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Research article

Regulatory effects of saponins from *Panax japonicus* on colonic epithelial tight junctions in aging rats

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ABSTRACT

Background: Saponins from *Panax japonicus* (SPJ) are the most abundant and main active components of *P. japonicus*, which replaces ginseng roots in treatment for many kinds of diseases in the minority ethnic group in China. Our previous studies have demonstrated that SPJ has the effects of anti-inflammation through the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways. The present study was designed to investigate whether SPJ can modulate intestinal tight junction barrier in aging rats and further to explore the potential mechanism.

Methods: Aging rats had been treated with different doses (10 mg/kg, 30 mg/kg, and 60 mg/kg) of SPJ for 6 mo since they were 18 mo old. After the rats were euthanized, the colonic samples were harvested. Levels of tight junctions (claudin-1 and occludin) were determined by immunohistochemical staining. Levels of proinflammatory cytokines (interleukin-1β and tumor necrosis factor-α) were examined by Western blot. NF-κB and phosphorylation of MAPK signaling pathways were also determined by Western blot.

Results: We found that SPJ increased the expression of the tight junction proteins claudin-1 and occludin in the colon of aging rats. Treatment with SPJ decreased the levels of interleukin-1β and tumor necrosis factor-α, reduced the phosphorylation of three MAPK isoforms, and inhibited the expression of NF-κB in the colon of aging rats.

Conclusion: The studies demonstrated that SPJ modulates the damage of intestinal epithelial tight junction in aging rats, inhibits inflammation, and downregulates the phosphorylation of the MAPK and NF-κB signaling pathways.

1. Introduction

The intestinal epithelium is the first line of defense against a detrimental invasion of food antigens, and microorganisms and their toxins, so the intestinal barrier is a vital component of gut homeostasis. Loss of intestinal epithelial integrity can lead to an increase of intestinal permeability, enabling noxious substances to pass through the intestinal mucosa [1]. Several studies have shown an increase of intestinal permeability to macromolecules in aged rodents [2,3], suggesting an age-associated impairment in intestinal epithelial barrier function. Further studies in *Drosophila* have demonstrated that a defective intestinal barrier can predict age-onset mortality, and it is important to emphasize that dysfunction of the intestinal barrier may have more severe consequences far beyond the gut [4]. Moreover, studies have shown that the intestine is an important target organ, via genetic interventions with respect to promoting longevity in both *Caenorhabditis elegans* and *Drosophila* [5,6]. A critical factor supporting intestinal barrier function is tight junctions (TJs), which mainly include zonula occludens, occludins, and claudins.

Although the mechanism of intestinal barrier dysfunction is not fully understood, proinflammatory cytokines have been reported to play a significant role in the process [7]. Factors that are known to modulate the expression of TJs include inflammatory cytokines,

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such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). In fact, low-grade, persistent but mild intestinal inflammation is frequently present in the elderly population, finally promoting low level of systemic inflammation [8,9]. It has been demonstrated that the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways play an important role in intestinal barrier dysfunction [10]. Activity of NF-κB in epithelial cells can trigger an inflammatory amplification cascade, and phosphorylation of the MAPK pathway also plays a crucial role in inflammation. The MAPK signaling pathways also play a pivotal role in the modulation of TJ integrity in some intestinal inflammatory diseases via phosphorylation. NF-κB activation in epithelial cells can initiate an inflammatory amplification cascade, and it is also of particular importance for the maintenance of epithelial barrier integrity. However, it is yet unknown whether intestinal epithelial barrier dysfunction in aging rats is associated with intestinal inflammation, and activation of the MAPK and NF-κB signaling pathways.

Panacis japonica rhizoma, derived from the dried rhizome of *Panax japonicus* (PJ), belongs to Araliaceae *Panax* family, and it is also named as the king of herbs in traditional Tujia and Hmong medicine, which replaces ginseng roots in the treatment of many kinds of diseases in minority ethnic groups. It distributes widely throughout the southwest region of China and Japan. Saponins from *P. japonicus* (SPJ) are the most abundant and main active components of PJ [11,12]. In our previous studies, we had verified that SPJ had anti-inflammatory effects, and could inhibit the expression of NF-κB and decrease the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 MAPK in myocardial infarction of rats [13]. Furthermore, we have found that SPJ could delay the cognitive impairment in D-galactose-induced aging rats [14]. Therefore, we hypothesized that SPJ might modify barrier dysfunction by reducing inflammation of the intestine in aging rats.

2. Materials and methods

2.1. Materials

PJ was obtained from Enshi Chunmuying Medicinal Materials Planting Base (Enshi, China). The voucher specimen was deposited at the Institute of Chinese Herb Medicine, Medical College of China Three Gorges University.

2.2. Extraction and analysis of total SPJ

Extraction and determination of SPJ were performed as described previously [14]. Briefly, root of dried PJ (1,000 g) was cut into small pieces and extracted with 60% ethanol (4,000 mL) for 2 h, and then the filtrate was collected. A further 4,000 mL 60% ethanol was added to the PJ residue and refluxed for another 2 h. All the filtrate was collected and decompressed to concentrate. Then the ethanol extract was dissolved in distilled water, extracted with n-butanol, and concentrated. The n-butanol extract was redissolved in water, and poured into a chromatographic column containing macroporous adsorption resin D101 (Tianjin Pesticide Factory, China), rinsed with water, and then eluted with 30%, 60%, and 90% ethanol in water. And last, we got ethanol extract in the range of 30–60% as a refined n-butanol extract. High-performance liquid chromatography analysis was performed on a SEYMC-Pack ODS-AQ column (4.6 mm × 250 mm, 5 μm YMC Co., Ltd. Japan) eluted with mobile phases of acetonitrile (A) and 5% acetic acid solution (B) at a flow rate of 1.0 mL/min. The elution program was as follows: 0–5 min, 5% A; 5–20 min, 5–30% A; 20–30 min, 30% A; 30–50min, 30–85% A; 50–60 min, 85% A. The sample injection volume was 10 μL, and the sample was tested by a UV detector.

2.3. Animals

Male Sprague-Dawley rats were supplied by the Animal Center of China Three Gorges University, Yichang, China. All experiments were approved by the Ethics Committee of China Three Gorges University, and were in accordance with the guidelines of the National Institutes of Health on the care and use of animals. The rats were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark cycle. The rats were fed *ad libitum*. Forty-eight rats were fed until they were 18 mo old, and then they were randomly divided into aging control group ($n = 12$) and SPJ-treated groups ($n = 36$). According to the record of Chinese Pharmacopoeia and our preliminary experiment, we chose 10 mg/kg, 30 mg/kg, and 60 mg/kg of SPJ as the appropriate doses. The rats in the SPJ-treated groups were given SPJ by oral gavage daily at three different concentrations consecutively for 6 mo until they were 24 mo old. Rats that were 6 mo old were used as the adult control group ($n = 12$).

2.4. Colonic morphology

After the rats were euthanized, colonic samples were harvested. Intestinal sample from each rat was fixed in 4% formaldehyde, embedded in paraffin, and sectioned into 4 μm-thick slices. Sections were deparaffinized in xylene, rehydrated with graded alcohol, and then stained with hematoxylin and eosin. Images were captured with 200 × magnifications via an Olympus microscope (Olympus, Shanghai, China).

2.5. Immunohistochemistry analysis

Colonic sample from each rat was fixed in 4% formaldehyde, embedded in paraffin, and sectioned into 4 μm-thick slices. Sections were deparaffinized in xylene, rehydrated with graded alcohol, and incubated with citrate buffer at 95–100°C for 20 min for antigen retrieval. After cooling to room temperature, the sections were incubated with 5% bovine serum albumin for 30 min at room temperature to block the nonspecific binding. Then the sections were stained with specific primary antibodies and incubated in a humidified chamber at 4°C overnight. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sections were subsequently treated with the corresponding secondary antibody for 30 min at room temperature. After rinsing sections with phosphate buffer saline three times, immunoreactivity was visualized with diaminobenzidine for 10 min. Images were acquired using a light microscope (Olympus). The semi-quantitative result of immunohistochemistry was based on the averaged value of three rats per group. Three separate slides of optical density at 20 × magnification were analyzed by Image J software (Rawak Software, Inc. Germany) for each rat.

2.6. Western blot

Samples of colon were collected and frozen in liquid nitrogen, and then stored at −80°C until analysis. Rat colon tissue proteins were extracted by a total protein extraction kit according to the instructions. Protein concentrations were analyzed by a bicinchoninic acid protein assay kit (Beyotime, Haimen, China). Total proteins (40 μg) from each tissue were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After being blocked in Tris buffered saline with Tween 20 containing 5% nonfat milk for 1 h at room temperature and washed three times in Tris buffered saline with Tween 20, the membranes were incubated with specific primary antibodies. The following antibodies were used for immunoblotting: anti-phospho-c-Jun amino-terminal protein kinase (anti-

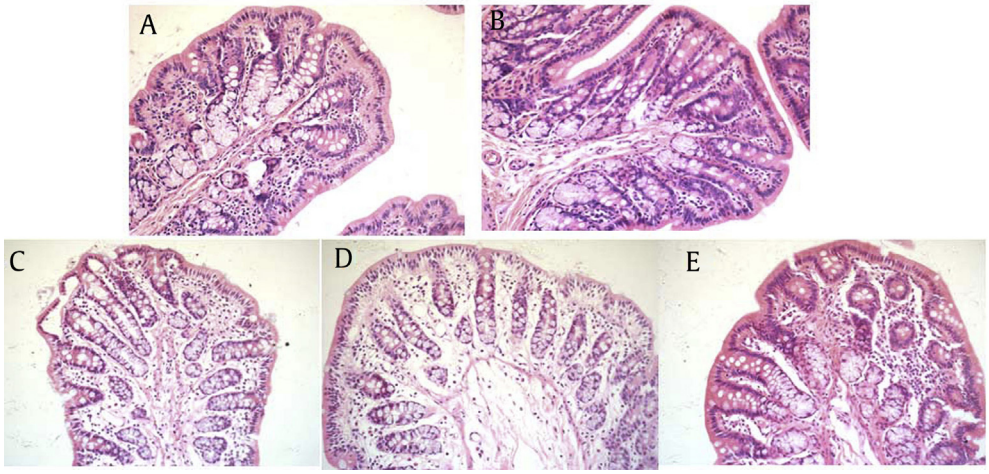

Fig. 1. Effect of aging on colonic mucosal histology (200×). (A) Adult group rats. (B) Aging group rats. (C) SPJ 10 mg/kg. (D) SPJ 30 mg/kg. (E) SPJ 60 mg/kg. SPJ, saponins from *P. japonicas*.

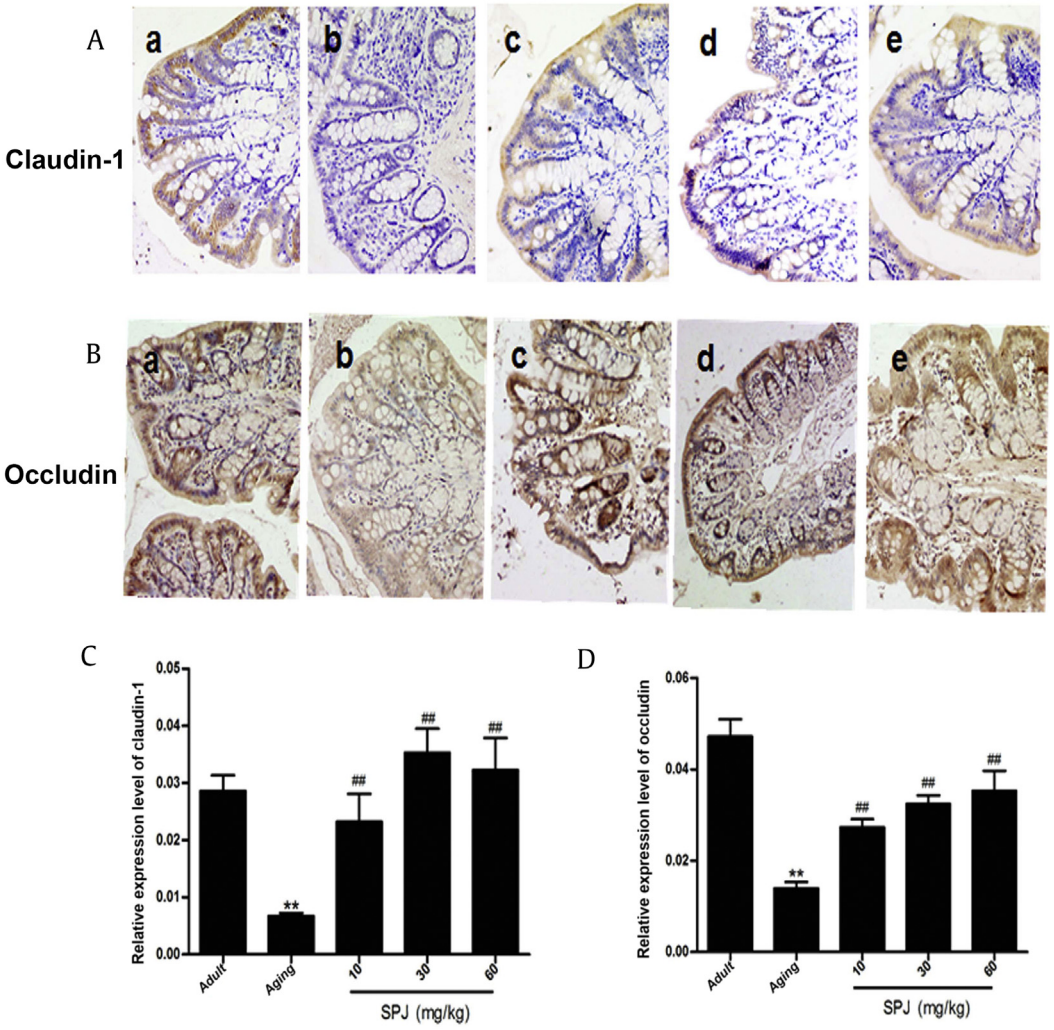

Fig. 2. Effects of SPJ on TJs in the colon of aging rats. (A) Expression of claudin-1 in the colon of rats by immunohistochemistry (200×). (B) Expression of occludin in the colon of rats by immunohistochemistry (200×). (C) Claudin-1 protein expression by IHC. (D) Occludin protein expression by IHC. Data are expressed as mean ± SEM ($n = 6$). The lowercase letters in the figure indicate the following: a, adult group rats; b, aging group rats; c, SPJ 10 mg/kg; d, SPJ 30 mg/kg; and e, SPJ 60 mg/kg. * $p < 0.05$ versus adult group rats. ** $p < 0.01$ versus adult group rats. # $p < 0.05$ versus aging group rats. ##$p < 0.01$ versus aging group rats. IHC, immunohistochemistry; SEM, standard error of the mean; SPJ, saponins from *P. japonicas*; TJ, tight junction.

phospho-JNK), anti-JNK, anti-phospho-p38, anti-p38 (Cell Signaling Technology, Boston, MA, USA); anti-phospho-ERK, anti-ERK1/2, anti-β-actin (Santa Cruz Biotechnology). After washing three times in Tris buffered saline with Tween 20, they were incubated with horseradish peroxidase-conjugated immunoglobulin G at room temperature for 1 h. Membranes were detected using an electrochemiluminescence kit (Beyotime) according to the manufacturer's instructions. Finally, membranes were detected by the Bioshine ChemiQ4800 mini imaging system (Bioshine, Shanghai, China). The relative quantity of proteins was analyzed by Image J software and normalized to that of loading controls.

2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean. SPSS software (version 13.0; IBM, Armonk, NY, USA) was used for statistical analysis. Data were analyzed by one-way analysis of variance. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Composition of total flavonoids

In our present extraction and purification method, the content of SPJ determined by HPLC–UV analysis was 92.5%, according to our previous study [14].

3.2. Effect of aging on colonic mucosal histology

The histological analysis (Fig. 1) revealed that there was no evidence of crypt atrophy or inflammation in the colon of aged rats when compared with the young samples. No marked differences were observed in the colon of aging rats by SPJ treatment.

3.3. Effects of SPJ on TJ expression in the colon of aging rats

To determine whether intestinal barrier is impaired in aging rats, we performed immunohistochemical staining to examine the expression of claudin-1 and occludin in the colon. As shown in Fig. 2, claudin-1 and occludin were obviously expressed in the colon of adult rats. By contrast, a significant decrease of claudin-1 and occludin was found in aging rats. The results suggested that impaired barrier integrity existed in aging rats. However, treatment with SPJ in aging rats significantly upregulated the expression of both proteins in the colon, indicating that SPJ can modify intestinal barrier integrity in aging rats.

3.4. Effects of SPJ on cytokine production in the colon of aging rats

Inflammatory cytokines can modulate intestinal permeability through regulation of TJs; thus, we measured the expression of IL-1β and TNF-α by Western blot in the colon (Fig. 3). We found that IL-1β and TNF-α were significantly increased in the colon of old rats compared with those in adult rats. Treatment of SPJ in aging rats

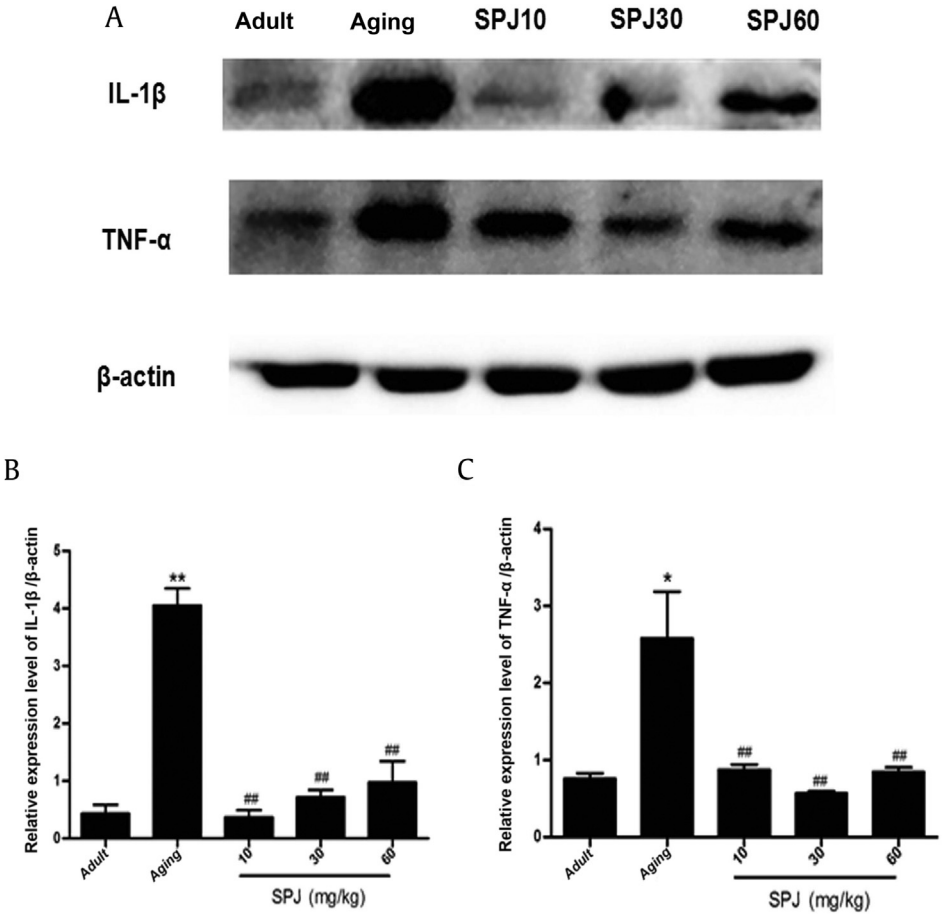

Fig. 3. Effects of SPJ on cytokine production in the colon of aging rats. (A) Expression of IL-1β and TNF-α in the colon of rats by Western blot. (B) Quantification of IL-1β/β-actin ratio. (C) Quantification of TNF-α/β-actin ratio. Data are expressed as mean ± SEM ($n = 6$). * $p < 0.05$ versus adult group rats. ** $p < 0.01$ versus adult group rats. # $p < 0.05$ versus aging group rats. ## $p < 0.01$ versus aging group rats. IL-1β, interleukin-1β; SEM, standard error of the mean; SPJ, saponins from *P. japonicas*; TNF-α, tumor necrosis factor-α.

significantly downregulated the expression of both proteins in the colon, suggesting that SPJ can modify intestinal barrier integrity in aging rats via inhibition of intestinal inflammation.

3.5. Effects of SPJ on the expression of NF-κB in the colon of aging rats

NF-κB plays an important role in inflammation. Accordingly, we determined the levels of NF-κB p65 subunit. The results showed that total NF-κB p65 subunit levels were increased significantly in the colon of aging rats (Fig. 4). However, SPJ markedly decreased the level of NF-κB p65 subunit.

3.6. Effects of SPJ on the expression of the MAPK signaling pathway in the colon of aging rats

To further explore the anti-inflammatory mechanisms underlying the protective effects of SPJ on intestinal barrier function in aging rats, expressions of JNK, ERK1/2, and p38 were explored. Expression levels of both total and phosphorylated proteins were assessed by Western blot, and the ratio of phosphorylated proteins to total proteins was used to evaluate the phosphorylation levels of these molecules to assess the involvement of the MAPK signaling pathways (Fig. 5). We found that phosphorylation of JNK, ERK1/2, and p38 increased significantly in the colon of aging rats, but the protein expressions of total ERK1/2 and p38 were not affected. However, it is interesting that the expression of total JNK also increased significantly in the colon of aging rats. SPJ reduced the activation of JNK, ERK1/2, and p38 MAPK, and the expression of total JNK in the colon of aging rats.

4. Discussion

The current study was undertaken to investigate the effects of SPJ on intestinal barrier integrity in aging rats and further to explore the potential mechanism. We discovered that there was a significant decrease in claudin-1 and occludin proteins in the colon samples of old rats compared with those in adult rats. We also examined an increased level of proinflammatory cytokines (IL-1β and TNF-α), in the absence of obvious inflammation as assessed by routine HE staining, suggesting that inflammatory cytokines modulate intestinal barrier integrity via regulation of TJ expression. We have demonstrated that treatment with three different doses of SPJ could significantly increase the expression of occludin and claudin-1 proteins in the old rat colon. Upon investigation of the potential mechanisms that may be responsible for the effects of SPJ on TJ expression of the colon in aging rats, we found that SPJ obviously decreased the expression of IL-1β and TNF-α, reduced the expression of NF-κB, and inhibited the activation of the MAPK signaling pathway in the colon of aging rats.

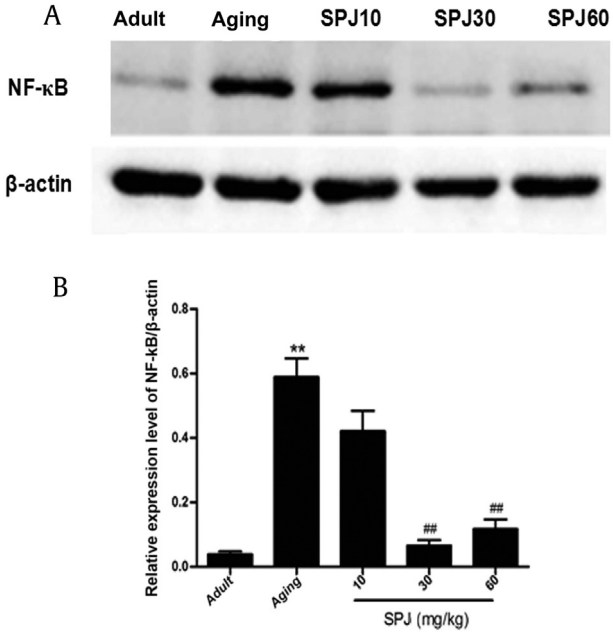

Fig. 4. Effects of SPJ on the expressions of NF-κB in the colon of aging rats. (A) Expression of NF-κB in the colon of rats by Western blot. (B) Quantification of NF-κB/β-actin ratio. Data are expressed as mean ± SEM ($n = 6$). * $p < 0.05$ versus adult group rats. ** $p < 0.01$ versus adult group rats. # $p < 0.05$ versus aging group rats. ## $p < 0.01$ versus aging group rats. NF-κB, nuclear factor kappa B; SEM, standard error of the mean; SPJ, saponins from *P. japonicas*.

The intestine is the largest exposed surface of the body and an important barrier to defend against the invasion of potentially harmful compounds and microorganisms. Intestinal barrier function is mainly maintained by the intercellular TJs between adjacent cells, which include claudins and occludins. Intestinal barrier dysfunction can increase intestinal permeability, and then noxious substances can pass through the intestinal mucosa. Several studies have discovered an increase of intestinal permeability to macromolecules in the colon of aging rodents, suggesting that intestinal barrier dysfunction occurs with age [15]. A recent study has also examined that colonic permeability was significantly higher in old baboons, with decreased expression of TJs, such as zonula occluden-1 and occludin [16]. Here, we found that the expression of the TJs occludin and claudin-1 decreased in the colon of old rats. SPJ enhanced the expression of both TJs, suggesting that SPJ can improve the barrier integrity loss of the colon in aging rats.

Disruption of the selective physical barrier finally results in chronic intestinal inflammation, and the increase of proinflammatory cytokines induced in intestinal TJ permeability seems to play an important role in the modulation of intestinal TJs [7,17]. Studies have shown that the proinflammatory cytokines such as TNF-α and IL-1β can cause disturbance of the intestinal TJ barrier, leading to a further increase in TJ permeability [18]. Inhibition of cytokine has an important protective effect against intestinal mucosal damage and development of intestinal inflammation [19,20]. Upregulation of proinflammatory cytokines was also found in the intestinal epithelium of aging mammals, and perhaps, it was a potential mechanism for TJ barrier dysfunction and enhanced intestinal permeability in aged animals [16]. Here, we also found an obvious increase of IL-1β and TNF-α in the colon of aging rats compared with that in adult rats, while SPJ obviously reduced the expression of proinflammatory cytokines in aging rats, suggesting that increased inflammation is linked to intestinal barrier dysfunction in aging rats and that regulation of intestinal TJ barrier dysfunction in aging rats by SPJ might be associated with the reduction of intestinal inflammation.

An increasing body of evidence suggests that the MAPK signaling pathway plays a crucial role in intestinal inflammation by sequential protein phosphorylation [21,22]. The three major MAPK signaling pathways include p38 MAPK, ERKs, and JNKs. Studies have discovered that the expression of P38 was increased in the colon of mice with dextran sodium sulfate-induced colitis and patients with inflammatory bowel disease [23], while p38 inhibitor suppressed the inflammation in DSS-induced colitis model via a decrease of IL-1β and TNF-α [24]. Activation of JNK or phosphorylation of ERK was induced by IL-1β or TNF-α in intestinal epithelial cells [25,26]. The three isoforms of MAPK have also been reported to mediate intestinal epithelial barrier dysfunction via modulation of TJ integrity [27,28]. Increased MAPK isoforms phosphorylation was found in ethanol-induced intestinal permeability increase.

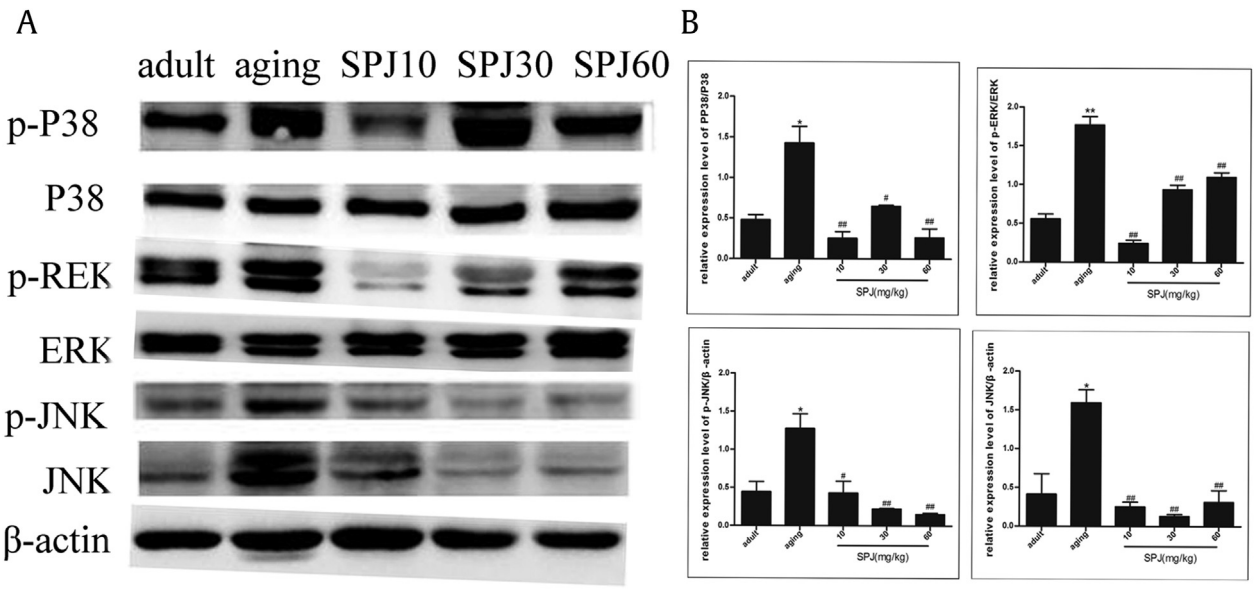

Fig. 5. Effects of SPJ on the expression of the MAPK signaling pathway in the colon of aging rats. (A) Expression of the MAPK signaling pathway in the colon of rats by Western blot. (B) Quantification of p-ERK/ERK, p-P38/P38, p-JNK/β-actin, and JNK/β-actin ratios. Data are expressed as mean ± SEM ($n = 6$). * $p < 0.05$ versus adult group rats. ** $p < 0.01$ versus adult group rats. # $p < 0.05$ versus aging group rats. ## $p < 0.01$ versus aging group rats. ERK, extracellular signal-regulated protein kinase; JNK, c-Jun amino-terminal protein kinase; MAPK, mitogen-activated protein kinase; SEM, standard error of the mean; SPJ, saponins from *P. japonicas*.

Pretreatment with MAPK inhibitors reversed the increased permeability induced by ethanol in Caco-2 monolayers. An important role is played by p38 in regulating burn-induced intestinal permeability, and inhibition of p38 attenuated intestinal barrier dysfunction by preventing the burn-induced alterations of TJs [29]. Inhibition of p38 MAPK can significantly attenuate butyrate-induced intestinal barrier dysfunction [30]. Studies have discovered that the MAPK signaling pathway plays an important role in modulating epithelial barrier function during intestinal inflammation [31–33]. However, the effects of the MAPK signaling pathway on epithelial barrier integrity during the aging process are unknown. Here, we also found an obvious increase in the phosphorylation of the of three MAPK isoform phosphorylation in the colon of aging rats compared with that in adult rats, while SPJ obviously reduced the expression of MAPK isoform phosphorylation in aging rats, suggesting that regulation of intestinal barrier dysfunction in aging rats by SPJ might be associated with decreased phosphorylation of MAPK isoforms.

NF-κB is of particular importance for maintenance of intestinal epithelial barriers, and activation of NF-κB in epithelial cells can lead to production of inflammatory cytokines, thereby triggering an inflammatory amplification cascade [34–37]. Studies have shown that constitutive intestinal NF-κB cannot induce inflammation unless accompanied by MAPK activation [38]. It has been shown that IL-1β increased the intestinal epithelial permeability and reduced the expression of TJ proteins, via activation of the NF-κB pathways in intestinal epithelial cells [39]. An increase of TJ permeability induced by TNF-α also required the activation of NF-κB in Caco-2 monolayers [40]. Here, we also found an obvious increase of NF-κB in the colon of aging rats compared with that in adult rats, while SPJ obviously reduced the expression of NF-κB in aging rats, suggesting that regulation of intestinal barrier dysfunction in aging rats by SPJ might be associated with a decrease of NF-κB, finally alleviating the inflammation.

It is worth mentioning that different doses of SPJ have different effects on the expression of different signaling proteins, and our results did not show the obvious dose–response relationship, suggesting that we did not find the best dose of SPJ. However, our study demonstrated that SPJ has a significant anti-inflammatory effect. Next, we will expand the range of SPJ dosage in rats and further investigate the mechanism of the effects of SPJ.

5. Conclusions

In conclusion, our study explored the protective effect of SPJ on intestinal barrier function in aging rats. Administration of SPJ significantly strengthened the expression of claudin-1 and occludin in the colon of aging rats. The mechanism of the effects of SPJ might include the decrease of inflammation, downregulation of NF-κB, and inactivation of MAPK. These results collectively suggest that SPJ can be a promising candidate for the treatment of intestinal barrier dysfunction in aging rats.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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