

ORIGINAL RESEARCH ARTICLE

Early acidification of stool and urine as a simple noninvasive screening tool for ulcerative colitis activity

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by recurrent inflammation of the colon. The absence of simple, noninvasive, and early biomarkers for disease onset and progression remains a major clinical challenge. This study aimed to evaluate the potential of pH values in biological samples as noninvasive indicators for the early disease-related changes in an experimental rat model of UC. Experimental colitis was induced in rats by administering dextran sulfate sodium, and the results were compared to those of healthy controls. Disease progression was monitored by evaluating disease severity scores and histopathological changes. Throughout the experimental period, stool and urinary pH were measured daily, while colonic and serum pH were assessed post-mortem. As the experiment progressed, the colitic rats exhibited marked weight loss and severe inflammatory changes in the colon. Despite the worsening disease, no significant alterations were observed in colonic or serum pH. In contrast, both stool and urinary pH values significantly decreased during the early phase of experimental colitis. However, these pH fluctuations did not consistently correlate with UC severity. In conclusion, our findings suggest that stool and urinary pH represent promising, noninvasive biomarkers for the early detection and monitoring of disease activity in UC.

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract characterized by persistent and recurrent inflammation, primarily affecting the large intestine.¹ The two major clinical subtypes, ulcerative colitis (UC) and Crohn's disease, are well recognized among clinicians.² Patients typically experience long-term abdominal pain, diarrhea, and repeated cycles of relapse and remission.³ Although the precise etiology of IBD remains unclear, genetic factors such as *NOD2/CARD15*, *IRGM*, and *ATG16L1* are believed to contribute to disease susceptibility.⁴⁻⁶ Defects or loss of function in these genes may lead to abnormal immune responses against commensal bacteria or luminal antigens, thereby initiating intestinal inflammation. Myeloid-derived

cells, including neutrophils and macrophages, play central roles in the onset and progression of IBD. Once activated in colonic tissue, these cells release excessive amounts of pro-inflammatory cytokines, leading to epithelial damage and disruption of the intestinal barrier. To suppress hyperactivated macrophages, immunosuppressive agents such as tacrolimus (FK506) are often administered, and clinical studies have demonstrated their efficacy in maintaining remission.^{7,8} Nevertheless, accurate diagnosis of IBD remains challenging due to the heterogeneity of pathological features, underscoring the need for enhanced diagnostic and therapeutic strategies.

Colonoscopy remains the gold standard for directly assessing mucosal changes in IBD patients; however, repeated procedures are physically burdensome, psychologically stressful, time-consuming, and costly. For decades, C-reactive protein (CRP) has been widely used as a general inflammatory marker.⁹ High-sensitivity CRP (hsCRP) enables improved detection of chronic inflammation, yet both CRP and hsCRP lack specificity for IBD.¹⁰ In contrast, stool calprotectin (S100A8/A9) has emerged as a more reliable biomarker, particularly in patients with severe UC.^{11–13} Although cytokines such as interleukin-6 and tumor necrosis factor- α have been examined in IBD, these markers are not disease-specific.^{14,15} Recently, novel serum biomarkers—including anti-glycan antibodies, leucine-rich α 2-glycoprotein, and pyruvate kinase M2—have been proposed, though each has limitations in sensitivity and specificity.^{16–18} As noted above, numerous biomarkers are used in clinical laboratory settings; however, serum-based biomarkers require blood sampling, which is somewhat invasive and inconvenient for routine monitoring.

Accordingly, there is a strong demand for simple and minimally invasive biomarkers for IBD patients. Among potential indicators, pH fluctuations in biological samples have long been of interest. For instance, in several UC patients, markedly low pH values have been observed in the proximal colon, accompanied by elevated lactic acid concentrations in stool.^{19,20} Notably, stool pH tends to decrease in proportion to disease severity in UC.²¹ Factors such as carbohydrate fermentation by gut microbiota, absorption of short-chain fatty acids (SCFAs) through the mucosa, alterations in mucosal bicarbonate secretion, and changes in intestinal transit time are thought to contribute to pH reduction within colonic tissue.²² Our previous study demonstrated decreased expression of type III carbonic anhydrase (CA) in the colonic tissue of dextran sulfate sodium (DSS)-induced UC model rats.²³ CA catalyzes the rapid, reversible conversion of carbon dioxide (CO_2) and water into bicarbonate ions (HCO_3^-) and protons (H^+)

via carbonic acid.²³ Therefore, reduced CA activity could logically result in decreased pH. However, despite these findings, pH changes in colonic tissue and other biological samples have not yet been incorporated into clinical laboratory diagnostics for UC.

Importantly, numerous established biomarkers for UC require specialized assays and are not always suited for frequent, real-time monitoring in daily life. In this context, pH measurement offers a distinct practical advantage: it is rapid, inexpensive, and can be repeated serially with minimal burden, potentially enabling point-of-care or home-based monitoring. Moreover, stool and urine pH may capture disease-related shifts in luminal metabolism and host acid–base handling (e.g., microbial fermentation, mucosal bicarbonate secretion, and systemic metabolic changes), thereby providing information that is complementary to inflammation-focused biomarkers.

We hypothesized that the pH values of serum, urine, stool, and colonic tissue decrease in response to disease progression in UC, and that these fluctuations could serve as useful indicators of disease severity. To test this hypothesis, we measured pH levels in various biological samples obtained from DSS-induced UC model rats. To our knowledge, no comprehensive analysis of pH variations across multiple biological matrices in experimental UC models has been reported. Thus, this study provides novel insights into the potential of pH as a noninvasive biomarker for disease activity and progression in UC.

2. Materials and methods

2.1. Materials

Dextran sulfate sodium (molecular weight 36,000–50,000), 5-aminosalicylic acid (5-ASA), carboxymethyl cellulose sodium salt (CMC-Na), and 1% eosin Y solution were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Mayer's hematoxylin solution was obtained from Muto Pure Chemicals Co., Ltd. (Japan). Anti-neutrophil elastase (NE) IgG, anti-type II CA (CA-II) IgG, and 3,3'-diaminobenzidine (DAB) were purchased from Cosmo Bio Co., Ltd. (Japan), and peroxidase-labeled anti-mouse IgG was obtained from Funakoshi Co., Ltd. (Japan). The LAQUAtwin pocket pH meter was purchased from HORIBA Advanced Techno Co., Ltd. (Japan). The Fisherbrand™ 150 handheld homogenizer motor and plastic disposable generator probes were obtained from Thermo Fisher Scientific Inc. (USA). Standard (10 × 10 mm) and custom-made (1 × 1 mm) wire mesh for rat cages were purchased from Shimizu Laboratory Supplies Co., Ltd. (Japan). All other reagents were obtained from Wakenyaku Co., Ltd. (Japan), Nacalai Tesque Co., Ltd. (Japan), and Bio-Rad Laboratories, Inc. (USA).

2.2. Protocol for animal experiments

Male Wistar rats (10 weeks old, 220–250 g, $n = 24$) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Prior to the experiment, the animals were acclimated for approximately one week with free access to a standard laboratory diet (MF, Oriental Yeast Co., Ltd., Japan) and distilled water (DW). During the experimental period, rats were housed individually in sawdust-lined plastic cages under controlled environmental conditions (temperature: 22 °C, relative humidity: 60%) with a 12 h light/dark cycle maintained by artificial illumination.

Rats were randomly divided into three groups ($n = 8$ per group): the normal control group (the N group), which received DW without DSS for 10 days and was administered 1% CMC-Na once daily by oral gavage; the DSS group, which received 3% DSS in drinking water for 10 days to induce colitis and was administered 1% CMC-Na once daily by oral gavage; and the DSS + ASA group, which received 3% DSS in drinking water plus 5-ASA (100 mg/kg/day) suspended in 1% CMC-Na and administered once daily by oral gavage for 10 days as a treatment model. The dose of 5-ASA was selected based on a previous report.²⁴ Throughout the experimental period, body weight and drinking fluid (DW or 3%DSS solution) consumption were measured daily at approximately 9:00 A.M., after which all stool and urine were removed from each cage. Newly excreted stool and urine during the following 2 h were collected immediately into closed tubes and promptly processed to prepare pH-measurement samples as described in the next section for subsequent analyses. Rats were housed individually in cages with a custom-made 1 × 1 mm wire mesh overlaid with a standard 10 × 10 mm wire mesh (Figure 1). This setup allowed for efficient separation and collection of stool retained on the upper mesh and urine collected beneath it. Disease activity index (DAI) scores were calculated based on established criteria, which evaluate body weight loss, stool consistency, and the degree of rectal bleeding, each scored from 0 to 4.²⁵ On day 10, rats were euthanized, and blood samples (3 mL per rat) were collected via cardiac puncture. The entire colon was then carefully excised, and its length was measured immediately.

2.3. Sample preparation

Blood samples were allowed to clot at room temperature and then centrifuged at 3,500 rpm for 15 min to obtain serum. Urine samples were centrifuged under the same conditions, and the supernatant was collected. Stool samples were prepared as suspensions according to a previously described method.²⁶ Briefly, 1 mg of stool was

mixed with 1 mL of physiological saline, incubated on ice for 15 min, homogenized using a handheld homogenizer, and centrifuged at 12,000 rpm for 10 min. The resulting supernatant was collected for analysis. The large intestine was divided into three equal segments, designated as the rectal (Rec), middle (Mid), and proximal (Pro) regions, starting from the distal end near the anus. These tissue sections were fixed in 10% formalin prepared in 0.1 M phosphate buffer (pH 7.4) for histological examination and subsequent paraffin embedding.

2.4. Microscopic observation of colonic tissues

The inflammatory condition of colonic tissues from each rat was evaluated using a light microscope (BIOREVO BZ-9000, Keyence Co., Ltd., Japan). Paraffin-embedded tissue sections (3 µm thick) were prepared and stained with hematoxylin and eosin (H & E) for general histopathological assessment. Tissue damage was evaluated using histological severity (HIS) scores based on H & E staining, with the extent of damage assessed according to established criteria on a 0–14 scale.²⁷ To summarize the criteria concisely, it involved assigning a score ranging from 0 to 14 based on inflammation severity, inflammation extent, crypt damage, and percent involvement. Immunostaining detection of NE or CA-II was performed using peroxidase-labeled antibodies as previously described.²⁸ Briefly, 3 µm-thick colonic sections were incubated overnight at 4 °C in a humidified chamber with anti-NE IgG or anti-CA-II IgG (5 µg/mL), followed by a 1-h incubation at room temperature with peroxidase-labeled anti-mouse IgG (5 µg/mL). NE or CA-II expression in colonic tissue was visualized as a brown color following reaction with DAB containing hydrogen peroxide. Images were captured using a microscope (BIOREVO BZ-9000, Keyence Co., Ltd., Japan).

2.5. Semi-quantitative analysis of carbonic anhydrase-II-positive area

Semi-quantitative analysis of NE or CA-II immunostaining was performed using ImageJ/Fiji (Version 1.54, NIH, USA). For each image, the region of interest (ROI) was defined as the tissue area, excluding slide background and annotations. The DAB channel (brown) was separated, and an identical threshold was applied across all groups to segment NE- or CA-II-positive signals. The percentage of these protein-positive areas (% positive area) was calculated as the area of threshold-positive pixels divided by the total ROI area (excluding background) and expressed as a percentage. All images were acquired using identical microscope settings (objective, exposure time, gain, and illumination) and processed using the same analysis parameters.

2.6. pH measurement of biological samples

The pH of all biological samples was measured using a pH meter (LAQUAtwin pocket, HORIBA Advanced Techno Co., Ltd., Japan). Prior to measurement, the instrument was calibrated according to the manufacturer's instructions. For colonic tissue, pH was determined by directly contacting the freshly dissected tissue surface with the pH electrode immediately after excision. The pH of serum, urine, and stool samples was measured immediately after preparation, following the procedures described in Section 2.3.

2.7. Clinical chemistry assays in rat serum

Twenty clinical chemistry parameters were measured in rat serum by Nagahama Life Science Laboratory, Oriental Yeast Co., Ltd. (Japan). To reduce individual variability, equal volumes of serum from six rats per group were pooled prior to analysis. Measurements were conducted using an analyzer (JCA-BM6050, JEOL Ltd., Japan) and included the following parameters: total protein (TP), albumin (ALB), glucose, blood urea nitrogen (BUN), creatinine (CRE), total bilirubin, uric acid, iron (Fe), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), creatine kinase, cholinesterase, total cholesterol (T-CHO), triglycerides (TG), low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDL-C), pyruvic acid (PA), and lactic acid (LA).

2.8. Statistical analysis

Data were presented as mean $\pm$ standard deviation. For time-course variables measured repeatedly in the same animals (e.g., body weight, DAI scores, and daily pH in urine and stool samples), statistical analyses were performed using two-way repeated-measures analysis of variance (ANOVA) with group and day as factors, including the group $\times$ day interaction. When significant effects were detected, Dunnett's post hoc test was used to compare multiple groups against the N group within each day, as appropriate. For single-time-point endpoints (e.g., immunostaining % positive area and pH values in colon and serum samples), one-way ANOVA followed by Dunnett's post hoc test was used. Statistical significance was set at $p < 0.05$. In addition, correlation analyses were performed to assess associations between excreta pH (urine and stool pH) and disease indices (DAI and HIS scores). Spearman's rank correlation coefficients were calculated and reported for each comparison. Correlation strength was interpreted based on the R value: <0.20 , negligible; 0.20 – 0.39 , weak; 0.40 – 0.59 , moderate; 0.60 – 0.79 , strong; and ≥ 0.80 , very strong.

3. Results

3.1. Drinking liquid consumption and pH

The volume and pH of the drinking solutions were measured to assess their potential influence on the pH of biological samples. No significant differences in liquid consumption were observed between the groups, whether the rats received DW or 3% DSS solution (Figure 2A). Similarly, the pH values of each liquid showed minimal variation (Figure 2B).

3.2. Changes in body weight and disease activity index scores

Body weight and DAI scores were recorded daily throughout the 10-day experimental period. Rats in the N group exhibited a steady increase in body weight, whereas the DSS group showed a marked decrease in body weight beginning on day 3 (Figure 3A). In addition, rats in the DSS + ASA group gained weight until day 5, but gradually lost weight thereafter (Figure 3A). DAI scores, assessed according to established criteria²⁵, gradually increased in the DSS group starting on day 4, whereas those in the DSS + ASA group exhibited a modest increase from day 6 onward (Figure 3B).

3.3. Changes in rat colon lengths

Gross examination revealed that colons from the N group appeared to maintain their normal length, whereas colons from the DSS group and the DSS + ASA group appeared slightly shortened (Figure 4A). However, quantitative analysis of mean colon lengths showed no significant differences between the three groups (Figure 4B).

3.4. Microscopic observation and evaluation of colonic inflammation

Histological analysis was performed to assess tissue damage and neutrophil infiltration in the colon. In the N group, normal colonic mucosa was observed, whereas the DSS group exhibited marked disruption of the epithelial structure along with extensive infiltration of immune cells (Figure 5A). On the contrary, H&E staining of colonic tissue in the DSS + ASA group suggested relatively mild mucosal damage (Figure 5A). NE, a marker of neutrophil activation, was detected via DAB immunostaining. NE-positive areas were prominent in the colonic tissue of the DSS group but were minimal in the N group and the DSS + ASA group (Figure 5B). HIS was evaluated on day 10 according to established criteria.²⁷ Rats in the DSS group showed increased HIS, whereas lower values were observed in the DSS + ASA groups (Figure 5C). Semi-quantitative analysis demonstrated that the NE-positive area (% positive area)

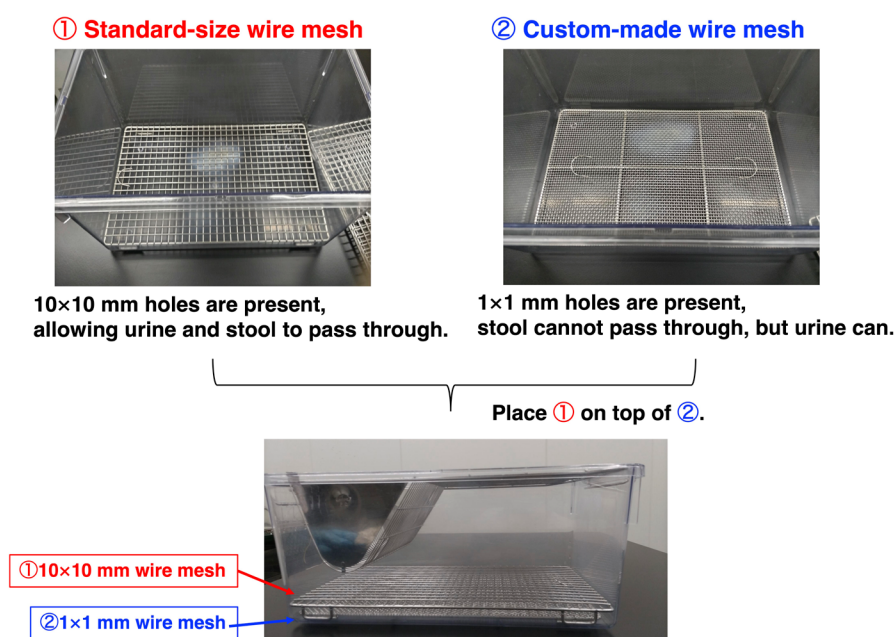


Figure 1. Installation of wire mesh for rat housing. A standard wire mesh with 10 × 10 mm openings was placed over a custom-made wire mesh with 1 × 1 mm openings, allowing for the separate collection of stool retained on the upper mesh and urine collected beneath it.

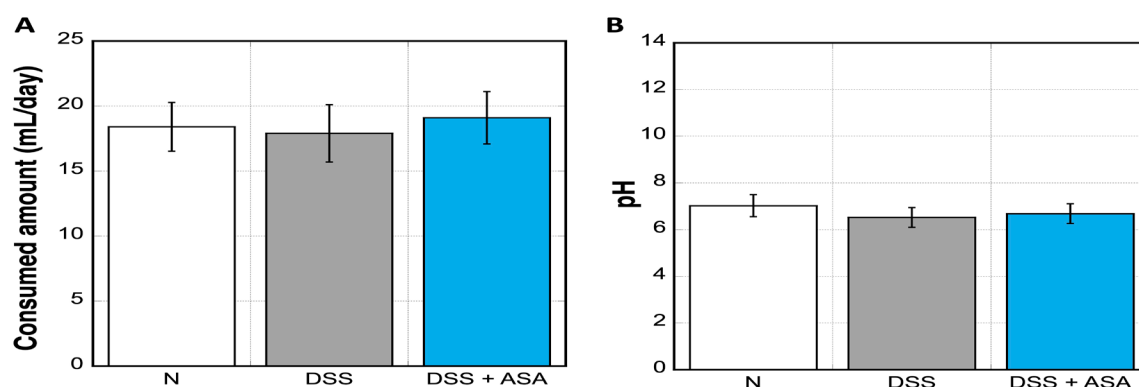


Figure 2. Consumption of drinking liquids and their pH values. (A) Daily liquid intake (mL/day). The N group received distilled water (DW) for drinking, whereas the DSS and DSS + ASA groups received a 3% dextran sulfate sodium (DSS) solution as drinking liquid. (B) pH values of DW and 3% DSS solution. For each liquid, measurements were performed six times on different days. The results are presented as mean ± standard deviation. Notes: The N group (white): normal rats without DSS exposure; The DSS group (gray): 3%DSS-induced colitis rats; The DSS + ASA group (light blue): 3%DSS-induced colitis rats with 100 mg/kg/day 5-aminosalicylic acid; $n = 8$ per group; No significant differences were observed among groups in liquid consumption and pH.

was significantly higher in the DSS group compared to the N group (Figure 5D). In contrast, the NE-positive area in the DSS + ASA group was comparable to that of the N group (Figure 5D).

3.5. Carbonic anhydrase-II expression in colonic tissues

Carbonic anhydrase-II expression in colonic tissues

was evaluated using DAB immunostaining. In the DAB images, CA-II immunoreactivity was readily observed in the DSS group, whereas staining was less prominent in the N and DSS + ASA groups (Figure 6A). Semi-quantitative image analysis of DAB signals, expressed as the percentage of CA-II-positive area under identical acquisition and thresholding conditions, showed a significant increase in the DSS group compared to the N and DSS + ASA groups (Figure 6B).

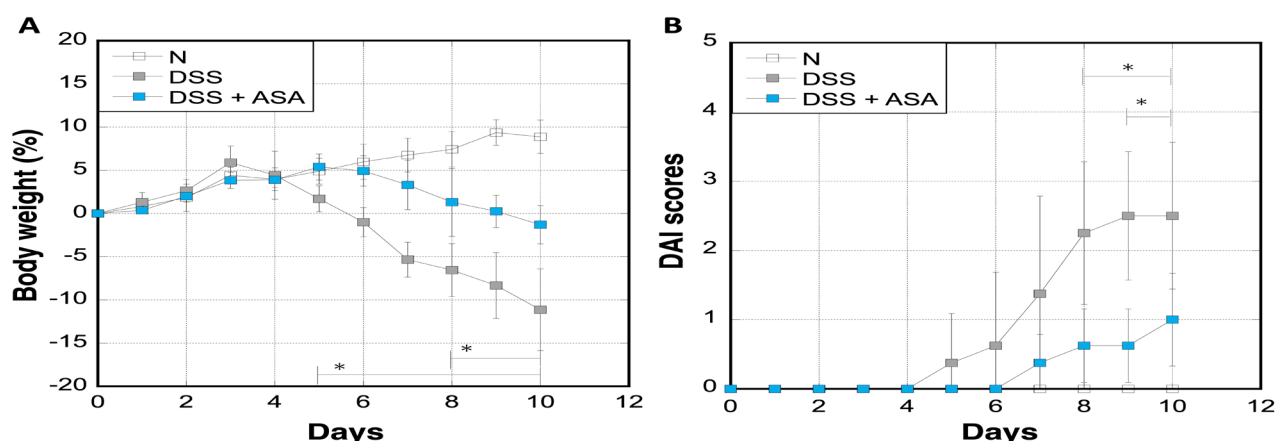


Figure 3. Changes in body weight and disease activity index (DAI) scores. (A) Percentage change in body weight relative to day 0, which was set as the baseline (0%). (B) DAI scores assessed according to previously established criteria.²⁵

Notes: Data are presented as mean $\pm$ standard deviation; * $p < 0.05$ (compared to N group); $n = 8$ per group.

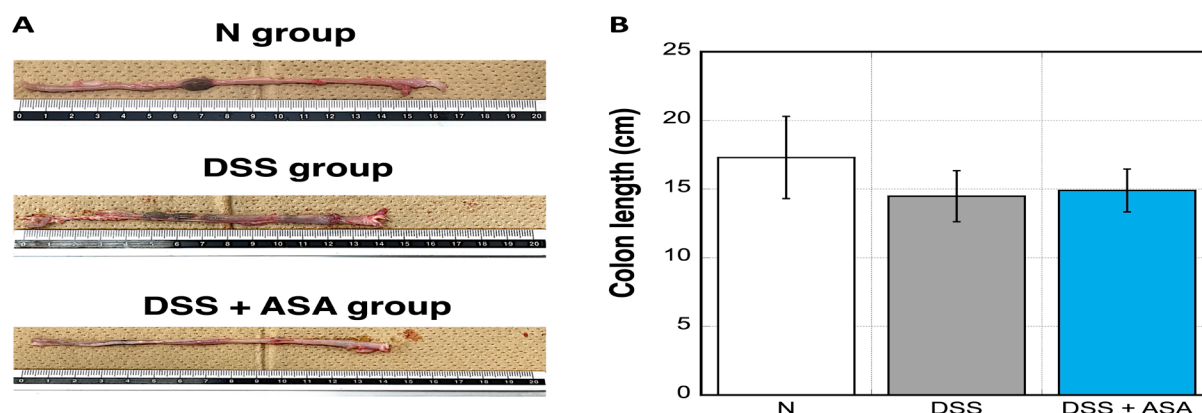


Figure 4. Changes in rat colon length. (A) Representative images of colon tissues from the N group (upper panel), the DSS group (center panel), and the DSS + ASA group (lower panel). (B) Quantitative measurement of colon length (cm) in the N group, DSS group, and DSS + ASA group.

Notes: Data are presented as mean $\pm$ standard deviation; $n = 8$ per group; No significant differences were observed among groups in colon length.

3.6. pH measurement results of colon tissue and serum

pH was measured in three distinct regions of the large intestine (Rec, Mid, and Pro), but no significant differences were observed between the three groups (Figure 7A). Similarly, serum pH values did not differ significantly between the groups (Figure 7B).

3.7. Fluctuations in urine pH

Urine pH was monitored daily over the 10-day experimental period, and average values were calculated. Throughout most of the study, the DSS group exhibited lower urinary pH compared to the N group, with several days showing statistically significant differences (Figure 8A). Meanwhile, the urine pH in the DSS + ASA group was comparable to that in the N group until day 7, but gradually decreased

thereafter and was significantly lower by day 10 (Figure 8A). The overall mean urine pH was significantly lower in the DSS group than in the N group and the DSS + ASA group (Figure 8B).

3.8. Fluctuations in stool pH

Stool pH was monitored daily over the 10-day experimental period, and average values were calculated. Stool pH decreased significantly during the early phase of the experiment (days 1–3) and subsequently returned to levels comparable to those in the N group (Figure 9A). In contrast, stool pH in the DSS + ASA group showed a gradual decrease from day 6 onward (Figure 9A). Nevertheless, the mean stool pH over the entire period was significantly lower in the DSS group compared to the other groups (Figure 9B).

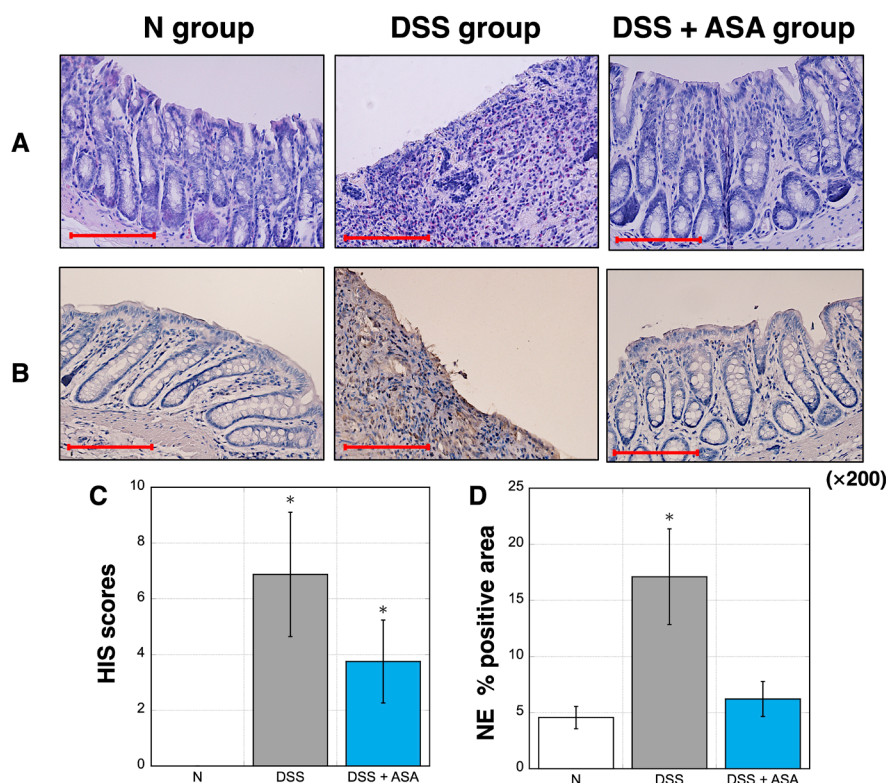


Figure 5. Histological assessment of colonic tissues in rats. (A) Representative hematoxylin and eosin (H&E)-stained images of colonic tissues from the N group (left), the DSS group (center), and the DSS + ASA group (right). Red scale bar: 200 μ m; magnification: 200 $\times$. (B) Representative 3,3'-diaminobenzidine (DAB) immunostaining images showing activated neutrophils in colonic tissues from the N group (left), the DSS group (center), and the DSS + ASA group (right). Scale bar: 200 μ m; magnification: 200 $\times$. (C) Histological severity (HIS) scores on day 10 after the start of the experiment, assessed according to the criteria.²⁷ (D) Semi-quantitative analysis of neutrophil elastase (NE) signals expressed as NE-positive area based on DAB immunostaining images. Notes: Data are presented as mean $\pm$ standard deviation; * $p < 0.05$ (N group vs. DSS group); $n = 8$ per group.

3.9. Correlation analyses between disease indices and excreta pH

To further evaluate whether excreta pH reflects disease severity, we examined the associations between pH values and disease activity indices using correlation analyses. In [Figure 10A](#) and [10B](#), neither urine pH nor stool pH showed a meaningful correlation with DAI scores ($R = 0.045$ and 0.144 , respectively). Similarly, in [Figure 10C](#) and [10D](#), no significant correlations were observed between HIS scores and urine or stool pH ($R = 0.141$ and 0.056 , respectively).

3.10. Clinical chemistry parameters in rat serum

Twenty clinical chemistry parameters were measured in the serum of experimental rats. ALB level, an indicator of nutritional status, was lower in the DSS group compared to the other groups ([Table 1](#)). Renal function markers, including BUN and CRE, were more favorable in the N group and the DSS + ASA group than in the DSS group. Liver function indicators, including AST, ALT, and LDH,

generally showed better values in the N group ([Table 1](#)). Fe, an indicator of anemia, was reduced in the DSS group. Lipid-related parameters, including T-CHO, TG, and HDL-C, were slightly elevated in the DSS group and the DSS + ASA group. Additionally, LA, which contributes to body fluid acidification, was slightly higher in the DSS and DSS + ASA groups, whereas PA was higher in the N group ([Table 1](#)).

4. Discussion

In this study, we conducted a detailed analysis of pH fluctuations in biological samples from UC model rats. Although previous reports have indicated that colonic pH in patients with UC is significantly lower than normal^{19,20}, there has been limited evidence regarding whether such pH changes are reflected in other biological samples. Notably, locally elevated pH resulting from increased glycolysis and lactic acid production has been observed not only in UC but also in cancerous lesions²⁶, suggesting that these metabolic changes could influence pH in biological

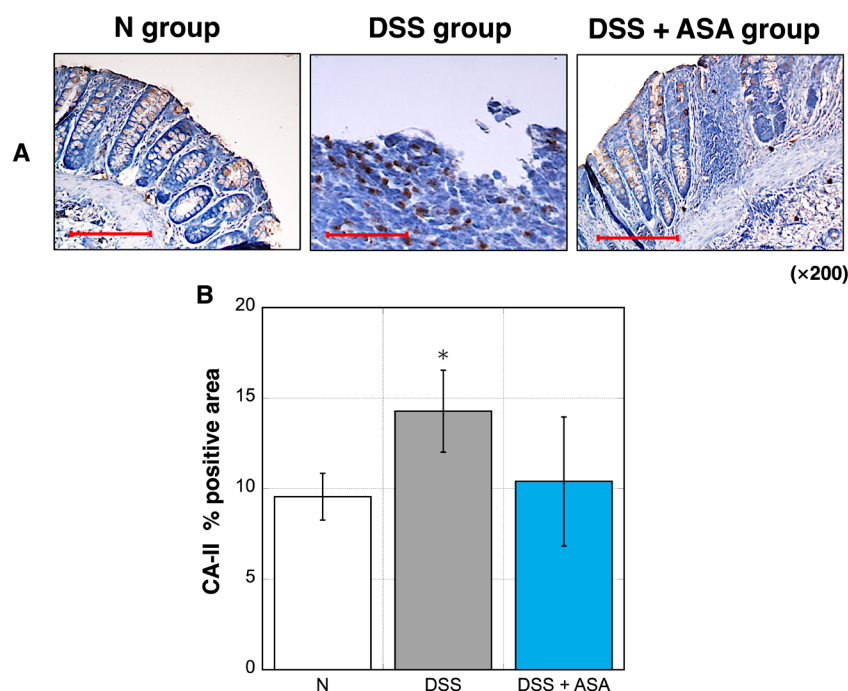


Figure 6. Analysis of type II carbonic anhydrase (CA-II) expression levels in rat colonic tissues. (A) Representative 3,3'-diaminobenzidine (DAB) immunostaining images showing CA-II in colonic tissues from the N group (left), the DSS group (center), and the DSS + ASA group (right). Red scale bar: 200 μ m; magnification: 200 $\times$. (B) Semi-quantitative analysis of CA-II signals expressed as %CA-II-positive area based on DAB immunostaining images. Notes: Data are presented as mean $\pm$ standard deviation; * $p < 0.05$ (N group vs. DSS group); $n = 8$ per group.

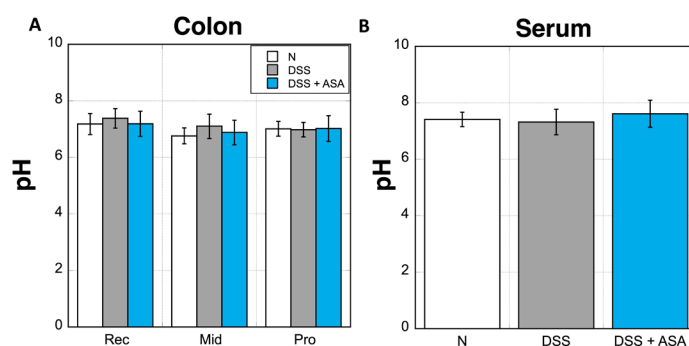


Figure 7. Average pH values of the colon and serum. (A) pH values of the three regions of the large intestine: rectal (Rec), middle (Mid), and proximal (Pro). (B) Serum pH values.

Notes: Data are presented as mean $\pm$ standard deviation; No significant differences were observed among groups in pH values of the colon and serum; $n = 8$ per group.

samples from UC patients. However, a comprehensive collection of multiple biological samples from UC patients is challenging, making animal studies a practical first step to gather supporting evidence.

First, the pH difference between DW and 3%DSS was insignificant, and the solution remained essentially neutral (Figure 2B). Given that fluid intake was comparable between the groups (Figure 2A), acidification of biological

samples due to consumption of the drinking solution was unlikely. The DSS group exhibited significant weight loss and elevated DAI scores, indicating that daily ingestion of 3% DSS effectively induced and exacerbated experimental colitis (Figure 3). Although mean colon length did not differ significantly, slight shortening in the DSS group suggested intestinal mucosal atrophy (Figure 4). Histological examination confirmed typical

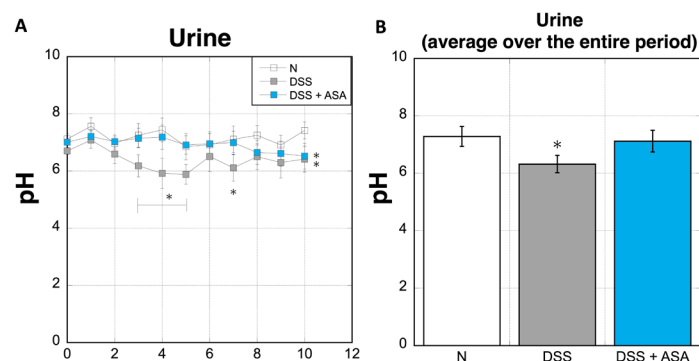


Figure 8. Urine pH fluctuations. (A) Daily urine pH values over the 10-day experimental period. (B) Average urine pH for the entire study period. Notes: Data are presented as mean $\pm$ standard deviation; * $p < 0.05$ (compared to N group); $n = 8$ per group.

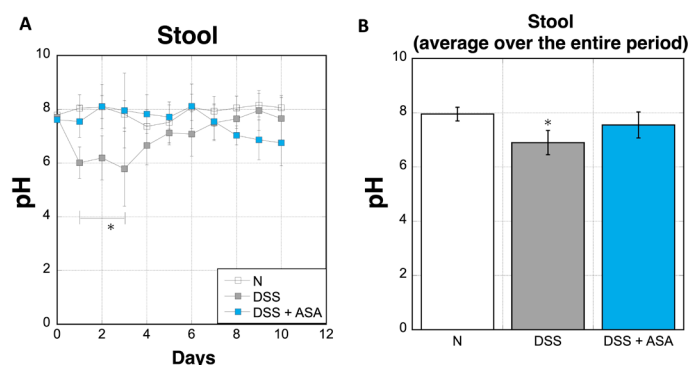


Figure 9. Stool pH fluctuations. (A) Daily stool pH values over the 10-day experimental period. (B) Average stool pH for the entire study period. Notes: Data are presented as mean $\pm$ standard deviation; * $p < 0.05$ (N group vs. DSS group); $n = 8$ per group.

UC pathology in rats of the DSS group, including loss of goblet cells, epithelial desquamation, and prominent neutrophil infiltration (Figure 5). In addition to the DSS group, we incorporated a treatment group (DSS + ASA) to assess whether pH fluctuations in biological samples are modulated by therapeutic intervention. Histologically, colonic tissues from the DSS + ASA group showed relatively mild mucosal damage compared to the DSS group, and NE immunoreactivity was minimal (Figure 5). Consistently, semi-quantitative image analysis demonstrated that the NE-positive area in the DSS + ASA group was comparable to that in the N group, supporting that 5-ASA attenuated DSS-induced inflammatory changes, particularly those associated with neutrophil activation (Figure 5D). Collectively, these findings demonstrate the onset and progression of experimental UC in the DSS group and the therapeutic effect on inflammation in the DSS + ASA group, supporting the rationale for comparing pH values in biological samples from each group.

We focused on CA-II because it is one of the most abundantly expressed CA isoforms in the gastrointestinal tract, and we anticipated that its expression would exhibit relatively large inflammation-associated changes.²³ Interestingly, CA-II expression was increased in the DSS group but not in the DSS + ASA group (Figure 6). CA-II catalyzes the rapid interconversion of CO_2 and $\text{HCO}_3^-/\text{H}^+$ and is broadly expressed in the gastrointestinal tract, where it supports epithelial acid-base handling and provides bicarbonate for transepithelial transport processes.²³ In the colon, bicarbonate transport is considered essential for maintaining epithelial and luminal pH homeostasis, including regulation of the mucosal surface pH that protects barrier function and mucus properties.²³ In this context, the increase in CA-II immunoreactivity observed in the DSS group may represent a compensatory epithelial response to inflammatory stress and acid-base disturbance, aimed at sustaining intracellular buffering and bicarbonate supply during acute mucosal injury (Figure 6). Notably, despite the inflammation-associated change in CA-II

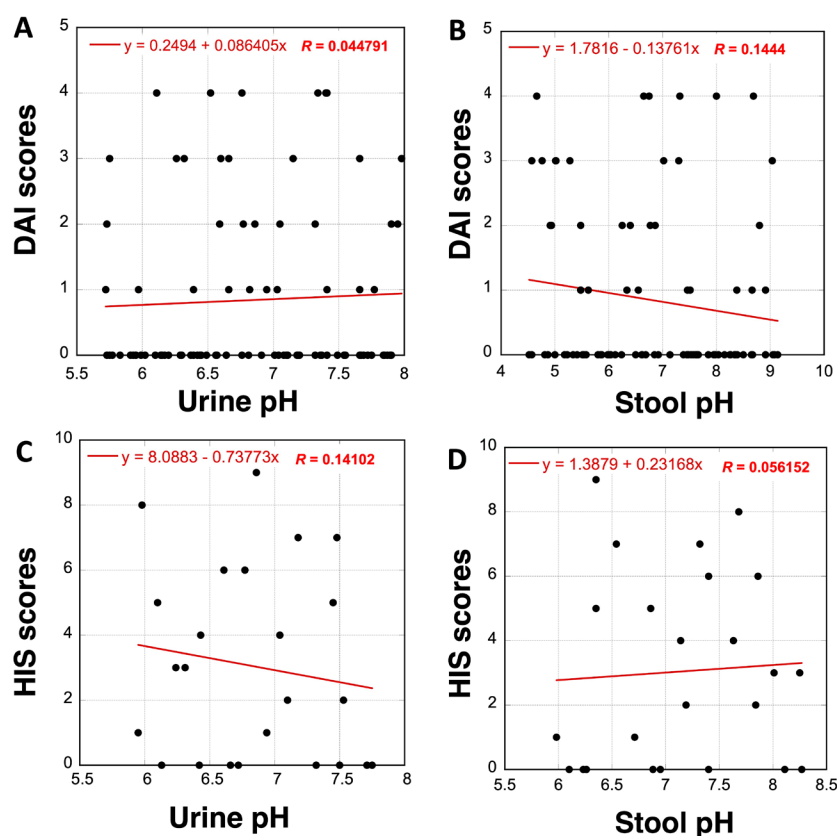


Figure 10. Correlation analyses between disease indices and excreta pH. Scatter plots showing the relationships (A) between disease activity index (DAI) scores and urine pH and (B) between DAI scores and stool pH, using pooled measurements across the 10-day experimental period (total $n = 88$). Scatter plots showing the relationships (C) between histological severity (HIS) scores and urine pH and (D) between HIS scores and stool pH, using measurements obtained on day 10 only (total $n = 24$). Each point represents an individual measurement. Spearman's rank correlation coefficient (R) is shown in each panel. No meaningful correlations were observed across these comparisons.

immunoreactivity, we did not detect a clear difference in colonic tissue pH among groups (Figure 7A). This apparent discrepancy from previous clinical observations of markedly low intraluminal colonic pH in active UC may be explained by methodological and biological factors.^{19,28} First, tissue and blood compartments are strongly buffered and tightly regulated; therefore, bulk tissue pH measured at a single time point may remain stable even when localized pH at the mucosal surface or within the lumen is altered. Second, pH within the colon is spatially heterogeneous and can vary across the luminal, mucus, and epithelial microenvironments; thus, a single-point measurement on freshly excised tissue may not capture focal or layer-specific acidification. Third, prior studies in humans often used capsule-type or intraluminal pH sensors, which interrogate luminal pH more directly and may be more sensitive than the compact pH meter approach used here.^{19,28} Finally, the 10-day acute DSS model and the timing of sampling may preferentially capture early-stage metabolic shifts in

excreta while allowing tissue and systemic pH homeostasis to be maintained. Consistent with this, serum pH also showed little difference between groups (Figure 7B), suggesting that systemic acid-base regulation prevented overt acidemia under the present experimental conditions. Reports of serum acidification in UC are rare; in severe cases, dehydration, electrolyte imbalance, and elevated lactate levels may contribute to decreased serum pH.²⁹ Consistent with this, clinical chemistry results indicated a slight increase in serum LA in the DSS group, which could potentially lower pH over time (Table 1). Despite elevated TP, lower ALB levels in the DSS group suggest increased globulin production associated with immune activation (Table 1). Additionally, indicators of impaired liver and kidney function and anemia progression imply that extending the experimental period may further exacerbate UC. These considerations suggest that a longer experimental period may reveal clearer differences in colonic and serum pH.

In the DSS group, urine pH decreased most markedly on days 3–5 and remained lower than that of the other groups thereafter (Figure 8A). Although the normal range for urine pH is broad, approximately 4.6–8.0, the values observed in UC rats were not extremely low. Urinary pH can fluctuate due to lifestyle factors, such as diet and exercise, as well as physiological variables. However, in controlled animal studies, these influences are minimal, suggesting that the observed changes reflect the pathological effects of UC. Therefore, rather than comparing against a standard reference, a decrease in an individual's urine pH relative to their normal daily levels may indicate acute colitis. Since urine pH did not correlate with increasing DAI scores, it appears to be a marker of early disease onset rather than of disease severity (Figures 3B and 8A). Regarding urine pH, the DSS + ASA group showed a distinct temporal pattern compared to the DSS group. Urine pH in the DSS + ASA group remained comparable to that of the N group until approximately day 7, but then gradually decreased and became significantly lower by day 10 (Figure 8A). We interpret this delayed change as consistent with a 5-ASA treatment effect that attenuated and slowed the progression of DSS-induced colitis, thereby postponing the downstream systemic acid-base alterations reflected in urine pH compared with the DSS group.

Stool pH (day 1–3) in the DSS group acidified earlier than urine pH (day 3–5) and gradually returned to levels similar to those in the N group (Figure 9A). This temporal disconnect is biologically plausible because these readouts capture different layers of the disease process. Excreta pH likely reflects rapid, upstream changes in the luminal environment and host acid-base handling that occur soon after barrier perturbation (e.g., shifts in microbial fermentation and organic acid load and/or transient alterations in epithelial bicarbonate secretion), while the DAI is a composite clinical index that increases once inflammation and mucosal injury progress to a threshold that produces overt symptoms. Therefore, early pH acidification may be more indicative of disease onset or early-phase activity than of subsequent progression in severity. In healthy adults, stool pH averages approximately 6.6 ± 0.85 , indicating slight acidity³⁰; however, reference values for rat stool are lacking. Compared to human reference values, the DSS group exhibited a modest decrease in stool pH, which may reflect UC onset. Similar to urine pH, stool pH showed little correlation with DAI scores (Figures 3B and 9B). Stool pH in the DSS + ASA group tended to decrease in the latter half of the experimental period (Figure 9A). As with urine pH, we interpret this delayed shift as reflecting the 5-ASA-mediated attenuation of DSS-induced colitis progression, resulting in a slower

emergence of inflammation-associated luminal and metabolic changes and, consequently, a later change in stool pH compared to the DSS group. These findings also imply that pH fluctuations in excreta are not solely determined by the extent of overt neutrophil-driven inflammation, and may also be influenced by treatment-related factors, including altered intestinal metabolism, shifts in gut microbial fermentation, and/or systemic metabolic effects. Although excreta pH values clearly decreased during the early phase of DSS exposure, the correlation analyses in Figure 10 showed negligible associations between urine/stool pH and conventional indices of disease severity (DAI and HIS). This lack of correlation suggests that excreta pH does not scale linearly with the magnitude of clinical symptoms or histological injury. Rather, the temporal profile observed in this study indicates that urine and stool pH tend to acidify before overt worsening of UC indices, supporting the concept that pH changes may reflect early-stage pathophysiological shifts that precede prominent tissue damage and symptomatic exacerbation.

While urine pH is commonly measured in clinical laboratories, stool pH is rarely assessed due to the practical challenges of sample collection. Consequently, acidification of urine and stool may serve as indicators of UC onset or early-stage disease rather than disease severity. We should avoid making predictive or diagnostic claims in a clinical sense: the present study was not designed to assess diagnostic performance (e.g., sensitivity/specificity or receiver operating characteristic [ROC] analyses) or to determine whether pH changes precede all other biological manifestations. Instead, our data support the concept that serial monitoring of excreta pH provides a simple, noninvasive readout that can sensitively capture early-stage disease-related alterations during experimental colitis. Future studies are needed to investigate the mechanisms underlying urine and stool acidification, which would further clarify their relationship to UC pathophysiology.

Our study has several limitations. First, this study used a 10-day acute DSS colitis model; therefore, our findings primarily reflect early-stage inflammatory responses and may not fully represent chronic and relapsing clinical UC. Given the strong buffering capacity and homeostatic regulation of tissue and blood pH, a longer observation period and/or chronic or relapsing DSS models may be required to detect stage-dependent changes in colonic and serum pH. Future studies that extend the duration and incorporate chronic/relapsing models will enable systematic evaluation of pH dynamics across disease progression and remission. Second, our pH measurements relied on a compact pH meter and single-point assessments for colonic tissue and serum, which may be less sensitive

Table 1. Clinical chemistry parameters in rat serum for each group

Parameters	N group	DSS group	DSS + ASA group
TP (g/dL)	7.2	7.1	6.9
ALB (g/dL)	5.1	3.9	4.1
GLU (mg/dL)	138	104	99
BUN (mg/dL)	22.1	48.3	29.5
CRE (mg/dL)	0.31	0.51	0.39
T-BIL (mg/dL)	0.02	0.02	0.02
UA (mg/dL)	2.9	3.7	3.1
Fe (µg/dL)	197	116	156
AST (IU/L)	138	489	411
ALT (IU/L)	45	188	101
ALP (IFCC) (IU/L)	178	272	278
LDH (IFCC) (IU/L)	1,020	3,182	2,710
CK (IU/L)	1,039	1,617	1,012
ChE (IU/L)	18	8	18
T-CHO (mg/dL)	68	99	101
TG (mg/dL)	62	98	105
LDL-C (mg/dL)	7	16	18
HDL-C (mg/dL)	29	22	31
PA (mg/dL)	2.14	1.02	1.11
LA (mg/dL)	102.2	110.2	107.6

Abbreviations: ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; ChE: Cholinesterase; CK: Creatine kinase; CRE: Creatinine; Fe: Iron; GLU: Glucose; HDL-C: High-density lipoprotein-cholesterol; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine; LA: Lactic acid; LDH: Lactate dehydrogenase; LDL-C: Low-density lipoprotein cholesterol; PA: Pyruvic acid; T-BIL: Total bilirubin; T-CHO: Total cholesterol; TG: Triglycerides; TP: Total protein; UA: Uric acid.

than other approaches, and methodological optimization (e.g., measurement timing, handling, and matrix-specific procedures) is warranted. In clinical practice, fecal calprotectin (S100A8/A9) and other established biomarkers are widely used to assess intestinal inflammation and, in various settings, correlate with endoscopic or histological activity.^{11–13} In contrast, the present study was not designed to benchmark the diagnostic performance of pH against these markers (e.g., sensitivity/specificity or ROC analyses). Rather, our data suggest that excreta pH may provide complementary information as a rapid, low-cost, and repeatedly measurable readout that tends to change early in the disease course, whereas its utility as a severity index appears limited (Figure 10). Third, while the DSS + ASA group suggested that therapeutic intervention

can delay pH fluctuations, medication in clinical UC is adjusted dynamically according to disease status. Given physiological and pathophysiological differences between animal models and patients, the observed pH trends should be interpreted as preclinical, hypothesis-generating findings. Future clinical studies are warranted to compare fecal and urinary pH among individuals with active UC, those in remission, and healthy controls, ideally using longitudinal sampling and concurrent assessment of established clinical markers to determine the translational applicability of excreta pH measurements. Finally, because mechanistic determinants of excreta pH (e.g., microbial fermentation, electrolyte balance, and renal acid–base handling) were not directly measured, future studies integrating these readouts are necessary

to clarify the biological basis of the observed pH shifts. Performing further analyses—integrating microbiome data (e.g., 16S rRNA sequencing/metagenomics) and metabolite measurements (e.g., SCFAs) with serial pH monitoring—will likely generate new evidence to elucidate the mechanisms underlying early pH changes.

From a practical standpoint, even if excreta pH does not provide robust severity grading, it may still have clinical utility as a simple adjunct readout. For example, serial urine and stool pH measurements could be used for low-burden, within-patient monitoring to detect early deviations from an individual baseline that may precede symptomatic worsening or relapse. In addition, because pH testing is inexpensive and can be repeated frequently, it could serve as a screening/triage signal to prompt earlier confirmatory assessments (e.g., fecal calprotectin and/or endoscopy) when symptoms are nonspecific. Thus, excreta pH is best considered a complementary parameter for early detection and longitudinal monitoring rather than a standalone marker of disease severity.

Beyond UC, numerous intractable diseases are autoimmune or chronic inflammatory conditions that currently require long-term management, imposing burdens on both patients and healthcare providers. Laboratory tests based on pH measurement could have broad applicability in chronic inflammatory diseases, offering a rapid, simple, and minimally invasive diagnostic approach. Such tests have the potential to reduce patient burden and support clinical decision-making. Future studies should carefully assess whether the pH fluctuations observed in animal models can also be detected in humans, particularly in UC patients. The novel UC severity screening system proposed here, which utilizes pH fluctuations in biological samples, holds promise for further development and potential clinical application.

5. Conclusion

Using a DSS-induced rat model of UC, we demonstrated that pH fluctuations in excreta can be detected noninvasively during the disease course. While colonic tissue and serum pH remained stable, both urine and stool pH exhibited decreases during the experimental period, with distinct temporal patterns across groups. Importantly, inclusion of the DSS + ASA treatment arm indicated that these pH changes can shift in timing under anti-inflammatory intervention, suggesting that excreta pH may reflect early and/or evolving pathophysiological alterations rather than simply mirroring conventional severity indices. Together, our findings support stool and urinary pH as simple, readily quantifiable candidates for early screening and longitudinal monitoring of UC activity, and warrant

further studies to clarify the mechanisms driving these pH changes and to evaluate their responsiveness to therapy in longer-term and clinical settings.

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Conflict of interest

The authors declare that they have no competing interests.

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Investigation: Kohki Okada, Ruku Sasaki

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Visualization: All authors

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Ethics approval and consent to participate

The animal experiments complied with the Animal Research: Reporting of *In Vivo* Experiments guidelines and were approved by the Animal Care and Use Committee of Kyoto Tachibana University (permission number: 22-07).

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available upon reasonable request from the corresponding author.

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