Arctiin alleviates functional constipation by enhancing intestinal motility in mice

YUJIN WANG[1], HUA JIANG[2], LIJUN WANG[2], HUIPING GAN[2], XINCHUN XIAO[2], LIANGWU HUANG[2], WENXIN LI[2] and ZONGRUN LI[2]

[1]College of Basic Medicine and [2]The First Clinical Medical College, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi 712046, P.R. China

Received July 22, 2022; Accepted January 30, 2023

DOI: 10.3892/etm.2023.11898

Abstract. Functional constipation (FC), a common symptom that is primarily associated with intestinal motility dysfunction, is a common problem worldwide. Arctiin (Arc) is a lignan glycoside isolated from the Chinese herbal medicine *Arctium lappa L*., which is a health food in China. The present study aimed to evaluate the laxative effects of Arc against FC in mice. A model of FC induced by loperamide (5 mg/kg) was established in male Institute of Cancer Research (ICR) mice. Arc was administered at a dose of 100 mg/kg as a protective agent. The faecal status, intestinal motility and histological analyses were evaluated. Furthermore, the levels of gastrointestinal motility-associated neurotransmitters, such as motilin (MTL), nitric oxide (NO), and brain-derived neurotrophic factor (BDNF) and the protective effect of Arc on interstitial cells of Cajal (ICC) were assessed. Arc treatment reversed the loperamide-induced reduction in faecal number and water content and the intestinal transit ratio in ICR mice. Histological analysis confirmed that Arc administration mitigated colonic injury. Moreover, Arc treatment increased levels of motilin and brain-derived neurotrophic factor while decreasing nitric oxide levels and ICC injury in the colon of FC mice. Arc decreased inflammation induction and aquaporin expression levels. Owing to its pro-intestinal motility property, Arc was shown to have a protective effect against FC and may thus serve as a promising therapeutic strategy for the management of FC.

Introduction

Functional constipation (FC) is a common bowel disorder, which affects 10-15% of the global population (1). In the past 30 years, the prevalence of FC in China has increased two-fold (2). FC is characterized by persistently difficult, incomplete or infrequent defecation without an identifiable organic cause (3). Among the effects of FC, the decreased health-associated quality of life and increased levels of depression are particularly notable and are associated with an elevated all-cause mortality rate (4).

The causes of FC are multifactorial and include diet, lifestyle, psychological factors, inflammation and oxidative stress. Pathophysiologically, the aforementioned factors result in dysfunctional motility of the colon, which is typically the primary cause of FC (5). Neurotrophic factors serve a vital role in affecting the enteric nervous system, which regulates intestinal motility by neurotransmitters, including excitatory transmitters such as motilin (MTL; a gastrointestinal hormone) and inhibitory transmitters such as nitric oxide (NO) (6,7). Furthermore, a previous study showed that brain-derived neurotrophic factor (BDNF) is expressed in the myenteric plexus (8). Exogenous BDNF treatment significantly promotes contraction of longitudinal muscle strips from the distal colon of mice (9). Interstitial cells of Cajal (ICC) that specifically express receptor tyrosine kinase (C-Kit) are mesenchymal cells that form a cellular network in the gastrointestinal musculature (10). As a ligand of C-Kit, stem cell factor (SCF) is key for maintaining the survival and development of ICC and ICC networks (11). The ICC are associated with the autonomic nerves, which primarily promote intestinal motility (12). Furthermore, a recent study reported that aquaporins (AQPs) are expressed in the colon and regulate faecal water content (13). Due to increasing healthcare demands with the concomitant complexity of FC, additional treatment methods are required.

Dietary and lifestyle changes are current methods for the management of FC in the majority of the population (14). However, patients who experience persistent FC are in need of pharmaceutical-based therapies. Over-the-counter medications, including stimulants and osmotic laxatives, are primarily used for FC treatment (15). However, chronic use of laxatives is discouraged given high levels of dissatisfaction and the low efficacy and safety (16). Thus, it is worth exploring novel therapies for the management of FC.

Arctiin (Arc), extracted from the seeds of *Arctium lappa*, is a lignan glycoside that is digested by intestinal microbes

Correspondence to: Mrs. Hua Jiang, The First Clinical Medical College, Shaanxi University of Chinese Medicine, Qindu, Xianyang, Shaanxi 712046, P.R. China
E-mail: jh20042022@126.com

Key words: functional constipation, arctiin, intestinal motility, interstitial cells of Cajal, aquaporin

and absorbed into the blood (17). Several studies have showed a wide spectrum of pharmacological activity attributed to Arc including neuroprotection, anti-inflammation and anti-oxidative stress properties (18-20). Xu *et al* (18) demonstrated that Arc decreases neuronal injury and ameliorates depression in mice. Furthermore, Arc inhibits inflammation in mice with colitis (21). Additionally, the anti-inflammatory effect of Arc is attributed to inhibiting NO production by decreasing inducible NO synthase (iNOS) activity (22). Extracted mixtures from the roots of *Arctium lappa* are reported to have laxative properties (23). However, the effects of Arc on FC remain unclear.

Thus, the present study aimed to determine if Arc influences FC. Changes in intestinal motility associated with altered neurotransmitter expression levels and changes in ICC were assessed using an FC mouse model established by administering loperamide. The results of the present study provide a theoretical mechanism through which Arc alleviates FC.

Materials and methods

Establishment of a loperamide-induced FC mouse model and Arc treatment. A total of 84 male Institute of Cancer Research (ICR) mice (age, 6 weeks, weighting 25-30 g) from the Laboratory Animal Centre of the School of Basic Medical Sciences (Xi'an Jiaotong University and Medical Laboratory Animal Centre of Air Force Medical University, Xi'an, China) were used after acclimatization to housing conditions (22±1˚C; 12/12-h light/dark cycle; relative humidity 45-55%) and free access to normal mice chow and water. The normal mice chow was purchased from Huanyu Biotechnology Co., Ltd. and consisted of corn, soybean meal, flour, fish meal, yeast powder, vegetable oil, amino acids, calcium hydrophosphate, salt, vitamins and trace elements, which corresponded to 18.0% crude protein, 4.0% crude fat, 5.0% crude fibre, 8.0% ash, 1.0% calcium, 0.6% phosphorus and other macro- and micronutrients. The mice were divided into four groups: Control; FC; lactulose and Arc (n=6/group). The mice from the lactulose and the Arc group received oral administration of 500 mg/kg lactulose (Shanghai Aladdin Biochemical Technology Co., Ltd.) or 100 mg/kg Arc (>98% purity; Shanghai Aladdin Biochemical Technology Co., Ltd.) daily for 14 days, respectively. An equal volume of pure water was administered by oral gavage to mice in the control group and the FC group. Induction of FC was performed as described previously (24). The mice from the FC, lactulose and Arc groups were administered loperamide (5 mg/kg; Shanghai Aladdin Biochemical Technology Co., Ltd.) orally by gavage on days 12-14. Alterations in water consumption and food intake were measured using a metabolic cage. Additionally, body weight was measured daily on days 12-15 and faeces and distal colon segments were collected on day 15. Mice were anesthetized with inhalation of isoflurane at 3% induction and 2% maintenance. Blood (1 ml) was collected from the post-cava and left to stand for 30 min at room temperature. Mice were sacrificed by exsanguination under anesthesia. Serum was collected following centrifugation at 1,111 x g at room temperature for 10 min. Other mice were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbital. In this current study, the criteria for humane endpoint euthanasia included inability to access food and water and obvious anxiety resulting from FC and no mouse was sacrificed according to these. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Shaanxi University of Chinese Medicine (approval no. SUCMDL20190314001).

Measurement of intestinal transit in FC mice. Determination of intestinal transit ratio (n=6 each group) and transit time (n=6 each group) was performed as described previously (25,26). Briefly, following 14 days of treatment, mice fasted with free access to water for 12 h. For the intestinal transit ratio analysis, the mice were orally administered a meal containing 20% charcoal (Shanghai Aladdin Biochemical Technology Co., Ltd.) and 10% gum arable (Shanghai Aladdin Biochemical Technology Co., Ltd.) at a volume of 0.1 ml/10 g. Mice were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbital 30 min after meal ingestion and the intestine from the pylorus to the rectum were collected immediately. The lengths of the total intestinal tract and the distance the charcoal meal had travelled from the pylorus were measured. The intestinal transit ratio was calculated as follows: Intestinal transit ratio (%)=length travelled by the charcoal meal/total length of intestine x100. The transit time was evaluated in mice following gavage of a 0.1 ml volume of 5% Evans blue (MilliporeSigma) in 1.5% methylcellulose (Shanghai Aladdin Biochemical Technology Co., Ltd.). Faeces were monitored at 10 min intervals for the presence of Evans blue staining. The time to the excretion of the first blue faeces was recorded.

Histopathological examination. Formalin (10%, at room temperature for 24 h)-fixed distal colon segments were embedded in paraffin (at 60˚C for 2 h) and sliced into serial 5-μm thick sections. Hematoxylin and eosin (H&E) staining (2 g/l hematoxylin and 3.5 g/l eosin) at room temperature for 5 min was used to assess the morphometric features. To measure colonic mucosa thickness, colon sections were stained with Alcian Blue Dye Solution (pH2.5) (Leagene Biotechnology) at room temperature for 30 min. Images were acquired with a light microscope (Olympus Corporation) at x200 magnification. Image-Pro Plus software (Version 6.0.0.260; Media Cybernetics, Inc.) was used to measure the thickness of muscle and mucosa.

Transmission electron microscopy. Following fixation of distal colon samples in 1% osmic acid at room temperature for 2 h, washing in phosphate buffer and dehydrating in an ethanol series, the colon samples were embedded in epoxy resin (SPI Supplies; Structure Probe, Inc.) at 37˚C overnight and polymerized at 60˚C for 48 h. The blocks were cut into ultra-thin 70-nm thick sections and stained with 2% uranyl acetate and 2.6% lead citrate at room temperature for 8 min. A transmission electron microscope (H-7650, Hitachi) was used to detect the morphology and structure of Cajal cells in the colon of mice at x20,000 magnification. The images were acquired with an accelerating voltage of 80 kV.

Assessment of MTL, BDNF and NO. Commercial ELISA kits for MTL (cat. no. CEA575Mu; Cloud-Clone Corp.) and BDNF [cat. no. EK2127; Multisciences (LIANKE) Biotech., Co., Ltd.] were used to measure their concentration in the serum and

Table I. Detection of body weight, feeding behaviour and faeces secretion in FC mice.

Variable		Control	FC	Lactulose	Arctiin
Body weight, g	Day 12	33.50±1.87	34.00±1.26	32.83±1.72	32.67±1.63
	Day 13	33.83±1.47	34.17±1.17	33.17±2.04	33.00±1.41
	Day 14	34.33±1.63	34.50±1.76	33.00±1.90	33.50±1.38
	Day 15	34.33±1.51	35.17±1.47	33.33±1.97	34.00±1.41
Feeding behaviour	Water intake, ml/24 h	6.17±1.47	6.50±1.52	7.17±0.98	6.83±0.75
	Food intake, g/24 h	5.17±1.72	4.83±0.75	5.33±1.21	5.00±0.63
Faeces	Number, n	20.50±3.45	4.00±0.89[a]	10.17±2.14[c]	7.83±1.47[b]
	Weight, g	0.47±0.09	0.11±0.02[a]	0.28±0.03[c]	0.22±0.04[c]
	Water content, %	67.18±6.38	49.59±5.79[a]	61.80±4.25[c]	59.27±1.06[b]

[a]P<0.01 vs. Control. [b]P<0.05 and [c]P<0.01 vs. FC. FC, functional constipation.

colon tissues of mice, according to the manufacturer's protocol. The levels of NO in the colon tissue were assessed using an NO assay kit (cat. no. A013; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Immunoblotting. The colon tissue was homogenized in RIPA lysis buffer (Beyotime Institute of Biotechnology). Following quantification of protein concentrations using a BCA protein assay kit (Beyotime Institute of Biotechnology), a total of 15 μg/lane (C-Kit, SCF, AQP-3, and AQP-9) or 30 μg/lane (nNOS, eNOS, and iNOS) protein was loaded on an 8% or 12% SDS-gel, resolved using SDS-PAGE and transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.). The membranes were blocked with TBST containing 0.15% Tween-20 and 5% BSA (BioFroxx, Inc.) and incubated with primary antibodies against neuronal NOS (nNOS; 1:1,000; cat. no. A2649; ABclonal Biotech Co., Ltd.), endothelial NOS (eNOS; 1:1,000; cat. no. A15075; ABclonal Biotech Co., Ltd.), iNOS (1:1,000; cat. no. A0312; ABclonal Biotech Co., Ltd.), C-Kit (1:1,000; cat. no. AF6153; Affinity Biosciences), SCF (1:500; cat. no. 26582-1-AP; ProteinTech Group, Inc.), AQP-3 (1:1,000; cat. no. A2838; ABclonal Biotech Co., Ltd.), AQP-9 (1:1,000; cat. no. DF9225, Affinity Biosciences) or β-actin (1:2,000; cat. no. 60008-1-Ig; ProteinTech Group, Inc.) overnight at 4˚C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000; cat. nos. SA00001-1 and SA00001-2; ProteinTech Group, Inc.) at 37˚C for 40 min. Protein bands were visualized using ECL-Reagent (7 Sea Biotech) and imaged. Gel-Pro-Analyzer software (Version 4.0, Media Cybernetics, Inc.) was used for quantitative analysis of blots.

Immunohistochemistry and immunofluorescence. Immunohistochemical analysis of the colon tissue was performed on 5-μm sections. Briefly, antigen retrieval was performed using a citric acid buffer and heating in an 800 W microwave for 10 min, blocked with BSA (1%, Sangon Biotech Co., Ltd.) for 15 min at room temperature and incubated with primary antibodies against C-Kit (1:100; cat. no. AF6153; Affinity Biosciences), SCF (1:100; cat. no. 26582-1-AP; ProteinTech Group, Inc.), AQP-3 (1:100; cat. no. A2838; ABclonal Biotech Co., Ltd.), or AQP-9 (1:100; cat. no. DF9225, Affinity Biosciences) at 4˚C overnight. For immunohistochemistry, following incubation with horseradish peroxidase-conjugated secondary antibody (1:500; cat. no. 31460; Thermo Fisher Scientific, Inc.) at 37˚C for 1 h, sections were stained with DAB) detection kit (MXB Biotechnologies Co., Ltd.) at room temperature for 3 min, examined by light microscopy (Olympus Corporation) at x400 magnification equipped with a digital camera (Olympus Corporation). For immunofluorescence, the sections were incubated with Cy3-conjugated secondary antibody (1:200; cat. no. A27039; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. DAPI (100 ng/ml, Shanghai Aladdin Biochemical Technology Co., Ltd.) was used for counterstaining at room temperature for 5 min. Images were captured with a fluorescence microscope (Olympus Corporation) at 400x magnification. The evaluation of immunofluorescent and immunohistochemical staining was performed using Image-Pro Plus software (Version 6.0.0.260, Media Cybernetics, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Total RNA in the distal colon tissues of mice was extracted using TRIpure™ (BioTeke Corporation) and quantified using a spectrophotometer (NanoDrop™ 2000; Thermo Fisher Scientific, Inc.). Following cDNA synthesis with Moloney murine leukaemia virus reverse transcriptase (BeyoRT™ II M-MLV, Beyotime Institute of Biotechnology) according to the manufacturer's instructions, RT-qPCR was performed on an Exicycler 96 PCR system (Bioneer Corporation) with initial denaturation at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 s, and extension at 72˚C for 15 s. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (27). The primer sequences were as follows: Tumor necrosis factor-α (TNF-α) forward, 5'-TCTCATTCC TGCTTGTGG-3' and reverse, 5'-CTTGGTGGTTTGCTA CG-3'; interleukin (IL)-1β forward, 5'-CTCAACTGTGAA ATGCCACC-3' and reverse, 5'-GAGTGATACTGCCTGCCT GA-3'; IL-6 forward, 5'-TAACAGATAAGCTGGAGTC-3' and reverse, 5'-TAGGTTTGCCGAGTAGA-3' and β-actin forward, 5'-CTGTGCCCATCTACGAGGGCTAT-3' and reverse, 5'-TTTGATGTCACGCACGATTTCC-3'.

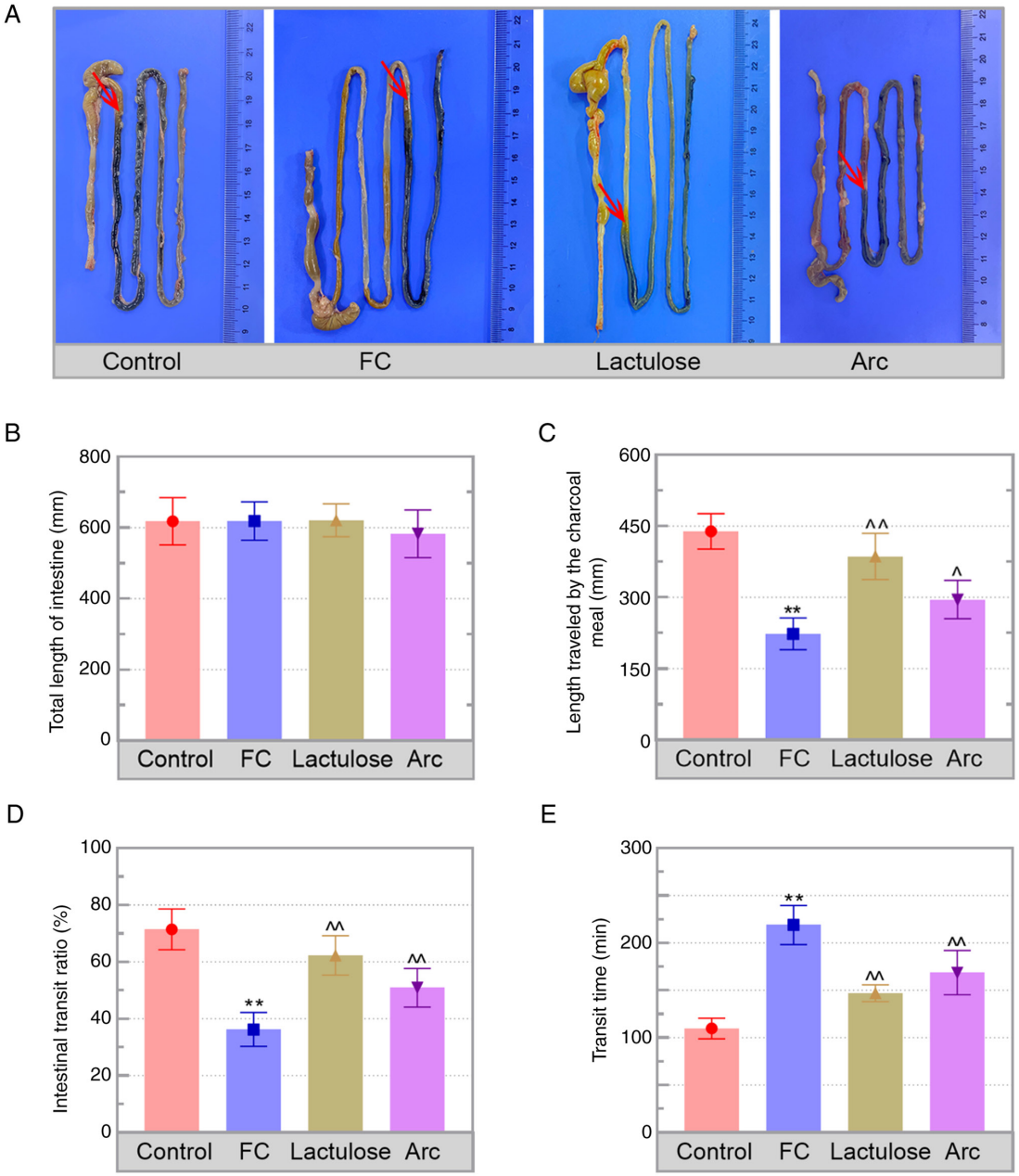

Figure 1. Arc mitigates loperamide-induced FC in mice. (A) Intestinal tract (the red arrows indicate the traveled location of the charcoal meal). (B) Total length of the intestine and (C) length traveled by the charcoal meal were measured. (D) Intestinal transit ratio and (E) transit time of mice. **P<0.01 vs. Control. $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. FC. FC, functional constipation; Arc, Arctiin.

Statistical analysis. The data are presented as the mean ± standard deviation (n=6) and analyzed using one-way ANOVA followed by post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Arc attenuates loperamide-induced FC in mice. FC was evaluated based on the changes in faecal matter, including defecation frequency and faecal moisture analysis. Loperamide was used to establish the FC model. Loperamide resulted in a significant decrease in faecal numbers (4.00±0.89), faecal weight (0.11±0.02 g) and water content (49.59±5.79%) in FC mice compared with that in the control mice (20.50±3.45, 0.47±0.09 g and 67.18±6.38%, respectively; Table I). Body weight and feeding behaviour did not vary between the control and FC groups. Additionally, the length travelled by the charcoal meal in the intestinal tract was shorter in loperamide-treated mice compared with that in the control (red arrows delineate the travelled location of the charcoal meal; Fig. 1A). Furthermore, no difference was observed in the total length of the intestine in each group, but the length traveled by the charcoal was shorter in FC mice than that of control (Fig. 1B and C). Measurement of intestinal transit showed a decrease in intestinal transit ratio and an increase in the transit time in the FC mice (Fig. 1D and E). The aforementioned results indicated that the FC model was established successfully. Arc treatment not only improved the faecal characteristics but also accelerated intestinal transit in FC mice.

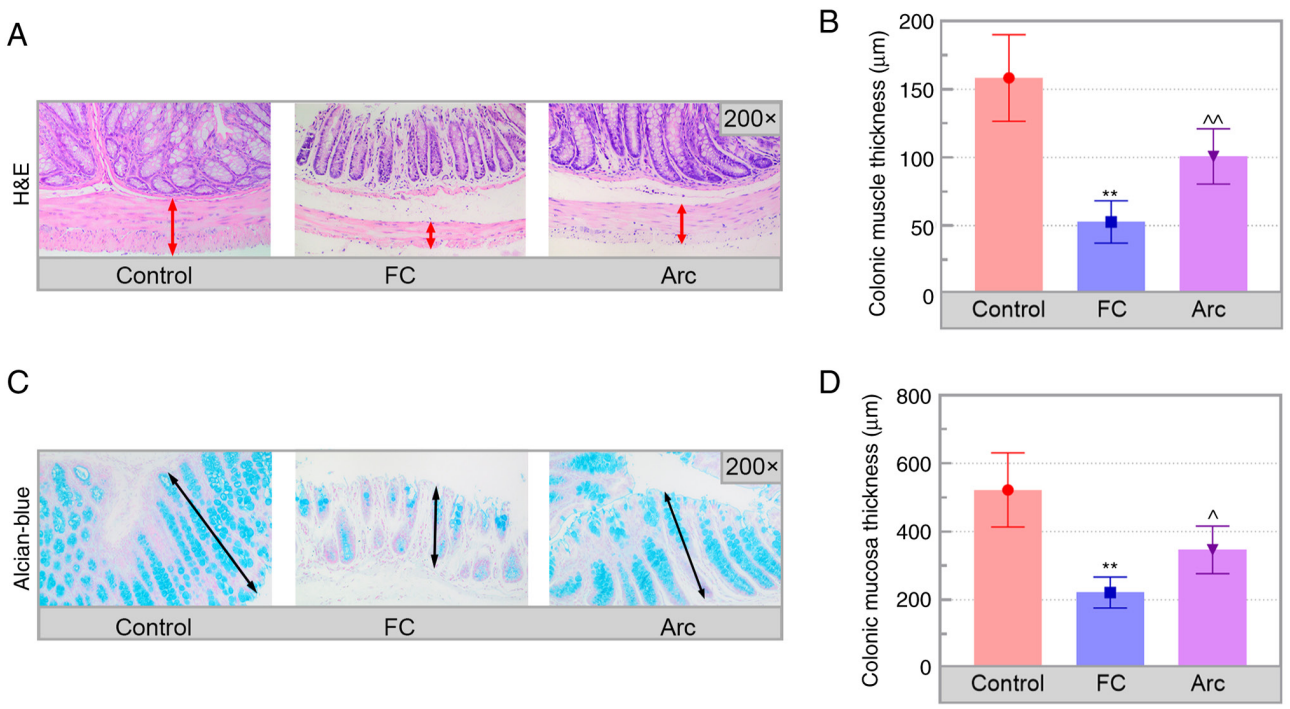

Figure 2. Arc relieves pathological damage to the colon. (A) H&E staining and (B) quantification of colonic muscle thickness (double-headed arrows delineate the muscular layer). (C) Alcian-blue staining and (D) quantification of colonic mucosa thickness (double-headed arrows delineate the colonic mucosa). **P<0.01 vs. Control. $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. FC. H&E, hematoxylin and eosin; Arc, Arctiin; FC, functional constipation.

Arc decreases pathological injury to the colon. Subsequently, changes in the colonic pathology in FC mice were assessed. H&E staining of the colon sections showed that the muscular layer was significantly thinner in the colon of FC mice compared with that in the control mice (Fig. 2A and B; double-headed arrows delineate the muscular layer). Furthermore, results of H&E staining could be confirmed by alcian blue staining, which provided more evidence on the protective effects of Arc on FC. The results of alcian blue staining revealed that the thickness of the colonic mucosa was significantly decreased in the FC group compared with the control group (Fig. 2C and D; double-headed arrows delineate the colonic mucosa). These changes were reversed in Arc-treated mice. Thus, Arc mitigated colonic damage in the FC mice.

Levels of gastrointestinal motility-associated neurotransmitters and inflammation in colon of FC mice are regulated by Arc. To investigate the underlying mechanism by which Arc attenuated FC, levels of excitatory (MTL) and inhibitory neurotransmitters (NO), as well as BDNF, were determined in the serum and colon tissue of mice. Arc ameliorated the decrease in MTL and BDNF levels, as well as the increase in NO observed in loperamide-treated mice compared with those in the FC mice (Fig. 3A-E). Western blot analysis showed higher expression of nNOS and eNOS and lower expression of iNOS in the Arc group compared with the FC group (Fig. 3F and G). Moreover, the mRNA levels of TNF-α, IL-1β and IL-6 were increased in FC mice compared with those in the control. Additionally, the inflammatory induction was significantly inhibited by Arc (Fig. S1A-C). These findings showed that Arc regulated levels of neurotransmitters and inflammation in FC mice.

Arc decreases ICC injury. The levels of C-Kit and SCF and the condition of mitochondria/ER were used to evaluate ICC injury. Immunohistochemical analysis showed that C-Kit and SCF were primarily present in the submucosa and muscle layers (Fig. 4A-C). Arc reversed the decrease in C-Kit and SCF levels in the colon of FC mice, which was confirmed by western blot analysis (Fig. 4D and E). Electron microscopy results showed that the ICC in the colon of control mice was rich in cell organelles, including endoplasmic reticulum and mitochondria. Injured ICC in the FC group were characterized by swollen mitochondria with disrupted cristae and partial cytoplasmic depletion. By contrast, the damage to the ICC in the Arc group was notably decreased (Fig. 4F). These results showed that Arc reduced ICC damage in the colon of FC mice.

Arc decreases the levels of AQPs. Based on the involvement of AQPs in FC progression (28), the expression levels of AQPs in the colon of mice were detected. Increased expression of AQP-3 and AQP-9 was observed in the colon of FC mice and this was significantly reduced by Arc treatment (Fig. 5A-E). Therefore, Arc downregulated the levels of AQPs in the colon of mice with FC.

Discussion

FC is a common gastrointestinal disorder that affects the quality of life of individuals worldwide (29). The present study aimed to evaluate the therapeutic effects of Arc on FC. *In vivo* experiments were used to investigate ICR mouse intestinal motility and changes in the ICC following induction of FC. To the best of our knowledge, the present study is the first to show that Arc alleviated loperamide-induced FC by decreasing pathological

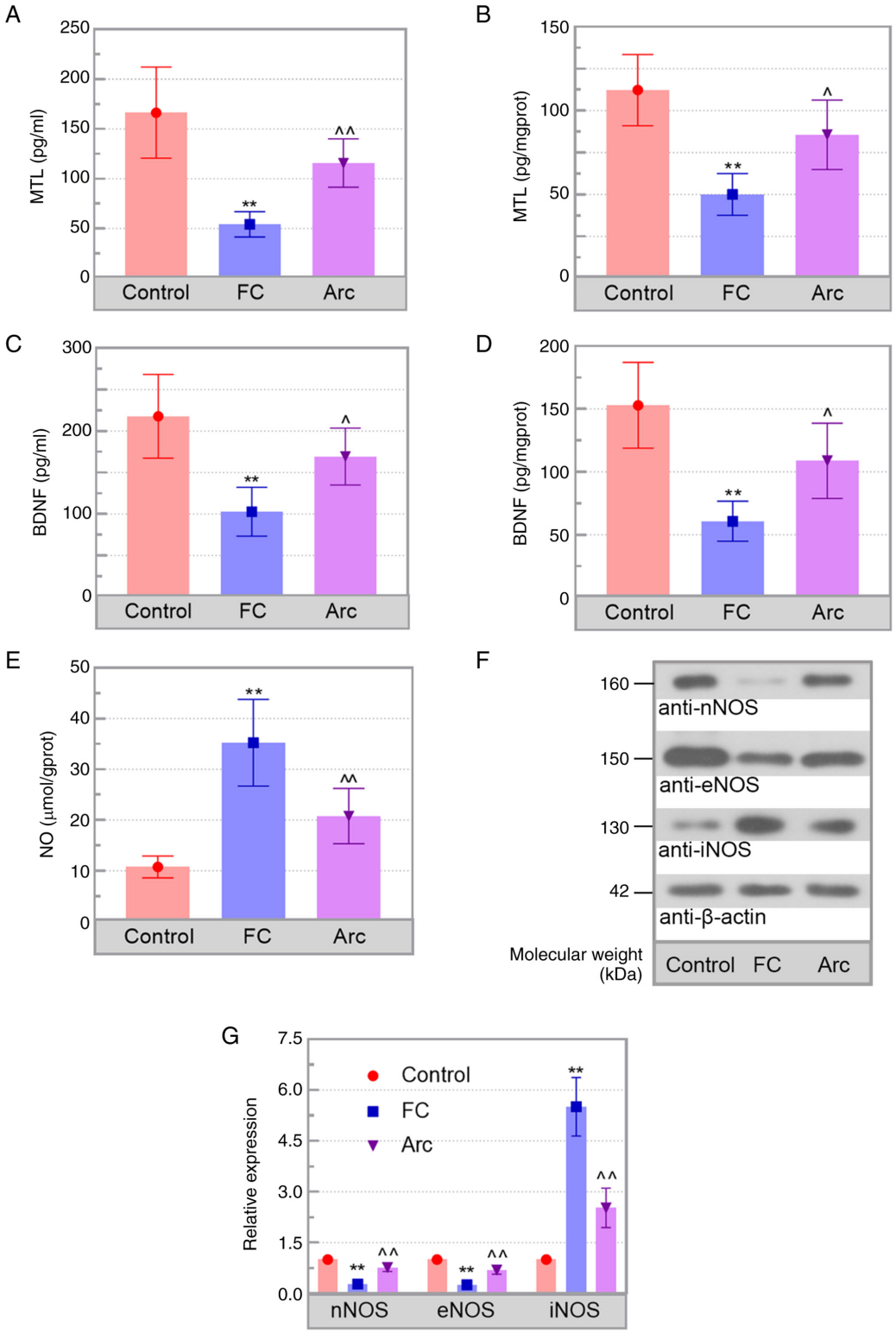

Figure 3. Arc regulates levels of gastrointestinal motility-associated neurotransmitters in the colon of FC mice. MTL concentrations in (A) serum and (B) colon tissues. BDNF concentrations in (C) serum and (D) colon tissue. (E) Levels of NO in the colon tissues. (F) Immunoblotting for nNOS, eNOS and iNOS and (G) quantification of the blots. **P<0.01 vs. Control. $^{^}$P<0.05 and $^{^^}$P<0.01 vs. FC. FC, functional constipation; MTL, motilin; BDNF, brain-derived neurotrophic factor; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; Arc, Arctiin.

damage and inflammation, regulating levels of gastrointestinal motility-related neurotransmitters and attenuating ICC injury in the colon of FC mice. It was also demonstrated that Arc could reverse the elevated levels of AQPs in the colon of mice with FC. Based on these findings, the protective effects of Arc on FC were shown and these results highlight the potential value of Arc in the treatment of FC (Fig. 6).

FC is a common gastrointestinal disorder that is associated with intestinal motility (30). Delayed gastrointestinal transit is associated with abnormal gut muscular movement, which

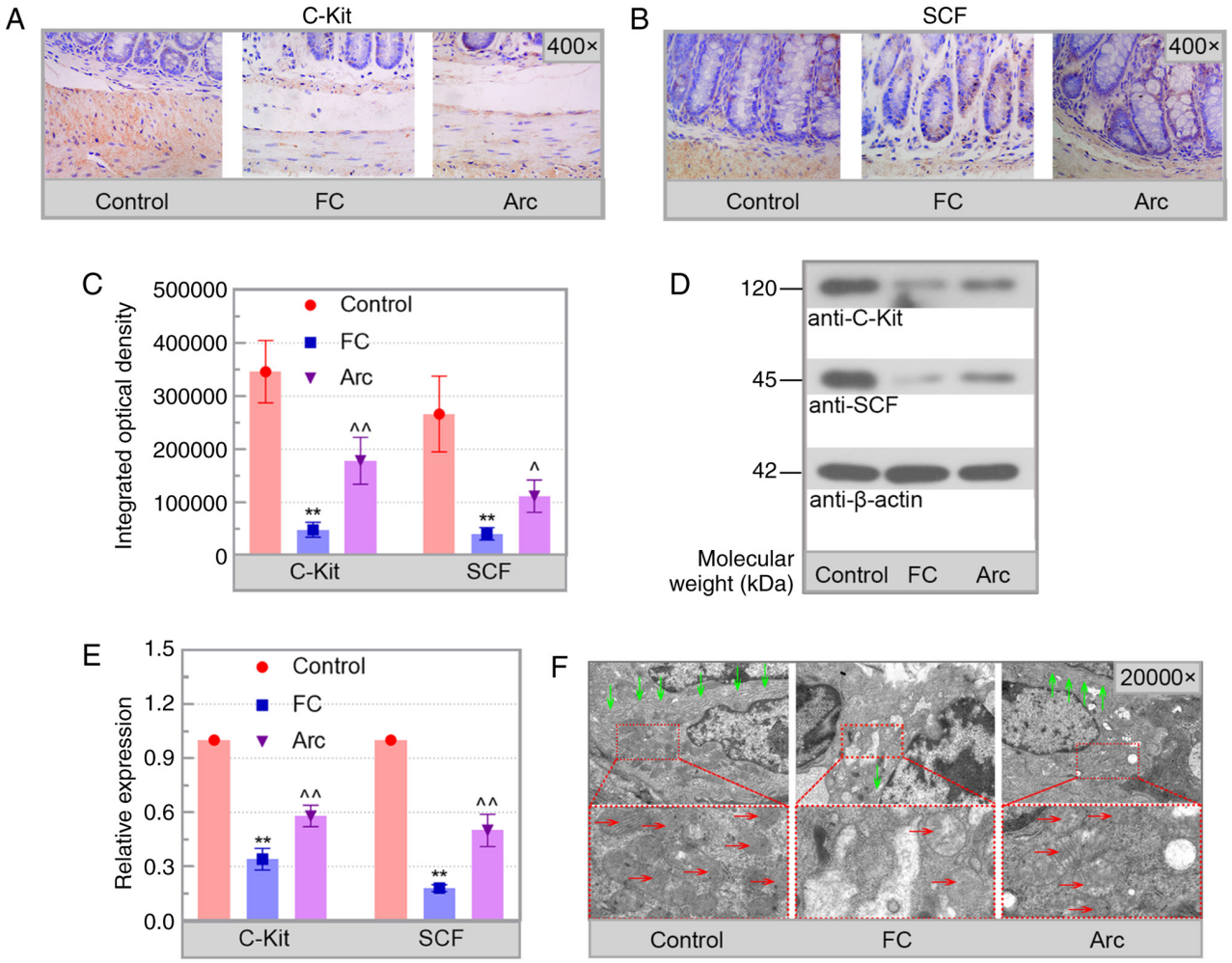

Figure 4. Arc alleviates ICC injury. Distal colon segments were used for detection of ICC injury. Immunohistochemical localization of (A) C-Kit and (B) SCF in colon tissues. (C) Evaluation of immunohistochemical staining. (D) Immunoblotting for C-Kit and SCF. (E) Densitometry analysis of immunoblots. (F) Electron micrographs of the ICC in the colons of mice. The green arrows delineate the endoplasmic reticulum while the red arrows delineate the mitochondria. **P<0.01 vs. Control. ^P<0.05 and ^^P<0.01 vs. FC. FC, functional constipation; Arc, Arctiin; ICC, interstitial cells of Cajal; SCF, stem cell factor; C-Kit, receptor tyrosine kinase.

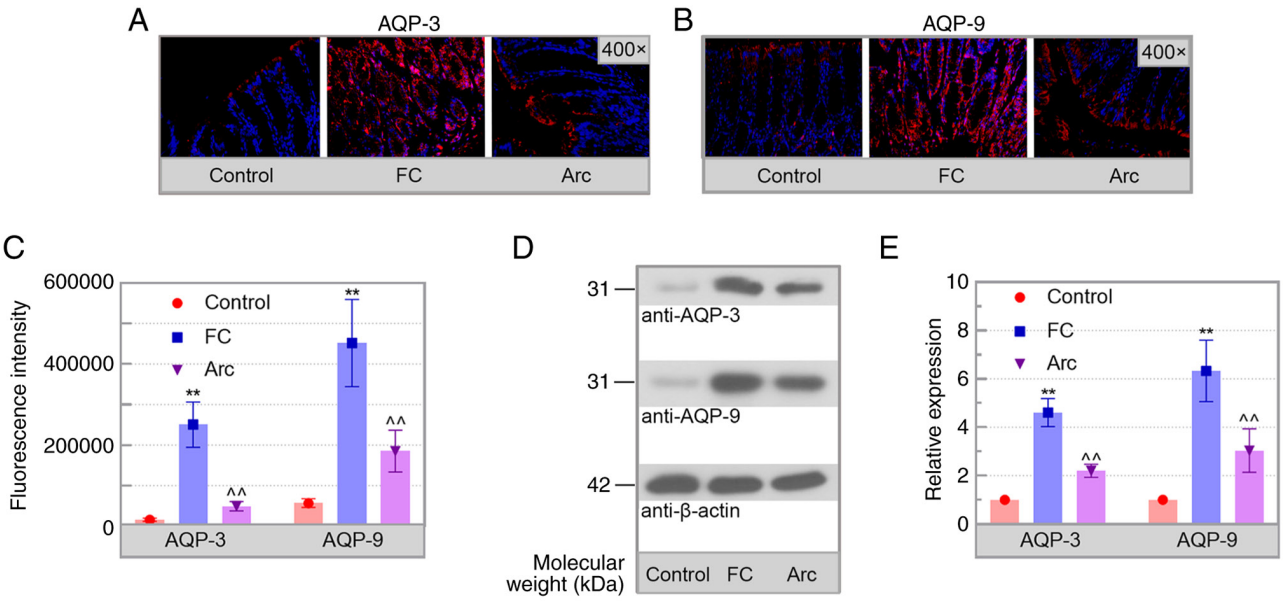

Figure 5. Arc reverses loperamide-induced elevation of AQP-3 and AQP-9. Immunofluorescence analysis of (A) AQP-3 and (B) AQP-9 in colon tissue. (C) Evaluation of immunofluorescent staining was performed using Image-Pro Plus software. (D) Western blot analysis of protein expression levels of AQP-3 and AQP-9. (E) Densitometry analysis of western blots. **P<0.01 vs. Control. ^^P<0.01 vs. FC. FC, functional constipation; Arc, Arctiin; AQP, aquaporin.

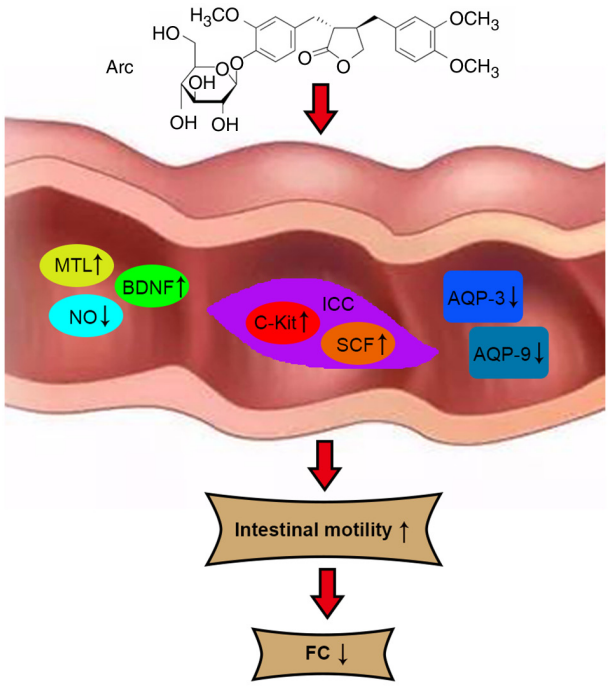

Figure 6. Hypothetical model of Arc-attenuated FC. Arc regulates levels of gastrointestinal motility-associated neurotransmitters and decreases ICC injury and AQP expression, alleviating FC. Arc, Arctiin; FC, functional constipation; ICC, interstitial cells of Cajal; SCF, stem cell factor; MTL, motilin; BDNF, brain-derived neurotrophic factor; NO, nitric oxide; AQP, aquaporin; C-Kit, receptor tyrosine kinase.

may be caused by insufficient inputs from the enteric neural circuitry (31). Therefore, neuromodulators regulating survival and development of neurons in the enteric nervous system regulate intestinal function (32). As one of the neurotrophin ligands, BDNF dose-dependently accelerates colon motility by varying the intestinal innervation structure (33). Conditional knockout of BDNF in the gut induces dopaminergic neuronal loss, motor dysfunction and constipation (34). Moreover, MTL enhances gastrointestinal motility via direct action on smooth muscle cells (35). By contrast, NO is the primary inhibitory neurotransmitter and levels of NO in the myenteric plexus serve a role in inhibiting the colonic motor complex (36). Synthesis of NO is catalyzed by NOS enzymes, including eNOS, nNOS and iNOS. The nNOS isoform is abundant in neural tissues and participates in pathological and physiological processes, such as nerve transmission and muscle contraction in the intestine (37). Under normal physiological conditions, NO is primarily produced from eNOS, which is abundant in endothelial cells where it regulates vascular homeostasis (38). Nevertheless, iNOS levels associated with excessive production of NO are increased in the colon of FC mice (39). Hence, regulating the levels of NOS can reduce NO content and mitigate FC. In the present study, the number and the water content of faeces were higher and the intestinal transit time was shorter in the Arc treatment group compared with the FC group. In addition, Arc increased the levels of BDNF, MTL, nNOS and eNOS while decreasing the levels of iNOS and NO in the colon of FC mice. Therefore, Arc treatment may relieve FC by regulating neuromodulators that enhance neural function, leading to elevated colonic contractility.

ICC, as mesenchymal cells, are pacemakers for the peristaltic reflex by which propulsive contraction in the gastrointestinal tract is achieved (40). ICC are rich in multiple organelles, including numerous mitochondria and abundant endoplasmic reticula (41). With a highly branched morphology and cellular network, ICC are an important component of the gastrointestinal tract (42). Additional evidence has shown that ICC contribute to intestinal motility by generating spontaneous electrical depolarization and increasing smooth muscle excitability (43). Furthermore, ICC differentiation and development rely on signals from C-Kit and its ligand SCF (44). Previous studies have ICC injury in the colons of patients and rats with FC (12,45). The present results revealed that C-Kit and SCF were mainly present in the submucosa and muscle layers, which is consistent with previous studies (46,47). Furthermore, a novel finding of the present study was that Arc prevented the increase in damage and increased the density of ICC in the colon of FC mice. Arc may promote intestinal motility by protecting against ICC injury and subsequently alleviating FC.

AQPs are key membrane proteins that primarily transport water across the plasma membranes (48). At present, 13 isoforms of AQP have been identified in mammals and these are divided into three groups. Water-selective AQPs, including AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8, are selectively permeable to water. Aquaglyceroporins, including AQP3, AQP7, AQP9 and AQP10, are permeable to water, glycerol and urea. Finally, super-aquaporins, including AQP11 and AQP12, are highly different from the aforementioned AQPs with low homology (49). AQPs are associated with transepithelial water movement in the intestinal tract and are key for maintaining body water homeostasis (50). Upregulation of AQP3 and AQP9 results in severe constipation by decreasing mucus secretion and faecal water content, as well as decreasing bowel peristalsis in rats (51). Multiple studies have showed that AQP-3 and AQP-9 are localized in the mucosal layer, including epithelial and goblet cells (52-55). Of note, suppression of AQP3 function in the colon results in diarrhoea (52). FC mice show increased levels of AQP3 and AQP9 compared with those in controls, which is consistent with the present results (56). Here, loperamide-induced elevation in AQP3 and AQP9 were decreased by Arc treatment. Therefore, Arc treatment may decrease AQP expression, thus relieving abnormal water transport and inhibiting the development of FC.

The present study has limitations. The symptoms of depression, which may be associated with constipation severity (57), were not investigated in the current FC model. Constipated mice show notable increases in immobility time of the forced swimming and tail suspension test (58). In addition, the specific effect of Arc on BDNF, MTL and NOS remains unclear. Further studies, including analyses of the symptoms of depression in FC mice and the mechanism of Arc on BDNF, MTL and NOS, should be performed to improve understanding of the function of Arc in the management of FC.

In conclusion, the present study showed that Arc promoted colonic contractility and decreased intestinal transit time by relieving colonic damage and ICC injury while downregulating

inflammation induction and AQP expression levels in the colon to mitigate FC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81973865).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW designed the study and wrote the manuscript. HJ designed the study and revised the manuscript. YW, LW, HG, and WL performed the experiments. YW, XX, LH, and ZL analyzed the data. YW, WL and ZL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Shaanxi University of Chinese Medicine (approval no. SUCMDL20190314001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Barberio B, Judge C, Savarino EV and Ford AC: Global prevalence of functional constipation according to the Rome criteria: A systematic review and meta-analysis. Lancet Gastroenterol Hepatol 6: 638-648, 2021.
2. Chen Z, Peng Y, Shi Q, Chen Y, Cao L, Jia J, Liu C and Zhang J: Prevalence and risk factors of functional constipation according to the Rome Criteria in China: A systematic review and meta-analysis. Front Med (Lausanne) 9: 815156, 2022.
3. Vilanova-Sanchez A and Levitt MA: Surgical interventions for functional constipation: An update. Eur J Pediatr Surg 30: 413-419, 2020.
4. Koloski NA, Jones M, Wai R, Gill RS, Byles J and Talley NJ: Impact of persistent constipation on health-related quality of life and mortality in older community-dwelling women. Am J Gastroenterol 108: 1152-1158, 2013.
5. Bharucha AE and Lacy BE: Mechanisms, evaluation, and management of chronic constipation. Gastroenterology 158: 1232-1249 e3, 2020.
6. Mori H, Verbeure W, Schol J, Carbone F and Tack J: Gastrointestinal hormones and regulation of gastric emptying. Curr Opin Endocrinol Diabetes Obes 29: 191-199, 2022.
7. Yip JLK, Balasuriya GK, Spencer SJ and Hill-Yardin EL: Examining enteric nervous system function in rat and mouse: An inter-species comparison of gastrointestinal motility. Am J Physiol Gastrointest Liver Physiol 323: G477-G487, 2022.
8. Lommatzsch M, Braun A, Mannsfeldt A, Botchkarev VA, Botchkareva NV, Paus R, Fischer A, Lewin GR and Renz H: Abundant production of brain-derived neurotrophic factor by adult visceral epithelia. Implications for paracrine and target-derived Neurotrophic functions. Am J Pathol 155: 1183-1193, 1999.
9. Chen FX, Yu YB, Yuan XM, Zuo XL and Li YQ: Brain-derived neurotrophic factor enhances the contraction of intestinal muscle strips induced by SP and CGRP in mice. Regul Pept 178: 86-94, 2012.
10. Yagasaki R, Shikaya Y, Kawachi T, Inaba M, Takase Y and Takahashi Y: Newly raised anti-c-Kit antibody visualizes morphology of interstitial cells of Cajal in the developing gut of chicken embryos. Dev Growth Differ 64: 446-454, 2022.
11. Iino S, Horiguchi K and Horiguchi S: c-Kit-stem cell factor signal-independent development of interstitial cells of Cajal in murine small intestine. Cell Tissue Res 379: 121-129, 2020.
12. Huizinga JD, Hussain A and Chen JH: Interstitial cells of Cajal and human colon motility in health and disease. Am J Physiol Gastrointest Liver Physiol 321: G552-G575, 2021.
13. Na JR, Kim E, Na CS and Kim S: Citric acid-enriched extract of ripe prunus mume (Siebold) Siebold & Zucc. Induces laxative effects by regulating the expression of aquaporin 3 and prostaglandin E2 in rats with loperamide-induced constipation. J Med Food 25: 12-23, 2022.
14. Rao SSC, Lacy BE, Emmanuel A, Müller-Lissner S, Pohl D, Quigley EMM and Whorwell P: Recognizing and defining occasional constipation: Expert consensus recommendations. Am J Gastroenterol 117: 1753-1758, 2022.
15. Camilleri M, Ford AC, Mawe GM, Dinning PG, Rao SS, Chey WD, Simrén M, Lembo A, Young-Fadok TM and Chang L: Chronic constipation. Nat Rev Dis Primers 3: 17095, 2017.
16. Schiller LR: Chronic constipation: New insights, better outcomes? Lancet Gastroenterol Hepatol 4: 873-882, 2019.
17. Lu Z, He B, Chen J, Wu LJ, Chen XB, Ye SQ, Yang WH, Shao ZY, Jin EG, Wang SJ, *et al*: Optimisation of the Conversion and Extraction of Arctigenin From Fructus arctii Into Arctiin Using Fungi. Front Microbiol 12: 663116, 2021.
18. Xu X, Zeng XY, Cui YX, Li YB, Cheng JH, Zhao XD, Xu GH, Ma J, Piao HN, Jin X and Piao LX: Antidepressive effect of arctiin by attenuating neuroinflammation via HMGB1/TLR4- and TNF-α/TNFR1-Mediated NF-κB activation. ACS Chem Neurosci 11: 2214-2230, 2020.
19. Liu X, Wang J, Dou P, Zhang X, Ran X, Liu L and Dou D: The ameliorative effects of arctiin and arctigenin on the oxidative injury of lung induced by silica via TLR-4/NLRP3/TGF-β signaling pathway. Oxid Med Cell Longev 2021: 5598980, 2021.
20. Fierascu RC, Georgiev MI, Fierascu I, Ungureanu C, Avramescu SM, Ortan A, Georgescu MI, Sutan AN, Zanfirescu A, Dinu-Pirvu CE, *et al*: Mitodepressive, antioxidant, antifungal and anti-inflammatory effects of wild-growing Romanian native Arctium lappa L. (Asteraceae) and Veronica persica Poiret (Plantaginaceae). Food Chem Toxicol 111: 44-52, 2018.
21. Hyam SR, Lee IA, Gu W, Kim KA, Jeong JJ, Jang SE, Han MJ and Kim DH: Arctigenin ameliorates inflammation in vitro and in vivo by inhibiting the PI3K/AKT pathway and polarizing M1 macrophages to M2-like macrophages. Eur J Pharmacol 708: 21-29, 2013.
22. Gao Q, Yang M and Zuo Z: Overview of the anti-inflammatory effects, pharmacokinetic properties and clinical efficacies of arctigenin and arctiin from Arctium lappa L. Acta Pharmacol Sin 39: 787-801, 2018.
23. Li K, Zhu L, Li H, Zhu Y, Pan C, Gao X and Liu W: Structural characterization and rheological properties of a pectin with anti-constipation activity from the roots of Arctium lappa L. Carbohydr Polym 215: 119-129, 2019.
24. Hayeeawaema F, Wichienchot S and Khuituan P: Amelioration of gut dysbiosis and gastrointestinal motility by konjac oligo-glucomannan on loperamide-induced constipation in mice. Nutrition 73: 110715, 2020.
25. Yan S, Yue YZ, Wang XP, Dong HL, Zhen SG, Wu BS and Qian HH: Aqueous extracts of herba cistanche promoted intestinal motility in loperamide-induced constipation rats by ameliorating the interstitial cells of cajal. Evid Based Complement Alternat Med 2017: 6236904, 2017.
26. Ge X, Ding C, Zhao W, Xu L, Tian H, Gong J, Zhu M, Li J and Li N: Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. J Transl Med 15: 13, 2017.

27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
28. Ikarashi N, Kon R and Sugiyama K: Aquaporins in the colon as a new therapeutic target in diarrhea and constipation. Int J Mol Sci 17: 1172, 2016.
29. Belsey J, Greenfield S, Candy D and Geraint M: Systematic review: Impact of constipation on quality of life in adults and children. Aliment Pharmacol Ther 31: 938-949, 2010.
30. Singh R, Zogg H, Ghoshal UC and Ro S: Current treatment options and therapeutic insights for gastrointestinal dysmotility and functional gastrointestinal disorders. Front Pharmacol 13: 808195, 2022.
31. Sanders KM, Koh SD, Ro S and Ward SM: Regulation of gastrointestinal motility-insights from smooth muscle biology. Nat Rev Gastroenterol Hepatol 9: 633-645, 2012.
32. Spencer NJ and Hu H: Enteric nervous system: Sensory transduction, neural circuits and gastrointestinal motility. Nat Rev Gastroenterol Hepatol 17: 338-351, 2020.
33. Chen F, Yu Y, Wang P, Dong Y, Wang T, Zuo X and Li Y: Brain-derived neurotrophic factor accelerates gut motility in slow-transit constipation. Acta Physiol (Oxf) 212: 226-238, 2014.
34. Ahn EH, Kang SS, Liu X, Cao X, Choi SY, Musazzi L, Mehlen P and Ye K: BDNF and Netrin-1 repression by C/EBPβ in the gut triggers Parkinson's disease pathologies, associated with constipation and motor dysfunctions. Prog Neurobiol 198: 101905, 2021.
35. Verbeure W, van Goor H, Mori H, van Beek AP, Tack J and van Dijk PR: The role of gasotransmitters in gut peptide actions. Front Pharmacol 12: 720703, 2021.
36. Balasuriya GK, Nugapitiya SS, Hill-Yardin EL and Bornstein JC: Nitric oxide regulates estrus cycle dependent colonic motility in mice. Front Neurosci 15: 647555, 2021.
37. Bulbul A, Bulbul T, Sevimli A and Yilmaz O: The effect of dietary supplementation of nitric oxide donor and inhibitor on nNOS expression in and motility of the small intestine of broilers. Biotech Histochem 88: 258-266, 2013.
38. Mattila JT and Thomas AC: Nitric oxide synthase: Non-canonical expression patterns. Front Immunol 5: 478, 2014.
39. Zhou X, Chen Y, Ma X, Yu Y, Yu X, Chen X and Suo H: Efficacy of Bacillus coagulans BC01 on loperamide hydrochloride-induced constipation model in Kunming mice. Front Nutr 9: 964257, 2022.
40. Koh SD, Drumm BT, Lu H, Kim HJ, Ryoo SB, Kim HU, Lee JY, Rhee PL, Wang Q, Gould TW, *et al*: Propulsive colonic contractions are mediated by inhibition-driven poststimulus responses that originate in interstitial cells of Cajal. Proc Natl Acad Sci USA 119: e2123020119, 2022.
41. Means SA and Cheng LK: Mitochondrial calcium handling within the interstitial cells of Cajal. Am J Physiol Gastrointest Liver Physiol 307: G107-G121, 2014.
42. Drumm BT, Cobine CA and Baker SA: Insights on gastrointestinal motility through the use of optogenetic sensors and actuators. J Physiol 600: 3031-3052, 2022.
43. Drumm BT, Rembetski BE, Huynh K, Nizar A, Baker SA and Sanders KM: Excitatory cholinergic responses in mouse colon intramuscular interstitial cells of Cajal are due to enhanced Ca^{2+} release via M_3 receptor activation. FASEB J 34: 10073-10095, 2020.
44. Tan YY, Ji ZL, Zhao G, Jiang JR, Wang D and Wang JM: Decreased SCF/c-kit signaling pathway contributes to loss of interstitial cells of Cajal in gallstone disease. Int J Clin Exp Med 7: 4099-4106, 2014.
45. Lee SM, Kim N, Jo HJ, Park JH, Nam RH, Lee HS, Kim HJ, Lee MY, Kim YS and Lee DH: Comparison of changes in the interstitial cells of cajal and neuronal nitric oxide synthase-positive neuronal cells with aging between the ascending and descending colon of F344 Rats. J Neurogastroenterol Motil 23: 592-605, 2017.
46. Chen LL, Zhu J, Schumacher J, Wei C, Ramdas L, Prieto VG, Jimenez A, Velasco MA, Tripp SR, Andtbacka RHI, *et al*: SCF-KIT signaling induces endothelin-3 synthesis and secretion: Thereby activates and regulates endothelin-B-receptor for generating temporally- and spatially-precise nitric oxide to modulate SCF- and or KIT-expressing cell functions. PLoS One 12: e0184154, 2017.
47. Liu YA, Chung YC, Shen MY, Pan ST, Kuo CW, Peng SJ, Pasricha PJ and Tang SC: Perivascular interstitial cells of cajal in human colon. Cell Mol Gastroenterol Hepatol 1: 102-119, 2015.
48. Yde J, Keely SJ and Moeller HB: Expression, regulation and function of Aquaporin-3 in colonic epithelial cells. Biochim Biophys Acta Biomembr 1863: 183619, 2021.
49. Ishibashi K, Tanaka Y and Morishita Y: The role of mammalian superaquaporins inside the cell: An update. Biochim Biophys Acta Biomembr 1863: 183617, 2021.
50. Zhu C, Chen Z and Jiang Z: Expression, distribution and role of aquaporin water channels in human and animal stomach and intestines. Int J Mol Sci 17: 1399, 2016.
51. Kon R, Ikarashi N, Hayakawa A, Haga Y, Fueki A, Kusunoki Y, Tajima M, Ochiai W, Machida Y and Sugiyama K: Morphine-Induced constipation develops with increased aquaporin-3 expression in the colon via increased serotonin secretion. Toxicol Sci 145: 337-347, 2015.
52. Ikarashi N, Kon R, Iizasa T, Suzuki N, Hiruma R, Suenaga K, Toda T, Ishii M, Hoshino M, Ochiai W and Sugiyama K: Inhibition of aquaporin-3 water channel in the colon induces diarrhea. Biol Pharm Bull 35: 957-962, 2012.
53. Ikarashi N, Ushiki T, Mochizuki T, Toda T, Kudo T, Baba K, Ishii M, Ito K, Ochiai W and Sugiyama K: Effects of magnesium sulphate administration on aquaporin 3 in rat gastrointestinal tract. Biol Pharm Bull 34: 238-242, 2011.
54. Ren Z, Zhang X, Fan H, Yu Y, Yao S, Wang Y, Dong Y, Deng H, Zuo Z, Deng Y, *et al*: Effects of different dietary protein levels on intestinal aquaporins in weaned piglets. J Anim Physiol Anim Nutr (Berl): May 9, 2022 (Epub ahead of print).
55. Okada S, Misaka T, Matsumoto I, Watanabe H and Abe K: Aquaporin-9 is expressed in a mucus-secreting goblet cell subset in the small intestine. FEBS Lett 540: 157-162, 2003.
56. Meng Y, Li X, Wang X, Zhang L and Guan J: Network pharmacological prediction and molecular docking analysis of the combination of Atractylodes macrocephala Koidz. and Paeonia lactiflora Pall. in the treatment of functional constipation and its verification. Animal Model Exp Med 5: 120-132, 2022.
57. Albiani JJ, Hart SL, Katz L, Berian J, Del Rosario A, Lee J and Varma M: Impact of depression and anxiety on the quality of life of constipated patients. J Clin Psychol Med Settings 20: 123-132, 2013.
58. Xu N, Fan W, Zhou X, Liu Y, Ma P, Qi S and Gu B: Probiotics decrease depressive behaviors induced by constipation via activating the AKT signaling pathway. Metab Brain Dis 33: 1625-1633, 2018.