


# Cyanidin-3-O-Glucoside Improves Colonic Motility During Severe Acute Pancreatitis by Inhibiting the H<sub>2</sub>S-Regulated AMPK/mTOR Pathway

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**Background:** Cyanidin-3-O-glucoside (C3G) is an important anthocyanin that can modulate digestive system functioning. Inflammation associated with severe acute pancreatitis (SAP) induces H<sub>2</sub>S production, which impairs the gastrointestinal (GI) system. We investigated the effects of C3G in attenuating SAP-associated colonic motility loss by examining the H<sub>2</sub>S level and activity of AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway.

**Methods:** A rat model of SAP was induced using sodium taurocholate, and the effect of C3G on colonic motility, H<sub>2</sub>S production, and the inflammatory response was investigated. AMPK/mTOR pathway changes were detected to assess the pathways by which H<sub>2</sub>S influences colonic motility in SAP-model rats. The mechanism underlying H<sub>2</sub>S function was further examined by subjecting colonic muscle cells (CMCs) to C3G, SAP plasma and an AMPK activator.

**Results:** Administering C3G improved colonic motility but suppressed the inflammatory response and H<sub>2</sub>S production in the SAP-model rats, which was associated with inhibiting the AMPK/mTOR pathway. Furthermore, activating the AMPK/mTOR pathway in CMCs promoted inflammation but suppressed Ca<sup>2+</sup> levels, even after administering C3G.

**Conclusion:** Administering C3G may improve SAP-associated colonic motility by inhibiting the H<sub>2</sub>S-mediated AMPK/mTOR pathway.

**Keywords:** AMP-activated protein kinase, AMPK, cyanidin-3-O-glucoside, colonic motility, severe acute pancreatitis, hydrogen sulfide

## Introduction

Acute pancreatitis (AP) is an inflammatory process that occurs in an otherwise healthy pancreas and exhibits wide clinical variation.<sup>1</sup> Although most cases of AP are mild, ~20% of patients with AP develop a severe form of the disease characterized by organ dysfunction,<sup>1</sup> known as severe acute pancreatitis (SAP).<sup>2</sup> Most patients with SAP experience a diverse range of severe symptoms,<sup>3</sup> but current management strategies overlook the key role of intestinal function during SAP development. Previous studies have demonstrated that bacterial infection and intestinal organ sepsis are important factors in SAP development,<sup>4,5</sup> thus, increasing research is being conducted regarding novel therapies to limit colonic injuries.<sup>6-9</sup>

Previous studies have reported that H<sub>2</sub>S is produced predominantly by cystathionine-γ-lyase (CSE) and other kinases in the transsulfuration pathway. Moreover, H<sub>2</sub>S initiates distinct biological responses in the human body,<sup>10-12</sup>

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including the gastrointestinal (GI) tract, where H<sub>2</sub>S is produced by both GI tissues and gut bacterial flora.<sup>13,14</sup> H<sub>2</sub>S production was also reported to inhibit GI motility in a fish model.<sup>13,14</sup> Substantial H<sub>2</sub>S is produced during SAP attacks, where it inhibits inflammation in the GI system during SAP progression.<sup>15,16</sup> Therefore, H<sub>2</sub>S produced during SAP attacks is hypothesized to be associated with impaired intestinal mobility, and modulating H<sub>2</sub>S production may be a novel strategy for managing SAP-associated colonic motility loss in clinical settings.

Previous studies investigating the events associated with colonic motility loss have furthered the development of novel therapeutic approaches. For example, dietotherapy is attracting increased attention for its efficacy in improving digestive system functioning with few adverse effects.<sup>17,18</sup> Moreover, anthocyanins, which belong to the flavonoid family, are widely distributed in vegetables and other foods that are part of the human diet<sup>19</sup> and may carry health benefits owing to their antioxidant and anti-inflammatory properties.<sup>20</sup> Anthocyanins also affect the intestinal system; thus, studies have focused on their potential to modulate and improve the microflora in the GI tract.<sup>21</sup> However, no previous studies examining the interaction between anthocyanins and the GI system have assessed the effect of anthocyanins on intestinal motility. As one of the most abundant natural anthocyanins, cyanidin-3-O-glucoside (C3G) contributes to modulating numerous biological processes, particularly those involved in immunoregulation.<sup>22–24</sup> C3G exerts its functions in the GI tract via multiple mechanisms; therefore, the present study was conducted to investigate the protective effect of C3G against SAP-induced colonic motility loss by focusing on the effect of C3G on H<sub>2</sub>S and its downstream pathways.

To evaluate the study's hypothesis, rats were injected with sodium taurocholate to induce SAP, then C3G was administered. The effects of C3G on colonic motility, H<sub>2</sub>S production, and inflammatory cytokine levels were detected to identify the exact effect of C3G on SAP-induced colonic motility loss. Moreover, the changing pattern of H<sub>2</sub>S-mediated AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling<sup>25</sup> was detected to elucidate the mechanism by which C3G restores colonic mobility. To validate the *in vivo* assay results, colonic muscle cells (CMCs) were isolated and subjected to plasma isolated from SAP-model rats, then C3G and the AMPK inhibitor, MK-3903, were used to elucidate the interaction between C3G and the H<sub>2</sub>S-mediated AMPK/mTOR pathway.

## Materials and Methods

### Chemicals and Antibodies

C3G (purity >98%; cat. no. HY-N0640) was purchased from MedChemExpress. Antibodies against CSE (cat. no. 12,217-1-AP) were purchased from ProteinTech Group, Inc. Antibodies against total (t)-AMPK (cat. no. ab32047), phosphorylated (p)-AMPK (cat. no. ab23875), total (t)-mTOR (cat. no. ab2732), p-mTOR (cat. no. ab109268) and GAPDH (cat. no. ab181602) were purchased from Abcam. Secondary antibodies (cat. no. A0277, goat anti-rabbit; cat. no. A0208, goat anti-rabbit; cat. no. A0216, goat anti-murine) were provided by Beyotime Institute of Biotechnology. MK-3903 (an AMPK activator) was purchased from Sigma-Aldrich (Merck KGaA). The AMPK activator, MK-3903 (cat. no. HY-107,988), was purchased from MedChemExpress.

### SAP-Model Rats and C3G Administration

Adult male Wistar rats weighing 200–250 g were housed per routine protocols and grouped into the sham, SAP or SAP + C3G groups, with n=10 rats per group. The sham group was induced with SAP without injecting the corresponding agents. The SAP group rats were anesthetized with 50 mg/kg body weight pentobarbital sodium, subjected to a laparotomy, then injected with 5% sodium taurocholate into the pancreatic and bile ducts using a microinjection pump (1 mL/kg, 0.1 mL/min) for 10 min. The incision was then sutured (the overall survival rate of the SAP-model rats was ~60% over a 24-hour period; [Figure S1](#)). The SAP + C3G group rats were gavaged with 100 mg/kg body weight C3G and injected with sodium taurocholate. Before the subsequent assays, all rats were injected with 10 mL normal saline and fasted for 24 h. All animal experiments were conducted in accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of Laboratory Animals of Southwest Hospital of Army Medical University (Ref no. A-20,170,505) and the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, MD, USA, or the European Guidelines on Laboratory Animal Care.

### Colonic Motility Measurements

Colonic motility was assessed by measuring fecal pellet output numbers before and after SAP induction<sup>26</sup> and detected 1 h prior to model induction and 4, 8, 12, 16, 20, and 24 h after model induction. Upon completing the measurements, the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg).

## Measurement of Serum H<sub>2</sub>S Levels

Serum H<sub>2</sub>S levels were measured according to a previous study.<sup>27</sup> Aliquots (75  $\mu$ L) of sera were mixed with 100  $\mu$ L distilled water and 300  $\mu$ L 10% trichloroacetic acid. The reaction was stopped with 150  $\mu$ L of 1% zinc acetate. N, N-dimethyl-p-phenylenediamine sulfate (20  $\mu$ M) in 7.2 M HCl and FeCl<sub>3</sub> (30  $\mu$ M; 133  $\mu$ L) in 1.2 M HCl were then added to the mixture and incubated for 15 min. The absorbance at 670 nm was measured, and the H<sub>2</sub>S concentration was calculated.

## Detecting the Inflammatory Response

After the colonic motility measurements, the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg body weight), and plasma and colonic muscle tissue samples were collected. Tumor necrosis factor (TNF)- $\alpha$  (cat. no. H052) and interleukin (IL)-6 (cat. no. H007) production in the samples was detected using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bio-engineering Institute Co., Ltd.) per the manufacturer's instructions.

## Western Blotting

Extracted protein was subjected to routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then primary antibodies against CSE (1:500), t-AMPK (1:2000), p-AMPK (1:1000), t-mTOR (1:2000), p-mTOR (1:1000), and GAPDH (1:1000) were incubated on polyvinylidene difluoride membranes at 4°C overnight. After incubation with secondary horseradish peroxidase-conjugated IgG antibodies (1:5000), the relative protein expression levels were calculated using Gel-Pro-Analyzer (Media Cybernetics, Inc.).

## Cell Preparation and Administration

CMCs were separated from the mucous membrane of each rat's proximal colon and cultured in solution containing 0.15% collagenase II, 0.1% trypsin inhibitor, and 0.25% fetal bovine serum at 37°C. CMCs were identified via immunofluorescence detection of calponin and  $\alpha$ -SMA (Figure S2), then treated with different combinations of 90 mM H<sub>2</sub>S solution,<sup>28</sup> 10  $\mu$ g/mL C3G and 5  $\mu$ mol/l MK-3903 for 24 h. Inflammatory responses in the CMCs were detected as described above.

## Ca<sup>2+</sup> Assay

CMCs (1 $\times$ 10<sup>6</sup>) were subjected to repeated freezing and thawing to release the intracellular components. The

suspension was then centrifuged at 3000 rpm for 20 min to collect the supernatant. The Ca<sup>2+</sup> concentration was detected using an ELISA kit (Shanghai Keshun Biotechnology Co., Ltd.) per the manufacturer's instructions.

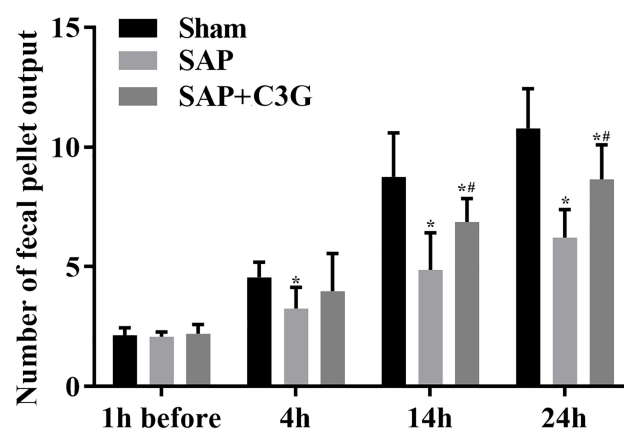
## Statistical Analysis

Data are presented as the mean  $\pm$  standard deviation. One-way analysis of variance followed by Duncan's post hoc multiple comparisons test were conducted. Statistical analyses were conducted, and graphs were created using GraphPad Prism 6 (GraphPad Prism Software, Inc.). P<0.05 was considered statistically significant.

## Results

### Administering C3G Improved Colonic Motility in SAP-Model Rats

The effect of C3G on colonic motility in the rats was assessed by measuring the fecal pellet output among the rats. Before inducing SAP, fecal pellet output numbers did not differ between the groups (sham vs SAP: P=0.993; sham vs SAP + C3G: P=0.991; SAP vs SAP + C3G: P=0.968; Figure 1). After inducing SAP, symptoms were induced via sodium taurocholate, and significant differences were detected between the sham and SAP groups after 4 h (P=0.04). C3G administration significantly alleviated colonic motility loss after 14 h (14 h: P=0.000; 24 h: P=0.000; Figure 1), indicating that C3G administration improved the colonic motility during SAP progression.



**Figure 1** C3G administration improved colonic motility in SAP-model rats. \*P<0.05 vs sham group; #P<0.05 vs SAP group.

**Abbreviations:** C3G, cyanidin-3-O-glucoside; SAP, severe acute pancreatitis.

## C3G Suppressed the Inflammatory Response and H<sub>2</sub>S Levels in SAP-Model Rats

Inducing SAP increased the levels of H<sub>2</sub>S (P=0.001; Figure 2A), TNF- $\alpha$  (P=0.000) and IL-6 (P=0.000; Figure 2B and C). H<sub>2</sub>S suppressed the inflammatory response; however, its anti-inflammatory effect was offset by its negative effect on colonic motility. Thus, C3G administration inhibited both H<sub>2</sub>S and inflammatory cytokine production (H<sub>2</sub>S: P=0.048; TNF- $\alpha$ : P=0.006; IL-6: P=0.004; Figure 2).

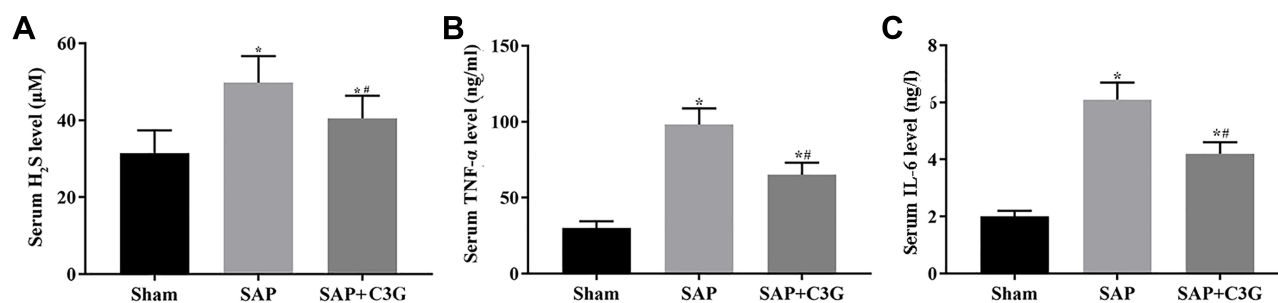
## Administering C3G Inhibited CSE Levels and Deactivated the AMPK/mTOR Signaling Pathway in SAP-Model Rats

To identify the mechanism by which SAP improved colonic motility, AMPK/mTOR pathway activity was detected. SAP increased the CSE expression (P=0.000) and activated the AMPK/mTOR pathway by increasing p-AMPK levels (P=0.000) and the p-AMPK/t-AMPK ratio (P=0.000) and

suppressing p-mTOR levels (P=0.000) and the p-mTOR/t-mTOR ratio (P=0.000; Figure 3). Moreover, C3G reversed the expression patterns of these indicators (CSE: P=0.001; p-AMPK: P=0.014; p-mTOR: P=0.035; p-AMPK/t-AMPK ratio: P=0.003; p-mTOR/t-mTOR ratio: P=0.018); thus, the effect of C3G on colonic motility was associated with inhibiting the H<sub>2</sub>S/AMPK/mTOR pathway.

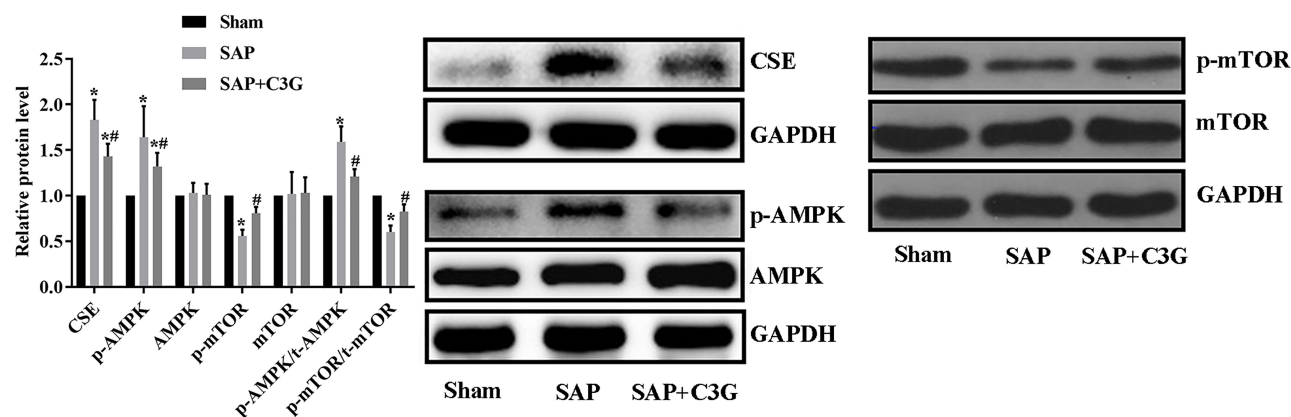
## Activation of AMPK/mTOR Blocked the Protective Effect of C3G Against SAP Plasma-Induced CMC Impairments

To further assess the pathway underlying the effects of C3G, CMCs were isolated and subjected to H<sub>2</sub>S solution, C3G, and an AMPK activator in different combinations. H<sub>2</sub>S administration increased CSE expression (P=0.000) and activated AMPK/mTOR signaling in CMCs (p-AMPK: P=0.000; p-mTOR: P=0.002; p-AMPK/t-AMPK: P=0.000; p-mTOR/t-mTOR: P=0.011; Figure 4A), which also induced cytokine production (TNF- $\alpha$ : P=0.000; IL-6: P=0.000; Figure 4B and C) and suppressed



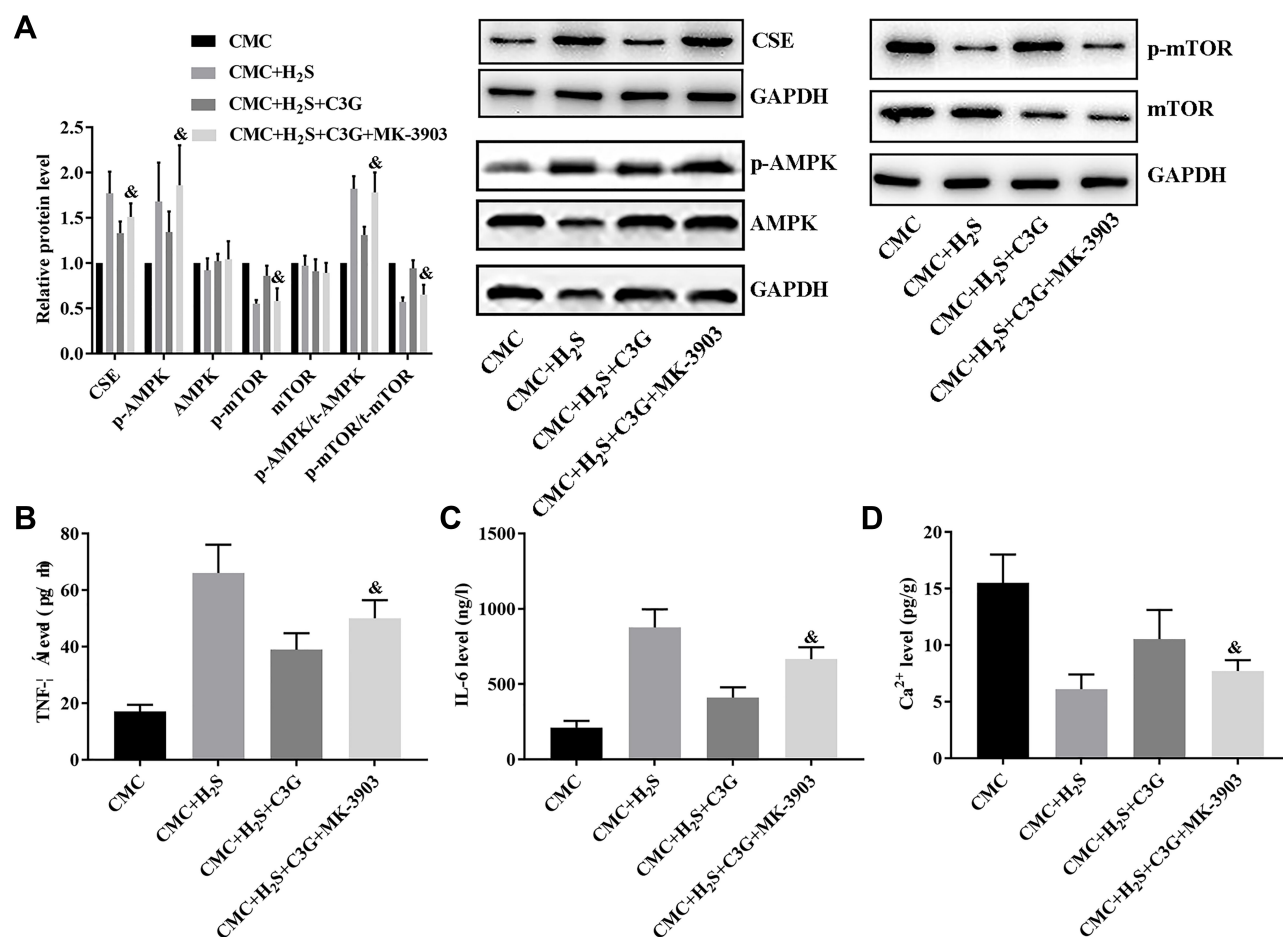
**Figure 2** C3G administration suppressed H<sub>2</sub>S production and cytokine levels in SAP-model rats. Serum (A) H<sub>2</sub>S, (B) TNF- $\alpha$  and (C) IL-6 levels. \*P<0.05 vs sham group; #P<0.05 vs SAP group.

**Abbreviations:** C3G, cyanidin-3-O-glucoside; SAP, severe acute pancreatitis; TNF, tumor necrosis factor; IL, interleukin.



**Figure 3** C3G administration suppressed CSE expression and deactivated the AMPK/mTOR pathway. \*P<0.05 vs sham group; #P<0.05 vs SAP group.

**Abbreviations:** C3G, cyanidin-3-O-glucoside; SAP, severe acute pancreatitis; CSE, cystathionine- $\gamma$ -lyase.



**Figure 4** AMPK activation offsets the effect of C3G on H<sub>2</sub>S-treated CMCs. (A) CSE levels and AMPK activity, (B) TNF- $\alpha$  levels, (C) serum IL-6 levels, (D) Ca<sup>2+</sup> accumulation. \*P<0.05 vs CMC + H<sub>2</sub>S + C3G group.

Ca<sup>2+</sup> accumulation (P=0.002; Figure 4D) in CMCs. C3G administration suppressed CSE expression (P=0.000) and AMPK/mTOR pathway activity (p-AMPK: P=0.000; p-mTOR: P=0.041; p-AMPK/t-AMPK: P=0.002; p-mTOR/t-mTOR: P=0.016), which contributed to inhibiting the inflammatory response (TNF- $\alpha$ : P=0.008; IL-6: P=0.000) and restored Ca<sup>2+</sup> accumulation (P=0.011). However, the AMPK activator impaired the protective effect of C3G against SAP plasma, which reactivated the AMPK/mTOR pathway by increasing p-AMPK (P=0.001) and p-AMPK/t-AMPK (P=0.004) levels but suppressing p-mTOR (P=0.043) and p-mTOR/t-mTOR (P=0.024) levels, which also contributed to increasing the CSE level (P=0.029; Figure 4A). These changes in CSE and AMPK/mTOR signaling increased TNF- $\alpha$  (P=0.041) and IL-6 (P=0.023; Figure 4B and C) production and decreased Ca<sup>2+</sup> production (P=0.025; Figure 4D). Thus, the protective effect of C3G against SAP-induced

colonic motility loss depended on H<sub>2</sub>S-mediated AMPK/mTOR pathway inhibition.

## Discussion

H<sub>2</sub>S is the third member of gasotransmitter family synthesized endogenously via the transsulfuration pathway, which is an important mechanism for providing cells with cysteine.<sup>29,30</sup> Being increasingly recognized as a functionally relevant mediator of a number of physiological functions, deficiencies in the H<sub>2</sub>S production can cause a chronic inflammatory response by inducing pro-inflammatory molecule production, thus resulting in development of various diseases.<sup>29</sup> Regarding the protective effects on GI system, H<sub>2</sub>S can decrease production of TNF- $\alpha$  and leukocytes.<sup>15</sup> However, the H<sub>2</sub>S is increasingly recognized as a functionally relevant mediator of a number of physiological functions. Tamizhselvi et al<sup>31</sup> revealed that H<sub>2</sub>S induced inflammation in AP rats. Therefore, the



functions and related mechanisms of H<sub>2</sub>S in GI diseases should be assessed.

Consistent with previous studies,<sup>16,32,33</sup> SAP symptoms initiated CSE synthesis and increased H<sub>2</sub>S levels. The enhanced release of H<sub>2</sub>S should be associated with a weakened inflammatory response, but it seemed that the anti-inflammation effects of H<sub>2</sub>S were blocked by its and suppressive effects on ed colonic motility during SAP progression. However, C3G administration suppressed the plasma cytokine levels and improved suppressed colonic motility in SAP rats. Therefore, an interaction was hypothesized to have occurred among C3G, H<sub>2</sub>S, and inflammation: H<sub>2</sub>S exerted an anti-inflammatory effect during SAP progression, but its positive effect was offset by its negative effect on colonic motility in SAP rats. C3G administration improved colonic motility by suppressing H<sub>2</sub>S production. In the meanwhile, the anti-inflammatory effects of C3G compensated for the lack in anti-inflammatory factors induced by the deficient production of H<sub>2</sub>S. Thus, applying C3G as a treatment agent for impairments associated with SAP not only improved the colonic motility loss but also contributed to the control of inflammatory response.

H<sub>2</sub>S regulates multiple pathways. In the present study, activity of the AMPK/mTOR pathway was detected to examine the signaling pathway mediating the effect of C3G. The results indicated that SAP and H<sub>2</sub>S solution induced AMPK/mTOR pathway activity both in vivo and in vitro, whereas C3G inhibited this activity. In addition, the CMCs were also treated with AMPK activator MK-3903. The set of the MK-3903 group was employed to validate that the effects of C3G were dependent on the inhibition of AMPK/mTOR pathway. Activation of the AMPK/mTOR pathway in CMCs impaired the protective effect of C3G against H<sub>2</sub>S, increased the cytokine production, and inhibited Ca<sup>2+</sup> accumulation in CMCs, indicating suppressed motility potential in the cells. The results clearly demonstrated the inhibition of AMPK/mTOR pathway was indispensable for the protective effects of C3G on SAP-induced colonic motility loss. Thus, the changes in AMPK/mTOR pathway partially explained the hypothesis we proposed above: the interaction between C3G and H<sub>2</sub>S influenced the activation of AMPK signaling, which finally led to the improved colonic motility and suppressed inflammatory responses associated with SAP initiation.

In conclusion, the in vivo and in vitro assay results demonstrated that administering C3G increased colonic

motility in rats by suppressing H<sub>2</sub>S production. Moreover, the effect of C3G depended on the H<sub>2</sub>S-mediated AMPK/mTOR pathway. H<sub>2</sub>S administration and AMPK activation impaired the motility potential of CMCs, even after C3G administration. However, the present study examined only the downstream pathways involved in the protective effect of C3G against SAP on the GI system. Therefore, further studies are required to improve our understanding of the mechanisms underlying C3G functions.

## Data Sharing Statement

Data will be provided when required.

## Ethics Approval

All animal experiments were conducted in accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of Laboratory Animals of Southwest Hospital of Army Medical University (Ref No. A-20,170,505) and with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, MD, USA, or the European Guidelines on Laboratory Animal Care.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no competing interests for this work.

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