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Hyaluronan 35 kDa enhances epithelial barrier function and protects against the development of murine necrotizing enterocolitis

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Abstract

Background—Disruption of tight junctions (TJ) predisposes to bacterial translocation, intestinal inflammation, and necrotizing enterocolitis (NEC). Previously studies showed that hyaluronan (HA), a glycosaminoglycan in human milk, maintains intestinal permeability, enhances intestinal immunity, and reduces intestinal infections. In this study, we investigated the effects of HA 35 kDa, on a NEC-like murine model.

Methods—Pups were divided into Sham, NEC, NEC + HA 35, and HA 35. Severity of intestinal injury was compared using a modified macroscopic gut scoring and histologic injury grading. The effect of HA 35 on intestinal permeability was determined by measuring FITC dextran and bacterial translocation. RNA and protein expression of TJ proteins (claudin 2, 3, 4, occludin, and ZO-1) were compared between the groups.

Results—Pups in the NEC + HA 35 group had increased survival and lower intestinal injury compared to untreated NEC. In addition, HA 35 reduced intestinal permeability, bacterial translocation, and proinflammatory cytokine release. Ileal expression of claudins 2, 3, 4, occludin and ZO-1 was upregulated in NEC + HA 35 and HA 35 compared to untreated NEC and shams.

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Author Contributions:

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Conclusion—These findings suggest that HA 35 protects against NEC partly by upregulating intestinal TJs and enhancing intestinal barrier function.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease that primarily affects premature infants of less than 32 weeks gestation. The incidence of NEC is between 7 to 12% and is gradually increasing in frequency, due in part to the increasing number of premature infants surviving each year [1]. Although the pathogenesis of NEC is still unclear, evidence suggest that prematurity, altered intestinal microbiome, and intestinal barrier dysfunction are major contributors to the disease. Disruption of intestinal barrier has been shown to be an important factor in the development of intestinal inflammation [2, 3], and in combination with an altered intestinal microbiome, can lead to translocation of bacteria and bacterial products, increased inflammation, intestinal necrosis, and in severe cases sepsis and death [4]. Thus, identifying strategies or interventions that protect intestinal integrity and modulate inflammation is critical in preventing NEC.

Evidence suggest that exclusive human milk (HM) feeding decreases the risk of NEC in premature infants [5, 6]. Several protective factors are present in HM including lactoferrin, oligosaccharides, and growth factors [7]. HM also contains glycosaminoglycans (GAGs) at 5–10 times the concentration of bovine milk [8]. Hyaluronan (HA), a GAG composed of repeating disaccharides of β- D-glucuronic acid and N-acetyl-β-D-glucosamine, is present in HM with the highest concentration during the first months after birth [9]. HA is produced at cell surfaces by hyaluronan synthases (HAS1, HAS2 and HAS3) generally as high molecular weight HA and can be degraded in response to injury [10]. The degraded fragments can either have pro- inflammatory or anti-inflammatory effects depending on the tissue environment or the size and molecular structure of the HA fragments [11].

Recently, HA from HM or purified HA of average molecular weight 35 kDa (HA 35) was shown to prevent intestinal bacterial infection and reduce intestinal inflammation in various mouse models mainly through maintaining the intestinal barrier defenses and function [9–13]. Since both factors are involved in the pathogenesis of NEC, we sought to determine the effect of HA 35 on survival, intestinal permeability, and histological injury in a NEC-like model. We then determined the effect of HA 35 on tight junction protein expression in ileal tissue. Finally, the effect of HA on proinflammatory cytokine release and bacteremia was compared between the groups.

MATERIALS AND METHODS:

All animal experiments (Protocol #04203AR) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Oklahoma Health Sciences Center (Protocol#101502–16-024), and performed according to recommendations in the Guide for the Care and Use of Laboratory Animals [14].

Experimental design and animal model of NEC

Pups were randomized to one of the following groups: 1) Sham, 2) NEC, 3) NEC + HA, and 4) HA alone. Sodium hyaluronate (Lifecore Biomedical, LLC, Chaska, MN) with a molecular weight of ~35,000 kDa (HA 35) was given to pups in the NEC+ HA and HA group by gavage at a concentration of 15mg/kg or 30mg/kg body weight once daily for three days prior to induction of NEC and one hour prior to bacterial administration (Figure 1). The doses and intervals were based on previous studies done in large intestinal inflammation models [9, 11].

NEC was induced using the Paneth cell disruption and *Klebsiella* infection NEC-like model [15, 16]. Crl:CD1(ICR) pups (Charles River) at P14 to P16 days of age received an intraperitoneal injection of dithizone (33 mg/kg) diluted in ethanol/ammonium hydroxide, followed by gavage administration of 1×10^8 CFU *Klebsiella pneumoniae* /kg (ATCC#10031, Manassas, VA). Pups were monitored for 10 hours after gavage for clinical illness and survival [17]. At the end of the experiment, surviving pups were euthanized, and blood and tissues were harvested for further analysis.

Macroscopic and microscopic evaluation of intestinal injury: Small intestines of pups were removed and visually inspected for macroscopic signs of NEC. Images were obtained using Amscope MU1803 microscope and digital camera (Irving, CA). Severity of gut injury was graded by a blinded investigator using a modified macroscopic gut scoring [17] with a 3-point scoring system based on gut color and dilatation as follows: 0: no discoloration or dilatation; 1: patchy discoloration; 2: extensive discoloration and gut dilatation. For histological injury scoring, the terminal ileum was removed and fixed in 10% formalin buffer, then paraffin-embedded and stained with hematoxylin and eosin for microscopic examination. Severity of intestinal injury was assessed based on a five point scoring system previously developed by Jilling et al [18]; Grade 0: intact; Grade 1: distal epithelial sloughing; Grade 2: mid villus sloughing; Grade 3: complete villus necrosis with preservation of the crypts; and Grade 4: transmural necrosis. Scores were based on the highest score observed on three to five sections in a specimen. A score of 2 or above was defined as NEC.

In Vivo Intestinal Permeability: To assess the effect of HA 35 on intestinal permeability *in vivo*, fluorescein isothiocyanate (FITC) labeled dextran (molecular weight 4,000) (Sigma-Aldrich Inc. FD4, St. Louis, MO) was used as previously described [19]. Mice pups from each group received 44mg/100gm body weight FD4, suspended in sterile PBS by orogastric gavage. Four hours later, pups were euthanized and serum levels of FD4 were measured by spectrophotofluorometry (Tecan, Maennedorf, Switzerland) at an excitation wavelength of 480 nm and emission wavelength of 520 nm after standard concentration curves were established.

Inflammatory cytokine quantification at plasma and intestine

Proinflammatory cytokines/chemokines **(**IL-1β, TNF-α, IL-6, IL-12 p70, IFN-γ, CXCL) and anti-inflammatory (IL-10) cytokines were measured in plasma and intestine using ProcartaPlex Mouse Cytokine & Chemokine Panel (bioscience, San Diego, CA, USA) based

on Luminex technology as per manufacturer's instructions. Small intestinal samples were homogenized using a bead beater (Next Advance INC, Troy, NY) in a buffer containing Calbiochem phosphatase and protease inhibitors (524625 and 535140, Millipore, Burlington, MA) and PMSF (93482, Sigma-Aldrich, St. Louis, MO). Samples were run on a BioPlex 200 (Bio-Rad, Hercules, CA) and results were calculated based on a 7-point standard curve for each analyte. Final cytokine levels were normalized to total protein concentration (mg/ml) and reported as picogram/ml for plasma and picrogram/mg for tissue levels.

Immunohistochemistry (IHC): IHC was performed according to manufacturer's protocol using Leica Bond-IIITM Polymer Refine Detection system (DS 9800). Polyclonal antibodies claudin 2, −3, −4 and Z0–1 (Catalog #36–4800 and PA5–28858, ThermoFisher, Waltham, MA) and occludin (Catalog #40–4700, Invitrogen, Carlsbad, and CA) were used. FFPE tissues were sectioned at desired thickness (4–8μm) and mounted on positively charged slides. The slides were dried overnight at room temperature and incubated at 60°C for 45 minutes followed by deparaffinization and rehydration in an automated multistainer (Leica ST5020). Subsequently, these slides were transferred to the Leica Bond-III™, treated for target retrieval at 100°C for 20 minutes in a retrieval solution, either at pH 6.0 or pH 9.0. Endogenous peroxidase was blocked using peroxidase-blocking reagent, followed by the selected primary antibody incubation for 60 minutes diluted at (Occludin-1:200, ZO-1, 1:100, Claudins 1:100). For the secondary antibody, post-primary IgG-linker and/or Poly-HRP IgG reagents was used. Detection was done using 3, 3′-diaminobenzidine tetra hydrochloride (DAB), as chromogen and counterstained with hematoxylin. Completed slides were dehydrated (Leica ST5020), and mounted (Leica MM24). Antibody specific positive control and negative control (omission of primary antibody) were parallel stained. IHC slides stained for the specific tight junction (TJ) protein were read by a blinded pathologist.

Real Time RNA PCR: RNA was isolated from the terminal ileum tissue using RNeasy Plus Mini Kit (74134, Qiagen, Germantown, MD) per manufacturer's instructions and reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (4368814, Applied Bio systems, Foster City, CA). Samples were run in triplicate on ZO-1 (Hs01551861_m1), occludin (Hs00170162_m1), claudin-4 (Hs00976831_s1), claudin-3 (Hs00265816_s1) and claudin-2 (Hs00252666_s1), and values were normalized to ACTB (Hs01060665_g1) using a ΔΔCt analysis of the TaqMan assays and reported as fold-change from sham.

Statistics: All data were analyzed and graphs created using Graph Pad Prism software version 6.00 for Windows (LA Jolla, California USA www.graphpad.com). Survival curves to assess mortality were obtained using the Kaplan Meier Survival Analysis. Data obtained from each group are presented as mean ± standard error of mean (S.E.M.) and analyzed using either unpaired t- test or one-way analysis of variance (ANOVA) with Turkey multi-comparison test, as appropriate. *P*-value of < 0.05 was considered significant.

RESULTS

Oral HA 35 reduces mortality and severity of NEC like intestinal injury

To determine if HA 35 protects against experimental NEC, mice pups were given HA 35 (15mg/kg or 30mg/kg), once daily, for three days prior to induction of NEC. NEC was induced using the Paneth cell ablation and *Klebsiella* Infection model as described above [15]. Pups were then monitored for a total of 10 hours for survival and signs of distress. Pups in the NEC + HA 35 had increased survival in a concentration dependent manner (Fig 2A) with a survival of 90% in the NEC + HA 35 at 30 mg/kg compared to 70% in the NEC + HA 35 at 15 mg/kg, and 50 % in untreated NEC with a p value 0.04 and 0.05 respectively.

Based on the mortality data, we next assessed the effect of HA 35 at only the 30 mg/kg dose on the incidence and severity of intestinal injury in the NEC model. Compared to the small intestines of pups in the sham group, the NEC group exhibited signs of inflammation, discoloration, and distension (Fig. 3). HA 35 resulted in improved overall gross appearance with reduced signs of dilation and discoloration (Fig-3 A) with a mean ± S.E.M. score of 2.125 ± 0.31 compared to 3.25 ± 1 in untreated NEC (p <0.01). (Fig-3C). H&E examination of the ileal section in the sham group showed normal, healthy villi and submucosal structure. In contrast, sections from the NEC group showed signs of moderate to severe injury with separation of the submucosa and in certain cases transmural necrosis (Fig.3 B). Notably, HA 35 administration in NEC was associated with attenuated histological injury (Fig.3 D) with a mean ± S.E.M. scoring of 1.2 ± 0.25 compared to 2.3 ± 0.15 in the NEC group (p <0.01).

HA 35 protects intestinal barrier function in NEC model

Disruption of the intestinal epithelial barrier predisposes to bacterial translocation, intestinal inflammation, and NEC [3, 20, 21]. Previous studies have shown that HA 35 maintains intestinal integrity and decreases intestinal permeability *in vivo* in various murine colitis model [12]. To determine the effect of HA 35 on intestinal permeability in NEC, serum levels of FITC-dextran were measured in all surviving pups 4 hours after administration of FD4. As expected, pups in the NEC group had elevated serum levels of FITC compared to shams with a mean ± S.E.M 5,274 ± 389 ng/ml versus 1,730 ± 250 ng/ml respectively p<0.0001, indicating an impaired barrier function in the NEC group (Fig. 2B). HA 35 treatment in NEC significantly decreased FITC levels in serum in a concentration-dependent manner with a mean ± S.E.M. of 1,554 ± 250 ng/ml in the NEC + HA 35 at 30 mg/kg vs 2,456 ± 212 ng/ml in the NEC+ HA 35 at 15mg/kg (p<0.000). Based on these results, we next determined the effect of HA 35 on bacteremia associated with the NEC model. In a separate experiment, blood culture was collected by intracardiac puncture an hour after oral *Klebsiella* administration. HA 35 treatment was associated with two-fold reduction in bacterial CFU/ml in the blood compared to untreated NEC, with a mean ± S.E.M. of 5,426 ± 378 CFU/ml compared to 12,722 ± 1519 CFU/ml in the untreated NEC group (Fig. 2C; p<0.0001). Live bacteria were undetected by culture methods in liver and spleens of pups from all groups (data not shown). Altogether, these results indicate that HA 35 administration in NEC leads to reduced intestinal permeability and bacteremia.

HA 35 decreases levels of NEC-induced inflammatory cytokines levels in pups with NEC

Cytokine/ chemokine dysregulation plays an important role in the pathogenesis of NEC as demonstrated in human and animal models of NEC where increased expression of inflammatory mediators such as tumor necrosis factor (TNF), interleukin (IL)-Iβ, IL-6, and IL-8 and others were seen in both intestinal tissues and plasma. Studies also suggest that cytokines released in NEC could further damage the epithelial barrier. We therefore determined the effect of HA 35 on pro-inflammatory and anti-inflammatory cytokine expression in plasma and intestines of all the groups. Pups treated with HA 35 at both doses, 15mg/kg and 30mg/kg, had lower plasma levels of IFN-γ (15mg/kg, not significant; 30mg/kg, p=0.0149), TNF- α (p= 0.0483, p=0.0018), Gro-α (p=0.0003, p<0.0001), IL-12ρ70 (p =0.0003), and IL-6, (p<0.0001) as compared to pups in the untreated NEC group respectively (Fig. 4). Cytokine expression in the intestine for TNF- α, IFN-γ, IL-6, CXCL, and IL-1β showed a lower trend for the HA treated groups but did not achieve statistical significance. No significant differences in anti-inflammatory cytokine expression (IL-10) was noted between HA-treated NEC and untreated NEC, both in the plasma and intestines.

HA 35 increases tight junction expression in NEC

Intestinal epithelial integrity is maintained by tight junctions (TJs) which play a major role in regulating intestinal permeability. Alterations in expression or localization of TJ proteins occurs in intestinal inflammation models such as inflammatory bowel diseases and NEC [3, 22]. Importantly, HA 35 administration maintained intestinal permeability and increased ZO-1 expression in both healthy and DSS-treated wild type mice [12]. We therefore sought to determine the effect of HA 35 on localization, intensity and expression of Claudins −2, −3, −4, occludin, and ZO-1. We found that in shams claudin −2 and ZO-1(Fig 5) staining was mainly located in the cytoplasm and apical region, while occludin, claudin −3 and −4 were localized in the cytoplasm and lateral walls (Fig. 5). Similar to other studies, staining intensity of occludin was noted to be decreased in the NEC group compared to shams, while claudin −2 staining, a pore-forming tight junction, was increased in NEC. Intensity staining and localization of ZO-1 was not affected in NEC compared to sham. Importantly, HA 35 treated pups had increased staining intensity for all tight junction proteins in the healthy and NEC challenged pups. Moreover, occludin and claudin −3 localization in HA treated groups were similar to shams, concentrated mainly in the cytoplasm and lateral cytoplasmic membrane at basal crypt regions. Claudin −4 staining was also increased in the HA treated pups and localized mainly at the tips of the villi in the lateral cytoplasmic membranous region. RNA expression by qPCR showed significantly up-regulated expression of all the tight junctions in the HA 35 treated in healthy and NEC+ HA 35 group compared to untreated NEC and shams. On the other hand, expression of claudin −2 and 3 was downregulated in the NEC group.

DISCUSSION

Altogether our study shows that oral HA 35 increases survival, reduces incidence and severity of intestinal injury in Paneth cell ablation and *Klebsiella* infection NEC model. HA 35 also reduced intestinal permeability, bacteremia, and systemic inflammatory response in

the NEC model. While the pathogenesis of NEC remains unclear, altered intestinal microbiome, immature intestinal defenses and barrier, and exaggerated inflammatory response play major roles in the disease [20]. Though increased intestinal permeability is a predisposing factor for developing NEC, intestinal inflammation itself can lead to increased gut permeability, resulting in a positive feedback loop. The net effect is increased inflammatory cytokine release, leukocyte infiltration, epithelial necrosis, and bacterial translocation across the lumen [4, 7, 23].

Research efforts have been ongoing to develop nutritional or pharmacological strategies that promote intestinal integrity and modulate intestinal inflammation in preterm infants. HM has been proven to be safe and protective in human studies [5]. Compared to formula, HM decreases intestinal permeability and enhances the underdeveloped physical barrier, likely due to its bioactive components such as lactoferrin, immunoglobulin, and oligosaccharides (HMOs). HM is also rich in GAGs (HA, chondroitin sulfate, heparin, heparan sulfate) early in lactation suggesting an important role of this component in the neonatal period [24]. We previously showed that chondroitin sulfate in HM protects against intestinal bacterial infection through a reduction in both invasion and translocation [25]. In addition, GAGs have been shown to play an important role in innate intestinal immunity as an antioxidant and by promoting microbial colonization of the intestine [26]. Specifically, HA is synthesized by one of the hyaluronan synthases and has either pro- or anti-inflammatory properties depending on the size and tissue environment. Mice pretreated *intraperitoneally* with HA <500 kDa are resistant to LPS-induced sepsis [27]. HA isolated from HM or commercially available HA 35 enhances intestinal innate immunity, decreases intestinal permeability in colitis models, and attenuates ethanol- induced gut and liver injury partly through maintaining intestinal barrier function [12, 24, 28]. Notably, of all the specific sized HA (HA 4.7kDa, 16kDa, 28kDa, 74kDa), HA 35 was the most potent inducer of TJ proteins and antimicrobial peptides expression in colonic epithelium *in vitro*. Importantly, large molecular weight (HA 2000 kDa) had no effect on TJ protein expression, suggesting that this effect is highly size specific [29]. Similarly, we found that prophylactic HA 35 was protective in NEC model and was associated with two-fold reduction in intestinal permeability as assessed by serum FITC level, and levels of bacteremia and systemic proinflammatory response. Several mechanisms could lead to the protective effect of HA in the NEC model we used in our study, including direct or indirect effects on bacteria such as competitive and direct binding of HA to bacteria, or inhibiting bacterial growth. In a clinical trial, the use of HA and chondroitin sulfate as a combination was superior to standard of care in preventing recurrence of urinary tract infection [30]. *In vitro*, high molecular weight HA (< 750 kDa) was shown to act as a bacteriostatic agent, with no bactericidal effects, mainly against gram positive bacteria [31][32]. Though not addressed in our study, HA 35 could be protective by acting as a bacteriostatic agent and dampening the effect of the *Klebsiella* challenge in the model, thereby reducing the levels of bacteremia, systemic inflammation, and mortality. It is also possible that the protective effects of HA 35 on NEC induced increase permeability and bacteremia is indirectly through reducing intestinal histological injury specifically cell necrosis and apoptosis.

Another potential protective mechanism of HA in NEC could be through its effect on TJs expression and localization. Claudins, occludin, and ZO-1, are complex transmembrane

proteins located at the apical and lateral ends of the cells and play a role in regulating selective intestinal permeability [22]. Increased endocytosis or decreased exocytosis of these proteins from cytoplasm to cytoplasmic membrane, or decreased expression can occur during intestinal inflammation resulting in disassembly of the junction, and translocation of antigens and bacteria [33]. Studies from humans and animal models of NEC confirm the importance of optimal localization and/or expression of these TJs in maintaining intestinal epithelial integrity and prevention of NEC [34]. Similar to others, we showed decreased intensity staining and altered localization of the barrier forming claudin −3, claudin −4, and occludin in the NEC group. Notably, HA 35 therapy was associated with increased staining intensity expression for most of the TJs studied in both heathy pups and pups challenged with NEC. These data support findings by others studies where HA 35 administration enhanced TJ expression in colonic epithelium of healthy and *Citrobacter* infected mice[12]. Similar to studies from human patients with NEC, staining intensity for claudin −2, a pore forming TJ, was higher and more localized within crypts in the NEC group compared to sham [2]. HA 35 prevented the increased localization of claudin- 2 in the NEC animals, however in contrast to the IHC staining data, gene expression for claudin - 2 was higher in the HA treated groups. However, this gene expression increase was not associated with increased intestinal permeability *in vivo*.

Our study has several limitations. First, the effect of HA on the intestinal microbiome was not evaluated. Similar to other glycans, HA is non-digestible and has been shown to prevent bacterial adhesion and inhibit bacterial growth [26, 35–38]. Moreover HA 35 administration was protective in a bacterial infection model by increasing β- defensin expression in intestinal epithelium *in vivo* and *in vitro* [10]. These data raise an intriguing possibility that HA could act as a prebiotic and could potentially alter the intestinal microbial composition of neonatal pups, thereby preventing the development of NEC. Second, our study did not address the mechanism by which HA 35 affect TJ expression in the NEC model. It's well known that HA exert it's effect by interacting with the signaling pathway receptors Toll-like receptor 4 and 2 (TLR4 and TLR2) and CD44 [9]. In an ethanol induced liver injury model, HA 35 given orally restored Toll-interacting protein (Tollip) and decreased TNF-α expression. Tollip is ubiquitously expressed protein that form a complex with the interleukin-1 receptor-associated kinase 1 (IRAK1) and impairs the activation of NF-κB. Tollip also decreases TLR4 and TLR2-mediated inflammation by direct interaction with the receptors [28]. Interestingly, NEC is characterized by an imbalance between TLR4 activation and its negative regulators. Specifically, TOLLIP expression is lower in both immature enterocytes [23] and in NEC models leading to the exaggerated inflammatory response in immature intestines as compared to term infants and adult. Third, only specific size HA 35 was used in our study based on the previous studies [9, 11, 24]. Data on lower molecular or higher molecular weight HA in the NEC model is currently unknown. Moreover, only used 15 and 30 mg/kg dosing was used in the study based on prior studies in larger intestinal models [9, 11, 24]. Further studies are needed to determine if higher doses are more beneficial or determinantal in NEC. Lastly, the Paneth cell disruption and *Klebsiella* infection model used in this study has some limitations. Similar to other murine NEC models, it does not encompass all the risk factors that contribute to NEC. However, it offers the advantage of challenging pups at P14 with the intestinal developmental age similar

to that of infants at highest risk if developing NEC [39]. In addition, it results in an end point of impaired barrier function, intestinal inflammation, bacterial dysbiosis, and histological injury similar to that seen in the classical hypoxia/hypothermia model and that of human infants with NEC [15, 40].

In conclusion, we have demonstrated that HA 35 administration prevents mortality and reduces intestinal permeability in a NEC-like intestinal injury model. This protective effect of HA 35 in the model was at least in part due to enhanced intestinal barrier function and preservation of the TJ expression and localization. These data support the concept that oral HA may be effective as a prophylactic treatment that promotes intestinal barrier function in premature infants and prevents the development of NEC. Further studies are needed to elucidate the mechanism of action, and determine its effects in other NEC models, and on the intestinal microbiome.

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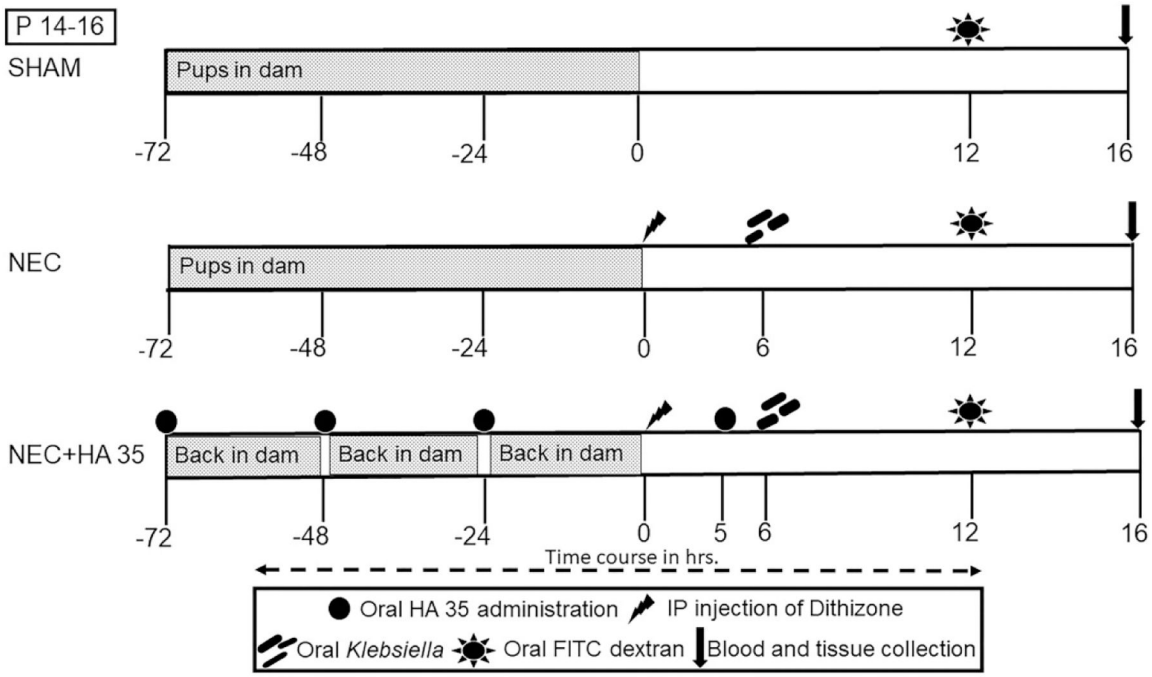

Figure-1: HA 35 administration in Dithizone/*Klebsiella* murine NEC model.

Pups were randomized to one of the following groups: Sham; NEC; NEC + HA, and HA alone. HA 35 was given to pups in the NEC+ HA and HA group by gavage at a concentration of 15mg/kg or 30mg/kg body weight once daily for three days prior to induction of NEC and one hour prior to bacterial administration. NEC was induced using the Paneth cell disruption and *Klebsiella* infection NEC-like model. *I.P.* injection of dithizone (33 mg/kg) was given at P14–16, followed by gavage administration of 1×10^8 CFU *Klebsiella pneumoniae* /kg. Pups were monitored for 10 hours after gavage for clinical illness and survival.

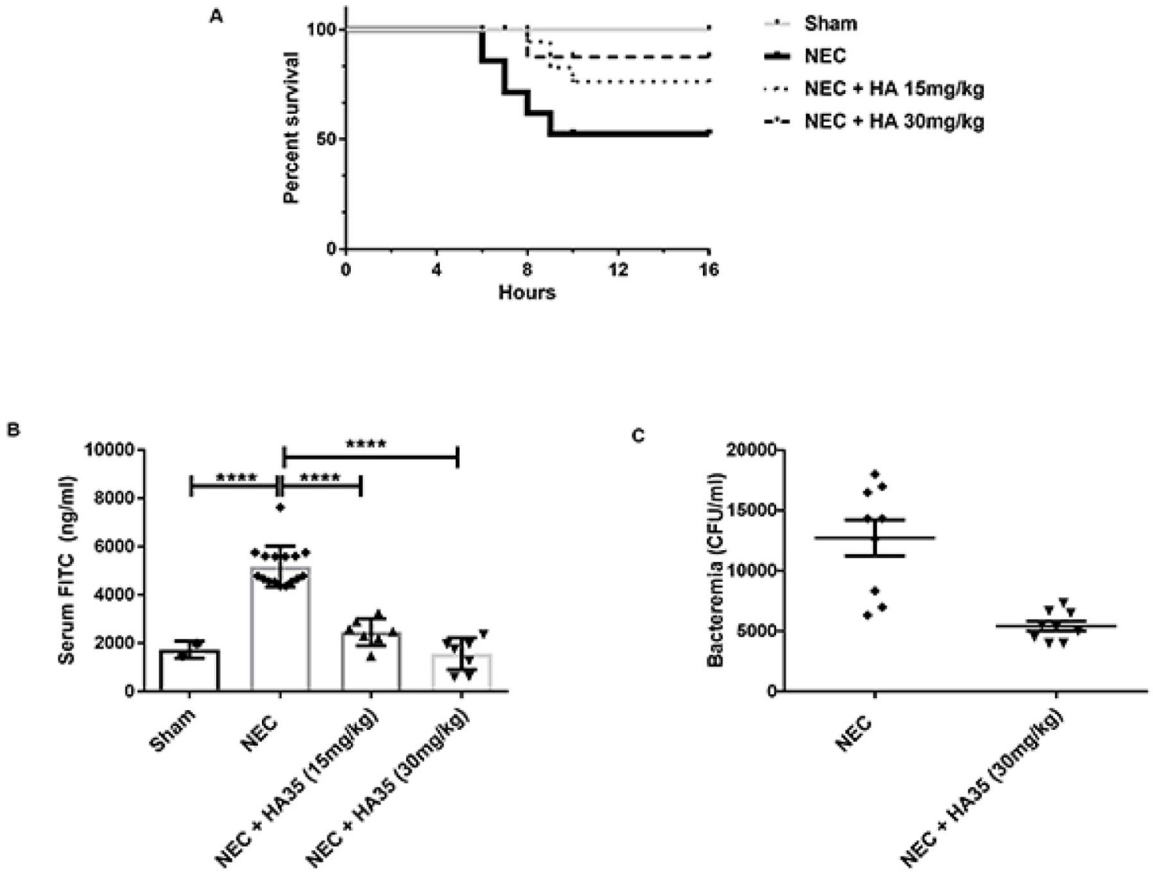

Figure 2. Kaplan-Meier survival curve (A) showing increased survival in HA administered groups.

Survival in low dose HA (15 mg/kg) at 70 %, high dose HA (30mg/kg) at 90 % as compared to untreated NEC at 50% (*p=0.05* & *p=0.04* respectively). (B) Serum FITC levels were lower in NEC + HA 35 treatment compared to NEC with mean ± S.E.M. of 1,554 ± 250 ng/ml in NEC + HA 30 mg/kg, 2,456 ± 212 ng/ml in NEC + HA 15mg/kg, and 5,274 ± 389 ng/ml in untreated NEC. **** *p<0.0001.* (C) Scatter plot of bacterial colony counts from blood cultures being significantly lower in NEC + HA 30mg/kg compared to untreated NEC (mean ± S.E.M of 5,426 ± 378 CFU/ml versus 12,722 ± 1519 CFU/ml respectively. ****p*<0.0001. One-way ANOVA with Turkey's multi-comparison test.

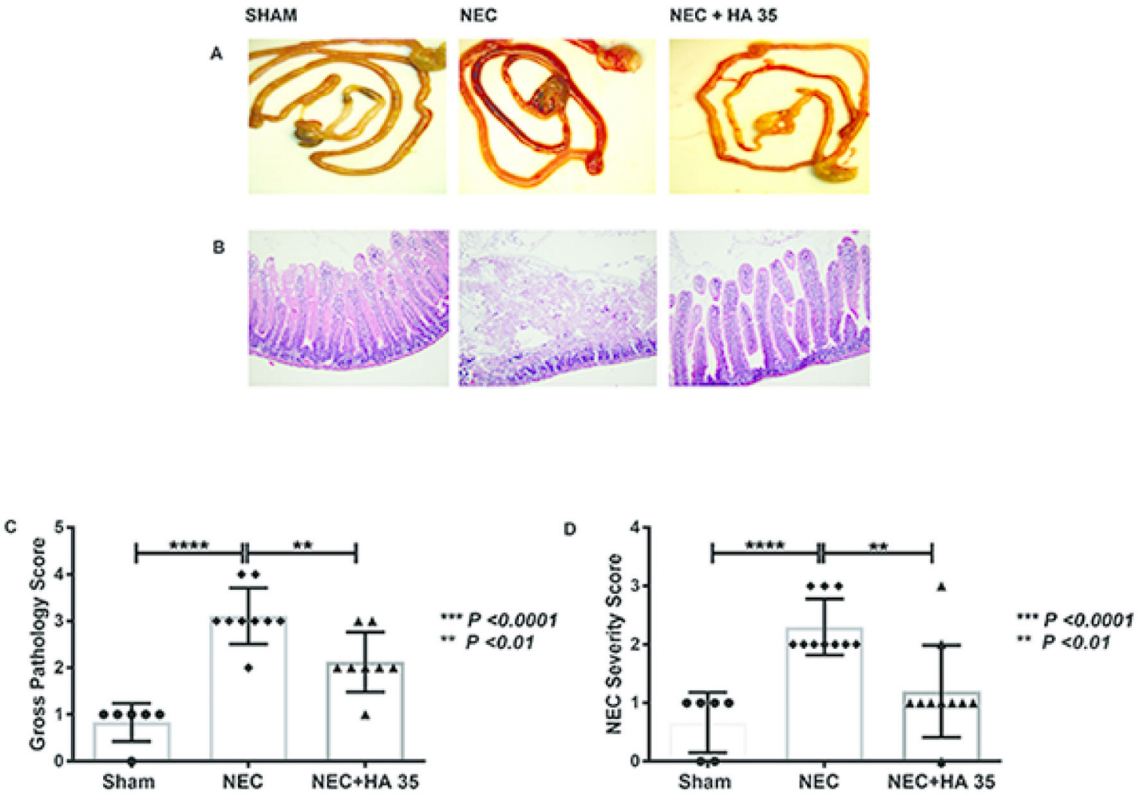

Figure-3: HA 35 reduces the severity of small intestinal injury in murine NEC.

Representative gross (A) and H&E images (B) of the small intestine from the groups. (C) HA 35 administration was associated with decreased gross appearance of injury with a mean ± S.E.M macroscopic score of 2.125 ± 0.31 compared 3.25 ± 1 in the NEC group. ** p <0.01. (D) Histological NEC severity scores of a mean ± S.E.M of 1.2 ± 0.25 in the NEC + HA 35 group versus 2.3± 0.15 in the untreated NEC group. ** p <0.01. Data are in mean ± S.E.M. Results are representative of at least ≥ six animals and at least two separate experiments. One-way ANOVA with Dunnet's multi-comparison test.

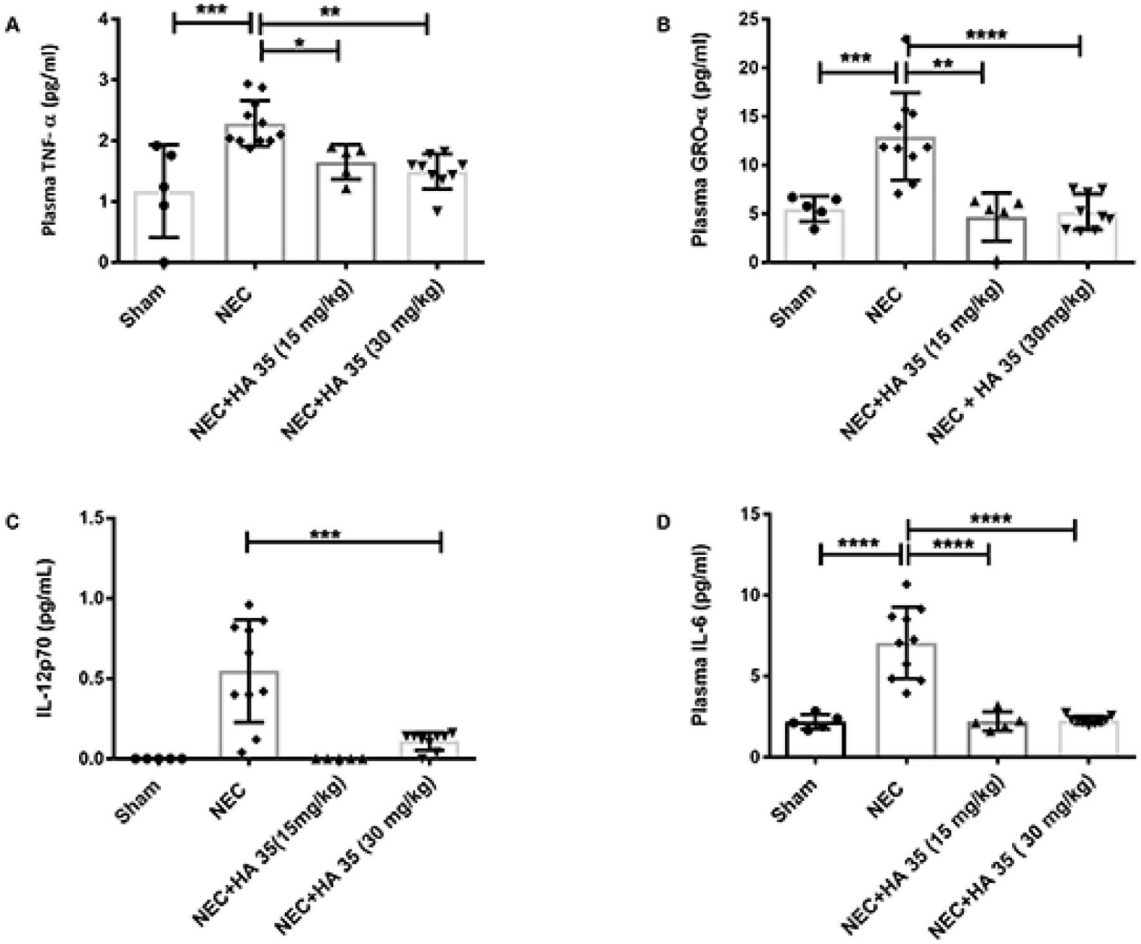

Figure-4: HA 35 administration decreases the systemic (plasma) pro-inflammatory cytokine release in NEC-like model.

Pro-inflammatory cytokine levels of TNF α (**p= 0.0483, **p=0.0018*), Gro-α *(*** p=0.0003, **** p<0.0001*), IL-12ρ70 (**** p =0.0003*), IL-6 (*****p<0.0001*) were decreased in NEC + HA 35 compared to untreated NEC. Data are in mean ± S.E.M. Results are representative of at least > six animals and at least two separate experiments. One-way ANOVA with Turkey's multi-comparison test.

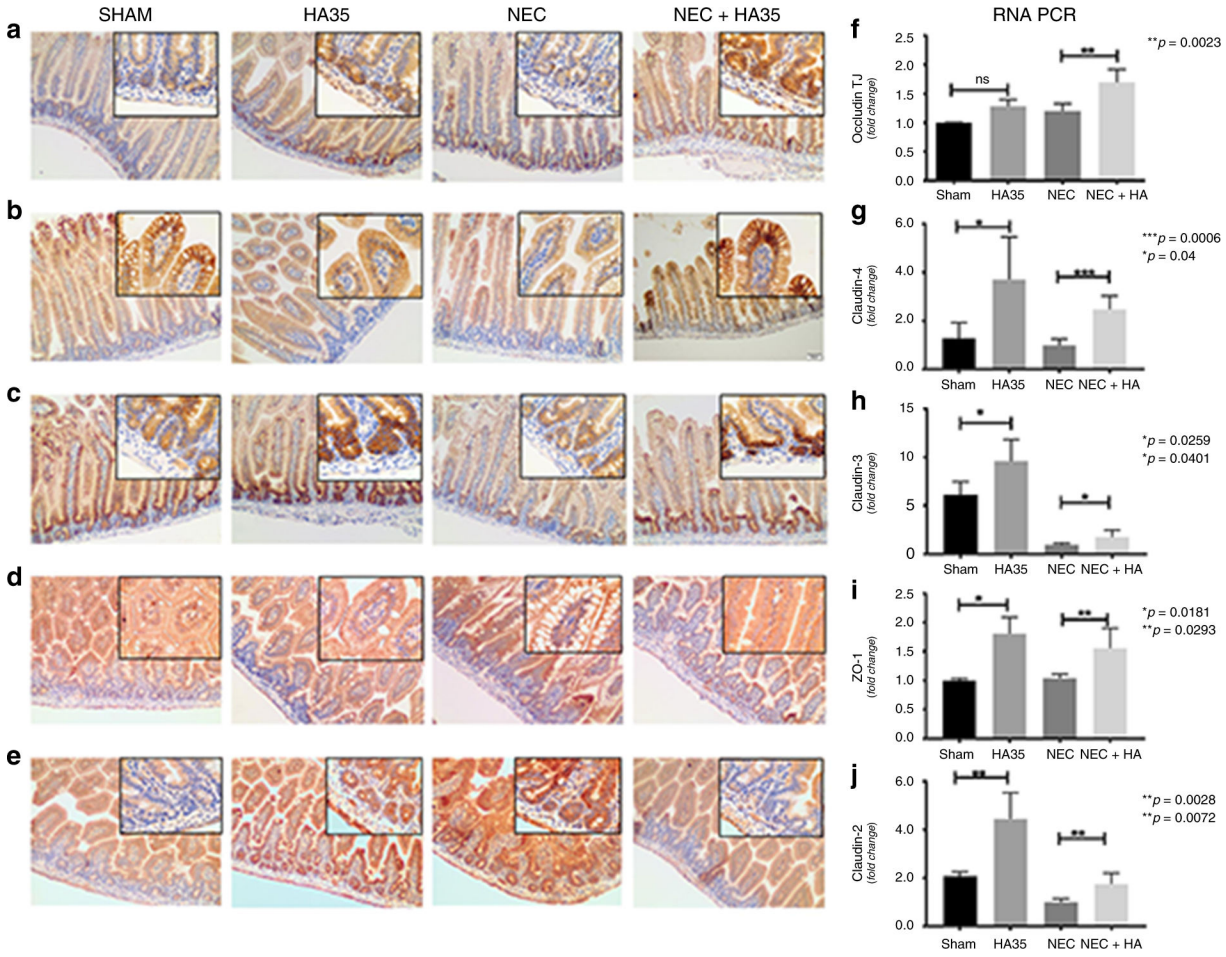

Figure-5: Immunohistochemical staining images (10x and 40x inserts) for TJ proteins and the corresponding qPCR RNA analysis.

HA 35 treated pups showed increased intensity of staining in healthy and NEC challenged pups for all TJ proteins. Insert 40 x images showing increased cryptal intensity of staining in HA 35 treated healthy and NEC challenged pups for TJ occludin (A) and claudin-3(C). Claudin −4 (B) staining was increased in the lateral cytoplasmic membranes of the tips of villi. Insert 40x images showing cytoplasmic staining for ZO-1 (D) in tips of villi in all groups and increased cytoplasmic cryptal staining for claudin −2 (E) noted in the NEC group. RNA PCR results showed upregulated expression of occludin, claudin −4, claudin −3, ZO-1, and claudin −2 in HA 35 and NEC + HA 35 groups compared to untreated NEC (F-J). Claudin −3 RNA expression was downregulated in the NEC group compared to sham (H), while RNA expression for ZO-1 and Claudin −2 was upregulated in HA 35 and NEC + HA 35 group compared to untreated NEC (I, J). Data represent mean ± S.E.M. Analysis was done by two-way ANOVA with Turkey's multi-comparison test.