18 +/- 37.11% HPE vs 42.73 +/- 39.63% p = 0.129; 75 +/- 23.45% HPE vs 57.78 +/- 19.68%, p = 0.195). Four of 6 piglets reached confluency by 4 days of treatment. (Figure 6A)

Bulk RNAseq analysis identified 3,772 DEGs in the HPE-treated compared to untreated neonatal ileal epithelial monolayers (Figure 6B). Targeted GSEA revealed significant treatment-induced enrichment for genes associated with wound healing, epithelial cell migration, and epithelial cell proliferation (Figure 6C-I). Genes upregulated by treatment and common to those three pathways included *integrin subunit beta 3* (*ITGB3*, logFC=1.36, p-val = 5.13e-05, adjP = 0.012*)*, the *delta member of the peroxisome proliferator activated receptor family* (*PPARD*, logFC = 0.71, p-val = 0.0002, adjP = 0.012*), WNT5a (*logFC = 1.32, p-val = 0.0002, adjP = 0.012), *WNT7a (*logFC = 1.21, p-val = 0.0032, adjP = 0.022), *apolipoprotein E* (*APOE*, 1.57, p-val = 0.00196, adjP = 0.018*)*, and *guanine adenine thymine adenine binding protein 2* (*GATA2*, 1.47, p-val = 0.0078, adjP = 0.034*)*.

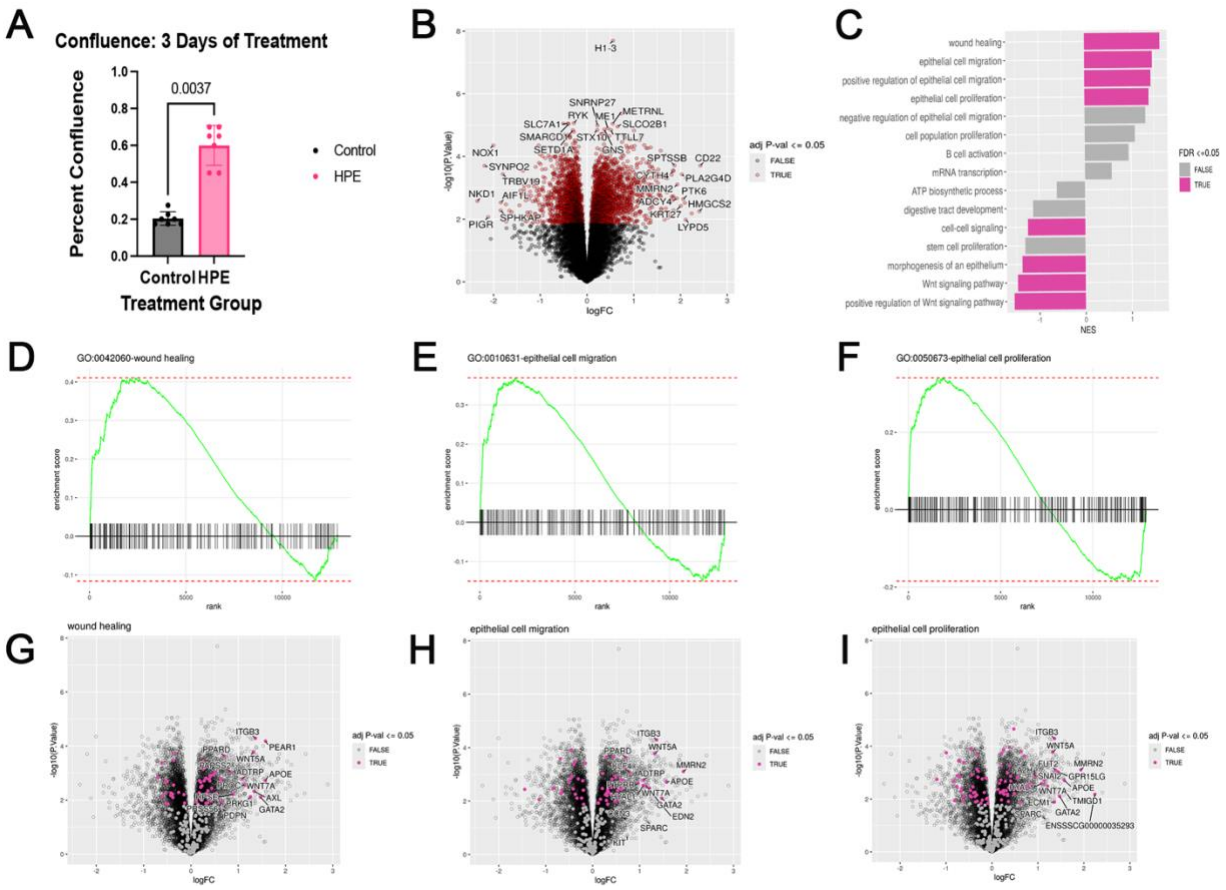

Figure 6: HPE increases transcriptomic evidence of wound healing in treated monolayers. HPE or cell culture media alone (control) was applied to single epithelial cells plated for monolayer development. A) Confluence was evaluated daily with a Zeiss Axio inverted microscope and compared. Paired T test. B) Volcano plot of gene expression changes in HPE-treated versus control samples. Higher log2FoldChange (logFC) values indicate HPE induced increases in expression. 3,722 significant DEGs (Adj P-value <= 0.05) are highlighted in red. C). Bar plot of targeted GSEA of pathways of interest with Normalized Enrichment Scores (NES) with significantly enriched pathways (Adj P-value <= 0.05), including wound healing, epithelial cell migration, and epithelial cell proliferation highlighted in magenta. D, E, F) Enrichment plots of these pathways depict the enrichment score (green line) relative to genes (black bars) ranked from highest to lowest log_2FoldChange (x-axis). G, H, I) Volcano plots highlighting (magenta) key upregulated DEGs for those three pathways. n= 6 pigs, 6 independent experiments, 2-3 replicates per pig.

Discussion

This work demonstrates that a regenerative human placental extract can treat and prevent NEC in *in* vitro and *in vivo* neonatal porcine models, respectively. *In vivo*, HPE pretreatment increased weight gain, decreased the number of intestinal segments impacted by NEC, and reduced gross and histological damage severity. *In vitro* assays evaluating post-injury HPE

application demonstrated that the overall mechanisms of action of HPE revolve around its ability to upregulate two primary elements of restitution: cellular migration and proliferation. These pathways were highlighted by both functional assays and transcriptomic analysis of epithelial monolayers following HPE application. Specifically, HPE accelerated scratch wound closure and increased the proportion of proliferative cells along the leading edges of these wounds. A significant upregulation of proliferation was also appreciated in the ileal crypts of piglets who received HPE and did not develop NEC, compared to those that did not receive treatment. Furthermore, transcriptomic pathway analysis revealed that HPE increased genetic signatures associated with epithelial proliferation, wound healing, and epithelial migration. These beneficial effects occurred in the absence of any indication of toxicity such as histomorphometry-related abnormalities, weight loss, or behavioral abnormalities. As HPE improved epithelial reparative capacity while remaining safe and well-tolerated, this product may serve as a novel therapeutic for the prophylaxis and remedy of the morbidities of NEC and severe prematurity in infants.

HPE may be able to help fill the current need for a treatment capable of directly enhancing epithelial recovery in NEC patients. The intensity of this need is demonstrated by the fact that current medical management techniques are unable to prevent the deterioration of clinical status in over 30% of patients who go on to require surgical resection of devitalized intestine.[31] For infants requiring surgery, mortality rates increase up to 50%.[32, 33] Dependent upon the amount of bowel that requires surgical removal, survivors may face life-long complications like short bowel syndrome and intestinal failure, which often require repeated surgeries and hospitalizations.[31, 33-35] Thus, HPE's ability to reduce both the overall severity of the affected segments of intestine and the number of affected segments, with prophylactic therapy, could significantly reduce the necessity of surgical intervention, or minimally, the amount of intestine requiring resection. Either result has the potential to significantly improve the likelihood of recovery and quality of life for NEC patients.

Human placental extract is a milieu of the many components found in the human placental disc. Many of the local factors that are synthesized, stored, and secreted by the placenta during pregnancy have been evaluated in experimental NEC preclinical models and intestinal cell culture studies and have demonstrated positive phenotypic outcomes (e.g., decreased NEC incidence, increased cellular proliferation, mediation of inflammatory response, improved wound healing). Literature has highlighted many critical growth factors including Hepatocyte Growth

Factor (HGF), Platelet Derived Growth Factor b (PDGFb), Fibroblast Growth Factor b (FGFb), and Epithelial Growth Factor (EGF), in addition to a variety of chemokines and cytokines which may contribute to the functional endpoints evaluated in these studies.[14-16, 20, 25]

HGF is the most predominant growth factor in the amniotic fluid of some species and is critical to development of the trophoblast.[16] Specific to the intestine, HGF increases intestinal epithelial cell proliferation in both animal models of NEC and fetal intestinal epithelial cell lines.[16, 36] PDGFb is also known to increase cell proliferation, play roles in the epithelial-mesenchymal transition, and is reduced in NEC-affected ileum.[37] Another inducer of proliferation and differentiation, FGFb, accelerates epithelial migration in *in vitro* models and enhances stem cell survival.[38, 39] EGF is also reduced in the serum of infants with NEC.[40] EGF receptor (EGFR) activating ligands, which protect against NEC, have been isolated in breastmilk and are expressed by placental stem cells.[18, 41, 42] Of note, in previous work by Radulescu and Yang, EGFR ligands have been unable to treat NEC after injury induction, as was modeled in our *in vitro* work.[43, 44] Complete profiling of HPE has not been pursued though these growth factors may be contributing to the impacts observed in this work.

As the product is cell-free, intact placental stem cells, associated with NEC recovery in other models, are not potential direct agents of the observed benefits.[8] However, the contents and products of these cells may survive processing. As hypothesized by Weis and colleagues, after appreciating that human placental stem cells promoted NEC-recovery without engraftment, the secretome of these cells may also be therapeutic.[8] Specifically, extracellular vesicles from placental stem cells alleviate other forms of experimental colitis in mice.[45] These cellular contributors are only one component of the many potential mechanisms contributing to the activity induced by HPE. To begin prioritizing which elements of this milieu may be promoting the beneficial effects of its application, we took a mechanistic approach through functional assays and transcriptomic analyses.

In our *in vitro* assessments of HPE, the lowest dose applied (0.075 mg/mL) most effectively encouraged epithelial migration. As such, this was the only dose explored in tight junction recovery and *in vitro* proliferation assessments. This dose was comparable to those used *in* vivo following corrections for physiological considerations such as convective washout.[46] While this dose was the most effective, others applied *in vitro* were non-inferior to control.

Future evaluations of doses below 0.075 mg/mL HPE will demonstrate if benefits may be achieved with less product.

Functional and transcriptomic findings aligned elegantly in that wound healing, epithelial cell migration, and epithelial cell proliferation were mutually increased. Amongst the many differentially expressed genes, *ITGB3, PPARD, WNT5a, WNT7a, APOE, and GATA2* stood out as upregulated elements of those three pathways. ITGB3 is a cell-surface protein which participates in cell adhesion and cell-surface signaling involved in epithelial migration in addition to preserving stemness in tumors.[47] Increased epithelial migration following HPE application, as observed in scratch assays, may represent the activity of ITGB3. In their work demonstrating that postnatal amniotic fluid administration decreases the incidence of NEC in neonatal piglets, Siggers and colleagues similarly found an upregulation of ITGB3 in piglets receiving protective amniotic fluid compared to colostrum.[20] PPARD functions as an integrator of transcriptional repression and nuclear receptor signaling and is associated with colorectal cancer tumor activity, including upregulation of PDGFb receptor activity.[48] *PPARg* has been evaluated in the context of NEC and repeatedly shown to be protective, resulting in less severe injury and nuclear factor kappa B (NFκB) activity when activated with an agonist prior to NEC-injury induction.[49, 50] Another PPAR family member, *PPARa,* reduces ileal permeability in experimental colitis, though has not been explicitly evaluated in NEC.[51]

Wnt5a and *Wnt7a* are members of the *Wnt* family which is critical to maintenance of the intestinal stem cell (ISC) niche and the proliferation that occurs there.[52] ISC proliferation is an important determinant of NEC severity. Severe NEC correlates with ISC depletion and, conversely, agents that support ISC expansion reduce NEC severity.[53] The Wnt family, and impairments in its β-catenin-dependent pathway, have been implicated in the development of NEC.[53] *Wnt5a* is a non-canonical Wnt essential for embryonic gastrointestinal development.[54] It has been identified as a key factor for promoting colonic crypt regeneration, which involves the activity of Ki67 and Sox9, after injury through its potentiation of transforming growth factor β (TGF-β) in addition to acting through stromal cells following irradiation injury.[55, 56] To our knowledge, *Wnt7a* has not been thoroughly explored in the intestinal epithelium in the context of injury and recovery though is increasingly expressed by intestinal epithelial cells following irradiation injury and mesenchymal stem cell-conditioned media treatment.[57] Supplementation with Wnt7b, amniotic fluid stem cells, or amniotic fluid

stem cell-derived extracellular vesicles, which differentially express genes of the Wnt/β-catenin pathway, decreases the severity of NEC.[17, 53] Interestingly, while HPE significantly increased *Wnt5a* and *Wnt7a* transcription, the overall *Wnt* signaling pathway was not upregulated by HPE treatment in developing monolayers. This is likely due to a lack in significant differential expression of the other members of the *Wnt* family, including *Wnt3,* which is recognized for its prominent role in maintaining the ISC niche.[58] Despite the lack of overall upregulation of Wnt signaling, HPE appears to support the ISC niche given its ability to upregulate $Ki67^{+}Sox9^{+}$ cells in treated piglets and increase the rate to confluency in stem cell-enriched developing monolayers. Further profiling of HPE and genetic screening of intact intestinal crypts from *in vivo* experimentation will likely shed light on this dynamic.

The role of *APOE* in the intestine is not well-established. A key regulator in lipoprotein metabolism, this protein limits proliferation in lymphocytes, endothelial cells, smooth muscle cells, and tumor cells.[59] Consistent with its limitation of lymphocyte proliferation, *APOE* is anti-inflammatory and able to regulate the activity of toll-like receptor 4 (TLR4) whose increased expression contributes to the pathogenesis of NEC.[60, 61] Additional exploration of the potential role of this gene in the activity of HPE is necessary prior to any mechanistic speculation. *GATA2* is critical for the development and maintenance of hematopoietic and trophoblastic stem cells.[62] In mappings of the intestine, *GATA2* has only thus far been identified in mast cells, and its interaction with intestinal epithelium after development remains unknown.[63] Of the genes shared between the pathways of interest, the roles of *APOE* and *GATA2* are currently the least certain, though the ability of *APOE* to reduce TLR4 expression in other disease settings is intriguing.[61] Overall, these genes of interest provide a potential springboard for additional explorations for how HPE may be enhancing intestinal epithelial reparative capacity.

Neonatal porcine models were employed in this study because of their increased translatability to human patients, compared to rodent models.[27, 64, 65] While porcine intestinal epithelial and vascular anatomy and intestinal microbiota are more similar to humans than rodents, there are some limitations to their usage.[64, 66] Generally, compared to human and rodent studies, transcriptomic analysis in pigs is limited by the reduced availability of annotated libraries. Despite this limitation, the notable differences in gene expression observed here suggest a substantial change induced by HPE. Additionally, while piglets are highly

translatable to neonatal humans, the time-course of NEC is not perfectly transferrable. In the neonatal porcine model, NEC induction begins immediately after birth whereas in humans NEC typically manifests at 2-4 weeks of age.[67],[68] Notwithstanding, the porcine neonatal model of NEC remains an informative and valuable tool and its response to HPE is encouraging for that which may be seen in human patients.[12, 65] Additional limitations of this work include the small sample size employed in *in vitro* work and the reductionist nature of those experiments. The combination of both *in vitro* and *in vivo* models was performed to complement each model's constraints.

In sum, HPE is capable of accelerating recovery from NEC-associated injuries at the cellular level and prevent injury from occurring at the whole-animal level. Mechanistically, this appears to occur through the upregulation of epithelial migration and proliferation. Transcriptomic evaluations added evidence of the upregulation of wound healing, as a summative effect of migration and proliferation, in addition to specific genes of interest. Additional, more complete proteomic profiling of the product is pending to provide a more comprehensive characterization of key HPE ingredients. Continued experimentation with HPE will include measurements of its ability to modulate TLR4 activation and the generalized inflammatory immune response which contributes to NEC injury.[1, 4, 6, 20] Supplemental toxicity testing is currently underway to confirm the safety and tolerance of HPE as appreciated in both utilized models here. Overall, the dual protective and therapeutic potential of HPE warrants additional exploration into its utility for addressing the current gap in interventions for the devastation caused by NEC.

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CHAPTER 4

Equine Placental Derivatives Accelerate *in vitro* Intestinal Epithelial Healing

Introduction:

As the most common emergency in equine medicine, colic takes an excessive toll on the equine industry.[1,2] Severe colic produces intestinal hypoxia which significantly damages the intestinal epithelial lining.[3] The intestinal mucosal barrier is formed by a single layer of columnar epithelial cells connected via junctional complexes which protect against the translocation of harmful luminal bacteria. When damaged during colic, this barrier can no longer prevent noxious substances from reaching the underlying blood supply, leading to severe downstream sequelae.[3] Recovery requires epithelial restitution, tight-junction re-establishment, and epithelial cell renewal.[4,5] Epithelial renewal is achieved by intestinal stem cell (ISC) proliferation from within the mucosal crypt.[6] Once hypoxic injury extends beyond 3 hours of injury, however, the intestinal crypt epithelium can be severely damaged.[7] This duration of severe colic is common in clinical settings.[8] Unfortunately, no treatments currently exist to specifically enhance these reparative processes or upregulate the ISC niche. Placental-derived accelerate recovery in investigational studies of intestinal disease, but none in the horse. [9-12]
The placenta produces, or is a conduit for, most of the beneficial factors found in amniotic fluid, shown to have anti-inflammatory and pro-maturate impacts on the intestinal epithelium in other species.[13, 14-16] Placental stem cells (PSC) are found within amniotic fluid and have accelerated intestinal epithelial recovery in other species and disease models.[9,12] Interestingly, these cells are able to enhance healing without engraftment, suggesting a paracrine mechanism.[12,17] One byproduct of PSC, extracellular vesicles (EV), have also been shown to accelerate epithelial recovery in murine intestinal injury models.[18] Additionally, in other species and in extra-gastrointestinal models, hypoxic preconditioning of EVs increases their reparative potential.[19]
In this work, two placental-derived therapies were evaluated for their ability to enhance equine epithelial repair *in vitro:* complex equine placental extract (ePE) and primed human placental stem cell-derived extracellular vesicles (EV). This approach was designed to firstly establish the general benefit of ePE and then, if successful, interrogate a specific constituent of this extract. In preliminary work in neonatal porcine *in vitro* and *in vivo* models, human placental-derived extract accelerated epithelial repair via increased restitution and proliferation, though had no impact on epithelial tight junction recovery. Thus, the objective of this project was to evaluate if ePE and, separately, naïve, hypoxia-primed, or LPS-primed EVs increase equine intestinal epithelial recovery *in vitro*. We hypothesized that ePE and EVs, primed and naïve, would

accelerate equine intestinal epithelial recovery *in vitro* through the mechanisms of restitution and proliferation. With further exploration, we hope to establish these agents for directly upregulating epithelial repair and thus an adjunct therapeutic for horses with severe colic.

Materials and Methods

Equine Intestinal Tissue Collection

This study was reviewed and approved by North Carolina State University's Institution Animal Care and Use Committee. Tissues were obtained from 4 breeds (Quarter Horse, Thoroughbred, Appendix, Morgan) of adult horses (ages 4 – 14 years) without history or clinical evidence of gastrointestinal disease who were subjected to euthanasia for reasons unrelated to this study. Immediately following euthanasia, multiple approximately 20 cm sections of mid-jejunum were obtained through a midline celiotomy and placed in cold phosphate buffered saline (PBS) on ice for transportation to the laboratory for crypt isolation.

Jejunal crypt isolation and organoid culture

Intestinal crypts from the jejunum were harvested as previously described.[20] To briefly review, segments of mid-jejunum were opened longitudinally along the antimesenteric border and separated into approximately 1 cm x 1 cm sections which were then incubated in cold phosphate buffered-saline (PBS) with 30 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, MO), 10 mM Y-27632 (Selleck Chemicals, Houston, TX), 1X Primocin (InvivoGen, San Diego, CA), 1X Antibiotic-Antimycotic (Gibco, ThermoFisher Scientific, Waltham, MA) and 1 mM dithiothreitol (DTT) (Sigma-Aldrich) for 30 minutes on ice with vigorous shaking every 5-10 minutes. Tissue was then incubated in pre-warmed PBS with 30 mM EDTA, 1X Primocin, 1X Antibiotic-Antimycotic and 10 mM Y-27632 for an additional 10 minutes. Tissues were then transferred to ice-cold PBS with 1X Antibiotic-Antimycotic and Primocin and shaken vigorously. The solution was sampled every 2-3 minutes and evaluated for the presence of crypts. Upon visualization of crypts, tissues were transferred to a new, identical wash to reduce debris.

Upon collecting a concentration of approximately 1 crypt/µL with minimal debris, crypts were pelleted by centrifugation at 200-300 x G for 5 minutes and plated for enteroid formation. Crypts were resuspended in growth factor reduced Matrigel (BD Bioscience, San Jose, CA) at a concentration of 50 crypts per 50 µl of Matrigel per well. Matrigel polymerized for 30 minutes at

37°C and was then overlaid with Advanced DMEM/F12 (ThermoFisher Scientific) containing 1x N-2 supplement (Life Technologies Corporation, Carlsbad CA), 1x-B27 supplement minus vitamin A (Life Technologies Corporation), 1x glutamax (Life Technologies Corporation,), 1X antibiotic-antimycotic and 1 mmol/L HEPES buffer (Life Technologies Corporation) supplemented with 100 ng/mL recombinant Noggin (Millipore Sigma, St. Louis, MO), 500 ng/ml recombinant human R-spondin (Sigma Aldrich), 50 ng/mL recombinant human EGF (Life Technologies Corporation), 100 ng/mL recombinant human Wnt3a, 10 mmol/L Y-27632, 10 mmol/L SB202190, 500 nmol/L LY2157299 (Selleck Chemicals), 2.5 µmol/L CHIR99201 (Sigma Aldrich) a glycogen synthase kinase 3 inhibitor, 500 nM A 83-01 (Tocris, Minneapolis, MN), 0.01 uM [Leu15]-Gastrin I human (Sigma Aldrich, St. Louis MO), and 0.01 M Nicotinamide (Sigma Aldrich). Enteroid growth was assessed daily. Growth factors and media were replaced every 48 hours (Hrs).

Jejunal Epithelial Monolayer Culture

Once mature enteroids had grown to provide adequate cell numbers for monolayer production, typically after 4-6 days in culture, they were harvested by depolymerizing the Matrigel matrix as previously described.[20,21] The plated cell concentration for monolayers was 40-50,000 cells/cm^2 as previously published.[21] Briefly, culture media was removed and the Matrigel matrix was washed with cold PBS while on ice. The Matrigel matrix was incubated on ice for 30 minutes in cell recovery solution (Life Technologies Corporation) with aggressive pipetting at the end of incubation to ensure collection of all enteroids within the well. The resultant enteroid-containing solution was centrifuged at 200-300 x G for 5 minutes. The supernatant was removed and the enteroid pellet resuspended in 100 µl of advanced DMEM/F12 media (ThermoFisher Scientific) per intended monolayer. Resuspended enteroids were vigorously pipetted using 100 and 10 µl pipette tips to dissociate the cells and subsequently plated in in 6.5 mm transwells with 0.4 uM pores (Corning 3470, Durham, NC) coated in 0.5% v/v Matrigel in DMEM-F12 as outlined by Van der Hee et al.[22] An additional 100 µl of advanced DMEM/F12 Media was added to the apical chamber of the transwell and 500 µl was added to the basal chamber with the same growth factor combination as that listed for enteroids. Monolayers were assessed daily using an inverted microscope at 10x and 20x for visual confluency. Once 100% visual confluency was achieved, monolayers were then evaluated for tight junction integrity (Transepithelial Electrical Resistance, TER) as described below.

ePE collection and application

ePE was manufactured by a proprietary, patented process developed for human placental tissue from term deliveries. Equine placental membranes (n =3) were acquired from post-parturient mares with uncomplicated pregnancies and deliveries immediately following foaling. Membranes were collected, inspected, and then thoroughly washed in cold PBS thrice prior to storage in a PBS-antibiotic combination for transport to the processing laboratory. At processing, the chorioallantoic region housing the chorionic girdle was mechanically dissected for continued processing. Tissue was then rinsed in sterile bioprocessing solutions and homogenized. Blood was removed using red cell lysis buffer with agitation. Following blood removal, a PBS solution containing a proprietary mixture of protease inhibitors, to protect cells and preserve proteins, was used for multiple rounds of rinsing with centrifugation. The washed tissue was processed to fine particle size to optimize cell lysis. Lysis of the cells within this mixture was accomplished utilizing a specified freeze/thaw procedure. Multiple tissues were combined and mixed to reduce individual component variability in the final product. Further cell lysis was accomplished by sonication and centrifugation to remove larger extracellular matrix components, resulting in a cell-free shelf-stable liquid suspension. This suspension was stored at -80C until experimentation, at which point it was gradually thawed at room temperature and then applied, apically, at the listed doses. Doses were selected based upon preliminary results using a human placental-derived extract in similar *in vitro* modeling using neonatal piglet ileal epithelial cells. For treatment, 0.075 mg/mL of FBS, as a protein-concentration control, or one of three different doses of ePE: low (0.05 mg/mL), middle (0.075 mg/ML), or high (0.15 mg/mL) was added apically to each monolayer.

EV collection and application

EV were collected by the Wake Forest Institute for Regenerative Medicine (WFIRM) from human placental tissue collected from the Wake Forest University teaching hospital. Placentas were rinsed with 0.9% saline and then processed to isolate single cells. Isolated placental cells were plated in a-MEM media supplemented with AmnioMax and Glutamax. Following cell expansion, cell selection for C-kit positive human placental stem cells (hPSC) cells was performed with a CD117 antibody (Cat No. 120099-672, Miltenyi Biotec, Germany) in a Miltenyi Mini Macs System. Selected hPSC were then expanded in culture and underwent sterility, endotoxin, mycoplasma, and Karyotype testing. Additional phenotypic testing of hPSC

confirmed CD29(þ), CD44 (þ), CD73(þ), CD105(þ), CD146(þ), SSEA-4(þ), HLA-ABC (þ), CD34([1]), CD45([1]), and HLA-DR([1]). hPSC were cultured to ~60% confluence, then media was swapped for fresh EV-depleted FBS media and primed through the addition of LPS (200ug/ul) or hypoxia (5% oxygen) or nothing (naïve) for 48H. This EV-enriched media was collected, ultra-filtered, and condensed using Amicon® Stirred cell (100kDa) and the Ultra-15 Centrifugal Filter (100kDa) to condense the media. Condensed media was then chromatographically separated (qEV2 / 35 nm Gen 2 column IZON Science LTD) into 15 fractions. Fractions 8-11 were pooled together to recover EVs in the range of 35-350nm. EVs were reconstituted in PBS and stored at a concentration of 2.50E+07 EV/uL. For treatment, 30 uL of each EV subtype (naïve, hypoxia, or LPS-primed), or PBS as the vehicle control, was added apically to each monolayer. PBS at an equal volume served as the control.

Monolayer TEER Assessment

From the point of visual confluency onward, transepithelial resistance (TER) was measured daily. Individual monolayer TEER measurements were normalized by comparison to a cell-free insert and then multiplied by the area of the insert (0.33cm^2). For all experiments, the EVOM Epithelial Volt/Ohm (TEER) Meter 3 was used to record resistance readings (World Precision Instruments, Sarasota, FL). Monolayers were deemed confluent when the TEER reached 1000 Ω/cm^2.[21,22]

For ePE experiments, the Endohm 6G symmetrical electrode chamber was used to measure TEER whereas, for EV experiments, the STX4 EVOM Electrodes were employed (Figure 1). Prior to all TEER measurements, media was removed and replaced in the apical chamber to ensure an exact 200 uL of media was present. New treatment was added to the apical chamber after TEER reading.

Following hypoxia exposure (2.7) TEER was measured to confirm injury prior to the scratch (2.8). Upon epithelial restitution to visual confluence, TEER was measured every 6 Hrs for 48 Hrs with media replacement and treatment at each measurement timepoint. Rate of TEER change was calculated using each monolayer as its own baseline (Figure 1).

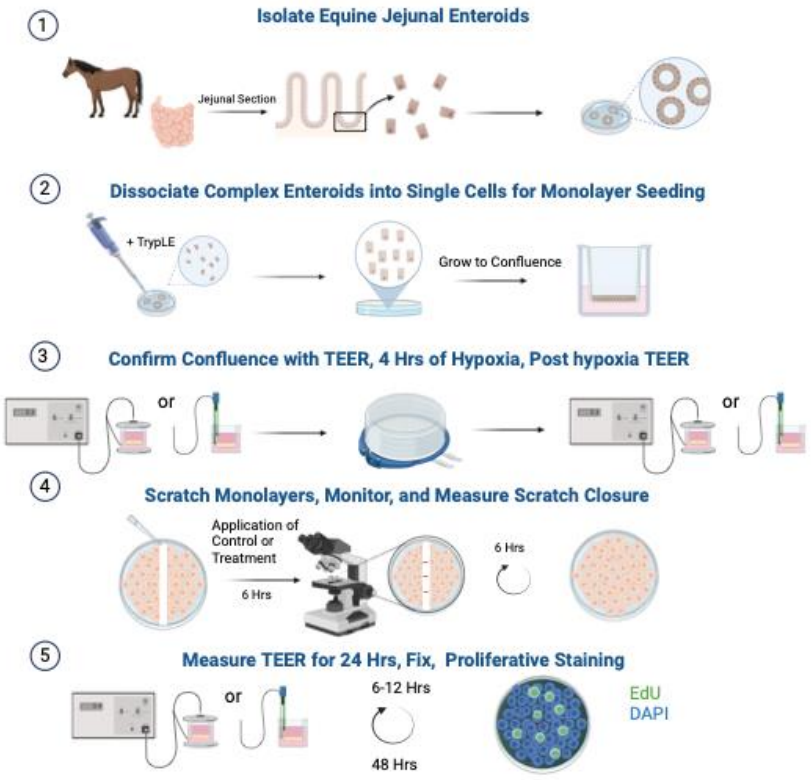

Figure 1: Experimental design. This figure demonstrates the procedural steps for development of confluent primary equine jejunal monolayers, exposure to hypoxia, scratch wound injury, and measurements of recovery including scratch-closure, Transepithelial Electrical Resistance (TER) recovery, and changes in cell proliferation.

Hypoxia Exposure

Once confluent, culture media and growth factors were refreshed, and the wells were placed in a Modulator Incubator System (Billups-Rothenberg, inc.; 1% O_2, 5% CO_2, balanced with N_2) for 4H, to parallel durations of hypoxic injury known to create significant intestinal epithelial injury reaching the crypt. Following hypoxia, monolayers were then scratched (see 2.6) to replicate epithelial loss.

Scratch Assay

Once visually confluent, a 200 uL pipette tip was used to create a longitudinal scratch wound across the center of the well. Following scratch, the wells were washed with media and then fresh media, growth factors, and treatment was applied. Monolayers were treated with control or treatment. Scratches were imaged at the same locations along leading edge of the scratch every 6 Hrs closure or 24 Hrs. To maximize visibility, new media was applied prior to each imaging timepoint with new growth factors and treatment added after imaging (Figure 1).

Immunofluorescent Analysis

For proliferative assessments, monolayers were incubated for 1 hour with 5-ethynyl-2'-deoxyuridine (EdU Click-iT, Thermo Fisher Scientific, Waltham, MA) following stabilization of their TEER, or 48H, as previously outlined.[23] Monolayers were then with fixed with 4% PFA for 15 minutes and submerged in PBS until further immunofluorescence analysis for EdU coloration (Alexa 488/ GFP) and nuclear staining (DAPI) (Figure 1). To quantify the number of actively proliferating cells, a grid was applied to each image and a set area was counted in the same six locations along the leading edge for each scratch. The number of cells expressing EdU was quantified for each set area.

Acquisition of Images

Images were taken using an inverted fluorescence microscope (Olympus IX83, Tokyo, Japan) fitted with a monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Shizuoka, Japan). Scratches were imaged using the bright field channel whereas immunofluorescence was assessed using appropriate color channels (DAPI, 488). The objective lenses used were X10, X20, and X40 (numerical apertures of 0.3, 0.45, and 0.6, respectively) (LUC Plan FLN, Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed with Prism software (GraphPad Software, La Jolla, CA). Outliers were removed with the robust regression and outlier removal tool. The normality of raw data was assessed with a Shapiro-Wilk normality test, followed by either a 2-way analysis of variance with Tukey's multiple comparisons, 2-way analysis of variance with Dunnett's multiple comparisons, or Kruskal-Wallis test. Statistical significance was defined as $P < .05$.

Results:

ePE Accelerates Epithelial Restitution in vitro

In severe colic, prolonged ischemia results in significant intestinal epithelial loss which requires cells to restitute to re-establish the epithelial barrier. To evaluate the impact of ePE on epithelial restitution, following ischemia, linear scratch wounds were created in confluent monolayers which were then treated. Six hours post-injury, control monolayers achieved a mean closure of 42.3% +/- 11.2% compared to ePE-treated cultures of 48.45% +/- 15.53% (0.05

mg/mL), 55.1% +/- 17.8% (0.075 mg/mL), and 41.71 +/- 12.44% (0.15 mg/mL) (n= 3-5) with a statistically significant difference between control and 0.075 mg/mL (p = 0.0273) (Figure 2A+ 2B). After 12 Hrs, all scratches, treated and control, were closed (data not shown).

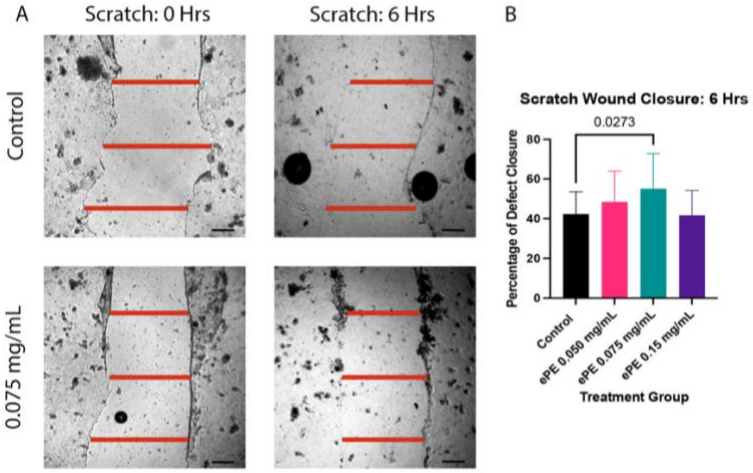

Figure 2: ePE accelerates jejunal scratch wound closure. Following scratch wound injury, control media, 0.05 mg/mL ePE, 0.075 mg/mL ePE, or 0.15 mg/mL ePE was applied to scratch wounds. 9 sites along the leading edge of the scratch wound were measured every 6 Hrs until closure. Compared to control, 0.075 mg/mL ePE significantly increased scratch closure at 6H. A) Representative images from a 0.075 mg/mL ePE treated scratch immediately post-scratch (Scratch: 0 Hrs) and 6 hours following (Scratch: 6 Hrs). B) Comparison of rate of scratch closure between treatments at T6. N= 3 horses. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons. Scale bar = 100 uM, all images taken at 10X.

ePE Improves Tight Junction Recovery

At 12 -18 Hrs after reaching visual confluency, all treated monolayers achieved greater TEER recovery compared to control (0.23 +/- 55.09%) though only 0.15 mg/mL ePE was statistically improved (0.15 mg/mL: 46.23 +/- 39.47%, p = 0.0321; 0.05 mg/mL: 44.04 +/- 41.39%; 0.075 mg/mL: 25.72 +/- 37.06%) (Figure 3A). Twenty-four hours following restitution, treatment continued to improve TEER recovery compared to control-treated wells (-0.36% +/- 24.72), however, only 0.075 mg/mL ePE achieved statistical significance (0.075 mg/mL: 21.5 +/- 25.00%, p= 0.0031; 0.05 mg/mL: 15.43 +/- 46.17%; 0.15 mg/mL: 33.88 +/- 22.72%) (Figure 3B).

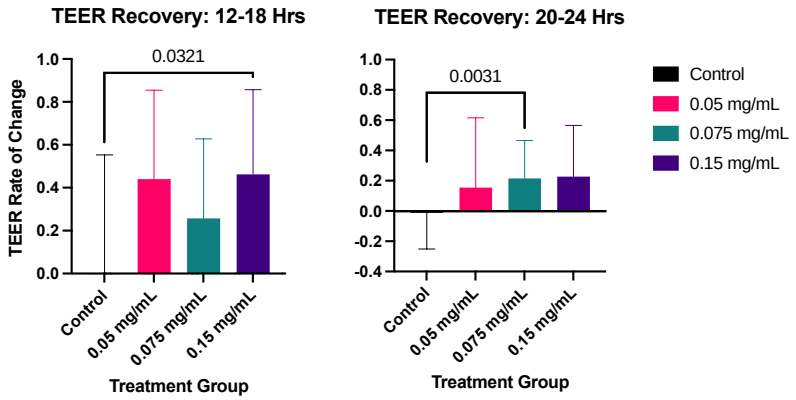

Figure 3: ePE increases tight junction recovery following hypoxia and scratch-wound injury. Following restitution, TEER of recovering monolayers was measured every 6 Hrs for up 48 Hrs. TEER measured at each timepoint was compared to pre-hypoxia TEER levels, and rate of change calculated. A) Compared to control, 0.15 mg/mL ePE increased TEER recovery at 12-18 Hrs B) 0.075 mg/mL ePE increased TEER recovery significantly at 20-24Hrs. n = 3 horses. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons tests.

LPS and Hypoxia-Primed Evs Accelerate Epithelial Restitution In Vitro

To next assess the impact of Evs, a potential component of ePE, on epithelial restitution, treated linear scratch wounds were monitored for rate of closure. Six hours post-injury, control monolayers achieved a mean closure of 61.82% +/- 26.76% compared to EV-treated cultures of 50.07 +/- 36.09% (naïve), 64.98% +/- 11.67% (LPS), and 59.87 +/- 8.64% (Hypoxia) (n= 2-3) with a statistically significant difference between control and LPS-primed EV ($p = 0.0036$) (Figure 4A+ B). At 12 Hrs, control monolayers were 87.70% +/- 12.52% closed compared to EV-treated cultures of 78.17 +/- 34.51% (naïve), 93.46% +/- 7.92% (LPS), and 97.52 +/- 3.51% (Hypoxia) (n= 2-3). Both the LPS and hypoxia-primed Evs achieved statistically significant difference compared to control ($p < 0.0001$) (Figure 4A + C).

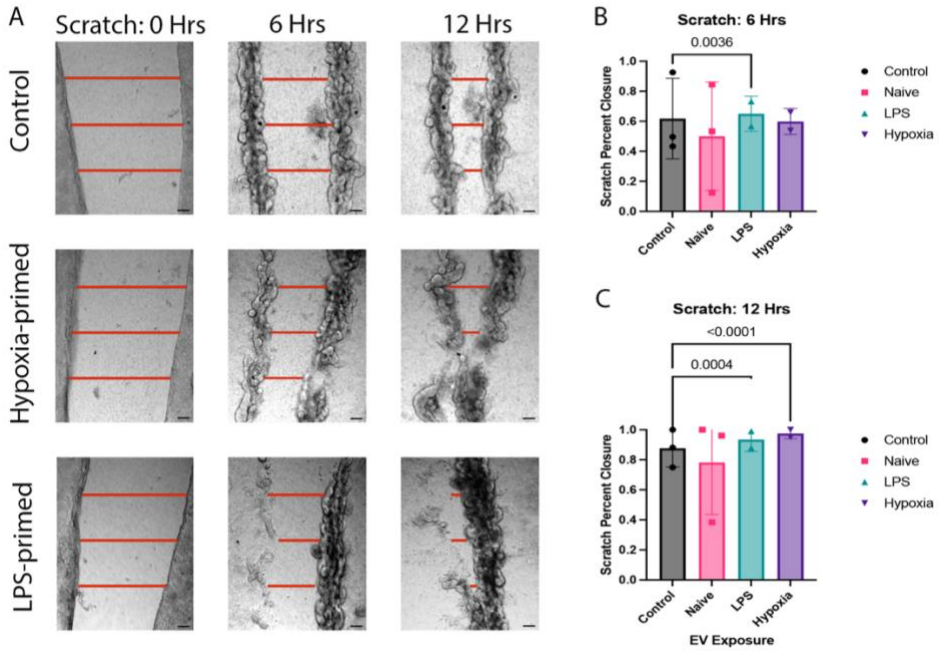

Figure 4: LPS and hypoxia-primed EVs accelerate jejunal scratch wound closure. Following scratch wound injury, control media, naïve, hypoxia-primed, or LPS-primed EVs were applied to scratch wounds. 9 sites along the leading edge of the scratch wound were measured every 6 Hrs until closure. A) Representative images from control, hypoxia-primed, and LPS-primed scratches immediately (0 Hrs), 6 Hrs, and 12 Hrs following scratch. B) Compared to control, LPS-primed EV increased rate of scratch closure at 6 Hrs. C). 12 Hrs following scratch, LPS and Hypoxia-primed EV increased the rate of scratch closure. N= 2-3 horses, 3 technical replicates per treatment per horse. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons test. Scale bar = 100 uM, all images taken at 10X.

LPS-Primed EVs Improve Early Tight Junction Recovery

Once restituted, TEER was measured every 6 Hrs for 48 Hrs to assess the reformation of the tight junctions between these now confluent cells. Evaluating all horses, averaged together by treatment, over time, there was no significant impact of EV application (Figure 5A). However, when comparing different treatments at each specific timepoint, 6H after visual confluence, treatment increased TEER (naïve: 13.84 +/- 21.24%; hypoxia: 5.05 +/- 28.11%; LPS: 28.56 +/- 16.16%) over control, which showed a weakening in TEER compared to baseline (-6.27 +/- 29.69%). These comparisons, however, only reached statistical significance for LPS-primed EV ($p = 0.0375$) (Figure 5B).

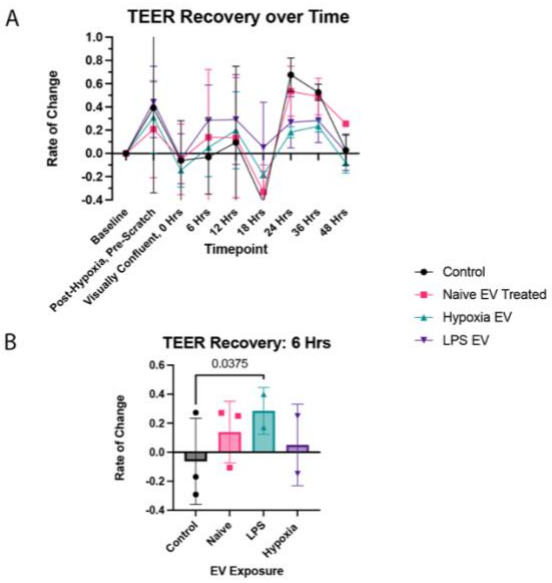

Figure 5: LPS-primed EVs increase TEER early in recovery. A) Comparison of all horses, averaged together per treatment, did not reveal a consistent effect of EV application over time B) However, 6 Hrs following visual confluency, LPS-primed EV increased TEER compared to all other treatment groups. n = 2-3 horses, 3 technical replicates per treatment per horse. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons test.

EVs May Not Impact Epithelial Proliferation

To evaluate if EVs may be inducing proliferation as a mechanism of recovery, confluent monolayers were EdU-pulsed to evaluate the number of cells in the S phase of proliferation. There was no significant impact of any of the EVs on proliferation, compared to control, suggesting this is not a mechanism leveraged by EVs to accelerate repair (Fig. 6A + B).

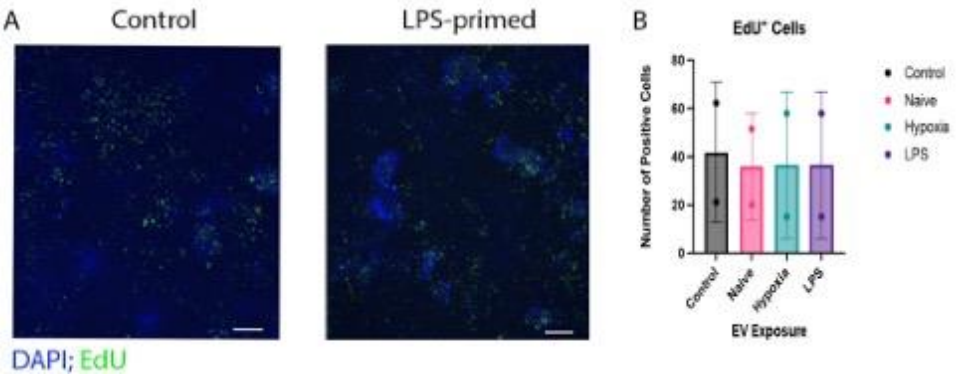

Figure 6: EVs do not increase equine jejunal proliferation *in vitro*. Following stabilization of TER, monolayers were EdU pulsed to evaluate cells in the S phase of proliferation. A) Representative images of pulsed monolayers with nuclear marker, DAPI, and EdU in Alexa 488/GFP. B) No significant differences in treated and untreated monolayers were identified. N = 2 horses, 3 technical replicates per treatment per horse. Statistical significance was determined by 2way ANOVA followed by Tukey's multiple comparisons test. Scale bar = 100 uM, all images taken at 10X.

Discussion:

This study demonstrates that acellular equine placental extract and primed placental-derived extracellular vesicles may enhance intestinal epithelial repair following intestinal injury akin to that which develops in severe ischemic colic. In our complex *in vitro* injury model, which combines hypoxia with epithelial cell loss, post-injury ePE and EV application resulted in enhanced cellular migration and tight junction recovery: two elements of restitution. Specifically, 0.075 mg/mL ePE accelerated scratch wound closure 6 Hrs after injury. Once wounds had restituted, 0.15 mg/mL ePE increased tight junction recovery early in the repair process (12 – 18 Hrs) but was later superseded by greater TEER recovery with the 0.075 mg/mL dosage at 20 – 24 Hrs. Thus, in consideration of multiple mechanisms of epithelial recovery, the mid-dose of ePE, 0.075 mg/mL, appears to be the most effective. Preliminary evaluations of EVs, as a suspected component of ePE, demonstrated that those primed with LPS enhanced epithelial restitution at 6 and 12 Hrs after scratch-wounding, with hypoxia-primed EVs also achieving accelerated closure at 12 Hrs. EV treatment-associated tight junction repair was only observed at 6 Hrs following restitution, and limited to LPS-primed EVs. These results suggest that LPS-priming best enables placental EVs to encourage epithelial recovery in our model. As ePE and EVs improved epithelial reparative capacity without demonstrating any evidence of cellular toxicity, continued exploration of these products with additional *in vitro* biological replicates, *ex*

vivo, and ultimately *in vivo* models is warranted to determine if they may expand therapeutic options for colic.

To confirm preliminary, encouraging trends associated with EV application, additional biological replicates will be performed. Additionally, evaluations of ePE for intact EV using fractionation and transmission electron microscopy are currently ongoing. Once isolated, equine placental EVs will be available for future experimentation, including LPS and hypoxia-priming, to confirm the benefits observed with human placental-derived EVs. Human EVs were utilized for this initial experimentation because of their ready availability and optimized collection protocol. If equine placental EVs demonstrate similar therapeutic promise, sourcing and culture optimization will be pursued. Following successful collection, the content of equine EVs will be profiled with proteomics to determine potential contributors to epithelial recovery. Even if intact EV are not located, their components could still be contributing to the milieu that is ePE. For this reason, characterization of the entire, complex content of ePE, through proteomic analysis, will also be undertaken.

Regardless of the contents of ePE and EVs, their enhancement of *in vitro* intestinal epithelial recovery is promising. Their strengthening of epithelial migration and tight junction recovery, and not proliferation, suggests that they likely leverage their effect through more differentiated cell populations, rather than the ISC niche.[5] Identification of this candidate effector cell population is advantageous for prioritizing pathways and mechanisms of action. Potential contributors to increased epithelial migration and tight junction formation, which have been found in other placental derivatives, include Fibroblast Growth Factor β (FGFβ) and Heparin binding EGF-like growth factor (HB-EGF).[24,25] FGFβ accelerates epithelial migration in *in vitro* models and enhances stem cell survival.[24,25] HB-EGF is also known to usher in the earliest cellular responses to injury, such as enterocyte migration, and proliferative stimuli.[26,27]

Interestingly, we did not see the proliferative upregulation in our experiments. As suggested by studies in other species and models of gastrointestinal epithelial injury treated with placental derivatives, protein targets associated with the observed recovery include integrin subunit beta 3 (ITGB3) and chemokine stromal cell-derived factor-1 (CXCL12). ITGB3, a cell-surface protein which participates in cell adhesion and cell-surface signaling upregulates epithelial migration.[28,29] CXCL12 has been demonstrated to drive intestinal epithelial cell migration following scratch wounds, as performed in our model.[9,30] These suggested elements

are only a sampling of those which could be contributing to the therapeutic effects appreciated following ePE and EV application, but are worth considering for future mechanistic inquiries. Primary equine jejunal monolayer cultures were employed in this study to provide an accessible model of the equine intestinal epithelium. Similarly produced monolayers have been previously demonstrated to recapitulate the *in vivo* equine intestinal epithelium in both cell constituents and response to challenges.[21,31] Additionally, monolayers have a polarized epithelium with easy access to the apical, or luminal, compartment.[21] ePE and EV were applied apically in these models to mirror ultimate *in situ* luminal application, where these products may be delivered locally to the damaged intestinal epithelial mucosa. While our injury model involved several types of damage and was performed with epithelium like that found *in vivo*, it is still clearly a reductionist platform. Additional work in further complex models, such as those involving the addition of inflammatory cytokines and co-culture with immunocytes and bacteria, presents another opportunity for improving the translatability of this *in vitro* work. Regardless, the equine jejunal monolayer model of colic remains a valuable tool and its response to ePE and EVs is encouraging for that which may be seen in the complex equine intestine.

Overall, ePE and EVs are capable of accelerating recovery from colic-associated injuries at the cellular level. Mechanistically, this appears to occur through the upregulation of epithelial migration and tight junction restoration. These elements of recovery are not specific to severe, ischemic colic, and thus these products may have potential applicability to inflammatory forms of colic as well.[32] Additionally, the production of ePE is scalable, with each equine placenta producing liters of extract, suggesting that it may ultimately be a cost-effective adjunct therapeutic. Further exploration of equine EV isolation and culture will provide greater insight into the clinical applicability of this treatment modality as well. In sum, the therapeutic potential of ePE and EVs encourage additional exploration into their utility for addressing the current gap in interventions for the devastation caused by severe colic.

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CHAPTER 5

Modulating Allograft Inflammatory Response Via Storage Method in Intestinal Transplantation

Abstract

Transplantation is the only treatment option for intestinal failure patients who cannot tolerate parenteral nutrition. Normothermic machine perfusion (NMP), thought to inhibit ischemia-reperfusion injury, is advancing solid organ transplantation with distinct benefits over traditionally employed cold storage (CS), but little is known regarding its application to intestinal transplantation (IT). Compared to CS, our results indicate that NMP-storage stabilizes most T cell populations by reducing natural killer cells. Following reperfusion, epithelial integrity and anti-apoptosis-associated proteins were increased (Myosin IXB and SON DNA and RNA binding protein, respectively). Upon NMP time-course analysis, most inflammatory biomarkers were reduced at 48 hours post-transplantation (*IFNG*, *IL21*, and *IL17*), along with concomitant increases in anti-inflammatory signatures (*IL22*, *FOXP3*, *CCR10*) and anti-apoptosis associated proteins (Hypoxia up-regulated protein 1, Regucalcin, Galectin, Spingosine-1-phosphate lyase, and FLVCR heme transporter 1). Finally, microbiome sequencing suggested that NMP successfully preserved the gut microbial populations throughout storage and after transplantation. Overall, NMP storage supported a favorable immune environment, epithelial integrity, and microbiota homeostasis in a porcine IT model and may ultimately reduce morbidity and mortality after small intestinal transplantation.

Introduction

Intestinal transplantation (IT) is the only therapeutic option for intestinal failure patients who cannot tolerate parenteral nutrition.[1,2] Unfortunately, IT, as it is currently performed, is associated with more significant immune reactivity and high rates of rejection. Consequently, compared to other organ transplantation, IT results in increased graft loss and reduced patient survival.[3-10] Acute graft rejection has been reported in up to 72% of recipients within 30 days of IT.[7,11] A significant factor of this rejection is the method of organ preservation prior to transplantation and the associated ischemia reperfusion injury (IRI).[4,8,11,12] Static cold storage (CS) is the current gold standard for intestinal graft preservation; however, this type of storage contributes to allograft IRI which, along with proinflammatory and T cell-mediated responses in the intestine, result in allograft rejection.[6,12-17]

In other abdominal organs like the liver, normothermic machine perfusion (NMP) has improved transplantation success.[18,19] This organ preservation technique is designed to preserve

normal physiological metabolism through circulating a blood-based, oxygenated, body temperature perfusate through the allograft.[20-22] In the liver, NMP reduces T cell inflammatory cytokine production, decreases natural killer cell populations associated with rejection, and increases regulatory T cells associated with immunotolerance.[17,23] However, NMP has not been extensively studied in the intestinal tract. The potential for NMP to modulate inflammatory responses in the intestine presents an exciting opportunity to improve IT outcomes.[24]

To combat intestinal rejection, immunological tolerance must be achieved. Current methods for rejection surveillance are limited to endoscopically obtained ileal biopsies through an ileostomy. However, these ileal samples represent a small portion of the entire graft. Thus, comparisons of both jejunal and ileal response to novel storage options, like NMP, over time are needed. This study presents a more comprehensive evaluation of the total graft response over extended durations and with expanded methods of surveillance. Through these evaluations, additional biomarkers for allograft health were identified.

In a previous study, we demonstrated that compared to CS, NMP increased two-day survival of pig IT recipients from 30 to 75%.[12] To better understand potential immunologic contributions to this difference, we combined flow cytometric evaluations, microbiome assessments, genomic analyses, and proteomic studies. We thereby studied changes in intestinal inflammation and host response in the different sections of the intestine (jejunum versus ileum) following NMP and, when available, CS-stored allografts. We hypothesized that NMP stabilizes overall immune cell populations, limits pro-inflammatory cohorts, reduces genetic and protein markers of inflammation, and increases markers of recovery, thus improving graft and recipient health.

Materials and Methods

Experimental animals

Yorkshire crossbred pigs, eight to ten-weeks-old, of either sex weighing between 15-30 kg were used (n=18). The Institutional Animal Care and Use Committee (IACUC) of North Carolina State University (IACUC Number 21-489) and the United States Army Medical Research and Development Command Animal Care and Use Review Office Animal studies approved all experimental activity.

Donor intestine procurement

Protocols for intestinal donor management including pig pre-operative medications, anesthesia induction, intraoperative medications, intestinal procurement, and euthanasia, were performed as previously published.[11,12] All grafts were intravascularly flushed with 1 L of 4ºC University of Wisconsin (UW) preservation solution immediately following procurement and underwent 1-hour of CS prior to beginning the 6-hour storage period. The donor infra-renal abdominal aorta and caudal vena cava were collected to serve as vascular extensions at graft implantation, if needed.

Storage protocols

Cold Storage (CS)

Following the initial 1 hour of CS described above, allografts undergoing only CS were maintained on ice and refrigerated at 4°C in UW solution for an additional 6 hours (CS_T6), as previously described.[12] Prior to implantation in the recipient, CS-stored intestine was flushed with 500 mL of 5% Albumin.

Normothermic Machine Perfusion (NMP)

After the initial 1 hour of CS, allografts stored by NMP were flushed with 5% albumin and then placed onto the proprietary perfusion machine (Functional Circulation, LLC, Northbrook, IL). This machine circulated a 34°C, oxygenated blood-based perfusate into the graft via the cranial mesenteric artery (CMA), with blood collected from the reservoir passing through a hemodiafiltration circuit for an additional period of 6 hours (NMP_T6), as described.[11,12]

Intestinal transplantation –implantation, postoperative management, euthanasia

Recipient pigs received identical preoperative medications and management as donors with the addition of tacrolimus (0.2 mg/kg subcutaneously) once per day starting 24 hours prior to surgery.[11] Additional dosing was determined based upon daily whole blood tacrolimus concentration measurement, with a target trough of 8 - 12 ng/mL.[25] Anesthesia and surgical approach were identical to that used for donor pigs. Recipient's innate intestine was resected and removed as previously described.[12] Vascular extensions, collected from the donor pig, were anastomosed end-to-side to the recipient's abdominal aorta and/or caudal vena cava followed by end-to-end anastomosis to the donor intestine vasculature. Five minutes after completion of reperfusion, methylprednisolone (20 mg/kg) was administered intravenously.[11,12] Following one

hour of *in vivo* reperfusion, biopsies were collected, and then end-to-end donor-to-recipient intestinal anastomosis was performed. Routine abdominal closure was then performed.

Postoperatively, pain management included subcutaneous bupivacaine at incision closure, transdermal fentanyl patch, lidocaine-ketamine constant rate infusion, and buprenorphine. Additional supportive care included gastroprotectants (omeprazole) and anti-emetics (maropitant), and anti-thrombolytics including heparin or enoxaparin, dose-adjusted to activated clotting times (ACT), as previously described.[12] Blood measurements of ACT, complete blood count, blood serum chemistry, and blood lactate were performed daily.[11] Immunomodulation was achieved with tacrolimus administration and tapering methylprednisolone. Beyond hematologic assessment, physical examinations, enteral feeding, fecal and urine production were closely monitored. Hill's Prescription Diet A/D Urgent Care wet food (Hill's Pet Nutrition, Topeka, KS) was offered every 6 hours following anesthetic recovery. At the designated 48-hour postoperative endpoint, or when noted to be in distress, animals were anesthetized with intramuscular xylazine (1.5 mg/kg) and ketamine (11 - 20 mg/kg) and euthanized with pentobarbital (100 mg/kg).

Tissue collection

Full thickness jejunal and ileal intestinal biopsies were collected prior to the initial 1-hour period of CS to serve as control (T0). Additional biopsies were collected at the end of preservation (CS_T6 or NMP_T6), 1 hour following graft anastomosis to the recipient blood supply and reperfusion (CS_T1RP or NMP_T1RP), and at the study endpoint of 48 hours following euthanasia (CS_T48 or NMP_T48). Sampling timepoints and processing are summarized in Figure 1.

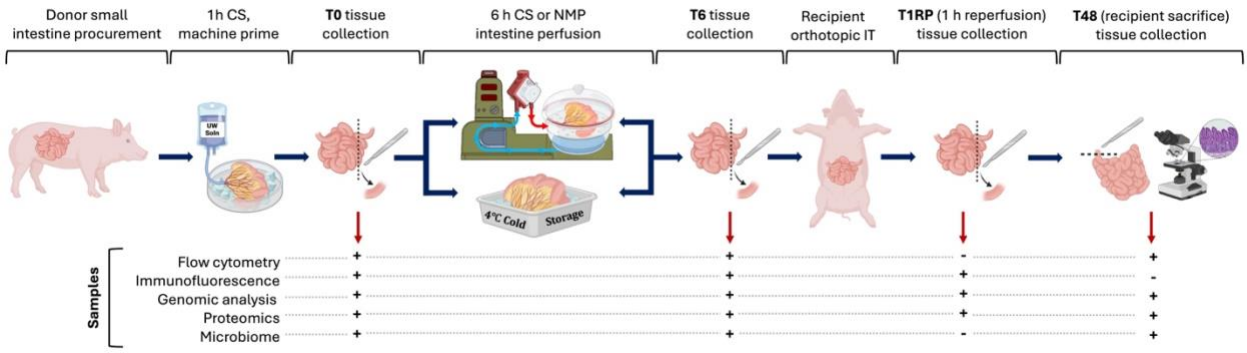

Figure 1. Experimental design of the different intestinal storage processes, timepoints, and samples collected. IT: intestinal transplant, CS: cold storage, NMP: normothermic machine perfusion, T0: control/baseline, T6: 6 h of storage, T1RP: 1 h after reperfusion, T48: 48 h post-transplant.

Tissue histomorphometric evaluation and immunofluorescence

Full-thickness sections of the jejunum and ileum were opened longitudinally along the anti-mesenteric border and fixed, embedded, and stained with hematoxylin and eosin as previously described.[12,26] Slides were evaluated by a boarded pathologist blinded to time and storage conditions. Scoring was performed using the Chiu/Park scale for grading intestinal IRI in addition to a clinically used graft rejection scoring system.[27,28] The scoring system evaluated intestinal injury via assessment of subepithelial bleb or Gruënhagen's space formation, epithelial surface erosion/denuded villi, and columnar attenuation using a scale of 0 to 4 (0, normal; 1, focal and mild; 2, diffuse/multifocal and mild; 3, diffuse and moderate; 4, diffuse and severe).

Tissue for immunofluorescent analysis was fixed, sectioned, and processed as in prior reports.[12] Immunofluorescence processing was performed for cleaved caspase 3 (CC3, rabbit, 1:500, Cell Signaling Technology, Danvers, MA), a marker for cellular apoptosis at all timepoints.[29,30] Alexa 555/Cy3.5 secondary antibody was diluted (1:500) and applied for 45 min at room temperature (RT). Slides were then counterstained with bisBenzimide H 33258 nuclear stain (1:1,000, Sigma-Aldrich, St. Louis, MO) prior to imaging. $CC3^+$ cells were counted per well-oriented villus of blinded images using ImageJ (1.54f) and reported as the total number of cells per villus axis.

Image acquisition

Images were captured using an inverted fluorescence microscope (Olympus IX83, Tokyo, Japan) fitted with a color camera (DP26, Olympus, Tokyo, Japan) and monochrome digital camera (ORCA-flash 4.0, Hamamatsu, Japan). Objective lenses X10 and X20 (numerical apertures of 0.3 and 0.45) (LUC Plan FLN, Olympus, Tokyo, Japan) were used for imaging.

Tissue processing and flow cytometry

To provide spatial context for changes in intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), these populations were isolated from tissues at T0, T6, and, when available, T48. Single cell suspensions were prepared from full thickness intestinal biopsies avoiding gut associated lymphoid tissue. Tissues were thoroughly rinsed with a Hank's Buffered Saline Solution (HBSS)-based wash (HBSS, HEPES 1 M, 7.5% sodium bicarbonate) and the serosa was removed. Tissue was then mechanically minced prior to placement in a pre-warmed pre-digestion buffer (HBSS wash, 1.23% Bovine Serum Albumin (BSA), 0.1% dithiothreitol (DTT, 1 M), 1% ethylenediaminetetraacetic acid (EDTA, 0.5 M)) at 27°C for 10 min under continuous rotation (250 rpm). This sample was mixed well and then filtered through a 110-μm

mesh to allow collection of the IEL-containing filtrate. IELs were then pelleted, washed, resuspended in flow cytometry buffer (FCM buffer: phosphate buffered saline (PBS), 2% fetal bovine serum (FBS), and 0.4% EDTA, 0.5 M), and stored on ice until antibody staining.

Remaining tissue was again incubated in pre-warmed pre-digestion buffer for another 10 min at 27°C under continuous rotation (250 rpm). Tissue was washed and then placed into a second pre-digestion buffer (HBSS wash, 1.25% BSA) for 10 min at 27°C under continuous rotation (250 rpm). Tissue was again washed before addition to RPMI-BSA media, Liberase TM (26 Wu/mL, Roche) and DNase (1 mg/mL) and incubated at 37°C under continuous rotation for 30 min at 225 rpm. Following incubation, tissue was dissociated using the program: m_intestine_01of the gentle gentleMACS™ Dissociator (Miltenyi Biotec, Cologne, Germany). The produced solution was filtered through a 100-μM filter and then centrifuged at 300 ×g for 10 min. The supernatant was removed, and the remaining pellet treated with 2 mL of 1X RBC lysis buffer for two min, which was then neutralized with RPMI-based media and then filtered via a 40-μm filter. The solution was centrifuged at 300 ×g for 10 min prior to resuspension in FCM buffer.

Cells were counted with a Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA) and 5.0 $\times 10^5$ cells were stained per antibody combination. Cells were stained with primary antibodies for 10 min at RT in the dark, washed with PBS, then stained with secondary antibodies under the same conditions. Stained cells were then fixed in 1% PFA for 10 min in the dark, then resuspended in FCM buffer with glycine prior to placement in FCM with EDTA and freezing at -20°C until analysis. Primary antibodies included PE-conjugated anti-NKP46 (VIVI-KMI, BIORAD, Hercules, CA), FITC-conjugated anti-CD3 (PPT3, BIORAD), Cy5/Alexa 647 conjugated anti-TCRγδ (PGBL22A, Kingfisher Biotech Inc, St. Paul, MN), anti-CD4 (74-12-4, mouse IgG2b, BEI Resources, Manassas, VA), anti-CD8b (PG164A, mIgG2a, Kingfisher Biotech Inc), and biotinylated anti-CD21a (BB6-11C9.6, Novus Biologicals, Centennial, CO). Secondary antibodies included anti-mIgG2b-BV421 (CD4, Jackson ImmunoResearch Laboratories, West Grove, PA), anti-mIgG2a-BV480 (CD8, Jackson ImmunoResearch Laboratories) and streptavidin (SA)-BV605 (CD21, BD Horizon Biosciences, San Jose, CA). A near-infrared live/dead fixable stain kit (ThermoFisher Scientific) was used as a viability dye. SpectraComp beads (Slingshot Bioscences, Emeryville, CA) were used for single stained compensation controls. Data was acquired using a 3-laser, 9-parameter Beckman Coulter CytoFLEX instrument (Beckman Coulter,

Loveland, CO) and analyzed using FlowJo software v10.8.1 or v10.9 (BD Biosciences, Franklin Lakes, NJ).

Mucosal scraping sampling

To collect mucosal scrapings, 1 cm x 1 cm sections of intestine were opened longitudinally and exfoliated to the level of the serosa using a razor blade. The serosa was discarded, and the remaining tissue was stored for future processing. The collected tissue was snap-frozen in liquid nitrogen and stored at -80°C.

Gene expression analysis

To evaluate the impact of NMP storage on genetic expression, isolated mucosal scrapings were collected for all timepoints (T0, T6, T1RP, T48) from 3 pigs who received NMP-stored allografts and survived until 48 hours. Total RNA was extracted (RNeasy Minikit, Qiagen, Valencia, CA), and yield and quality were determined in a NanoDrop (ThermoFisher Scientific, Waltham, MA) by the 260/280 nm ratio (2.03-2.07).

At processing, 5 µL of total RNA were thawed and then hybridized to a custom code set including 50 genes (7 housekeeping genes and 43 target genes) associated with intestinal epithelial and lymphocyte response to injury and recovery (Table 1). Hybridization was completed over 18 hours at 65ºC following the NanoString Gene Expression CodeSet RNA Hybridization Protocol (NanoString, Seattle, WA). Probe–target complexes were matched and immobilized in the nCounter cartridge, which was then placed in nCounter® MAX/FLEX system to acquire images and process data, following the manufacturer's protocol. Gene-expression levels were then tabulated in a comma-separated value format. The raw digital count of expression was exported from nSolver v4.0 software for downstream analysis. Raw digital counts underwent quality control processing and were normalized based upon manufacturer provided standards.

Table 1. Custom NanoString code set (50 probes) applied to the samples collected in this study.

N°	Gene	Accession Number	N°	Gene	Accession Number
1	BCL6[a]	NM_001315701.1	26	IFNG[a]	NM_213948.1
2	CCR10[b]	NM_001044563.1	27	IL10[b]	NM_214041.1
3	CCR4[c]	XM_003361791.2	28	IL12[a]	NM_001145986.1
4	CCR5[a]	NM_001001618.1	29	IL13[b]	NM_213803.1
5	CCR6[a]	XM_013992405.1	30	IL17[a]	NM_001005729.1
6	CCR7[a]	NM_001001532.3	31	IL2[a]	NM_213861.1
7	CCR9[b]	NM_001001624.1	32	IL21[a]	NM_214415.1
8	CD103[c]	NM_001315722.1	33	IL22[b]	XM_021091967
9	CD27[a]	NM_001278763.1	34	IL4[a]	NM_214123.1
10	CD28[c]	NM_001287410.1	35	IL5[a]	NM_214205.1
11	CD39[b]	NM_214153.1	36	IL6[a]	NM_001252429.1
12	CD4[b]	NM_001001908.2	37	IL8[a]	NM_213867.1
13	CD40L[a]	NM_214126.1	38	PRF1[a]	XM_003483492.4
14	CD45[c]	XM_003130596.4	39	RORγt[c]	XM_003355171.4
15	CD69[a]	NM_214091.1	40	TBET[a]	XM_005669122.2
16	CD72[c]	NM_001097493.1	41	TRGC[c]	XM_013978233.1
17	CD73[b]	XM_001927095.4	42	TGFB[a]	NM_001244288.1
18	CD8B[a]	NM_001348770.1	43	TNF[a]	NM_214022.1
19	CTLA4[b]	NM_214149.1	44	B2M[d]	NM_213978.1
20	CXCR3[a]	XM_003135179.5	45	GAPDH[d]	NM_001206359.1
21	EOMES[a]	XM_003132081.4	46	GUSB[d]	NM_001123121.1
22	FOXP3[b]	NM_001128438.1	47	HMBS[d]	NM_001097412.1
23	GATA3[b]	NM_001044567.1	48	HPRT1[d]	NM_001032376.2
24	GZMA[a]	NM_001143709.1	49	PGK1[d]	NM_001099932.2
25	GZMB[a]	NM_001143710.1	50	TUBB[d]	NM_001044612

[a] pro-inflammatory targets, [b] anti-inflammatory targets, [c] unclassified targets *housekeeping genes

Proteomics

To determine changes in gene expression due to NMP and CS, as well as differences between intestinal segments and timepoints, we analyzed the proteome of mucosal scrapings using shotgun proteomics.[31] All available timepoints (T0, T6, T1RP, T48) were analyzed. To extract proteins from the mucosal scrapings we first homogenized mucosal tissue in 100 µL of PBS with Roche cOmplete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) using an Omni International tissue homogenizer with hard tissue plastic tips (Omni International). Homogenized samples were then placed in a Lysing Matrix E Tube (MP Biomedical) with 1.5 mL SDS-lysis buffer (5% (w/v) SDS, 100 mM Tris-HCL pH 7.6) and bead beat in a Bead Ruptor Elite 24 (5 cycles for 45 s at 6.45 m/s with 1 min dwell time between cycles). Proteins were then extracted using an S-Trap protocol.[32] Lysates were heated for 10 min at 95ºC and then briefly vortexed, before centrifuging for 5 min at 21000 ×g. Lysate, 140 µL per sample, was placed into an

Eppendorf tube, and 6.4 µL of 500 mM DTT was added to each sample and incubated at 95ºC for 10 min. After cooling for 5 min, 12.7 µL of 500 mM indole-3-acetic acid (IAA) was added, and samples were shaken in a thermomixer for 1 min and then incubated at RT in the dark for 30 min. After incubation, samples were acidified with 16 µL of 12% phosphoric acid, followed by 1050 µL of binding buffer (100 mM TEAB in 90% methanol) and vortexed. Then 600 µL of each sample was loaded onto S-Trap mini columns (ProtiFi, Fairport, NY). S-Trap columns were centrifuged at 4000 ×g for 1 min. The flow through was dumped and then another 600 µL of sample was added to each trap column and centrifuged at 4000 ×g for 1 min. Then samples in the S-Trap columns were washed 3 times using 400 µL of binding buffer and 1 min of centrifugation at 4000 ×g. The S-Trap columns were transferred to new collection tubes. 1 µg of Pierce Trypsin Protease MS-Grade (ThermoFisher Scientific) suspended in 40 µL of digestion buffer (50 mM TEAB). Omnisolv LC-MS water was added to each S-Trap to digest the protein into peptides. Samples were incubated overnight at 37ºC. After digestion, 80 µL of digestion buffer was added to each sample and the samples were incubated 37ºC for 10 min followed by a 4000 ×g centrifugation for 1 min. Then 80 µL of the first elution buffer (0.2% formic acid) was added to each sample followed by a 4000 ×g centrifugation for 1 min. 80 µL of second elution buffer (50% acetonitrile (ACN), 0.2% formic acid) was then added to each sample followed by a 4000 ×g centrifugation for 1 min. To remove ACN and concentrate the peptides, sample eluents were placed on a SpeedVac (ThermoFisher Scientific Savant SPD120 Vacuum Concentrator) for 1 hour and 15 min. Peptide concentration was determined using a MicroBCA Protein Assay Kit (ThermoFisher Scientific). Peptides were measured using an Exploris 480 mass spectrometer coupled to an Ultimate 3000 RSLCnano system with a C18 PepMap100 pre-column (ThermoFisher Scientific) and a 75 cm x 75 µm analytical EASY-Spray PepMap RSLC C18 column analytical column. Briefly, 1 µg of peptides was loaded per column, desalted, and then separated along a 140-min reverse phase gradient on the analytical column before being emitted onto the mass spectrometer by electrospray. Peptides were measured on the mass spectrometer using a data-dependent acquisition method. The method contained a full scan at resolution 60000, a scan range of 380-1600 m/z, an AGC target of 300% (3.0 x 106 charges), and a maximum injection time of 200 ms for every 15 MS2 scans fragmented using an HCD collision energy of 27%, measured at a resolution of 15000, an AGC target of 100% (1.0 x 105 charges), a maximum injection time of 50 ms, and a 25 min exclusion list.

For protein identification, raw MS data was searched against the *Sus scrofa* proteome (UP000008227) from UniProt clustered at 95% ID using cd-hit and a list of contaminants from the cRAP database using the Proteome Discoverer software (v2.3) from ThermoFisher Scientific.[33-35] Briefly, peptides were identified using the Sequest HT and Percolator nodes at a 5% FDR, protein groups were identified by strict parsimony principles in the consensus step using a 5% FDR, and protein groups were quantified by area under the curve MS1 intensity using unique plus razor peptides. Protein group abundances were normalized by total sum scaling and exported for statistical analysis to the Perseus (v1.6.14.0) analysis software.[36]

Microbiome

DNA isolation and Whole Genome Shotgun (WGS) sequencing were performed by the University of North Carolina, School of Medicine, Microbiome Core Facility (Chapel Hill, NC). For DNA isolation, mucosal scraping samples were transferred to a 2 mL tube containing 200 mg of 106/500 μm glass beads (Sigma-Aldrich) and 0.6 mL of Qiagen ATL buffer (Valencia, CA), supplemented with 60 mg/mL lysozyme (ThermoFisher Scientific). The suspension was incubated at 37°C for 1h with occasional agitation. The suspension was agitated for 20 min on a Digital Vortex Mixer at 3000 rpm. Subsequently, the suspension was supplemented with 600 IU of Qiagen proteinase K and 0.3 mL of Qiagen AL buffer followed by a 70°C incubation for 1 h. After a brief centrifugation, supernatant was aspirated and transferred to a new tube containing 0.5 mL of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents and eluted in DNase free water.

For WGS sequencing,[37] 5 ng of genomic DNA were processed using the Nextera XT DNA Sample Preparation Kit (Illumina). Target DNA was simultaneously fragmented and tagged using the Nextera Enzyme Mix containing transposome that fragments the input DNA and adds the bridge PCR (bPCR)-compatible adaptors required for binding and clustering in the flow cell. Next, fragmented and tagged DNA was amplified using a limited-cycle PCR program. In this step, index 1(i7) and index 2(i5) were added between the downstream bPCR adaptor and the core sequencing library adaptor, as well as primer sequences required for cluster formation. The thermal profile for the amplification had an initial extension step at 72°C for 3 min and an initial denaturing step at 95°C for 30 s, followed by 15 cycles of denaturing at 95°C for 10 s, annealing at 55°C for 30 s, a 30 s extension at 72°C, and final extension for 5 min at 72°C. The DNA library was then purified using Agencourt® AMPure® XP Reagent. Each sample was quantified and normalized before

pooling. The DNA library pool was loaded on the Illumina platform reagent cartridge (Illumina) and the Illumina instrument.[38]

Sequencing outputs from the Illumina NextSeq 4000 platform were converted to fastq format and demultiplexed using Illumina BCL Convert v4.2.4 (BCL Convert ver 4.2.4 (2023), Illumina, Inc. San Diego, CA, USA) [A]. Quality control of the demultiplexed sequencing reads was verified by FastQC (v. 0.11.8. 2018, Babraham Institute, Cambridge, UK) . Adapters were trimmed using Trim Galore (2017, Babraham Institute, Cambridge, UK). The resulting paired-end reads were submitted to Kraken2[39] for taxonomic classification with respect to a database targeting *Sus scrofa* gut species. An estimate of taxonomic composition including host was produced from these results using Bracken 2.5.[40] Reads classified as *Leishmania* spp. by Kraken2 were further filtered by alignment to the genomes of the *Leishmania* spp. and to *Sus scrofa* using Bowtie2.[41] Reads aligning to the *Leishmania* spp., but not aligning to the *Sus scrofa* reference, were included in the read count for the appropriate *Leishmania* spp.

Statistics

Statistical analyses were performed using Prism 10 for macOS v. 10.3.1 (GraphPad Software, La Jolla, CA) software, where not detailed. A Shapiro-Wilk normality test was performed on raw data, and different tests were applied depending on data normality and the specific comparisons (NMP *vs* CS) or NMP over time. Statistical significance was defined with an alpha threshold and confidence level of 0.05.

NanoString nCounter

Results from gene expression levels obtained in NanoString nCounter experiments and normalized in nSolver software were exported into R (version 4.4.0, released 2024-04-24) and analyzed using the differential expression testing with linear mixed models for repeated measures (DREAM) package.[42] In the fitted linear model, individual gene counts were treated as the dependent variable, while time points (T0, T6, T1RP, and T48) served as fixed effects. To improve normality assumptions, gene counts were transformed using base 2 logarithms, and the log2-transformed values were then weighted to stabilize variance among different gene expression levels before fitting the linear model. The Kenward-Roger method was employed to address small sample size issues (n=3), enhancing statistical power.[43] To evaluate the overall time effect, F-tests were conducted applying the Benjamin-Hochberg (BH) method to adjust the p-values and preventing the risk of false discovery rates (FDR).[44] An overall time effect was considered

statistically significant if the adjusted p-values were less than 0.05. Following the F-tests, pairwise comparisons with the baseline (T0) were performed using t-tests for the three combinations T6 *vs* T0, T1RP *vs* T0, T48 *vs* T0. The BH method was again utilized to adjust the p-values for the risk of FDR. The difference between two time points was considered statistically significant if the adjusted p-value was less than 0.05.

Proteomics

For proteomic analysis a t-test was used to compare T1RP CS relative to NMP with a BH for FDR control. For the time course analysis in the NMP experiments, differences between time points were tested using ANOVA followed by BH for FDR control. Significance was determined by $q < 0.05$.

Microbiome

Alpha diversity was measured by Evenness Pielou, Chao1, Shannon's entropy, and Simpson's diversity indexes. Comparisons between NMP samples over time were conducted by RM one-way ANOVA or Friedman test depending on data normality. Beta diversity as measured by Bray-Curtis dissimilarity was performed using QIIME2.[45] Absolute abundances were compared through the analysis of compositions of microbiomes with bias correction (ANCOM-BC)[46] that provided control of FDR and confidence intervals for differential abundance of each taxon. Additional visualization was provided by Microbiome Analyst.[47]

Results

NMP reduces tissue damage in the jejunum

Histological assessment was performed to compare the degree of tissue injury incurred with different storage methods and at different timepoints (T0, T6, T1RP, T48) in the jejunum and the ileum (Figure 2). Here, these evaluations are expanded to include additional animals, a clinically utilized graft rejection scoring system and an assessment post-transplantation.[12] In the jejunum (Figure 2A), after one hour of reperfusion (T1RP), multiple measurements of damage were significantly elevated in CS compared to control, including Chiu/Park scores (T1RP: mean 3.2 +/- 0.8367 *vs* T0 0.2 +/- 0.6325, p = 0.0405), subepithelial bleb grade (T1RP: 2.2 +/- 0.4472 *vs* T0: 0.2 +/- 0.6325, p = 0.0216), and denuded villi grade (T1RP: 2.66 +/- 0.8944 *vs* T0: 0 +/- 0, p = 0.0216). Additionally, jejunal subepithelial bleb grade at T1RP was significantly higher in CS *versus* NMP (CS: 2.2 +/- 0.4472 *vs* 1.0 +/- 0.7071, p = 0.0125). Conversely, jejunal columnar attenuation grade was elevated in NMP *versus* CS at T6 (NMP: 1.4 +/- 1.342 *vs* CS: 0 +/- 0, p =

0.0479), suggesting increased epithelial flattening and potential restitution with NMP storage. In the ileum (Figure 2B), NMP storage induced increased damage markers only at the end of the storage period (T6) compared to CS, including Chiu/Park, which resolved in all subsequent timepoints.

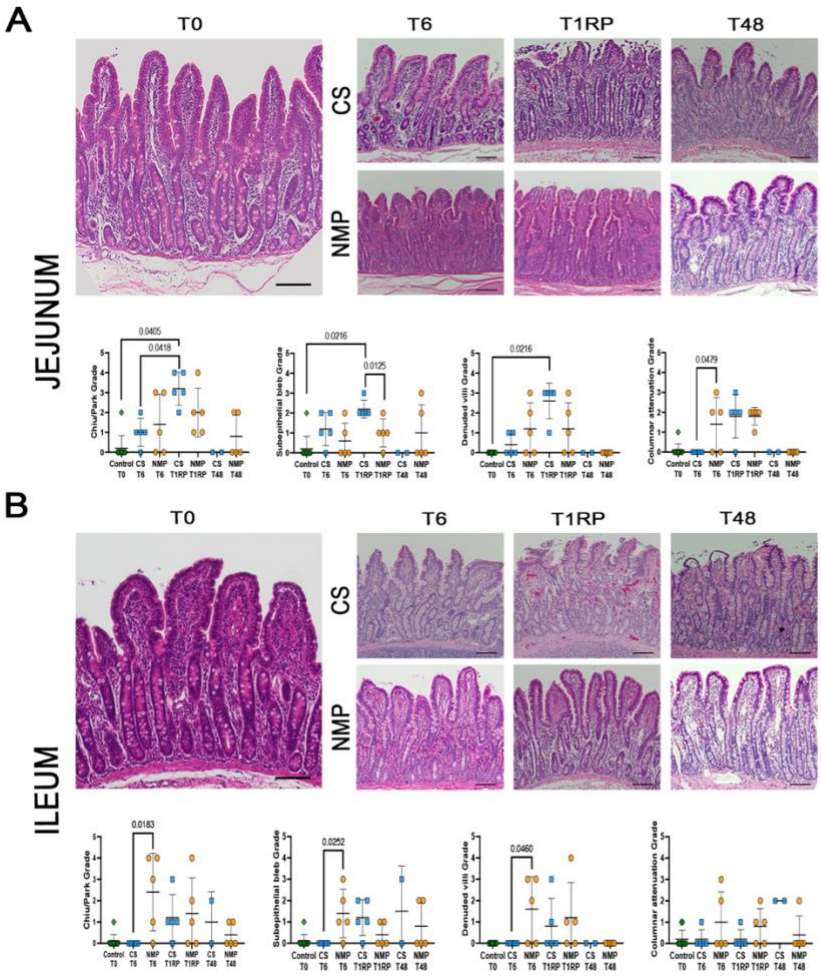

Figure 2. Histology and injury grading of jejunum and ileum following CS or NMP. Hematoxylin and eosin-stained sections of T0 (control), after 6 hours of CS or NMP (CS-T6, NMP-T6), after 1 hour of reperfusion posttransplant (T1RP), and 48 hours following transplantation (T48), were evaluated for differences in intestinal injury following each storage method. (A) CS-T6 and CS-T1RP jejunum showed evidence of significant damage compared with control (T0), consistent with mild to moderate villous epithelial loss. (B) NMP-T6 ileum had evidence of mild to moderate epithelial damage that appeared to resolve at all subsequent timepoints. T0 n =10 pigs, CS (T6, T1RP) n = 5 pigs, NMP (T6, T1RP) n = 5 pigs, and NMP (T48) n = 5 pigs. Original magnification: 10X, scale bar: 100 μm. Unpaired t-test or Mann-Whitney test, depending on data normality, were used to compare CS *vs* NMP at each time point. NMP or CS over time were compared, depending on data normality, by RM one-way ANOVA followed by Tukey or Friedman followed by Dunn's for multiple comparisons. Only significant differences ($p<0.05$) are shown with the corresponding p-value.

NMP preserves overall T cell populations but reduces specific rejection-associated lymphocytes

Flow cytometry was performed on samples from the 16 pigs with tissue available at T0 and T6 (CS: 10 pigs, NMP: 6 pigs) and at T48 (CS: 2 pigs, NMP: 4 pigs). Due to increased fatality following CS, and challenges processing jejunum, there were insufficient jejunal samples at T48, for intraepithelial or lamina propria lymphocytes (IEL or LPL), to facilitate statistical evaluation at this timepoint.

For total T cell populations ($CD3^+$), the percent of $CD3^+$ IELs in NMP stored tissue at each timepoint was not significantly different from T0 control. In comparison to CS, NMP preserved jejunal $CD3^+$ IELs at T6 (62.74 +/- 10.77% *vs* 32.97 +/- 24.18%, $p< 0.0001$), and in the ileum at both T6 (51.31 +/- 16.65% *vs* 42.77 +/- 24.37%, $p< 0.0001$) and T48 (57.73+/- 19.8% *vs* 34.21 +/- 26.11%, $p<0.0001$) (Figure 3A and B). Reductions in IEL $CD3^+$ populations identified following CS were likely linked to epithelial barrier breakdown and loss of cells into the lumen. There was no significant difference between the two storage methods for any $CD3^+$ LPL population at these timepoints (data not shown, d.n.s.).

Evaluating $\gamma\delta^+$ T cell populations, NMP reduced the $\gamma\delta^+$ IELs in the ileum, compared to CS, at both T6 (28.70 +/- 10.85% *vs* 44.5 +/- 23.01%, $p < 0.0001$) and T48 (16.68 +/- 4.29% *vs* 22.37 +/- 6.65%, $p < 0.0001$) (Figure 3E and F). Jejunal $\gamma\delta^+$ IEL were not significantly different at T6. No significant difference between the storage types was found for $\gamma\delta^+$ LPL populations (d.n.s.). Natural killer cells were significantly reduced in the LPL of NMP-stored jejunum (2.38 +/- 1.30% *vs* 4.33 +/- 2.09, $p = 0.045$) and ileum (3.05 +/- 1.51% *vs* 5.64 +/- 2.61%, $p = 0.001$) at T6 compared to baseline (T0) values (Figure 3G and H). The reduction of NK in NMP-stored jejunum may be relevant given the known role of NK cells in acutely rejecting allografts.[48] There were no significant differences in NK cell numbers between storage types at T6 or T48. Additionally, $CD4^+$, $CD8^+$, $CD3^+CD4^-CD8^-$ and $CD21^+$ cell populations were not significantly different between storage conditions at different timepoints, sections of intestine, or subpopulations of lymphocytes (d.n.s.).

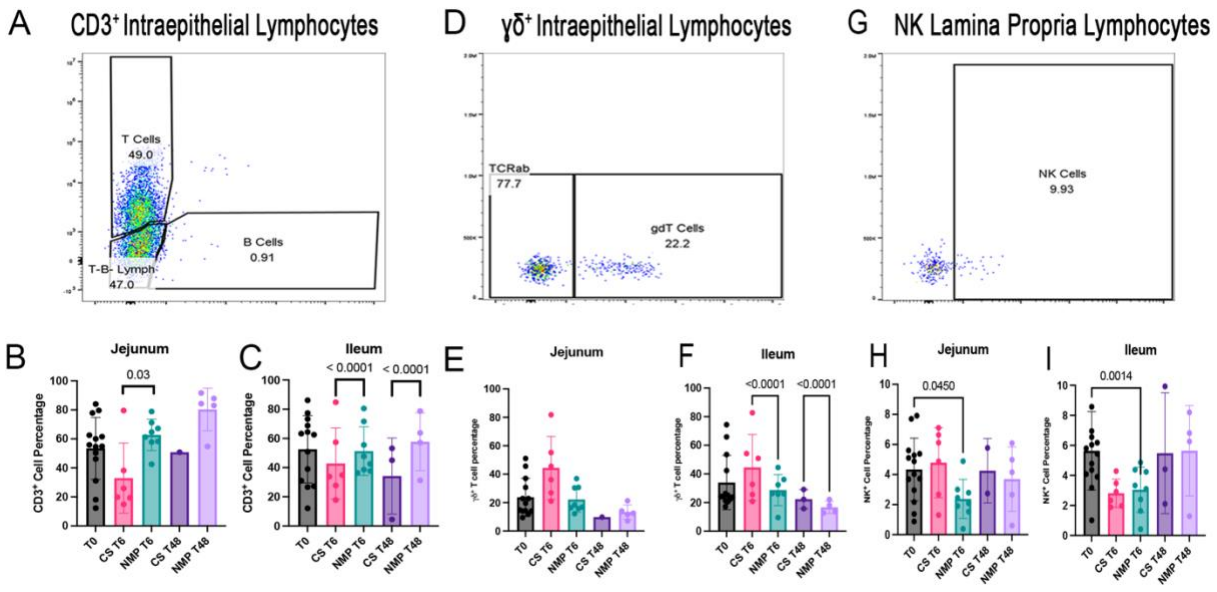

Figure 3. Flow cytometric analysis of critical leukocyte populations. A: Representative gating approach for CD3$^+$ IEL T cell populations. B + C: Quantification of CD3$^+$ IEL populations in the jejunum and ileum. One-way ANOVA with Kruskal-Wallis Test (jejunum) or Holm-Šídák's multiple comparisons test (ileum) D: Representative gating for γδ$^+$ IEL T cell populations. E + F: Quantification of γδ$^+$ IEL in the jejunum and ileum. One-way ANOVA with Tukey's multiple comparisons test (jejunum) or Holm-Šídák's multiple comparisons test (ileum) G: Representative gating approach for NK$^+$ LP cell populations. H + I: Quantification of NK$^+$ LP cell populations in the jejunum and ileum. One-way ANOVA with Tukey's multiple comparisons test (jejunum and ileum). n= 2-8 pigs.

Proteomics reveals critical differences following allograft reperfusion in apoptosis and barrier function proteins associated with storage method.

In total we quantified 8,305 protein groups with a protein level false discovery rate (FDR) below 5%. To determine if there were any significantly different proteins between CS and NMP storage, we analyzed intestinal samples at T1RP because that was the intervention time point at which we had enough replicates to do the analysis (n >3). In the jejunum we identified three proteins that were significantly different at an FDR ≤ 5% (t-test, BH FDR calculation) (Figure 4A). The two proteins significantly increased in NMP versus CS were Myosin IXB (Myo9B; Uniprot ID A0A8W4FPY8) and the SON DNA and RNA binding protein (SON; Uniprot ID A0A5G2RFH3). Dihydropyrimidinase like 4 (DPYSL4; UniProt ID A0A5G2QSW3) was more highly expressed following CS compared to NMP. Myosin IXB, an unconventional myosin molecule, contributes to actin remodeling, and thus epithelial tight junction integrity, within epithelial enterocytes.[49] Individuals homozygous for mutations in this protein are significantly

more at risk for Celiac's disease.[49,50] Furthermore, knockdown of Myo9B leads to increased tight junction permeability and reduced cellular migration, suggesting that the increased expression of Myo9B in NMP may correlate to improved barrier function and epithelial integrity.[50] The nuclear speckle protein, SON, binds DNA, RNA, and promotes pre-mRNA splicing, specifically assisting at weak splice sites.[51] Its target-RNAs are often associated with DNA repair, replication, and the cell cycle, usually impacting mitotic progress.[51] This protein is also a cell cycle regulator gene involved in preventing apoptosis.[52] As this protein was increased in mucosal scrapings, it is impossible to isolate which cells may be experiencing increased replication following NMP, though, our previous findings suggest that the epithelial intestinal stem cells, which are critical for epithelial recovery and repair, are a potential candidate.[12] Increased in CS jejunum, DPYSL4 enables filamin binding activity and thus also plays a role in cell migration, in addition to neurodevelopment.[53] DPYSL4 has also been suggested to be a tumor suppressor when expressed.[54] This protein, however, has not been extensively studied in the intestine, though its upregulation during reperfusion in CS allografts, is intriguing. Besides these three proteins, we identified 118 additional proteins in jejunum with a p-value below 0.05, suggesting that a more powered experiment could reveal even more proteins of interest for further investigation. For example, Filament A (FLNA) had a greater that 7 log2 fold-change increase in NMP and is a wound healing protein (t-test p-value = 0.04), and Filamin binding LIM protein 1 (FBLIM1; migfilin) was also increased in NMP relative to CS (t-test p-value = 0.003).

In the ileum, we identified three significant differentially expressed proteins (Figure 4B). In NMP relative to CS, procollagen C-endopeptidase enhancer (PCOLCE;UniProt ID I3LEE6) and RAB3 GTPase activating non-catalytic protein (RAB3GAP2; UniProt ID A0A287BB18) were decreased, while casein kinase 2 alpha (CSNK2A2; UniProt ID A0A287BB18) was increased. Procollagen C-protein enhancing protein (*PCOLCE*), is a secretory glycoprotein that boosts the activity of procollagen C-protease and stimulates extracellular matrix remodeling and has been shown to play a role in tumor growth and metastasis in multiple cancers.[55] High expression of *PCOLCE* has been associated with poor prognosis in cancer patients and is associated with immune infiltration including in gastric cancer.[56] Casein Kinase, made up of alpha and beta subunits, has dual functionality with involvement in cell growth/proliferation as well as the suppression of apoptosis. Interestingly, within the intestinal epithelium, CK2 is considered a key regulator of homeostasis and promotes wound healing through inhibition of apoptosis during

inflammatory conditions.[57] Similar to the jejunum, we identified 85 proteins in ileum with p-values below 0.05 that could be worth investigating in a more powered experiment (Figure 4B). For example, programmed cell death protein 4[58] (Uniprot ID A0A480HSI3; PDCD4) was elevated in CS relative to NMP with a t-test p-value of 0.001. Although no known role of these proteins has yet been identified in intestinal transplantation, their identification may be useful in prognosticating intestinal transplant outcomes in the future.

Due to the differences identified in key cells associated with inflammation between CS and NMP-stored intestine by the flow cytometry analysis, we also investigated the abundance of known markers of inflammation, S-100A8/A9 (Calprotectin; humans)[59] and Lipocalin-2 (LCN2; mice)[60] across the study, even though they were not significant in our unsupervised analysis. Both of these proteins were overall significantly different in abundance across all the conditions in both the jejunum and ileum by ANOVA ($p < 0.05$; Figure 4C-F). Elevated fecal calprotectin has been associated with intestinal transplant rejection, although as a marker alone, it is not sufficient to distinguish rejection from other causes of inflammation.[61] Calprotectin began to rise following reperfusion in both storage methods, but this trend was not significant by multiple comparison analysis (Figure 4C). Calprotectin levels were found to be elevated in CS-ileum at T48, though conclusions are limited by low animal survival (Figure 4D). Lipocalin-2 is considered a master mediator of intestinal and metabolic inflammation with known pro- and anti-inflammatory activities.[60] In both the jejunum and ileum, LCN2 levels began to be elevated at T1RP, with significance reached at T48 in allografts stored by both methods in the jejunum and only CS ileum (Figure 4E and 4F).

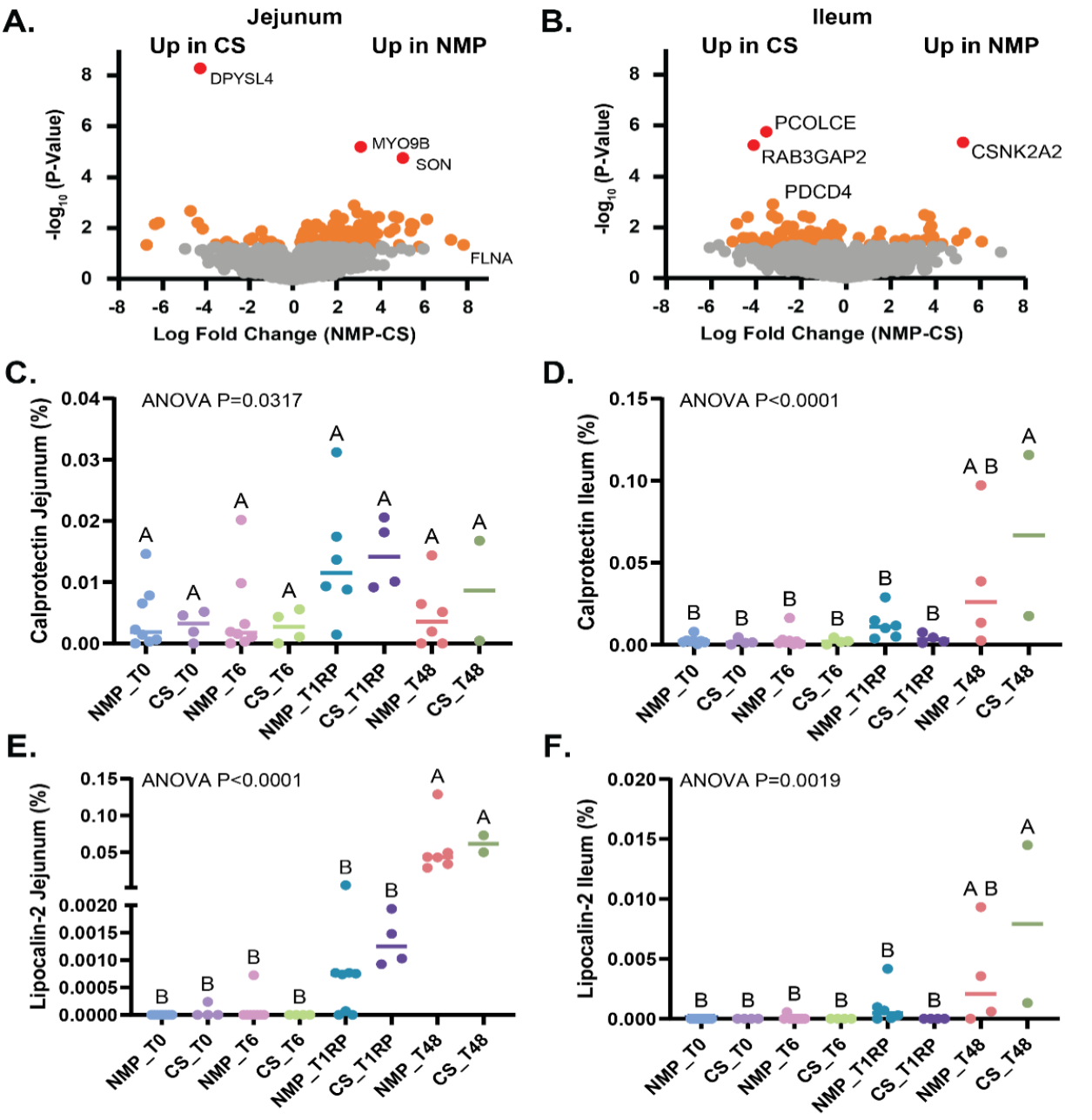

Figure 4. Proteomic analyses of jejunum and ileum in CS and NMP pre- and post-intestinal transplant. (A-B) Volcano plots depicting all the proteins significantly different between NMP and CS at T1RP for the jejunum and ileum reveal inflammation and apoptosis signals. Significant proteins are marked in red and had a BH corrected q < 0.05 for a t-test. Proteins with p-values < 0.05, but that did not pass the BH FDR are shown in orange. (C-F) Dot plots of the abundance of inflammation markers calprotectin (S-100A8/A9) and lipocalin-2 (LCN2) in the jejunum and ileum. Lines represent the media and letters that do not overlap represent significant differences by ANOVA multiple comparison test followed by Tukey ($p < 0.05$), (n=2-8 pigs).

Pro- and anti-inflammatory gene signatures are modified in NMP-stored allografts following reperfusion and transplantation

Given the preservation of general T cell populations seen on flow cytometry in NMP stored intestine, we next sought to further identify functional capacity of those T cells through gene expression. While there is a paucity of studies that broadly evaluate inflammatory pathways associated with intestinal transplant outcome, the genes selected for analysis in this study were chosen and grouped by those historically associated with pro-inflammatory or anti-inflammatory processes (Figure 5). In our study, a thorough comparison between storage methods was hindered by the high mortality rate in animals transplanted with CS allografts (70%).[12] Therefore, we focused all subsequent analyses on the time course of NMP-stored allografts from T0 to T48 to more fully interrogate the changes that occurred over the progression of storage and following transplantation in these tissues. To assess changes in inflammatory signatures, targeted genomic analysis (nanoString nCounter) was applied to RNA extracted from intestinal epithelial mucosal scrapings from three NMP-stored allografts who survived until the study endpoint (T48). Differential gene expression patterns were identified for each section of the intestine and each timepoint with a notable increase in most gene biomarker expression following 1 hour of reperfusion. This decreased by 48 hours post-transplant (Figure 5A and B). Interestingly, despite an apparent increase in gene expression following reperfusion in the ileum, few genes reached statistical significance (Figure 5B, D, F, H). In the jejunum, however, multiple genes classified as pro- and anti-inflammatory were statistically increased (Figure 5C, E, G). In our study, *IL6* was the only gene found to be elevated following NMP-storage that remained increased at all timepoints in the jejunum. Despite the historical categorization of IL-6 as a pro-inflammatory cytokine, multiple studies have demonstrated its role in upregulating the intestine's regenerative potential as well as inhibiting acute inflammatory responses via prevention of ischemia reperfusion injury following intestinal transplantation.[62,63] This is particularly relevant in light of our previous work[12] that identified increased epithelial cellular proliferation in immunohistochemical analyses of tissues and crypt cultures derived from 6-hour NMP-stored intestine as well as at T1RP. We also found the expression of *IL2*, *IL5*, and *IL21* significantly increased in jejunum and, although they have been traditionally classified as pro-inflammatory cytokines, all have been shown to suppress intestinal inflammation,[64] with IL-2 and IL-5 administered therapeutically to prevent allograft rejection.[65,66]

Known key mediators of rejection in solid organ transplantation include granzymes (GZM) and perforin (PRF).[67] GZMB and PRF have been suggested as diagnostic markers in acute rejection of intestinal allografts[68] while graft-versus-host disease (GVHD) in hematopoietic stem cell transplant is promoted by GZMA-producing T-helper cells.[69] Importantly, none of these genes were significantly increased at any time point in NMP-stored intestine and, furthermore, *GZMA* expression was downregulated at T48 in both the jejunum and ileum, although statistical significance was not reached (Figure 5G).

In the cohort of analyzed genes considered pro-inflammatory and associated with either intestinal transplant rejection or GVHD[70], *TNF, CD69, IFNG, IL21* and *IL17* expression all increased following reperfusion (Figure 5E). However, all except *TNF* exhibited a downward trend in expression by 48 hours of transplantation, suggesting a reduction in inflammation (Figure 5G). The research is limited;[71,72] however, clinical studies have shown that administration of anti-TNFα therapy (infliximab) abrogated acute rejection in intestinal transplant patients unresponsive to other immunomodulatory therapies .[73,74] Furthermore, the persistence of TNFα/IL17 double positive T cells in grafts of patients experiencing intestinal rejection resistant to traditional immunomodulatory therapy has been identified,[73] however, IL-17 blockade has also been associated with increased inflammation. This is likely due to its dual role in maintaining mucosal homeostasis and tolerance to commensal bacteria through key pathways including *IL22* and *IL10* which were both found to be significantly elevated in our analyses (Figure 5E).[75,76]

Levels of the remaining cytokines - *IL12*, *TBET*, and *TGFB* - were found to be significantly elevated at T1RP but all decreased to the T0 baseline by T48 (Figure 5G). While *IL12* and *TBET* have been generally associated with pro-inflammatory pathways[77] and intestinal transplant rejection,[78] respectively, it is known that manipulation of the bowel during procurement is an inevitable source of cellular inflammation that may be potentiated during reperfusion[79] and could be the source of the varied results at T48, with some pro-inflammatory mediators remaining elevated and other returning to baseline. The multi-faceted actions of TGFβ have been shown in the intestine to have numerous protective and anti-inflammatory effects, including suppressing inflammatory macrophage cytokines[80] as well as upregulating homeostatic innate lymphoid cell (ILC) populations of both the regulatory and group 3 (ILC3) subtypes.[81] Perhaps most interestingly, TGFβ has also been shown to prevent differentiation of naïve T cells into the rejection-associated inflammatory Th1[82] and effector T cell phenotypes via inhibition of both IL-

12 and Tbet.[83] The correlated upregulation of some of these cytokines may warrant further investigation into whether some of these very early inflammatory products are being counteracted by tolerogenic cytokines like TGFβ. Further still, TGFβ has been implicated in promoting the regulatory T cell (Treg) phenotype[84,85]—crucial for mitigating inflammation[86] and preventing rejection and GVHD[87,88] via FOXP3 upregulation, and, along with IL-6 and IL-22, has also been associated with ISC renewal via the STAT3 signaling pathway.[89,90] This, along with our data showing *TGFB*'s coinciding up- and downregulation with the pro-inflammatory markers *IL12* and *TBET*, all suggest a more nuanced role for *TGFB* in the context of intestinal transplantation and offer an interesting avenue for future study.

The expression of multiple genes associated with anti-inflammatory pathways, including *IL13* and *GATA3*, and some specifically related to successful intestinal transplantation, such as *CCR10*,[91] *IL22*,[92,93] *CTLA4*,[94] and *FOXP3*,[88] were all significantly upregulated at T1RP (Figure 5E and 5F) and remained so in the jejunum after 48h of transplant (Figure 5G). Of these, IL-13 is a known mediator in pathways regulating ISC differentiation and epithelial regeneration.[95] Interestingly, GATA3 promotes ILC2 and Th2 differentiation, both of which produce IL-13,[95] further supporting our holistic view of a regenerative intestinal environment. In this vein, the elevation of IL-22 is of particular interest given its known role in promoting intestinal stem cell-mediated epithelial regeneration, both independently and synergistically with other cytokines like IL-6.[96,97] Similarly, *CCR10* is associated with ILC3s[98] critical to barrier integrity, immune regulation, and ISC renewal.[95] The downregulation of *CCR10* has specifically been associated with rejection and graft failure (+REF).[91,94] In light of these previous works, our data warrant further inquiry as to whether the increased organoid growth and proliferation noted at 6 hours of NMP storage and following reperfusion in our earlier study were attributed to such key cytokine and immune cell signaling pathways.[12] Lastly, both CTLA4 and FOXP3 are known to promote regulatory T cells, reduce inflammatory signaling, and are associated with transplantation success.[99,100] As mentioned previously, FOXP3 induces regulatory T cell differentiation through TGFβ as opposed to RORγt, which promotes Th17 differentiation. Although numerous studies have shown that both cell types can be beneficial for intestinal healing,[101] overabundance of Th17s is considered inflammatory and increases rejection risk, whereas higher levels of Tregs are directly implicated in transplantation success. Furthermore, Zhou et al. found that TGFβ-induced FOXP3 can prevent excessive Th17 differentiation but is contingent upon a balance between FOXP3 and

RORγt signaling.[102] Our data show significant upregulation of the Treg-associated transcription factors *FOXP3* and *CTLA4* at both T1RP and T48, whereas the Th17-associated *RORγt* never reached significant upregulation at any measured timepoint. In light of the preservation of $CD3^+$ T cells shown by our flow cytometry data, these gene expression findings altogether suggest an overall functional anti-inflammatory trend of regulatory T cells in the NMP-stored intestinal environment.

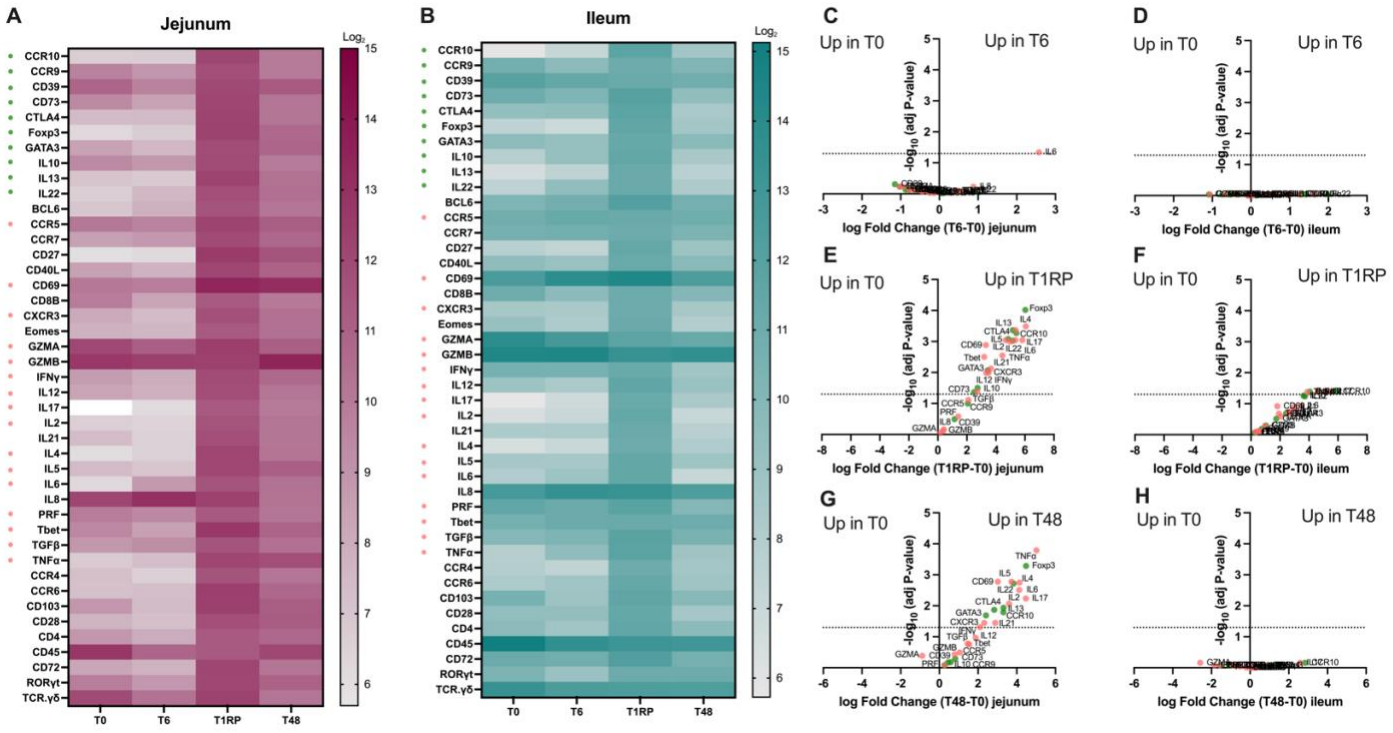

Figure 5. NanoString genomic analysis performed on NMP-stored intestine over time (n = 3 pigs). The expression (log_2 of nCounter® values) of the 43 genes corresponding to immune targets in the codeset is shown in heatmaps by intestinal segment (A: jejunum; B: ileum). Significant differences with the baseline (T0) in transplant-relevant genes are represented in volcano plots by time point for jejunum (C, E, G) and ileum (D, F, H). Red and green dots represent genes historically associated with pro-inflammatory or anti-inflammatory processes, respectively (see text for interpretations).

Proteomics reveals critical changes in apoptotic signaling across time and between intestinal segments in NMP-stored allografts (Figure 6)

We initially analyzed the overall profile of the proteome of the NMP samples using principal component analysis (Figure 6A). We found that overall and at T0 the principal component representing the largest amount of the variance was separated semi-randomly across samples, while the second largest variance was due to segmental differences between the ileum and jejunum across all time points (Figure 6A). However, when each timepoint was analyzed separately, the greatest source of variance was found between jejunum versus ileum and this difference increased between T6 and T48, suggesting an increasingly distinct jejunum and ileum proteome at T1RP and T48 relative to T0 and T6.

Over the included time points, we identified 32 proteins different across time in the jejunum (Figure 6C) (ANOVA; BH $q < 0.05$). 13 of these proteins were significantly increased at T48 relative to all the other time points. This included HYOU1 (UniProt ID F1SAI8),[103] Regucalcin (RGN; UniProt ID F1RWY0),[104] Galectin (LGALS3; UniProt ID A0A5G2QFK8), Sphingosine-1-phosphate lyase (SGPL1; UniProt ID A0A287BEZ7),[105] FLVCR heme transporter 1 (FLVCR1 ; UniProt ID F1S2X6),[106] which are all anti-apoptosis proteins. Two serpins were also among the 13 proteins. Serpins are serine protease inhibitors, which block the delivery of apoptotic signals.[107] DEDD and the three fibrinogens were also significantly more abundant in T1RP and T48 than in T6 and T0 in addition to angiotensinogen and another serpin containing protein. Death effector protein (DEDD; F1S193) is an apoptosis signaling protein,[108] while fibrinogens (Fibrinogen gamma (FGG; UniProt ID A0A5G2QUU1) and alpha (FGA; UniProt ID F1RX36) act as anti-apoptosis factors.[109] Four proteins were increased in T6, T1RP, and T48 relative to T0: two Globin domain containing proteins and alpha-2-macroglobulin, which were also increased in these time points in the ileum, and an Ig-like domain containing protein.

In the ileum, we identified 23 significantly different proteins with some redundancy with those expressed in the jejunum (Figure 6B) (ANOVA; BH $q < 0.05$). Among these proteins BROX (UniProt ID A0A287AAM4), angiotensinogen (AGT; UniProt ID A0A287AJ51), and serpin domain-containing protein (LOC100153899; UniProt ID A0A287AT48) were each significantly increased in abundance at T48 relative to the other time points (Tukey HSD; $q < 0.05$). BROX is highly enriched in an immortalized cancer cell line, indicating that it may be a proliferation gene.[110]

Similar to that found in the jejunum DEDD, FGG and FGA were increased in abundance at T1RP and T48 relative to T0 and T6. An additional 8 proteins (another fibrinogen, serpin and protease inhibitor) were also elevated in T6, T1RP, and T48 relative to T0. No proteins were identified that were uniquely more abundant at T0 over all the other time points. Together, these results indicate modulation of pathways associated with apoptosis signaling at T1RP and T48 in both the jejunum and the ileum in the NMP samples.

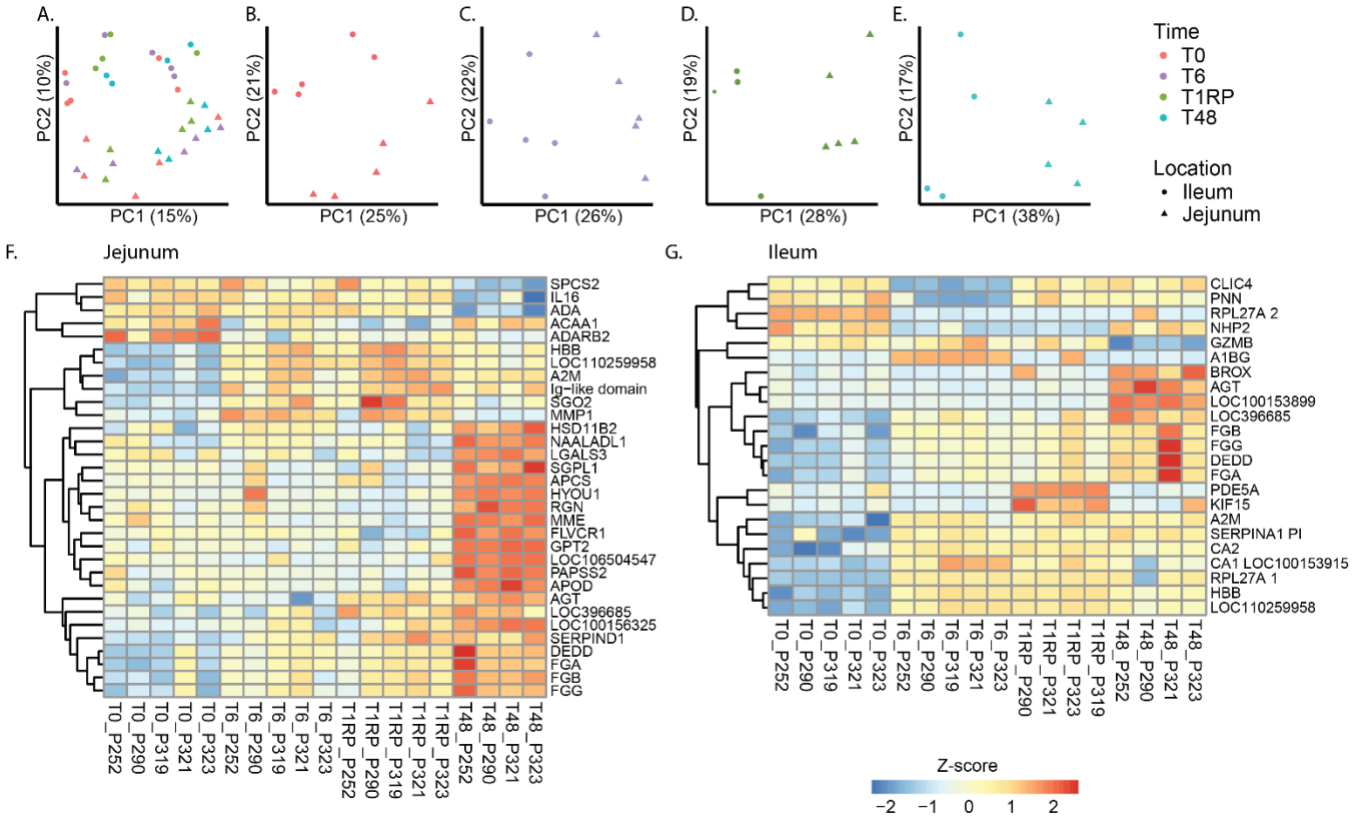

Figure 6. Proteomic analyses of NMP-stored jejunal and ileal mucosal scrapings over time (n= 4--5 pigs). (A-E) Principal component analysis of NMP mucosal scraping overall, and at each time point. (F) A heatmap of the z-scored abundance of the 32 proteins significantly different in abundance across time in NMP jejunum mucosal scrapings (ANOVA, BH q < 0.05). (G) A heatmap of the z-scored abundance of the 23 protein significantly different in abundance across time in NMP ileum mucosal scrapings (ANOVA, BH q < 0.05). Heatmaps were rendered in R using pheatmap. Clustering by row was done using the Ward D2 method.

NMP reduces intestinal epithelial apoptosis over the course of storage

Arising from our proteomic analyses were multiple proteins elevated in NMP-stored intestine associated with modulation of pathways associated with cell death. The caspase pathway is a single, but critical, mechanism by which cells undergo controlled death. We therefore

evaluated changes in cells undergoing apoptosis over time in tissues that underwent NMP-storage. Consistent with gene expression and proteomics analyses, there was progressive reduction in the number of cleaved caspase 3 (CC3) expressing cells reaching a statistically significant reduction at 48 hours following transplantation in both the jejunum and the ileum (Figure 7; Jejunum T0 3.033 +/- 3, T48 0.2333 +/- 0.4302; Ileum T0 2.433 +/- 2.176, T48 0.5 +/- 0.6823).

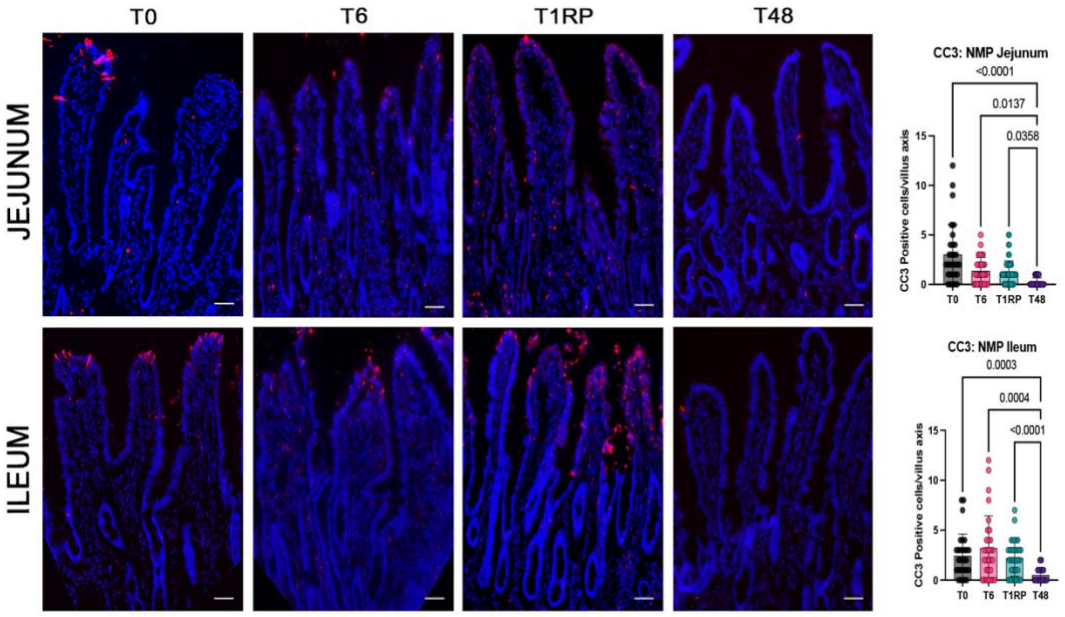

Figure 7. Immunofluorescence assessment of apoptosis in NMP-stored jejunum and ileum. Immunofluorescence of mucosal biopsies was performed for cleaved caspase 3 (CC3) to identify evidence of cell death over the course of NMP storage (n=3 pigs). Throughout storage (T6) and reperfusion (T1RP), there was a progressive reduction in the number of apoptotic cells that was significantly reduced 48 hours following transplantation (T48). Statistical comparisons were made by RM one-way ANOVA followed by Tukey or Friedman followed by Dunn's for multiple comparisons. Only significant differences ($p<0.05$) are shown with the corresponding p-value.

NMP storage preserves the microbial populations of transplanted intestines

The intestinal microbiota of jejunal and ileal sections pre- and post-transplant were analyzed for NMP-storage experiments using mucosal scrapping samples and whole genome shotgun sequencing (Figure 8). While several studies have helped define a 'core' microbiota for swine,[111] this study offers a broader characterization of the largely unknown inhabitants of the pig's intestine. Most bacterial populations identified in jejunum and ileum at the phylum level belonged to Pseudomonadota (formerly Proteobacteria) and Bacillota (formerly Firmicutes). Meanwhile, Ascomycota was the predominant phylum in the fungal gut microbiota, Cossaviricota was the most

abundant phylum of viruses and Apicomplexa was the largest occurring phylum of parasitic protists (Figure 8A and E).

The absence of a distinct microbiota profile in the jejunum and ileum prevented a clear differentiation between these sections based on microbial composition (Figure 8B and F). Furthermore, the alpha diversity (Figure 8C and G) and the beta diversity (Figure 8D and H) of the samples over time showed no significant differences between time points (T0, T6, T48) in either jejunum or ileum. However, our findings did reveal a high degree of interindividual variation across samples and between experimental animals, which is common in gut microbiota studies.[112] The most abundant species sequenced in both jejunum and ileum were aerobic or facultative anaerobic microorganisms. Likely, the exposure to oxygen during surgical and perfusion procedures in the typically anaerobic intestinal environment resulted in the detection of strict anaerobes only at very low abundance.

Abundant species identified in most samples included *Klebsiella pneumoniae* and *Escherichia coli*, which are part of the normal intestinal flora, but are also recognized opportunistic pathogens whose overgrowth in ileal effluents has been linked to rejection in small bowel transplantation.[113] In this study, these two species from the Enterobacteriaceae family were found abundant from T0 in some of the pigs, but they did not succeed in colonizing the intestines by T48 (Figure 8). Other pathogenic bacteria found abundant in the samples included *Staphylococcus aureus, Salmonella enterica*, and *Pseudomonas aeruginosa*. However, little is known about their potentially harmful effects in intestinal transplants. Meanwhile, the commensal bacteria *Enterococcus faecalis* has been seen to colonize mice models of allogeneic bone marrow transplantation with acute GVHD.[114] Nevertheless, several strains of enterococci, including *E. faecium* have probiotic potential with particularly interesting effects through the gut-brain axis.[115,116] The fungi *Aspergillus oryzae* found abundant in most samples from ileum and jejunum is commonly regarded as a beneficial mold and even suggested as a probiotic that exerts anti-tumor effects in pancreatic cancer.[117]

Overall, our analysis revealed that the microbial populations present in the jejunum and ileum during NMP storage (T6), and post-transplant (T48) resembled those found pre-transplant (T0). As small bowel graft rejection is associated with significant changes in the intestinal microbiota,[113] this suggests that NMP storage effectively preserved the donor intestine's microbiome throughout the storage period and after transplantation.

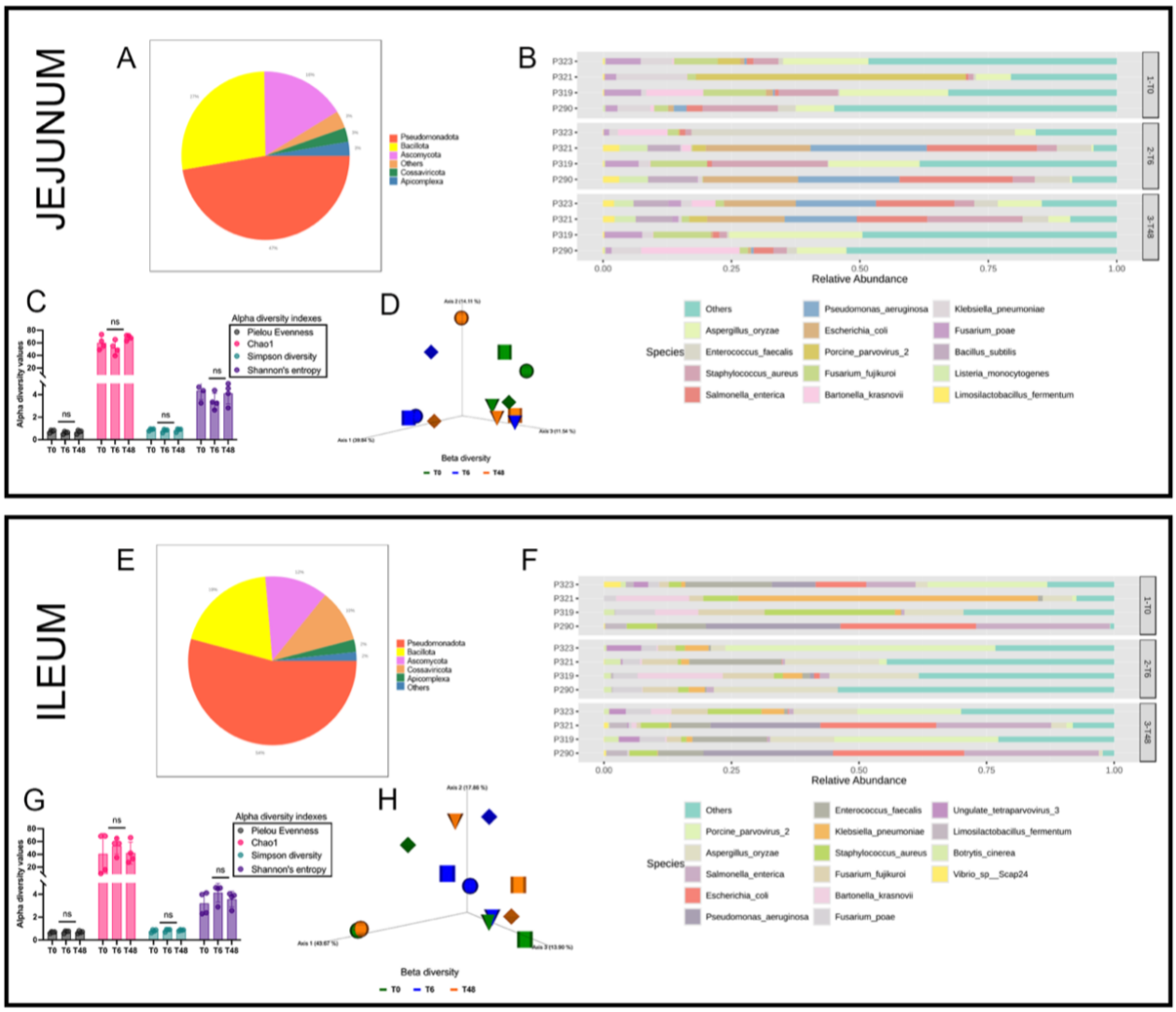

Figure 8. Whole genome shotgun sequencing analysis over time for NMP-stored jejunal (top) and ileal (bottom) mucosal scrapings (n=4 pigs). (A, E) Taxonomic analysis of the intestinal sections at the phyla level. (B, F) Classification of each sample at the species level. (C,G) Alpha diversity indexes (Pielou Evenness, Chao1 richness, Simpson diversity, and Shannon's entropy), ns: no significant differences ($p<0.05$) in RM one-way ANOVA or Friedman (depending on data normality). (D, H) Emperor representation of the beta diversity as measured by Bray-Curtis dissimilarity. Colors represent time points T0 (green), T6 (blue), T48 (orange); while shapes represent the different animals. PERMANOVA was used for comparisons and significance was not reached.

DISCUSSION

The findings presented here reflect early efforts to profile the host inflammatory response to different methods of storage during intestinal transplantation. High rates of rejection, mediated by the immune system, continue to haunt the field of intestinal transplantation.[11,12] In this work, we utilized a porcine model to examine the immunological effects of NMP compared to CS, hypothesizing that NMP mitigates inflammation-associated allograft injury thus improving organ preservation. We report flow cytometric, genomic, and proteomic analyses to profile allograft response over time. Compared to previous studies which used smaller or isolated segments of intestine without blood-based perfusion and limited profiling tools, this work is unique in its inclusion of extensive profiling tools and the use of a highly translatable large animal model with prolonged durations of storage and ultimate transplantation.[118,119] These efforts are an extension of our previous work that demonstrated a superiority of NMP to CS in preserving the intestinal epithelial allograft regenerative potential and viability.[120]

The evaluation of specific lymphocyte populations with flow cytometry showed that NMP preserved overall $CD3^+$ T cell populations in the jejunum and ileum more effectively than CS. Rejection is typically considered T-cell mediated; more specifically, $CD8^+$ cytotoxic T cells are responsible for that process.[5,121] Yet, there was no significant difference in $CD8^+$ populations between NMP and CS. The γδ subpopulation of intraepithelial T cells were reduced in the ileum following NMP. However, the role of these populations in IT is unclear.[24] Perhaps the most relevant findings for the potential to reduce allograft rejection was the reduction of natural killer cells in NMP-stored allografts.[17] Overall, these findings imply that NMP may be able to reduce lymphocytes associated with inflammation and rejection which has the potential to contribute to improved allograft health and transplantation outcome.

Proteomic analysis provided further functional insight into the impacts of the different storage approaches. The jejunum showed its greatest differential protein expression at T1RP with the proteins that were most highly expressed in NMP, compared to CS, contributing to improved barrier function and epithelial tight junction integrity as well as DNA repair and cellular replication.[49,50] [122] In our analyses, the use of mucosal scrapings provided a general overview of proteomic changes occurring in the tissues, however this approach prevented more specific localization of protein origin. In the limited proteomic studies done to date, measurements of fecal

calprotectin is used to identify rejecting allografts [59]. In assessing this protein here, across both sections of intestine, the increase in calprotectin in CS compared to NMP suggests reduced viability in those tissues. In future studies, proteomic analysis of specific cell types, with a special interest in epithelium compared to lymphocytes, may provide greater insight into localized functional trends within the tissues.

In our focused approach to evaluate mucosal changes in tissues undergoing NMP-storage, genomic profiling provided insight into the potential implications of the shifting cell populations initially identified in flow cytometry. Unsurprisingly, several of the most highly expressed genes in NMP-stored jejunum related to lymphocyte activity included those with both pro- and anti-inflammatory activity. [24,123,124] Many of the genes thought to modulate inflammation associated with intestinal allograft rejection reduced in expression by 48 hours suggesting that this waning of inflammation may be a potential benefit of NMP as the intestine proceeds through storage and transplantation. This notion is further supported by the maintained expression of cytokines such as IL-4, among others, that are thought to contribute to immunotolerance [125]. Further proteomic analysis of NMP-stored intestine provided additional insight into mechanisms by which NMP may be protective, with the increase anti-apoptotic signatures in both intestinal segments. This benefit appears to be localized, in part, to the epithelium thereby contributing to the maintenance of intestinal barrier function. As is necessary to elucidate cellular dynamics of the pathways identified, future efforts will profile the changes in gene and protein expression with more prolonged recovery times to determine if NMP storage reduces the onset of allograft rejection.

Of potentially more immediate clinical implication, proteomic analysis demonstrated that the jejunum and ileum do not identically respond to storage and transplantation. As such, these sections are not of equal utility for evaluating the allograft following transplantation. From our results, it appears that the jejunum is preferrable for earlier monitoring (< 48 hours) whereas the ileum begins to manifest evidence of inflammation after this timepoint. Current rejection monitoring typically employs ileal biopsies, for all timepoints, given the accessibility via ileostomies created during surgery.[10] These findings indicate that jejunal biopsy should instead be considered in the early postoperative period.

Finally, maintenance and stability within the microbiome is critical to intestinal homeostasis. NMP-storage appeared to preserve the donor intestine's microbiome throughout storage and following transplantation which may contribute to reduced immunogenicity of an allograft.

Preliminary conclusions from this work demonstrate that NMP may reduce inflammatory lymphocyte populations and their cytokines over time, upregulate markers known to target intestinal epithelial stem cell proliferation, and support improved barrier function. Because of the improved survival following NMP, most of the available data at the 48-hour study endpoint derived from NMP-stored allografts. While comparisons with CS would be interesting, the demonstrated inferiority of this method suggests that additional efforts and time are better directed at understanding and improving allograft response to NMP. With continued efforts, we hope to further elucidate the value of NMP in improving intestinal transplantation in extended recovery timepoints to determine if the changes identified are protective against rejection.

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CHAPTER 6

Dissertation Summary and Future Directions

This evaluation of novel biomimetics demonstrates that significant intestinal epithelial healing can be achieved through extrapolations from inherent physiological entities like the placenta and normothermic perfusion. The need for novel therapeutics to increase current treatment options for severe intestinal injury is emphasized by the stagnant survival rates in the diseases of interest discussed here: Necrotizing Enterocolitis, colic, and intestinal allograft failure. The lack of ongoing research into targeted treatment agents, as discussed in Chapter 1, also stresses the necessity of these efforts. Work thus far indicates that all these approaches upregulate intestinal epithelial proliferation and the intestinal stem cell niche as an element of their induced recovery. Evaluations of NMP storage demonstrate that this device may also reduce inflammation associated with injury. The addition of inflammatory analyses to the evaluations of HPE and ePE will help complete our understanding of their utility in their respective intestinal diseases as well.

Future research will explore which elements within these biological entities may be the biggest contributor to the observed beneficial effects. As often as appropriate, highly translatable large animal models, as discussed in Chapter 2, will be employed to best represent intestinal epithelial healing. For the placental extracts in Chapters 3 and 4, this will involve proteomic screening and analysis. Further evaluations for the NMP device, as introduced in Chapter 5, will include completion of ongoing analyses regarding allograft inflammation and the addition of proteomic analysis of separate lymphocyte and epithelial populations. Spatial technologies, such as RNAscope, will also be explored to reveal where signaling changes are occurring in relation to tissue injury. Lastly, in a combinatorial effort, plans to evaluate novel therapeutics, such as HPE, ePE, and EVs, in advanced ex vivo settings, like the NMP device, where injury and recovery can be manipulated, will expand opportunities for further development of these novel therapeutics.