## Contents

**EDITORIAL**

2618 Exosomal microRNAs in hepatocellular carcinoma, rising research filed in hepatocellular carcinoma  
*Zheng BH, Ni XJ, Liu HB*

2621 Transjugular intrahepatic portosystemic shunt for recompensating decompensated cirrhosis?  
*Karagiannakis DS*

2624 Metadherin-driven promotion of cancer stem cell phenotypes and its effect on immunity in hepatocellular carcinoma  
*Todorović N, Amedei A*

2629 Validation of adherence prediction system for lifestyle interventions in nonalcoholic fatty liver disease  
*Chisthi MM*

2633 Impact of lifestyle interventions on pathogenesis of nonalcoholic fatty liver disease  
*Ezzat WM*

**REVIEW**

2638 Latest insights into the global epidemiological features, screening, early diagnosis and prognosis prediction of esophageal squamous cell carcinoma  
*Zhao YX, Zhao HP, Zhao MY, Yu Y, Qi X, Wang JH, Lv J*

**ORIGINAL ARTICLE**

**Retrospective Cohort Study**

2657 Development and validation of a new prognostic model for patients with acute-on-chronic liver failure in intensive care unit  
*Zhu ZY, Huang XH, Jiang HQ, Liu L*

**Observational Study**

2677 Excess non-COVID-19-related mortality among inflammatory bowel disease decedents during the COVID-19 pandemic  

**Basic Study**

2689 Kinesin 26B modulates M2 polarization of macrophage by activating cancer-associated fibroblasts to aggravate gastric cancer occurrence and metastasis  
*Huang LM, Zhang MJ*
2709  Hydrogen-rich water alleviates constipation by attenuating oxidative stress through the sirtuin1/nuclear factor-erythroid-2-related factor 2/heme oxygenase-1 signaling pathway

LETTER TO THE EDITOR

2726  Colorectal cancer screening: The value of early detection and modern challenges
Li J, Li ZP, Ruan WJ, Wang W

2731  Downstaging strategies for unresectable hepatocellular carcinoma
Karachaliou GS, Dimitrokallis N, Moris DP
ABOUT COVER
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Basic Study

Hydrogen-rich water alleviates constipation by attenuating oxidative stress through the sirtuin1/nuclear factor-erythroid-2-related factor 2/heme oxygenase-1 signaling pathway


Abstract

BACKGROUND
Constipation, a highly prevalent functional gastrointestinal disorder, induces a significant burden on the quality of patients' life and is associated with substantial healthcare expenditures. Therefore, identifying efficient therapeutic modalities for constipation is of paramount importance. Oxidative stress is a pivotal contributor...
to colonic dysmotility and is the underlying pathology responsible for constipation symptoms. Consequently, we postulate that hydrogen therapy, an emerging and promising intervention, can serve as a safe and efficacious treatment for constipation.

**AIM**
To determine whether hydrogen-rich water (HRW) alleviates constipation and its potential mechanism.

**METHODS**
Constipation models were established by orally loperamide to Sprague-Dawley rats. Rats freely consumed HRW, and were recorded their 24 h total stool weight, fecal water content, and charcoal propulsion rate. Fecal samples were subjected to 16S rDNA gene sequencing. Serum non-targeted metabolomic analysis, malondialdehyde, and superoxide dismutase levels were determined. Colonic tissues were stained with hematoxylin and eosin, Alcian blue-periodic acid-Schiff, reactive oxygen species (ROS) immunofluorescence, and immunohistochemistry for cell growth factor receptor kit (c-kit), PGP 9.5, sirtuin1 (SIRT1), nuclear factor-erythroid-2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1). Quantitative real-time PCR and western blot analysis were conducted to determine the expression level of SIRT1, Nrf2 and HO-1. A rescue experiment was conducted by intraperitoneally injecting the SIRT1 inhibitor, EX527, into constipated rats. NCM460 cells were induced with H₂O₂ and treated with the metabolites to evaluate ROS and SIRT1 expression.

**RESULTS**
HRW alleviated constipation symptoms by improving the total amount of stool over 24 h, fecal water content, charcoal propulsion rate, thickness of the intestinal mucus layer, c-kit expression, and the number of intestinal neurons. HRW modulated intestinal microbiota imbalance and abnormalities in serum metabolism. HRW could also reduce intestinal oxidative stress through the SIRT1/Nrf2/HO-1 signaling pathway. This regulatory effect on oxidative stress was confirmed via an intraperitoneal injection of a SIRT1 inhibitor to constipated rats. The serum metabolites, β-leucine (β-Leu) and traumatic acid, were also found to attenuate H₂O₂-induced oxidative stress in NCM460 cells by up-regulating SIRT1.

**CONCLUSION**
HRW attenuates constipation-associated intestinal oxidative stress via SIRT1/Nrf2/HO-1 signaling pathway, modulating gut microbiota and serum metabolites. β-Leu and traumatic acid are potential metabolites that upregulate SIRT1 expression and reduce oxidative stress.

**Key Words:** Hydrogen-rich water; Constipation; Sirtuin1; Oxidative stress; Gut microbiota; Serum metabolites

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**INTRODUCTION**
Constipation is a highly prevalent functional gastrointestinal disorder characterized by infrequent stools (fewer than 3 bowel movements per week), dry and hard stools, or difficulty in passing stools, which may significantly affect the quality of life and impose a remarkable economic burden[1]. Currently, constipation is initially managed through the administration of dietary fiber and suitable stimulants or osmotic laxatives, with subsequent consideration of gut secretagogues or prokinetic agents, if necessary[2]. However, these approaches have limitations, such as restricted long-term utility, high recurrence rates, challenges in achieving complete resolution, and potential impacts on the physical and mental well-being of patients[3]. Therefore, identifying suitable intervention measures is a top priority for the treatment
study on the treatment of constipation

Hydrogen therapy is an emerging and promising strategy that involves the use of molecular hydrogen as a new, safe, and effective therapeutic agent that exhibits remarkable therapeutic effects in many oxidative stress-related diseases owing to its bio-reductivity and homeostatic regulation abilities[4]. Hydrogen-rich water (HRW), a specialized type of water containing a precise concentration of dissolved hydrogen molecules (H₂), has been reported to possess potent antioxidant, anti-inflammatory, and anti-apoptotic properties[5]. Oxidative stress is a key contributor to colonic dysmotility and leads to constipation[6]. Antioxidants, such as inulin, play a beneficial role in improving chronic constipation symptoms[7]. Thus, in this study, HRW was explored as a potential treatment with various advantages for constipation.

Mammalian SirT1 (SIRT1) is an NAD+-dependent deacetylase that primarily regulates various physiological functions, including cellular stress response, energy metabolism and inflammatory response[8]. By promoting the nuclear translocation of nuclear factor-erythroid-2-related factor 2 (Nrf2), SIRT1 can enhance the cellular antioxidant capacity; however, Nrf2 can stimulate the transcriptional expression of heme oxygenase-1 (HO-1) to increase the intracellular antioxidant stress response. The combined effects of these three factors help maintain the oxidative balance in cells and protect them from oxidative stress damage via the inhibition of reactive oxygen species (ROS) production[9]. HRW was found to alleviate other diseases such as liver/renal injury and amyloid β-induced cytotoxicity, by reducing oxidative stress via SIRT1 regulation[10-12]. Thus, we hypothesized that HRW can relieve constipation via a mechanism involving the SIRT1/Nrf2/HO-1 signaling pathway to alleviate oxidative stress.

To test this hypothesis, we confirmed the activity of HRW in rats with loperamide-induced constipation (Lop). Thereafter, 16S rDNA gene sequencing and serum metabolomic analyses were conducted, and a SIRT1 inhibitor was used to determine whether HRW could relieve constipation symptoms and elevate oxidative stress levels in model rats. Finally, the serum metabolites, β-leucine (β-Leu) and traumatic acid, were screened to determine their ability to increase SIRT1 expression and reduce oxidative stress level in NCM460 cells. Overview of the workflow is shown in Figure 1.

**MATERIALS AND METHODS**

**Materials and chemicals**

HRW (H₂ > 3.0 ppm) was provided by the Bauer Technology Company, Ltd. (Nanjing, China). Loperamide and β-Leu were purchased from Yuanye Biotechnology (Shanghai, China). Traumatic acid was purchased from Aladdin Biotechnology (Shanghai, China). EX527 (HY-15452) was purchased from MCE (NJ, United States). The MitoSOX staining kit (M36008, Invitrogen) was purchased from Thermo Fisher Scientific (Waltham, MA, United States). ROS assay kit (S0033S), lipid peroxidation malondialdehyde (MDA) assay kit (S0131S), and total superoxide dismutase (SOD) assay kit with WST-8 (S0101S) were purchased from Beyotime Biotechnology (Nanjing, China). Primary antibodies against cell marker. The carbon propulsion rate was calculated as follows: (Transit length of carbon/full length of the small intestine) × 100%. Serum, small intestine, and colon samples from all rats were collected and stored at -80 °C for other analysis.

**Animal husbandry and experiment**

Eighteen male Sprague-Dawley (SD) rats (8-wk old) were obtained from Nanjing Junke Biological Engineering Co. Ltd (Jiangsu, China) and housed under specific pathogen-free conditions. The rats were housed under a controlled temperature of 22 ± 1 °C, a humidity level of 55% ± 5%, and a 12 h light-dark cycle throughout the experiment.

To establish a model of constipation, rats were administered loperamide, as described by Wang et al[13]. Following one week of acclimatization, rats were randomly divided into three experimental groups (n = 6): Normal (Nor), Lop, and HRW intervention. The process of animal experimentation is interpreted in Figure 2A. In the initial seven-day phase, the Nor group received clean water, while the Lop and HRW groups were orally administered 3 mg/kg/day loperamide. From day 8-14, the Nor and Lop groups were administered distilled water, while the HRW group received HRW. The total 24 h fecal output was recorded every other day, and the fecal water content was measured on day 7 and 14.

At the end of the 14-d animal study, a 12 h fasting period was carried out prior to the oral administration of 2 mL of activated carbon (5%). After 30 min, the rats were exsanguinated and separated to retrieve intact small intestine. The entire length of the small intestine, measured from the pylorus to the cecum, served as an indicator of the overall length, whereas the distance from the pylorus to the front edge of the carbon suspension indicated the transit extent of the carbon marker. The carbon propulsion rate was calculated as follows: (Transit length of carbon/full length of the small intestine) × 100%. Serum, small intestine, and colon samples from all rats were collected and stored at -80 °C for other analysis.

In a subsequent animal experiments, an additional group of rats was administered HRW and intraperitoneal injections of the SIRT1 inhibitor, EX527 (10 mg/kg qd)[14]. The remaining experimental procedures were performed as mentioned above.

**Histopathological examination and immunohistochemical staining**

A portion of the distal colon was fixed with 4% polyformaldehyde for histopathological examination. The fixed tissue was embedded in paraffin and sliced into 5 μm sections, and stained with hematoxylin and eosin (H&E) and Alcian blue-periodic acid-Schiff (AB-PAS). For immunohistochemical (IHC) staining, the slices were blocked with 5% goat serum, and stained with primary antibodies against c-kit (1:300), PGP 9.5 (1:5000), Nrf2 (1:500), HO-1 (1:1000), and SIRT1 (5 μg/mL).
Polymeric secondary antibodies coupled to horseradish peroxidase and diaminobenzidine were used to visualize the stained sections. The tissue specimens were counterstained with hematoxylin and examined using a light microscopy. ImageJ software was used for the analysis, and the average optical density of the images was calculated.

**Gut microbiota analysis**

Microbial DNA was extracted from colon samples using the PureLink™ genomic DNA kit (K182002; Thermo Fisher, Invitrogen, MA, United States). The DNA sample quality was assessed through agarose gel electrophoresis and quantification using a DNA detection kit (B17661; AAT Bioquest). The V3-V4 variable regions of the microbial DNA were expanded and sequenced on the Illumina NovaSeq platform using forward (5'-CCTAYGGGRBGCASCAG-3') and reverse (5'-GGACTACNNGGGTATCTAAT-3') primers. Statistical analyses and visualization of the sequencing data were refined to enhance the accuracy and clarity of the results. In particular, six samples from each experimental group were used to evaluate gut microbiota.

**Serum non-target metabolomics analysis**

Serum non-targeted metabolomics analysis was conducted using ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry. An ExionLC™ AD system coupled with a QTRAP™ system was employed for data
Figure 2 Hydrogen-rich water alleviates loperamide-induced constipation in rats. A: Flow chart of the establishment of the rat model and drug administration; B: Total feces excreted within 24 h; C: Fecal water content on day 7 and 14; D and E: Propulsion rate of carbon through the small intestine of rats; F: Hematoxylin and eosin staining, Alcian blue-periodic acid-Schiff staining, immunohistochemical staining of cell growth factor receptor kit (c-kit) and PGP 9.5; G: Thickness of the mucus layer; H: Mean optical density of c-kit; I: Number of enteric neurons. *P < 0.05 vs normal group (Nor), **P < 0.01 vs Nor, *P < 0.05 vs loperamide-induced constipation group (Lop). Scale bar: 100 μm. Nor: Normal group; Lop: Loperamide-induced constipation group; HRW: Hydrogen-rich water intervention group; H&E: Hematoxylin and eosin; AB-PAS: Alcian blue-periodic acid-Schiff; c-kit: Cell growth factor receptor kit.

acquisition. The samples were eluted on a Waters Acuity UPLC HSS T3 C18 column under the optimized conditions. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The UPLC elution conditions were optimized as follows: Isocratic elution at 5%–90% B (0–11 min), 90% B (11–12 min), 90%–5% B (12–12.1 min), and 5% B (12.1–14 min). The flow rate was 0.4 mL/min, the temperatures of the column was maintained at 40 °C, and the sample injection volume was 2 μL. The pre-processed data underwent principal component analysis, and metabolites were identified using the Human Metabolome Database. Differential metabolites were selected based on the screening criteria, variable importance in the projection (VIP) ≥ 1 and P < 0.05. Pathway analyses were performed using
Chen KD et al. Study on the treatment of constipation

the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

**Detection of ROS, MDA, and SOD in rats**

ROS levels in the colon were measured via MitoSOX staining (M36008; Thermo Fisher Scientific, Invitrogen, MA, United States). MitoSOX was excited by a laser at 510 nm and data were collected at the FSC, SSC, and 580 nm (FL2) channels. The levels of MDA and SOD in the serum or colon tissue of rats were determined using the lipid peroxidation MDA assay kit (S0131S) and total SOD assay kit with WST-8 (S0101S) procedure.

**Quantitative real-time PCR**

Total RNA was extracted from colon tissues using the fastpure cell/tissue total RNA isolation kit (RC112-01, Vazyme). The total RNA was reverse transcribed to cDNA utilizing reverse transcription kits (R302-01, Vazyme). qRT-PCR was performed using the SYBR Green qPCR master mix (Q121-02, Vazyme) and cDNA as the template. The Applied Biosystems™ 7500 Fast qPCR System (A30299; Thermo Fisher, Invitrogen, MA, United States) was used for qRT-PCR. The primer sequences for all target genes are listed in Table 1. Normalization of the fluorescence intensity was accomplished using GAPDH as an internal reference, and data analysis was performed using the 2^(-ΔΔCT) method.

**Western blot analysis**

The colon tissue was lysed using a lysis buffer including phosphatase inhibitor (1:100), protease inhibitor (1:1000), and phenylmethanesulfonylfluoride (PMSF, 1:100). The lysate was then centrifuged (4000 g, 4 °C, 10 min) and the supernatant was collected. The protein concentration in each sample was determined using the BCA approach. Following adjustment of the protein concentration and addition of the loading buffer, all samples were denatured by boiling in a metal bath at 100 °C for 10 min. Sample proteins were divided on 10% Vazyme premix gel and transferred onto polyvinylidene fluoride (PVDF) membranes. Following a 1 h blocking step, the PVDF membranes were incubated with primary antibodies overnight at 4 °C, washed four times, and incubated with the corresponding secondary antibodies for 2 h at room temperature. Western blot images were captured utilizing the Tanon 5200 imaging system and analyzed using ImageJ software.

**Statistics**

All data are expressed as mean ± SD. Statistical analyses were performed using the GraphPad Prism software (version 9). Differences between two groups were evaluated using a two-tailed t test, whereas differences among three groups were analyzed using one-way ANOVA with Tukey's multiple-comparison test. A P value of less than 0.05 was considered to indicate statistically significance. The statistical methods of this study were reviewed by Ao Wang from the Department of medical statistics, the Affiliated Hospital of Nanjing University of Chinese Medicine.

**RESULTS**

**HRW alleviates loperamide-induced constipation in rats**

In this study, the effect of HRW was explored using a Lop model. A flow chart of the study is provided in Figure 2A. After seven days, the 24 h fecal total weight and fecal water content were found to decrease, indicating successful model construction of 18 SD rats (n = 6/group; Figure 2B and C). Following ad libitum consumption of HRW for seven days, the total amount, water content of the 24 h feces, and the charcoal propulsion rate increased compared to those in the untreated model animals (Figure 2B-E). Based on H&E staining, HRW improved the colonic villi and mucosal integrity by reducing looseness and breakage, restoring ileal epithelial integrity, and increasing muscle layer thickness in constipated rats. AB-PAS staining revealed a decrease in mucus layer thickness in constipated rats, which was reversed by HRW. Intestinal cells of Cajal, which are responsible for digestive tract motility and are closely associated with constipation, exhibited increased expression of the mast/stem c-kit following HRW consumption[16]. In addition, IHC staining of PGP9.5, a neurons marker[17], indicated that HRW increased the neuron count in constipated rats (Figure 2F-I). Overall, HRW can alleviate loperamide-induced constipation in rats.
Table 1 Primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>Human</td>
<td>CAAGAGGCGCGGAGAACATG</td>
<td>CGGCCCATTGCTCTTTCC</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Human</td>
<td>TTCGCCAGCCAGGACACATC</td>
<td>ACGGGAATGTCGCCCGAAAA</td>
</tr>
<tr>
<td>HO-1</td>
<td>Human</td>
<td>CAAGACGCCTCTACCGGCTGG</td>
<td>CGGGTGTAGGCGGTCCTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Human</td>
<td>CAATGCCCTCCTGGACACCA</td>
<td>GATGTTCTGAGACGCGC</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Rat</td>
<td>CCGGACAGTCTTCCGACATC</td>
<td>GGCACCTAGGACACGACAAGGA</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Rat</td>
<td>CGGATCTGGTCTGGCAGGTT</td>
<td>ACAAAGTGGTCCTGCTGCC</td>
</tr>
<tr>
<td>HO-1</td>
<td>Rat</td>
<td>AGAGCCTGTGCTGGCGTGGTT</td>
<td>TGAGACGGCCTACGCGAGGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rat</td>
<td>TGTGGATGGCCCTCCTGGAA</td>
<td>TGACCTGCCCACAGCCTTG</td>
</tr>
</tbody>
</table>

HRW modulates the gut microbiota in constipated rats

High-throughput sequencing of the 16S rDNA gene V3-V4 region of the gut microbiota revealed significant diversity and composition differences between the intestinal microbiota of rats treated with loperamide and that of Nor rats. PCoA indicated a clear separation between the Nor and the Lop groups. Notably, a partial correction trend was observed for constipated rats after intervention with HRW (Figure 3A). Based on the alpha diversity metrics, the Lop group had higher values on chao 1, dominance, and observed_otus indexes of the gut flora relative to the Nor group. Following HRW intervention, these indices decreased, resulting in a partial decrease in the richness and evenness of the gut flora (Figure 3B-D). The microbiota community structure at the phylum level is shown in Figure 3F. The identified microorganisms were mainly *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria,* with *Firmicutes* accounting for the largest proportion. Compared to the Nor rats, loperamide-induced constipated rats had a lower abundance of *Firmicutes* (92.58% vs 86.70%). *Bacteroidetes* (5.51% vs 7.50%) and *Actinobacteria* (0.57% vs 3.03%) were more abundant in model rats than Nor rats. Compared with the Lop group, the abundance of *Firmicutes* (86.70% vs 91.77%) increased whereas that of *Bacteroidetes* (7.50% vs 5.26%) and *Actinobacteria* (3.03% vs 0.81%) decreased in the HRW group. The dominant species in each group were selected and analyzed at the genus level to better understand the intestinal flora. As shown in Figure 3F and G, the relative abundances of *Allobaculum* (1.48% vs 9.95%), *Prevotellaceae_UCG-003* (0.01% vs 2.11%), and *Dubosiella* (0.05% vs 2.42%) were significantly increased whereas the relative abundances of *Romboutsia* (11.88% vs 3.95%) and *Clostridium_UCG-014* (4.73% vs 2.13%) were significantly decreased in the Lop group compared with the Nor group. Compared to the Lop group, the abundance of *Romboutsia* (3.95% vs 14.07%) and *Clostridium_UCG-014* (2.13% vs 3.30%) increased whereas the abundance of *Allobaculum* (9.95% vs 4.24%), *Prevotellaceae_UCG-003* (2.11% vs 0.05%), and *Dubosiella* (2.42% vs 0.30%) decreased in the HRW group. Finally, the differential flora between these groups were screened using a t test, with P < 0.05 as the threshold. Five bacteria, namely *Romboutsia, Parasutterella, Frisingicoccus,* and *Eubacterium_nodatum_group,* and *Oscillospiraceae_UCG-005,* were identified (Figure 3H). Altogether, HRW can regulate gut flora disorder in constipated rats.

HRW modulates the serum metabolism in constipated rats

UPLC-QTRAP-tandem mass spectrometry was employed to detect metabolites in rat serum. PCA revealed a distinct separation and significant alterations in metabolomic profiles between the Nor and Lop groups. However, a remarkable trend of callback was observed in the HRW group compared to the Lop group (Figure 4A). Orthogonal partial least squares discriminant analysis indicated alterations in the metabolites profiles were altered. Based on the screening criteria, VIP ≥ 1 and P < 0.05, 244 differential metabolites were identified between the Nor and Lop groups, and 174 differential metabolites were found between the Lop and HRW groups. Of these, 90 differentiated metabolites were present in all three groups, including oxidized lipids (20%), organic acids and their derivatives (20%), and small peptides (15.6%; Figure 4B and C). KEGG enrichment analysis revealed that these differential metabolites were significantly enriched in the arachidonic acid metabolism, bile acid biosynthesis, and androstenedione metabolism pathways (Figure 4D). As shown in the radar map, the contents of β-Leu and traumatic acid were most significantly altered in the model and HRW groups (Figure 4E), suggesting that HRW may play a role in relieving constipation by altering metabolism in vivo. Therefore, the efficacies of β-Leu and traumatic acid need to be evaluated.

HRW alleviates constipation by attenuating oxidative stress through the SIRT1/Nrf2/HO-1 signaling pathway

Increased oxidative stress levels are intimately connected with the occurrence and development of constipation. Oxidative stress, defined as a disturbance in the pro-/antioxidant balance, is harmful to cells due to excessive ROS generation. MDA, a product of lipid peroxidation, can indicate the severity of oxidative stress. SOD is an important antioxidant enzyme that removes ROS. Immunofluorescence (IF) staining revealed that ROS expression increased in constipated rats; however, this overexpression was markedly inhibited by HRW (Figure 5A). Compared to the control group, loperamide stimulation significantly increased the expression of MDA and decreased the expression of SOD in the model group. The expression levels of MDA and SOD were reversed by HRW (Figure 5B and C).
The decrease in SIRT1, Nrf2, and HO-1 causes an imbalance in intracellular oxidation, resulting in cellular oxidative stress damage[9]. The expression levels of these markers in the colon tissue of rats were further detected using an IHC assay. The levels of SIRT1, Nrf2, and HO-1 were markedly decreased in the constipated group; however, a remarkable increase was observed in the HRW group (Figure 5D). The expression levels of SIRT1, Nrf2, and HO-1 in the colon tissue of rats were validated using qRT-PCR and western blotting analysis. Notably, the results tended to be consistent with those of IHC staining (Figure 5E-H). The original western blots of Figure 5H are shown in the Supplementary Figure 1A.

**Inhibition of SIRT1 expression counteracts the regulatory effect of HRW in loperamide-induced constipated rats**

To confirm that SIRT1 mediates the regulatory activity of HRW in constipation, loperamide-induced constipated rats were intraperitoneally injected with EX527, a SIRT1 inhibitor, to suppress SIRT1 expression. A flow chart illustrating the modeling and drug administration process is shown in Figure 6A. The regulatory effect of HRW in constipated rats was significantly counteracted in rats treated with the SIRT1 inhibitor according to the total amount of feces excreted within 24 h (Figure 6B), water content of feces (Figure 6C), carbon end propulsion distance (Figure 6D and E), and the expression of ROS, MDA, and SOD in the colons of rats (Figure 6F-H). Similarly, the suppressed expression of SIRT1 impeded the improvement of SIRT1 signaling (SIRT1, Nrf2, and HO-1) (Figure 6I-L) following HRW intervention. The original western blots of Figure 6L are shown in the Supplementary Figure 1B. Overall, the results indicate that SIRT1 is a crucial target by which HRW alleviates constipation in loperamide-induced rats.

**Expression of SIRT1 is increased by the serum metabolites, β-leucine and traumatic acid, in NCM460 cells, leading to a reduction in H2O2-induced oxidative stress**

The serum metabolites, β-Leu and traumatic acid, were found to significantly increase after HRW intervention in loperamide-induced constipated rats. To validate whether these two metabolites were the basis for the effectiveness of HRW, NCM460 cells (a normal human colon mucosal epithelial cell line) was used. Based on the CCK-8 assay, administering appropriate concentrations of β-Leu and traumatic acid (20 μM, 50 μM, and 100 μM) did not affect normal cell growth (Figure 7A and B). Notably, H2O2 significantly induced excessive ROS in NCM460 cells; however, this induction was reversed by treatment with β-Leu and traumatic acid, suggesting that treatment with β-Leu and traumatic acid had stimulating rather than toxic effects on the viability of NCM460 cells. Following inhibition of SIRT1 expression, the effects of β-Leu and traumatic acid on the alleviation of oxidative stress were abolished (Figure 7C-F). Similarly, the variation in the expression levels of SIRT1 was confirmed via western blotting. Compared to the control group, H2O2 stimulation significantly decreased SIRT1 expression in the model group, which was increased in the β-Leu- and traumatic acid-treated groups, respectively. Of note, the expression of SIRT1 was reversed by the combination treatment of metabolites and EX527 (Figure 7G and H). The original western blots of Figure 7G and H are shown in the Supplementary Figure 1C.

**DISCUSSION**

Constipation is a prevalent gastrointestinal disorder characterized by difficulty in defecation and impaired intestinal motility. However, the current treatment options, such as oral laxatives, secretagogues, and prokinetic agents, have limitations, including short-term efficacy, high recurrence rates and inadequate curative effects[3]. The main approach for constipation management involves sufficient water intake. Magnesium and sulfate-rich water has been demonstrated to have beneficial laxative effects[21]. Hydrogen therapy has emerged as a promising therapeutic strategy owing to its biological reducibility and homeostatic regulatory abilities[4]. In fact, hydrogen therapy is a safe and effective therapeutic agent that has shown significant efficacy in various oxidative stress/inflammation-related diseases, including improving

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**Figure 3** Hydrogen-rich water modulates the gut microbiota in constipated rats. A: PCoA map of the microbiota; B-D: Alpha diversity index; E and F: Bar plots illustrating species richness at the phylum and genus levels; G: Phylogenetic tree depicting genus-level species; H: Heat map displaying differential flora. Nor: Normal group; Lop: Loperamide-induced constipation group; HRW: Hydrogen-rich water intervention group.

**Figure 4** Hydrogen-rich water alleviates constipation in loperamide-induced rats. The original western blots of Figure 4H are shown in the Supplementary Figure 1B. Overall, the results indicate that SIRT1 is a crucial target by which HRW alleviates constipation in loperamide-induced rats.

**Figure 5** The expression levels of SIRT1, Nrf2, and HO-1 in the colon tissue of rats were further detected using an IHC assay. Notably, the results tended to be consistent with those of IHC staining (Figure 5E-H). The original western blots of Figure 5H are shown in the Supplementary Figure 1A.

**Figure 6** The regulatory effect of HRW in constipated rats was significantly counteracted in rats treated with the SIRT1 inhibitor according to the total amount of feces excreted within 24 h (Figure 6B), water content of feces (Figure 6C), carbon end propulsion distance (Figure 6D and E), and the expression of ROS, MDA, and SOD in the colons of rats (Figure 6F-H). Similarly, the suppressed expression of SIRT1 impeded the improvement of SIRT1 signaling (SIRT1, Nrf2, and HO-1) (Figure 6I-L) following HRW intervention. The original western blots of Figure 6L are shown in the Supplementary Figure 1B. Overall, the results indicate that SIRT1 is a crucial target by which HRW alleviates constipation in loperamide-induced rats.

**Figure 7** The serum metabolites, β-Leu and traumatic acid, were found to significantly increase after HRW intervention in loperamide-induced constipated rats. To validate whether these two metabolites were the basis for the effectiveness of HRW, NCM460 cells (a normal human colon mucosal epithelial cell line) was used. Based on the CCK-8 assay, administering appropriate concentrations of β-Leu and traumatic acid (20 μM, 50 μM, and 100 μM) did not affect normal cell growth (Figure 7A and B). Notably, H2O2 significantly induced excessive ROS in NCM460 cells; however, this induction was reversed by treatment with β-Leu and traumatic acid, suggesting that treatment with β-Leu and traumatic acid had stimulating rather than toxic effects on the viability of NCM460 cells. Following inhibition of SIRT1 expression, the effects of β-Leu and traumatic acid on the alleviation of oxidative stress were abolished (Figure 7C-F). Similarly, the variation in the expression levels of SIRT1 was confirmed via western blotting. Compared to the control group, H2O2 stimulation significantly decreased SIRT1 expression in the model group, which was increased in the β-Leu- and traumatic acid-treated groups, respectively. Of note, the expression of SIRT1 was reversed by the combination treatment of metabolites and EX527 (Figure 7G and H). The original western blots of Figure 7G and H are shown in the Supplementary Figure 1C.
Figure 4 Hydrogen-rich water modulates serum metabolism in constipated rats. A: PCA plot of metabolites; B: Sector diagram illustrating the secondary classification of differential metabolites; C: Heat map of the differential metabolites; D: Kyoto Encyclopedia of Genes and Genomes enrichment map of the differential metabolites; E: Radar plot depicting the differential metabolites. Nor: Normal group; Lop: Loperamide-induced constipation group; HRW: Hydrogen-rich water intervention group.

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Sepsis-related encephalopathy[22], promoting of diabetic wound healing[23], and preventing or treating of cardiovascular diseases[24]. Notably, HRW, which is a specialized type of water that can be consumed orally, and contains a precise concentration of dissolved H₂, has been reported to possess potent antioxidant, anti-inflammatory, and anti-apoptotic properties. In this study, HRW was found to relieve constipation symptoms by regulating intestinal flora disorders and serum metabolic abnormalities. Therefore, HRW may provide a new auxiliary method for the clinical treatment of constipation.
constipation with advantages, such as convenient drinking, low price, and high acceptance.

Oxidative stress refers to the accumulation of free oxygen radicals to levels that exceed the ability of the antioxidant defense system, leading to oxidative damage to cells and tissues[26]. Oxidative stress can increase intestinal mucosal inflammation, destroy the intestinal barrier, reduce the mucus layer thickness, and affect intestinal peristalsis and water absorption[6]. Oxidative stress can also affect the function of the intestinal nervous system, inhibit the contraction of intestinal smooth muscles and the activity of ganglion cells, interfere with intestinal nerve conduction, slow intestinal peristalsis, and promote constipation[27]. H₂ in HRW can rapidly diffuse through the transmembrane to reach the cell, react with toxic ROS, and prevent oxidative damage[5]. In the present study, HRW reduced the levels of ROS and MDA and increased the SOD content in constipated rats to reduce oxidative stress. HRW can also increase the thickness of the intestinal mucosal layer, increase the number of ICC and intestinal nerve cells, and alleviate the symptoms of constipation.

SIRT1, a member of the NAD⁺-dependent deacetylase family, has been extensively explored as a potential therapeutic target to attenuate oxidative stress and inflammation-induced disorders[8]. The deficiency of SIRT1 markedly increases ROS and inflammatory reactions[28], while modulation of the SIRT1/NF-kappaB pathway can suppress NLRP3 inflammasome and oxidative stress[29]. Increasing evidence has suggested that SIRT1 play a role in intestinal disease[30]. SIRT1

Figure 5 Hydrogen-rich water alleviates constipation by attenuating oxidative stress through the sirtuin1/nuclear factor-erythroid-2-related factor 2/heme oxygenase-1 signaling pathway. A: Reactive oxygen species expression in the colon of rats; B: Serum malondialdehyde levels in rats; C: Serum superoxide dismutase levels in rats; D: Representative images of immunohistochemical staining of sirtuin1 (SIRT1), nuclear factor-erythroid-2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in the colon of rats; E-G: MRNA of SIRT1, Nrf2, and HO-1 in the colon of rats; H: Protein expression of SIRT1, Nrf2 and HO-1 in the colon of rats. *P < 0.05 vs normal group (Nor), **P < 0.01 vs Nor, ***P < 0.05 vs loperamide-induced constipation group (Lop), ****P < 0.01 vs Lop. Scale bar: 100 μm. Nor: Normal group; Lop: Loperamide-induced  constipation  group; HRW: Hydrogen-rich  water intervention  group; ROS: Reactive oxygen species; SOD: Superoxide dismutase; MDA: Malondialdehyde; SIRT1: Sirtuin1; Nrf2: Nuclear factor-erythroid-2-related factor 2; HO-1: Heme oxygenase-1.
Figure 6 Inhibition of sirtuin1 expression counteracts the regulatory effect of hydrogen-rich water in loperamide-induced constipated rats. A: Flow chart illustrating the modeling and drug administration process; B: Total amount of rat feces excreted in 24 h; C: Water content of rat feces; D and E: Propulsion rate of carbon through the small intestine of rats; F: Reactive oxygen species expression in the colon of rats; G and H: Malondialdehyde and superoxide dismutase expression in the colon of rats; I-K: MRNA expression levels of sirtuin1 (SIRT1), nuclear factor-erythroid-2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in the colon of rats; L: Protein expression levels of SIRT1, Nrf2 and HO-1 in the colon of rats. *P < 0.05 vs normal group (Nor), **P < 0.01 vs Nor, ***P < 0.005 vs loperamide-induced constipation group (Lop), ****P < 0.01 vs Lop, *****P < 0.05 vs hydrogen-rich water (HRW), ******P < 0.01 vs HRW. Scale scale: 100 μm. Nor: Normal group; Lop: Loperamide-induced constipation group; HRW: Hydrogen-rich water intervention group; SIRT1: Sirtuin1; Nrf2: Nuclear factor-erythroid-2-related factor 2; HO-1: Heme oxygenase-1; ROS: Reactive oxygen species; MDA: Malondialdehyde; SOD: Superoxide dismutase.
Figure 7 Expression of sirtuin1 in NCM460 cells is increased following treatment with β-leucine and traumatic acid, resulting in a reduction of H$_2$O$_2$-induced oxidative stress. A and B: CCK-8 assay of β-leucine (β-Leu) and traumatic acid in NCM460 cells; C and D: Reactive oxygen species (ROS) expression in response to β-Leu intervention; E and F: ROS expression in response to traumatic acid intervention; G: Sirtuin1 (SIRT1) expression following the administration of β-Leu and EX527; H: SIRT1 expression in response to the administration of traumatic acid and EX527. *P < 0.01 vs control group; **P < 0.01 vs H$_2$O$_2$; ***P < 0.01 vs β-Leu; ****P < 0.01 vs Traumatic acid. β-Leu: β-leucine; TA: Traumatic acid; ROS: Reactive oxygen species; Con: Control group.

can regulate intestinal inflammation in aging mice by altering the gut microbiota[31], and achieve mitochondrial homeostasis through the NAD$^+$-SIRT1 pathway to protect the intestinal barrier function in patients with severe malnourishment[32]. There are also reports emphasizing the dual role of SIRT1[33]; SIRT1 inhibitors can alleviate radiation-induced intestinal stem cell death, promote crypt recovery, and improve the survival rate of mice[34]. In this study, low SIRT1 expression was observed in the colons of constipated rats. Following intervention with HRW, the expression level
Figure 8 Schematic of the mechanism. Hydrogen-rich water alleviates constipation by attenuating intestinal oxidative stress through the sirtuin1/nuclear factor-erythroid-2-related factor 2/heme oxygenase-1 signaling pathway and modulating gut microbiota and serum metabolites. SIRT1: Sirtuin1; Nrf2: Nuclear factor-erythroid-2-related factor 2; HO-1: Heme oxygenase-1.

of SIRT1 was upregulated. EX527, a SIRT1 inhibitor, also verified that HRW alleviates oxidative stress and improves constipation symptoms by regulating the SIRT1/Nrf2/HO-1 signaling pathway.

Two serum metabolites, β-Leu and traumatic acid, which can relieve constipation, were screened using non-target metabolomics. At present, studies revealing the correlation between β-Leu and oxidative stress, which deserves further exploration, are lacking. Traumatic acid reduces oxidative stress and enhances collagen biosynthesis in human skin fibroblasts[35]. In addition, traumatic acid can reduce the growth of breast cancer cells by influencing apoptosis through oxidative stress[36]. Overall, traumatic acid is an organic acid derivative that holds promise for the treatment of various chronic diseases. In this study, both of β-Leu and traumatic acid could attenuate H2O2-induced ROS in NCM460 cells. Further, the SIRT1 inhibitor validated their ability of these metabolites to decrease oxidative stress reaction, thereby regulating SIRT1 expression in vitro. In the future, the efficacy and safety of β-Leu and traumatic acid in animals will need to be further refined and validated. Hence, HRW alleviates constipation in rats by increasing the serum levels of β-Leu and traumatic acid and SIRT1 expression by reducing oxidative stress levels.

Five different bacteria, namely Oscillospiraceae_UCG-005, Romboutsia, Eubacterium_nodatum_group, Frisingicoccus and Parasutterella were identified using 16s rDNA sequencing. Romboutsia is associated with the production of short-chain fatty acids, which are important fuels for intestinal epithelial cells and play an important physiological function in intestinal motility and host metabolism[37]. Eubacterium sp. and Oscillospora sp. can produce butyrate, which plays a crucial role in enhancing colonic motility and suppressing intestinal inflammation[37,38]. In the present study, HRW was significantly enriched in potentially beneficial bacteria (Oscillospiraceae_UCG-005, Romboutsia and Eubacterium_nodatum_group). In contrast, a positive correlation has been found between Parasutterella abundance and inflammation in constipated rats[39]. Constipation is a prevalent non-motor symptom observed in patients with Parkinson’s disease[40]. Frisingicoccus exhibits elevated abundance within the intestinal tract of patients diagnosed with Parkinson’s disease and is closely associated with neuroinflammation and motor function[41]. Similarly, HRW was found to reduce certain pathogenic intestinal microbes (Frisingicoccus and Parasutterella).

CONCLUSION

Altogether, HRW could reshape intestinal microbiota disorders, regulate serum metabolism abnormalities, and alleviate constipation by regulating the SIRT1/Nrf2/HO-1 signaling pathway to reduce oxidative stress levels in constipated rats (Figure 8). Notably, β-Leu and traumatic acid were identified as potential metabolites for the treatment of constipation as they increased SIRT1 expression and decrease the level of oxidative stress.
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FOOTNOTES

Author contributions: Chen KD, Chen YG and Zhang W conceptualized and designed the research; Chen KD, Wang KL, Chen C, Wang YJ, Chen ZP and He LH performed the experiments; Chen KD, Zhu YJ, Tang WW and Chen YG analyzed the data; Chen KD and Wang KL drafted the manuscript; Zhang W and Chen YG reviewed and edited the manuscript; All authors approved the final manuscript.

Chen KD proposed, designed, conducted experiments, performed data analysis and prepared the first draft of the manuscript. Wang KL was responsible for animal care, medication administration, and drafting initial manuscripts. Both authors have made crucial and indispensable contributions towards the completion of the project and thus qualified as the co-first authors of the paper. Both Zhang W and Chen YG have played important and indispensable roles in the experimental design, data interpretation and manuscript preparation as the co-corresponding authors. Zhang W conceptualized, designed, and supervised the whole process of the project. She searched the literature, revised and submitted the early version of the manuscript with the focus on the association between constipation and oxidative stress. Chen YG was instrumental and responsible for data re-analysis and re-interpretation, comprehensive literature search, preparation and submission of the current version of the manuscript with a focus on gut microbiota and serum metabolites as the predictors of constipation and on potential underlying mechanisms. Zhang W and Chen YG applied for and obtained the funds for this research project. This collaboration between Zhang W and Chen YG is crucial for the publication of this manuscript.

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