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Research Article

Comparison of Interleukin-6 Levels After Rectal Irrigation With Metronidazole and Normal Saline Versus Normal Saline Alone in Male Wistar Rats With a Hirschsprung-Associated Enterocolitis Model

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ABSTRACT

Background: Hirschsprung-Associated Enterocolitis (HAEC) is a serious complication of Hirschsprung disease characterized by intestinal inflammation. Rectal irrigation with systemic antibiotics is the standard management, but efficacy remains limited. Metronidazole, an effective anaerobic antibiotic, has immunomodulatory properties that may reduce inflammation. This study evaluates whether topical metronidazole combined with normal saline irrigation reduces IL-6 levels more effectively than saline alone.

Method: An experimental study using 20 male Wistar rats (2-3 months, 250-300g) was conducted. HAEC model was induced using benzalkonium chloride (BAC) 0.1% applied topically to the sigmoid colon. Rats were randomized into two groups: Control group (K) receiving rectal irrigation with normal saline 0.9% (10 mL/kg/day, once daily), and Treatment group (P) receiving combination irrigation with metronidazole 7.5 mg/kg/day plus normal saline 10 mL/kg/day (twice daily). Both interventions began on day 7 post-induction and continued until day 14. Serum IL-6 levels were measured by ELISA on day 14. Data were analyzed using independent samples t-test.

Results: The combination group demonstrated a mean IL-6 level of 4.47 ± 1.54 pg/mL versus 5.63 ± 2.04 pg/mL in the saline-only group (mean difference -1.15 pg/mL, 95% CI: -2.85 to 0.54; t = -1.430, df = 18, p = 0.170). Normal distribution was confirmed in both groups (Shapiro-Wilk: W = 0.904, p = 0.243 and W = 0.879, p = 0.126, respectively), and homogeneity of variance was confirmed by Levene's test (F = 0.043, p = 0.837).

Conclusion: Combination irrigation with metronidazole demonstrated a numerically lower mean IL-6 level compared with saline alone (4.47 ± 1.54 vs. 5.63 ± 2.04 pg/mL; Cohen's d = 0.64); however, this difference was not statistically significant under the current experimental conditions (p = 0.170). Given the unequal irrigation frequency between groups and the limited statistical power of this pilot study, these findings should be regarded as hypothesis-generating rather than evidence of therapeutic efficacy, warranting larger, adequately powered trials with equal irrigation frequency and longer intervention duration.

Keywords: Enterocolitis; Rectal irrigation; Hirschsprung disease; Inflammation; Interleukin-6; Metronidazole

INTRODUCTION

Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of ganglion cells in the intrinsic nerve plexuses (Meissner's and Auerbach's plexuses) of the distal gastrointestinal tract, resulting in an aganglionotic segment and functional obstruction.[1] While the incidence of Hirschsprung disease is approximately 1 in 5,000 live births, the emergence of Hirschsprung-Associated Enterocolitis (HAEC) represents one of the most serious and potentially fatal complications of this disease.[2,3]

HAEC is an acute inflammatory condition of the colon characterized clinically by explosive diarrhea, abdominal distension, fever, lethargy, and signs of systemic toxicity including dehydration and sepsis.[4] The incidence of HAEC in children with Hirschsprung disease varies widely between 5.2% and 56%, depending on the diagnostic criteria employed.[5,6] HAEC can occur both preoperatively and postoperatively, with reported incidence rates ranging from 6-60% overall and 25-42% in the postoperative period.[7] The mortality rate associated with HAEC ranges from 1-10%, with the highest mortality observed in neonates before definitive surgical correction.[8]

The pathogenesis of HAEC is multifactorial and involves four primary mechanisms: (1) intestinal dysmotility resulting in fecal stasis and bacterial overgrowth, (2) dysbiosis of the microbiota, (3) dysfunction of the intestinal barrier, and (4) abnormal mucosal immune response.[9,10] The disruption of normal neural regulation in Hirschsprung disease leads to impaired motility, which facilitates bacterial stasis and the proliferation of pathogenic organisms, particularly anaerobes such as Clostridium difficile, which are strongly associated with HAEC development.[11]

Current management of HAEC is primarily supportive and non-specific, including fluid resuscitation, bowel rest, systemic broad-spectrum antibiotics, and rectal irrigation with normal saline.[9,12] Metronidazole is the first-line antibiotic of choice, given its excellent activity against anaerobic bacteria including gram-negative organisms such as Bacteroides fragilis and gram-positive organisms such as Clostridium difficile.[13] However, systemic administration of metronidazole is associated with potential adverse effects including peripheral neuropathy, neurotoxicity, and gastrointestinal intolerance[14].

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that plays a central role in mediating the inflammatory response in enteric disorders. Elevated serum IL-6 levels are associated with greater HAEC severity and poorer clinical outcomes.[15,16] Notably, higher IL-6 concentrations are observed in the aganglionotic segments of Hirschsprung disease, reflecting the disrupted

neural regulation of immune responses.[17] Therapeutic interventions that reduce IL-6 levels have been shown to ameliorate inflammation in various models of inflammatory bowel disease.

Mechanistically, the pathway linking HAEC to systemic inflammation can be conceptualized as a sequential cascade: HAEC induction leads to dysbiosis and local mucosal inflammation, which promote anaerobic bacterial overgrowth; this anaerobic bacterial burden, in turn, drives IL-6 production by intestinal epithelial and immune cells, culminating in a systemic inflammatory response.[10,18,19] HAEC-associated inflammation, however, involves multiple overlapping pathways beyond IL-6, including IL-1β, TNF-α, IFN-γ, and broader mucosal immune activation.[20] IL-6 was selected as the primary outcome marker in this study rather than these other mediators because of its established correlation with HAEC severity and clinical outcomes,[15,17] its comparatively rapid and well-characterized kinetics following an inflammatory stimulus,[21] and the availability of a validated rat-specific ELISA assay; nonetheless, this represents a deliberately narrowed assessment of a broader inflammatory process, a limitation that is revisited in the Discussion.

While rectal irrigation with normal saline has demonstrated some benefit in reducing the incidence and severity of HAEC, the therapeutic effects remain limited.[22,23] The combination of topical irrigation with an antimicrobial and immunomodulatory agent may enhance therapeutic efficacy by simultaneously addressing both the microbiological and inflammatory components of HAEC pathogenesis. Metronidazole has been shown to possess immunomodulatory properties in addition to its antimicrobial effects, including the capacity to reduce pro-inflammatory cytokine production.[24,25]

To our knowledge, no previous studies have evaluated topical rectal irrigation with a combination of metronidazole and normal saline in the context of HAEC. However, animal models of BAC-induced HAEC and studies of systemic metronidazole therapy have each been reported separately. The novelty of the present study therefore lies specifically in (1) the topical, rather than systemic, route of metronidazole delivery, and (2) the evaluation of its putative immunomodulatory effect through its impact on serum IL-6 as a marker of systemic inflammation, distinguishing it from prior work examining saline irrigation or systemic antimicrobial therapy in isolation. The objective of the present study was to compare IL-6 levels in an experimental HAEC model treated with rectal irrigation using a metronidazole-normal saline combination versus normal saline alone, hypothesizing that the combination therapy would result in lower serum IL-6 levels, a marker of reduced systemic inflammation.

METHODS

This experimental study utilized a randomized, post-test-only control group design and was executed following ethical clearance from the Review Board of the Faculty of Medicine at Udayana University (Approval No. 0843/UN14.2.2VII.14/LT/2025). All handling and surgical procedures strictly adhered to the National Institutes of Health guidelines to ensure laboratory animal welfare and minimize distress. Based on the Federer formula for experimental design, and accounting for a potential 20% attrition rate, twenty healthy male Wistar rats (*Rattus norvegicus*) were enrolled. The subjects, aged 2 to 3 months and weighing between 250 and 300 grams, were obtained from Udayana University's animal facilities. The rodents were housed in groups of two to three per iron enclosure under

standardized environmental parameters, including an ambient temperature of 21°C, a 12-hour alternating light-dark cycle, and unrestricted access to standard laboratory chow and water. A mandatory seven-day acclimation period preceded any interventions to allow the animals to physiologically stabilize.

Following a 12-hour fast with ad libitum water access, the surgical induction of Hirschsprung-associated enterocolitis (HAEC) was performed on day zero. The subjects were anesthetized using 5–10 mg/kg of intramuscular ketamine. Employing strict aseptic techniques, the anterior abdomen was sterilized with 10% povidone-iodine before executing a 3-cm midline laparotomy. Once the sigmoid colon was exposed, an aganglionotic segment was chemically induced by circumferentially wrapping a 1 × 2 cm sterile gauze soaked in 0.1% benzalkonium chloride (BAC)—a cationic surfactant—around the bowel, roughly 1 cm distal to the descending colon junction. A plastic barrier was positioned beneath the intestine to protect surrounding structures from chemical exposure. To maintain tissue saturation, three additional drops of the BAC solution were administered every five minutes over a 15-minute window. The gauze was subsequently removed, the affected tissue was thoroughly flushed with 0.9% sterile saline, and the abdominal wall was closed using 3.0 non-absorbable multifilament sutures. Based on the foundational model described by Sato et al. (1978), this chemical denervation technique reliably mimics clinical HAEC presentations, typically manifesting as abdominal distension, reduced fecal output, and diarrhea by the third post-operative day.

By day seven post-induction, upon visual confirmation of HAEC symptoms, the rodents were allocated into two equal cohorts of ten using a computer-generated randomization sequence. The control cohort was subjected to once-daily rectal lavages utilizing 0.9% normal saline at a dosage of 10 mL/kg/day (roughly 5 mL per subject). Conversely, the intervention cohort received a twice-daily therapeutic irrigation comprising 7.5 mg/kg/day of dissolved metronidazole combined with 10 mL/kg/day of normal saline. All irrigations were performed between days 7 and 14 using a 5-or 8 French feeding catheter. To ensure maximum comfort and adequate colonic distribution, the lavage fluids were warmed to match standard rodent physiological temperatures (35.9–37.5°C). It is important to note a potential methodological limitation: the variation in lavage frequency (once versus twice daily) introduces a mechanical confounder, as the physical clearance of inflammatory debris may independently affect outcomes. Consequently, any observed variations in inflammatory markers cannot be exclusively attributed to the pharmacological efficacy of metronidazole.

At the conclusion of the 14-day protocol, the animals were humanely euthanized using a high-dose injection of sodium thiopental (200 mg/kg). Blood samples ranging from 0.5 to 1.0 mL were immediately drawn via retro-orbital venipuncture into EDTA-coated tubes, centrifuged at 3000 rpm for 10 minutes, and the resulting serum was cryopreserved at -20°C. Interleukin-6 (IL-6) concentrations were subsequently quantified in duplicate utilizing a rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kit (Bioassay Technology Laboratory). The assay demonstrated a sensitivity of 1.5 pg/mL, with intra- and inter-assay coefficients of variation remaining well below 10% and 15%, respectively. Concurrently, immediate necropsies were performed to photographically document macroscopic colonic pathologies, including gas distension, fecal impaction, edema, and mucosal inflammation. Because these macroscopic observations were recorded descriptively rather than through a

validated quantitative scoring system or histopathological ganglion cell validation, structural severity could not be statistically compared between the two cohorts. Statistical analyses were ultimately conducted using SPSS. After confirming data normality and variance homogeneity via the Shapiro-Wilk and Levene's tests, an independent-samples t-test (two-tailed) was applied to compare mean IL-6 differences between the groups. Statistical significance was set at $p < 0.05$, augmented with Cohen's d for effect size and a post hoc power analysis to better contextualize the statistical robustness of the sample size.

RESULTS

All 20 animals (10 in each group) completed the entire protocol without any dropouts or deaths. HAEC symptoms, including decreased stool output, abdominal distension, and diarrhea, were confirmed in all animals by day 3-4 post-induction, validating the successful establishment of the HAEC model in both groups.

Necropsy performed on day 14 revealed characteristic findings consistent with HAEC in all animals. The proximal colon demonstrated significant dilatation relative to the aganglionotic distal segment, with a clear transition zone between the dilated proximal and normal-caliber distal segments. Prominent fecal impaction and gas distension were observed in the proximal colon. Histopathologic findings included mucosal hyperemia, varying degrees of edema, mucosal thickening, and evidence of inflammatory infiltration. In several animals, focal necrosis and increased friability of the bowel wall were noted, indicating active inflammatory processes. One animal in the control group showed adhesions between intestinal segments, consistent with a chronic inflammatory response. These macroscopic features confirmed that the induced HAEC model successfully replicated the pathologic changes observed in human Hirschsprung-associated enterocolitis.

Table 1 shows the individual IL-6 concentrations measured in all 20 experimental animals. The saline + metronidazole group tended to exhibit lower IL-6 values than the saline group, although considerable variability was observed within both groups.

Table 1. The detailed IL-6 measurements for all 20 animals

	1	2	3	4	5	6	7	8	9	10
Salin + Metronidazole	2.256	6.169	3.271	5.831	4.478	2.546	6.314	3.609	5.976	4.285
Salin	6.217	8.343	1.966	5.638	5.444	5.976	8.15	2.546	6.169	5.831

Table 2 shows that the saline + metronidazole group had a lower mean IL-6 level (4.47 ± 1.54 pg/mL) than the saline group (5.63 ± 2.04 pg/mL). Despite this numerical difference (mean difference = −1.15 pg/mL), the difference was not statistically significant ($t = -1.430$, $p = 0.170$).

Table 2. Descriptive Statistics for IL-6 Levels

Group	n	mean ± SD (pg/mL)	Mean difference	value t	df	p-value
Salin + Metronidazole	10	4,47 ± 1,54	-1,15	-1,430	18	0,170
Salin	10	5,63 ± 2,04				

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For normality and variance testing, the Shapiro-Wilk test indicated normality in both groups: Saline + Metronidazole group ($W = 0.904$, $p = 0.243$) and Saline-only group ($W = 0.879$, $p = 0.126$). Levene's test for equality of variances yielded $F = 0.043$ ($p = 0.837$), confirming homogeneity of variance between groups. Both assumptions for the independent samples t-test were therefore satisfied.

Although the combination group (saline + metronidazole) demonstrated a lower mean IL-6 level (4.47 ± 1.54 pg/mL) compared to the saline-only group (5.63 ± 2.04 pg/mL), the difference of 1.15 pg/mL did not achieve statistical significance ($t = -1.430$, $df = 18$, $p = 0.170$). The 95% confidence interval crossed zero (-2.85 to 0.54), indicating that the true population difference could be zero. The corresponding effect size was Cohen's $d = 0.64$, conventionally interpreted as a medium-to-large effect; however, given the wide confidence interval and small sample size, this estimate is imprecise and should not be interpreted as evidence of efficacy in the absence of statistical significance. The distribution of individual IL-6 values by group, including the wide within-group variability underlying the non-significant result, is shown in Figure 1.

DISCUSSION

The central question of this study was whether adding topical metronidazole to standard rectal saline irrigation would meaningfully reduce systemic IL-6 levels in a rat model of HAEC. Our findings showed a numerically lower IL-6 in the combination group (4.47 ± 1.54 pg/mL) relative to the saline-only group (5.63 ± 2.04 pg/mL); however, this 20% descriptive difference did not translate into statistical significance (mean difference -1.15 pg/mL, 95% CI -2.85 to 0.54; $p = 0.170$). Interpreting these findings requires careful consideration of several methodological and biological factors discussed below.

The benzalkonium chloride (BAC)-induced HAEC model used in this study successfully replicated the characteristic clinical and pathologic features of human Hirschsprung-associated enterocolitis, including abdominal distension, decreased stool output, diarrhea, and macroscopic evidence of colonic dilatation with mucosal inflammation. The development of these symptoms by day 3-4 post-induction is consistent with previous reports utilizing the same model.[26] The demonstration of dilated proximal colon with a clear transition zone and aganglionotic distal segment, combined with histologic evidence of inflammation, confirms the validity of this model for studying therapeutic interventions in HAEC. However, definitive histopathological confirmation of aganglionosis — that is, microscopic verification of the absence of ganglion cells in the distal segment — was not performed in this study; therefore, model validation relies on clinical and macroscopic criteria consistent with prior reports rather than on independent histological confirmation. This represents a limitation in model validation that should be addressed in future work.

Interleukin-6 is a pleiotropic cytokine that plays a central role in regulating inflammatory responses throughout the body. In the context of gastrointestinal inflammation, IL-6 is produced by epithelial cells, lamina propria cells, and infiltrating immune cells in response to pathogenic stimuli, including bacterial antigens and lipopolysaccharides (LPS) from gram-negative organisms.[19] In acute inflammation, IL-6 rises rapidly within 3-6 hours of stimulation

and typically peaks within 12-24 hours, making it an excellent marker of the inflammatory response to therapeutic interventions.[21]

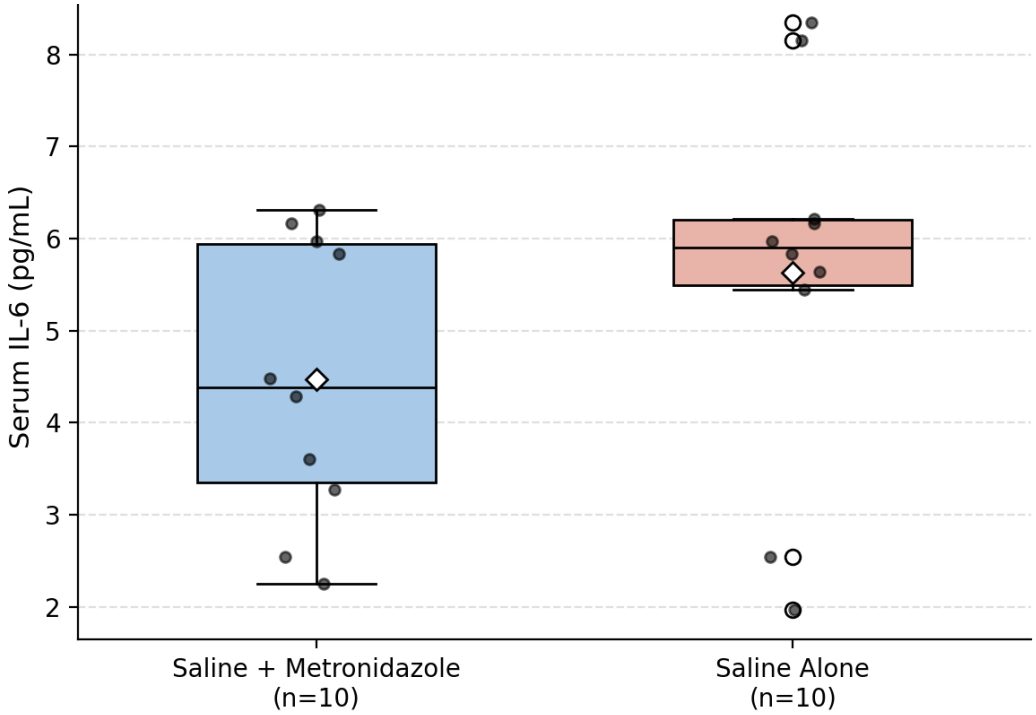

Figure 1. Boxplot of serum IL-6 levels (pg/mL) by group, with individual animal values overlaid. Diamonds indicate group means; horizontal lines within boxes indicate medians.

In HAEC specifically, elevated IL-6 levels have been shown to correlate with increased disease severity and poor clinical outcomes.[15,16] The aganglionotic segments in Hirschsprung disease lack normal cholinergic innervation, which results in impaired suppression of Th17 cells and a relative predominance of IL-6-mediated inflammatory pathways.[27] Higher IL-6 concentrations in aganglionotic bowel segments compared to ganglionated segments have been documented, and persistent elevation of IL-6 in the post-operative period is associated with increased risk of post-operative enterocolitis episodes.[27] Therefore, therapeutic strategies that reduce IL-6 production have the potential to ameliorate the inflammatory cascade and reduce disease severity.

Metronidazole is a 5-nitroimidazole prodrug that becomes bactericidal only under anaerobic conditions, when reduction of its nitro group generates reactive intermediates that fragment bacterial DNA, leading to cell death via strand breakage rather than enzyme inhibition.[13,28] This oxygen-dependent activation is what makes metronidazole selectively toxic to anaerobes while sparing aerobic host tissues. In clinical and experimental settings, the drug has been used for over four decades against a broad range of obligate anaerobes, including the gram-negative Bacteroides fragilis and gram-positive Clostridium difficile — both implicated in HAEC — with consistently favorable pharmacokinetics when delivered orally, intravenously, or, as in the present study, topically.[28,29]

The microbiota in HAEC is characterized by dysbiosis, with increased proportions of Bacteroidetes and Clostridium species and decreased Lactobacillus populations.[12,30] Clostridium difficile, in particular, is strongly associated with HAEC pathogenesis and is an

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obligate anaerobe highly susceptible to metronidazole. At the dose used in this study (7.5 mg/kg/day), metronidazole achieves effective concentrations against anaerobic pathogens and has been previously shown to reduce bacterial colonic burden in HAEC models.

The intervention demonstrated a non-significant trend toward lower IL-6 in the combination group (1.15 pg/mL difference, representing approximately a 20% descriptive reduction). While this directionally aligns with previous literature reporting immunomodulatory properties of metronidazole beyond its antimicrobial effects,[20,21] the lack of statistical significance ($p = 0.170$) precludes a conclusion about an anti-inflammatory effect from the present data. The contributing factors discussed below should therefore be read as exploratory considerations rather than confirmed explanations for the observed trend.

Limited statistical power due to the small sample size ($n = 10$ per group) is a plausible contributor to the non-significant finding. However, low power reflects statistical uncertainty and should not be interpreted as implying a hidden therapeutic effect. The sample size calculation using the Federer formula suggested a minimum of 16 animals (20 with a 20% dropout buffer), but it does not account for the anticipated effect size of the intervention. A post-hoc power analysis based on the observed effect size (Cohen's $d = 0.64$) indicates that the study was powered at only approximately 27% to detect a difference of this magnitude at $\alpha = 0.05$; achieving 80% power would require approximately 40 animals per group. This confirms that the study was substantially underpowered, which limits confidence in the null result but is not, by itself, evidence in favor of a true effect; post-hoc power should therefore be regarded as a contextual caveat rather than the central justification for the observed trend. Future studies should use an a priori, effect-size–based sample size calculation rather than relying on the Federer formula alone.

The doses and treatment duration employed in this study were selected based on clinical protocols and previous experimental designs. The metronidazole dose (7.5 mg/kg/day) reflects the maintenance dosing used in human pediatric patients, and the treatment duration (7 days) represents a reasonable timeframe for evaluating early inflammatory responses. However, higher doses or longer treatment duration may be necessary to achieve a statistically significant reduction in IL-6 levels. The twice-daily irrigation schedule in the treatment group compared to once-daily in the control group provides more frequent drug delivery but also introduces a confounding variable (frequency of irrigation itself may have anti-inflammatory effects through mechanical lavage). Consequently, the observed difference in IL-6 levels between groups cannot be attributed solely to the pharmacologic effect of metronidazole, since the mechanical irrigation itself was unequally distributed between arms.[33] This design limitation should be addressed in future studies by equalizing irrigation frequency between groups (for example, by administering a matched sham irrigation to the control group).

The pathogenesis of HAEC is multifactorial and involves complex interactions among intestinal dysbiosis, barrier dysfunction, and abnormal mucosal immunity. While metronidazole effectively reduces the anaerobic bacterial burden, the inflammatory cascade in HAEC is driven by multiple mediators, including IL-1β, TNF-α, IFN-γ, and others, in addition to IL-6.[30] IL-6 represents only one component of this complex inflammatory milieu. Measuring IL-6 alone may not fully capture the breadth of the anti-inflammatory effects of

metronidazole therapy. Other cytokines and inflammatory mediators may show more dramatic responses to intervention, whereas IL-6 might remain relatively elevated despite improvements in other inflammatory markers.

The route of metronidazole administration employed in this study (topical via rectal irrigation) differs from conventional systemic administration. While topical delivery minimizes systemic toxicity, it also results in lower peak concentrations in systemic circulation compared to intravenous or oral administration. The significantly lower systemic bioavailability of topically applied metronidazole may limit its immunomodulatory effects on systemic IL-6 production. Additionally, serum IL-6 levels reflect systemic inflammation, whereas the primary site of HAEC pathology is the intestinal mucosa. Local mucosal IL-6 production might be more dramatically affected by topical therapy than circulating IL-6 levels. This explanation remains speculative, as this study did not directly measure mucosal tissue drug concentrations or local IL-6 production; future studies incorporating paired tissue and serum sampling are needed to test this hypothesis.

HAEC remains a major contributor to morbidity and mortality among children with Hirschsprung disease. Should future, adequately powered studies confirm a clinically meaningful anti-inflammatory effect of topical metronidazole irrigation, this approach could represent a comparatively low-cost adjunct to existing management strategies. However, the present findings are preliminary and not statistically significant, and claims of reduced antimicrobial resistance, cost savings, or reduced reliance on systemic antibiotics are not directly supported by this study. They should not be inferred from the current data.

Although the difference did not reach statistical significance, the consistent directional trend toward lower IL-6 levels in the combination treatment group, together with the medium-to-large effect size (Cohen's d = 0.64), suggests the study may have been underpowered to detect a true effect; in the absence of statistical significance, however, the 1.15 pg/mL (20%) difference cannot itself be interpreted as clinically meaningful or as evidence of efficacy.[31] The lower standard deviation observed in the treatment group (SD = 1.54 vs. 2.04) may indicate reduced inter-animal variability, but, given the small sample size, this difference should be interpreted cautiously and should not be construed as evidence of therapeutic stabilization of the inflammatory response

The observed trend provides biological plausibility, rather than evidence of efficacy, to justify larger, adequately powered trials with equal irrigation frequency between arms, histological and tissue-level inflammatory endpoints, and measurement of additional cytokines, to more definitively evaluate whether topical metronidazole therapy contributes to reduced systemic inflammation in HAEC. Given the high morbidity and mortality associated with HAEC and the limitations of current standard management, continued research into more effective therapeutic strategies remains clinically important, even though the present results alone do not establish therapeutic benefit.

This study has several notable strengths. It used a well-characterized, BAC-induced HAEC model with a randomized control-group design and a clearly defined translational objective relevant to pediatric surgical practice. The intervention was grounded in a biologically plausible mechanistic rationale linking topical antimicrobial therapy to reduced systemic

inflammation, and the analysis incorporated effect-size and confidence-interval reporting in addition to hypothesis testing, allowing for a more complete and transparent interpretation of a non-significant result.

Several limitations should be considered when interpreting these findings. First, the unequal irrigation frequency between groups (once versus twice daily) represents an important confounding variable that precludes attributing the observed trend solely to metronidazole. Second, only a single inflammatory marker (serum IL-6) was assessed; tissue IL-6, histological inflammation scores, and additional cytokines (e.g., IL-1β, TNF-α, IFN-γ) were not measured and may have provided a more complete picture of the inflammatory response. Third, histopathological confirmation of aganglionosis via ganglion-cell verification was not performed, limiting definitive validation of the HAEC model, and macroscopic findings were recorded descriptively rather than with a standardized quantitative scoring system. Fourth, the relatively short intervention period (7 days) and small sample size (n = 10 per group, post-hoc power ≈27%) limited statistical power. Finally, as an animal model, these findings cannot be directly extrapolated to human clinical practice without further translational validation.

CONCLUSION

Combination irrigation with metronidazole demonstrated a lower mean IL-6 level compared with saline alone; however, this difference was not statistically significant under the current experimental conditions. In this rat model of Hirschsprung-Associated Enterocolitis, the mean IL-6 level in the combination group (4.47 ± 1.54 pg/mL) was approximately 20% lower than in the saline-only control group (5.63 ± 2.04 pg/mL; mean difference 1.15 pg/mL, 95% CI -2.85 to 0.54, p = 0.170, Cohen's d = 0.64).

These findings should not be interpreted as evidence of therapeutic efficacy of topical metronidazole irrigation in HAEC. Future studies should include a larger sample size with adequate a priori statistical power (an effect-size–based calculation suggests approximately 40 animals per group), equal irrigation frequency between treatment arms, histological and tissue-level inflammatory endpoints with ganglion-cell confirmation of the model, measurement of multiple cytokines (e.g., IL-1β, TNF-α, IFN-γ) alongside IL-6, and a longer follow-up duration, to more definitively evaluate the therapeutic potential of this approach.

Given the significant morbidity and mortality associated with Hirschsprung-Associated Enterocolitis and the limitations of current standard management strategies, continued research into rational combinations of antimicrobial and anti-inflammatory approaches remains warranted. The present findings provide a preliminary, hypothesis-generating basis — rather than confirmatory evidence — for future, adequately powered investigations of metronidazole-enhanced rectal irrigation in HAEC management.

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Declarations

Authors' contributions

A.A.A contributed to the study conception and design, data collection, data analysis and interpretation, and drafting of the manuscript. I.B.M.S. contributed to the research design, supervision of data analysis, critical revision of the manuscript for important intellectual content, and final approval of the version to be published. K.S. contributed to data interpretation, methodological input, and critical review of the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Coran AG, Adzick NS, Krummel TM. *Pediatric Surgery*. 7th ed. Elsevier; 2012.
2. Dolgin SE, Hamner CE. *Surgical Care of Major Newborn Malformations*. 2012.
3. Puri P, Nakamura H. Epidemiology and clinical characteristics of Hirschsprung's disease. In: Puri P, ed. *Hirschsprung's Disease and Allied Disorders*. Springer; 2019:167-174. doi:10.1007/978-3-030-15647-3
4. Lewit RA, Kuruvilla KP, Fu M, Gosain A. Current understanding of Hirschsprung-associated enterocolitis: Pathogenesis, diagnosis and treatment. *Semin Pediatr Surg*. 2022;31(2):151162. doi:10.1016/j.sempedsurg.2022.151162
5. Gershon MD, Rodriguez L, Arbizu RA. Hirschsprung's disease associated enterocolitis: A comprehensive review. *World J Clin Pediatr*. 2023;12(3):68-76. doi:10.5409/wjcp.v12.i3.68
6. Hagens J, Reinshagen K, Tomuschat C. Prevalence of Hirschsprung-associated enterocolitis in patients with Hirschsprung disease. *Pediatr Surg Int*. 2022;38(1):3-24. doi:10.1007/s00383-021-05020-y
7. Lewit RA, Kuruvilla KP, Fu M, Gosain A. Current understanding of Hirschsprung-associated enterocolitis: Pathogenesis, diagnosis and treatment. *Semin Pediatr Surg*. 2022;31(2):151162. doi:10.1016/j.sempedsurg.2022.151162
8. Lewit RA, Veras LV, Cowles RA, Gosain A. Reducing underdiagnosis of Hirschsprung-associated enterocolitis: A novel scoring system. *J Surg Res*. 2021;261:253-260. doi:10.1016/j.jss.2021.01.026
9. Gosain A, Barlow-Anacker AJ, Erickson CS. Impaired cellular immunity in the murine neural crest conditional deletion of endothelin receptor-B model of Hirschsprung's disease. *PLoS ONE*. 2015;10(6):e0128822. doi:10.1371/journal.pone.0128822
10. Li S, Zhang Y, Li K. Update on the pathogenesis of the Hirschsprung-associated enterocolitis. *Int J Mol Sci*. 2023;24(5):4602. doi:10.3390/ijms24054602
11. Chantakhow S, Khorana J, Tepmalai K. Alterations of gut bacteria in Hirschsprung disease and Hirschsprung-associated enterocolitis. *Microorganisms*. 2021;9(11):2241. doi:10.3390/microorganisms9112241
12. Gosain A, Frykman PK, Cowles RA. Guidelines for the diagnosis and management of Hirschsprung-associated enterocolitis. *Pediatr Surg Int*. 2017;33(5):517-521. doi:10.1007/s00383-017-4065-8
13. Löfmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis*. 2010;50(Suppl 1):S16-S23. doi:10.1086/647939

14. Ceruelos E, Camacho A, Orueta R. Adverse effects of metronidazole. *Med Clin (Barc)*. 2019;152(12):474-485.
15. Bennibol E, Kim M, Sanchez A. Elevated expression of IL-6 and TNF-α in segments of Hirschsprung disease-associated enterocolitis. *J Pediatr Surg*. 2023;58(4):815-821.
16. Keck B, Feurle A, Illing S. IL-6 and IL-1β mediated intestinal inflammation in Hirschsprung-associated enterocolitis. *J Pediatr Surg*. 2021;56(8):1439-1447.
17. Keck B, Feurle A, Illing S. IL-6 and IL-1β mediated intestinal inflammation in Hirschsprung-associated enterocolitis. *J Pediatr Surg*. 2021;56(8):1439-1447.
18. Gosain A, Brinkman AS. Hirschsprung's associated enterocolitis. *Curr Opin Pediatr*. 2015;27(3):364-369. doi:10.1097/MOP.0000000000000210
19. Tanaka T, Kishimoto T. The biology and medical implications of interleukin-6. *Cancer Immunol Res*. 2014;2(4):288-294. doi:10.1158/2326-6066.CIR-14-0022
20. Ji H, Liu Y, Ye F. Regulatory role of neural-immune axis in Hirschsprung disease. *Front Immunol*. 2023;14:1287906. doi:10.3389/fimmu.2023.1287906
21. Alhendi A, Naser SA. The dual role of interleukin-6 in Crohn's disease pathophysiology. *Front Immunol*. 2023;14:1295230. doi:10.3389/fimmu.2023.1295230
22. Lu C, Xie H, Li H. Feasibility and efficacy of home rectal irrigation in neonates and early infancy with Hirschsprung disease. *Pediatr Surg Int*. 2019;35(11):1245-1253. doi:10.1007/s00383-019-04552-8
23. Marty TL, Seo T, Matlak ME, Sullivan JJ, Black RE, Johnson DG. Rectal irrigations for the prevention of enterocolitis in Hirschsprung's disease. *J Pediatr Surg*. 1995;30(3):319-322. doi:10.1016/0022-3468(95)90047-0
24. Alkhashab MM, Abdalfattah DM, Al-Rawee RY. Metronidazole gel foam influence on salivary tumor necrosis factor and interleukin-6 with lower third molar extraction (randomized control clinical study). *Oral Health Dent Sci*. 2020;4(3):1-4.
25. Kazempour M, Izadi H, Chouhdari A, Rezaeifard M. Anti-inflammatory effect of metronidazole in hospitalized patients with pneumonia due to COVID-19. *Iran J Pharm Res*. 2021;20(3):532-540. doi:10.22037/ijpr.2021.114567.14917
26. Sato Y, Pringle KC, Soper RT. Rectal biopsy as a diagnostic tool for enterocolitis associated with Hirschsprung's disease. *J Pediatr Surg*. 1978;13(2):155-162.
27. Keck B, Feurle A, Illing S. IL-6 and IL-1β mediated intestinal inflammation in Hirschsprung-associated enterocolitis. *J Pediatr Surg*. 2021;56(8):1439-1447.
28. Dingsdag SA, Hunter N. Metronidazole: An old dog, new tricks? *Future Microbiol*. 2018;13(9):1027-1039. doi:10.2217/fmb-2018-0059
29. Löfmark S, Edlund C, Nord CE. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis*. 2010;50(Suppl 1):S16-S23. doi:10.1086/647939
30. Pierre JF, Barlow-Anacker AJ, Erickson CS. Intestinal dysbiosis and bacterial enteroinvasion in a murine model of Hirschsprung's disease. *J Pediatr Surg*. 2014;49(8):1242-1251. doi:10.1016/j.jpedsurg.2014.02.049