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Puerarin ameliorates 5-fluorouracil-induced intestinal mucositis in mice by inhibiting JAKs

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Running title: Puerarin ameliorates 5-FU-induced intestinal mucositis

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Abbreviations:

5-FU, 5-fluorouracil; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; COX-2, cyclooxygenase-2; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FD-4, fluorescein isothiocyanate-4 kD dextran; iNOS, inducible nitric oxide synthase; IL, interleukin; JAK2, Janus kinase 2; JAK-STAT, Janus kinase-signal transducer and activator of transcription; MLCK, myosin light chain kinase; SOCS3, suppressor of cytokine signaling 3; SPR,

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surface plasmon resonance; STAT3, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α, SD, standard deviation.

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Abstract

Intestinal mucositis, resulting from 5-fluorouracil (5-FU)-based chemotherapy, subjects patients to great pain and hampers cancer treatment progress. Puerarin, the major active ingredient in *Pueraria lobata*, exerts anti-inflammatory and anti-oxidative effects. However, whether puerarin has an effect on 5-FU-induced intestinal mucositis remains unknown. We established a mice model of intestinal mucositis through the intraperitoneal injection of 5-FU, and then injected puerarin (50 and 100 mg/kg) intraperitoneally for 7 consecutive days. Routine parameters, such as body weight, food intake, and diarrheal incidence, were examined to evaluate the effects of puerarin on intestinal mucositis in mice. The intestinal barrier's functions were also evaluated by measuring the serum recovery of fluorescein isothiocyanate-4kD dextran in this study. The expression levels of inflammatory cytokines, inflammatory mediators, oxidative reactions, as well as apoptotic marker proteins, were determined to elucidate the underlying mechanisms of puerarin on intestinal mucositis. The model mice presented symptoms and histopathological changes typical of 5-FU-induced intestinal mucositis. In addition to vigorous inflammatory reactions, oxidative reactions and cell apoptosis, Janus kinase (JAK) was markedly activated. Puerarin decreased the expression levels of those of inflammatory mediators, oxidative reactions, and apoptosis-related proteins in 5-FU-induced mucositis by blocking the activation of JAK. Puerarin decreased inflammation, oxidative reactions and apoptosis, and protected intestinal barrier functions, to ameliorate 5-FU-induced intestinal mucositis by inhibiting the activation of JAK. This study provides novel insights into the pathological mechanisms of, and treatment alternatives for, 5-FU-induced intestinal mucositis.

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Significance statement

This study reveals the mechanism responsible for the protective effects of puerarin in 5-fluorouracil-induced intestinal mucositis. Puerarin inhibits the activation of JAK, thereby suppressing inflammation, oxidative reactions, cell apoptosis, and protected intestinal barrier functions, to ameliorate 5-FU-induced intestinal mucositis. Overall, our results suggest that puerarin can serve as a potential natural JAK inhibitor in the treatment of 5-FU-induced intestinal mucositis.

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1.Introduction

As a first-line chemotherapeutic agent, 5-fluorouracil (5-FU) is widely used in cancer treatments (Huang et al., 2019). The most common adverse reaction in the course of chemotherapy, 5-FU-induced intestinal mucositis is characterized by diffuse intestinal mucosal barrier disruption, and patients present with diarrhea, weight loss, and malnutrition as the main symptoms (Gan et al., 2020). These symptoms seriously increase the risk of infection and worsen disease prognosis, even causing death. The severity of the symptoms affects the cancer treatment process, and many patients are forced to postpone or interrupt chemotherapy (Zhang et al., 2019). According to statistics, more than 80% of patients worldwide who have undergone chemotherapy with 5-FU suffer from intestinal mucositis, and the incidence is increasing annually (Hamouda et al., 2017).

The 5-FU-induced intestinal mucositis may be caused by the disruption of the intestinal mucosal barrier, which is associated with disruptions in cytokine production and increased levels of inflammatory mediators and apoptosis-related proteins (Jain et al., 2017). At present, complementary and alternative medicines based on anti-diarrheal agents, anti-inflammatory drugs, and intestinal mucosal protective agents are the main treatments to alleviate 5-FU-induced intestinal mucositis symptoms (Deng et al., 2017). It is necessary to explore the pathological mechanisms of 5-FU-induced intestinal mucositis and screen drugs for etiological treatments.

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway has been implicated in the molecular pathogenesis of intestinal inflammatory diseases, resulting in most strong phosphorylation associated with human intestinal diseases as well as in mice models (Tang et al., 2020). JAK-STAT pathway is the primary pathway for transcription factors that participate in pro-inflammatory cytokine responses in intestinal mucositis (Danese et al., 2019; Wang et al., 2020).

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Interleukin (IL)-6 is an essential cytokine in 5-FU-induced intestinal mucositis. When bound to a receptor, IL-6 enhances the phosphorylation of JAK and activates downstream STAT3, which regulates the transcription of downstream target genes, such as iNOS, COX-2, Bax, and Bcl-2, and participates in the regulation of inflammation, oxidative reactions and apoptosis(Chang et al., 2020). These processes are closely associated with a strong inflammatory response, oxidative reactions, intestinal epithelial cell apoptosis, and altered intestinal mucosal barrier functions (Lin et al., 2017). Therefore, the inhibition of JAK may be a potential way to treat 5-FU-induced intestinal mucositis.

Pueraria lobata is widely used in traditional Chinese medicine to treat gastrointestinal diseases, because of its excellent curative effects. Puerarin, an isoflavone component purified from *P. lobata*, possesses an extensive spectrum of biological activities, including anti-inflammatory, anti-tumor, anti-apoptosis, and immunomodulatory (Li et al., 2018; Li et al., 2019; Ma et al., 2020). Puerarin injected intraperitoneally has a high absorption level, may be absorbed by the intestine in a short time, and is not metabolized into other compound forms (Yamamoto et al., 2019). Puerarin may regulate the JAK2-STAT3 pathway (Wei et al., 2017). It maintains the integrity of the intestinal mucosal barrier and improves intestinal barrier functions by up-regulating the expression levels of tight junction proteins and by inducing goblet cells to generate mucin (Jeon et al., 2020). However, the effects of puerarin on 5-FU-induced intestinal mucositis and its potential mechanisms have not been extensively studied.

According to the docking results of the pre-experiment, puerarin has a strong affinity for JAK. The present study aimed to examine the effects of puerarin on 5-FU-induced intestinal mucositis in a mice model and to uncover potential mechanisms involving the JAK-STAT pathway. A widely accepted mice model for studying 5-FU-induced intestinal mucositis was established by intraperitoneally injecting 5-FU for 7 consecutive days.

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2.Materials and methods

2.1 Reagents

The fluorouracil injection was purchased from XUDONG HAIPU Pharmaceutical Co., Ltd. (Shanghai, China). Puerarin (98% purity) was purchased from Melonepharma Co., Ltd. (Dalian, China). AG490 and fluorescein isothiocyanate-4 kD dextran (FD-4) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam (Cambridge, UK). The specific antibodies against JAK1 (#3332), p-JAK1 (Tyr1034/1035) (#3331), JAK2 (#3229), p-JAK2 (Tyr1007/1008) (#3771), JAK3 (#8863), p-JAK3 (Tyr980/981) (#5031), TYK2 (#35615), p-TYK2 (Tyr1054/1055) (#68790), STAT3 (#9139), p-STAT3 (Tyr705) (#9131), COX-2 (#4842), iNOS (#13120), Bax (#2772), Bcl-2 (#3498) used for western blotting and immunohistochemistry were purchased from Cell Signaling Technology Inc. (Beverly, USA). Antibody against myosin light chain kinase (MLCK) (EP1458Y) was obtained from abcam. Cell counting kit-8 was provided by Biotool (Shanghai, China). Apoptosis was determined using an Annexin V-fluorescein isothiocyanate apoptosis detection kit, which was obtained from BD Pharmingen (Heidelberg, Germany).

2.2 Animals

In total, 50 male C57BL/6 mice (18–22 g, aged 6–8 weeks, SPF grade) were obtained from the laboratory animal center, Dalian Medical University [Certificate of Conformity: No. SYXK (Liao) 2018-0007] and maintained under specific pathogen-free conditions. After acclimation for 7 days, the mice were randomly divided into five groups containing seven mice each: (1) mice in the normal group received intraperitoneal injection with saline; (2) mice in the 5-FU group received 75 mg/kg 5-FU by intraperitoneal injection; (3) mice in the AG490 group received 75 mg/kg 5-FU and 8 mg/kg AG490 by

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intraperitoneal injection; (4) mice in the puerarin low-dose group received 75 mg/kg 5-FU and 50 mg/kg puerarin by intraperitoneal injection; and (5) mice in the puerarin high-dose group received 75 mg/kg 5-FU and 100 mg/kg puerarin by intraperitoneal injection. We measured the body weight, total food intake, and disease activity index of each mouse daily.

2.3 Cell culture

Rat small intestinal crypt epithelial cells, IEC-6, and human colorectal adenocarcinoma cells, Caco-2, were purchased from the Cell Bank of the Shanghai Institute (Shanghai, China). Both the IEC-6 and Caco2 cell lines used in this study were evaluated prior to formal experiments. In the pre-experiment, no significant interspecies variations in the JAK2–STAT3 signaling pathway that might influence the results of the current study were observed. The two cell lines were fostered in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% non-essential amino acids, and they were maintained at 37℃ with 5% CO_2. The culture medium for the IEC-6 cells contained 0.1 U/mL bovine insulin. IEC-6 and Caco-2 cells were cultured in Dulbecco's modified eagle medium supplemented with 2 mM 5-FU and maintained at 37℃ with 5% CO_2. Both the IEC-6 and Caco-2 lines were plated in 96-well plates (100 μL/well, 1×10^4 cells) and cultured at 37℃ with 5% CO_2 for 12 h. Then, IEC-6 cells were treated with a gradient dilution of puerarin (0, 10, 20, 40, 80, 160, and 320 μM) and cultured for 24 h. Caco-2 cells were treated with a gradient dilution of puerarin (0, 50, 100, and 200 μM) and cultured for 4, 6, 12, and 24 h. Cell counting kit-8 reagent was added to each well, incubated for 3.5 h in the dark, and then cell numbers were measured at 450 nm using a microplate reader.

2.4 Assessment of intestinal mucositis symptoms

The diarrheal severity was scored as follows: 0 points, normal stool; 1 point, soft stool; 2 points, wet

and unformed stool; and 3 points, watery stool with severe perianal staining of the coat. After the mice were sacrificed, the colonic mucosal damage index was evaluated (Wang et al., 2017). The numerical scores were determined as follows: (1) ulceration and inflammation: 0 points, normal; 1 point, focal congestion without ulceration; 2 points, ulceration without congestion or thickening of the intestinal wall; 3 points, presence of inflammation and ulceration at one site; 4 points, two sites of ulceration and inflammation; 5 points, portion of the colon extending beyond 1 cm of major injury; and 6–8 points, injury extending beyond 2 cm along the length of the colon, with the score increasing by 1 point for each additional 1 cm of injury; and (2) presence of adhesions: 0 points, normal; 1 point, minor adhesions; and 2 points, major adhesions. Colon samples from each group of mice were obtained. The length of colonic tissue from each group was photographed and measured. Subsequently, 1-cm-long fecal-free colon specimens were fixed in 4% paraformaldehyde for a week and embedded in paraffin to prepare sections. Colon sections were stained with hematoxylin and eosin or used for other immunohistochemical studies.

2.5 Assessment of inflammation, oxidative stress and epithelial barrier functions

Mice were perfused with 0.9% saline solution transcranial, the perfusion rate was 2.5 ml/min, until the perfusate cleared. Distal colon tissue at a distance of 3 cm from anus was isolated from mice, and were used in the evaluation of cytokines and protein expression(Kamphuis et al., 2017; Siri et al., 2019). Total protein and cytokine were isolated from distal colon tissue using a protein extraction kit (Solarbio, Beijing, China, R0100). Blots were transferred to polyvinylidene difluoride membranes and gently shaken overnight at 4°C with specific antibodies. Detection and quantification were performed on a Bio-Rad ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, USA). Cytokine levels were measured using ELISA kits in accordance with the manufacturer's instructions. Levels of glutathione

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(GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) were determined using assay kits (Solarbio, Beijing, China) according to the instructions. Intestinal barrier functions were measured in vivo. Mice were gavaged with 80 mg/mL FD-4 and fasted, but allowed watered ad libitum, for 3 h. After 3 h, sera from each group were collected and measured separately using a plate reader.

2.6 Annexin V assay

After different treatments, intestinal epithelial cells and Caco-2 cells were collected. The obtained cells were lysed in cold trypsin, and stained with fluorescein isothiocyanate-membrane coupling protein V and propidium iodide, following the operating protocol. Apoptotic cells were quantified by flow cytometry using the procedure.

2.7 Molecular docking

The molecular docking was performed with Autodock 4. The crystal structure of human JAK1 (PDB ID: 5E1E), JAK2 (PDB ID: 4GL9), JAK3 (PDB ID: 3PJC), TYK2 (PDB ID: 6VNV) were downloaded from RCSB Protein Data Bank and defined as receptor. After the removal of water molecules, hydrogen atoms were added and incomplete residues of the side chain were corrected. The energy minimization of the protein structure was achieved. Puerarin was defined as the ligands. The structure of puerarin energy minimization was performed and docked into binding site without constraint. Subsequently, the puerarin molecule was inserted into the binding pocket of JAK1, JAK2, JAK3, TYK2 based on the binding mode.

2.8 Surface plasmon resonance (SPR) assay

Biacore T200 instruments (GE Healthcare, USA) were used to assess the binding affinity of puerarin to recombinant human JAK2 protein (ab42619, abcam, UK) using SPR. After conditioning with 350 mM ethylene diamine tetraacetic acid (EDTA) and 50 mM NaOH, 50 μg/mL of JAK2 was captured on the

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surface of NTA chip at a 10 μL/min flow rate in 0.05% Tween-20. A series of puerarin concentrations was injected into the flow system and analyzed independently. The association time was set to 90 s, whereas the dissociation time was set to 120 s. The chip surface was regenerated with 350 mM EDTA and 50 mM NaOH, and JAK2 was captured for a concentration series. Prior to the analysis, double-reference subtractions were performed to eliminate bulk refractive index variations, injection noise, and data drift. The binding affinity was determined by global fitting to a Langmuir 1:1 binding model.

2.9 Measurement of complete blood picture

Blood was obtained by cardiac puncture and stored at 4°C in tubes with EDTA. Leukocytes, lymphocytes, and monocytes in the blood were counted using a SYSMEX XE-2100 Hematology Analyzer (SYSMEX, Kobe Japan).

2.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Prism Software, Inc., USA). A one-way analysis of variance was used for data comparisons. Student's t-test was used for data comparisons between two groups. Data were expressed as means ± standard deviations. Further evaluations were performed using the Kruskal–Wallis rank sum test. All the experiments were repeated at least three times, and statistical significance was indicated by P-value < 0.05.

3.Results

3.1 Puerarin dose selection and toxicity in normal cells and mice

The chemical structure of puerarin is shown in Supplemental Figure 1A. We examined the cytotoxicity of puerarin in IEC-6 cells in vitro to determine the effective non-toxic dose for subsequent experiments. Exposure to 5-320 μM puerarin for 24 h had no effect on the viability of IEC-6 cells (Supplemental Figure 1B). The intraperitoneal injection of puerarin (20, 80, and 320 mg/kg) for 7 consecutive days

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had no effect on mouse body weight (Supplemental Figure 1C), food intake (Supplemental Figure 1D), the colon weight to length ratio (Supplemental Figure 1E), or cytokine profiles, including tumor necrosis factor (TNF)-α, IL-1β, and IL-6) (Supplemental Figure 1F–H), in colon tissue. Furthermore, the intraperitoneal injection of puerarin (up to 320 mg/kg) exerted no significant effect on the expression of JAK2, p-JAK2, STAT3, or p-STAT3 in control mice (Supplemental Figure 1I, J). On the basis of these pre-experiments, we used <320 mg/kg and <320 μM puerarin for in vivo and in vitro experiments, respectively, in the current study (Supplemental Figure 1).

3.2 Effects of puerarin on 5-FU-induced intestinal mucositis in mice

The effects of puerarin on 5-FU-induced intestinal mucositis were investigated in a mouse model. Mice in the intestinal mucositis group had markedly lower body weights and food intakes, and higher diarrheal and colonic mucosal damage index scores, compared with those in normal group, as well as higher colon weight-to-length ratios (Figure 1 A-F). In the 5-FU group, 5-FU induced shortened villi, the loss of crypt architecture, and intense inflammatory infiltration. After 7 days of intraperitoneal administration, puerarin (50 and 100 mg/kg) reversed the pathological changes in the intestinal mucositis models, suggesting that puerarin significantly ameliorated the intestinal mucositis symptoms. JAK2/3 inhibitor AG490 (8 mg/kg) also exerted similar treatment effects on 5-FU-induced intestinal mucositis.

3.3 Effects of puerarin on 5-FU-induced inflammatory responses and oxidative stress

Intestinal mucositis induced by 5-FU was often accompanied by inflammatory response. A western blot analysis showed that levels of inflammation-related proteins (COX-2 and iNOS) in colon tissues increased after the injection of 5-FU (Figure 2A, B). An ELISA revealed that pro-inflammatory cytokine levels, including those of IL-1β, IL-6, and TNF-α, increased significantly in intestinal

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mucositis models (Figure 2C-E). In 5-FU-induced intestinal mucositis group, increased level of MDA and decreased levels of antioxidant enzymes, including GSH and SOD were observed (Figure 2F-H). All the changes in intestinal mucositis were significantly reversed by puerarin and AG490.

3.4 Effects of puerarin on 5-FU-induced intestinal barrier injury

A significant increase in the serum recovery of FD-4 in the intestinal mucositis group compared with the sham group was found after intraperitoneally injecting 5-FU for 7 consecutive days (Figure 3A). The level of the intestinal epithelial barrier dysfunction-related protein myosin light chain kinase (MLCK) significantly increased in the mucositis group (Figure 3B). Unlike the normal group, the Bax-to-Bcl-2 ratio significantly increased in the 5-FU-induced intestinal mucositis group (Figure 3C). All these results suggested the intestinal barrier injury in 5-FU-induced intestinal mucositis group. The effects of puerarin on the apoptosis of epithelial cells were determined using flow cytometry (Figure 3D). The apoptosis rate in the 5-FU-treated cells significantly increased compared with in the sham group. After the administration of puerarin and AG490, the tight junction protein recovered, epithelial cell apoptosis was reduced, and barrier function impairment improved.

3.5 Potential mechanisms of puerarin in the treatment of 5-FU-induced intestinal mucositis

The 3-D diagram of molecular docking revealed that puerarin has a high affinity for JAK2, with hydrogen bonds at Lys-943 at a distance of 1.9 Å/2.6 Å (Figure 4A). According to SPR results, puerarin targeted JAK2, with a binding affinity of 12.06 μM (Figure 4B). The qRT-PCR showed that puerarin had no significant effect on the JAK2 mRNA expression level (Figure 4C). The levels of related proteins were measured using western blotting. In 5-FU-induced intestinal mucositis model mice, the activation of p-JAK2 and p-STAT3 increased significantly, whereas puerarin administration down-regulated the expression levels of p-JAK2 and p-STAT3 (Figure 4D, E). The JAK-STAT downstream

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protein SOCS3 was also suppressed by puerarin in 5-FU-induced intestinal mucositis model mice (Figure 4F, G). The similar effects were also seen in AG490 group.

The regulation of JAK2 by puerarin was also studied in IEC-6 cells. IEC-6 cells were treated with 100 μg/ml 5-FU for 12 h to establish an in vitro 5-FU-induced intestinal mucositis model, and both puerarin and AG490 were added for 12 hours after the treatment of 5-FU. We observed significantly elevated levels of JAK2 (Figure 5A) and STAT3 (Figure 5B) phosphorylation, and a high expression level of downstream SOCS3 (Figure 5C). These changes were accompanied by a high Bax-to-Bcl-2 ratio (Figure 5D), as well as high expression levels of the inflammation-related proteins COX-2 (Figure 5E) and iNOS (Figure 5F). After treatment with puerarin (120 μM), the excessive activation of the JAK2-STAT3 signaling pathway was attenuated and inflammation, as well as apoptosis-related protein levels, were restored. The presence of AG490 (50 μM) alone showed similar therapeutic effects. Puerarin exerted protective effects was partially influenced by AG490, however, no significant difference was observed.

3.6 Effect of puerarin on the inhibition of JAKs

The 3-D diagram of molecular docking revealed that puerarin also have high affinity with JAK1, JAK3, TYK2 (Figure 6A-C). The result of western blotting shown that the level of p-JAK1, p-JAK3, p-TYK2 were significantly increased in mucositis model mice. Puerarin inhibited the activation of JAK1, JAK3, TYK2, however, only the phosphorylation of JAK3 was inhibited by AG490 (Figure 6D-F). Despite puerarin has inhibitory effects on JAK1, JAK2, JAK3, and TYK2, the inhibitory rate of JAK2 was higher than that of other JAKs (Figure 6G).

4.Discussion

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In intestinal mucositis model mice, JAKs was significantly activated. The intraperitoneal injection of puerarin significantly alleviated intestinal mucositis symptoms in 5-FU-induced intestinal mucositis model mice. Inflammation and intestinal barrier dysfunction are particularly critical events in 5-FU-induced intestinal mucositis (Carvalho et al., 2021). Both in vivo and in vitro, puerarin significantly alleviated inflammatory responses and restored the intestinal barrier functions in the intestinal mucositis models by inhibiting the activation of JAK. Oxidative stress can also induce the activation of JAK signaling pathway (Charras et al., 2019; Varyani et al., 2019; Chakrabarti and Visweswariah, 2020), however, antioxidants like N-acetyl-L-cysteine does not significantly inhibit JAK activation compared with JAK inhibitor(Zhou et al., 2017). Overall, these results suggest that puerarin suppressed JAK to exert a protective role against the intestinal mucositis induced by 5-FU.

Puerarin is a major active ingredient extracted from *P. lobata*. The bioavailability of oral administration of puerarin is very low (Yue et al., 2007; Anukunwithaya et al., 2018). Intraperitoneal injection of puerarin has a better bioavailability and safety with a high level of absorption, which can be absorbed by the intestine within a short period of time (Yamamoto et al., 2019). It has been demonstrated that puerarin modulates the JAK2-STAT3 signaling pathway to exert a protective effect on astrocytes during deprivation-reoxygenation injuries (Wei et al., 2017). Puerarin also relieve experimental colitis by reducing oxidative stress and enhancing tight junctions between intestinal epithelial cells (Jeon et al., 2020). Thus, puerarin may be a potential small molecule inhibitor in the treatment of 5-FU-induced intestinal mucositis that functions by targeting JAK. In this study, puerarin was found to inhibit the phosphorylation of JAK, resulting in the alleviation of intestinal mucositis in the models. These findings provide a potential mechanism of action by which puerarin alleviates intestinal mucositis.

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JAK-STAT signaling pathway is involved in the pathogeneses of intestinal diseases, and especially JAK inhibitors, which are effective for treating inflammatory bowel disease (Danese et al., 2019). JAK2-STAT3 is the major pathway of transcription factors involved in the proinflammatory cytokine responses of intestinal mucosal inflammation (Xue and Li, 2020). JAK2 was over-activated in 5-FU-induced intestinal mucositis models, which were accompanied by increased levels of inflammatory mediators (COX-2, iNOS, TNF-α, IL-6, and IL-1β), oxidative reactions and intestinal barrier dysfunction. The symptoms of intestinal mucositis were significantly alleviated after the intraperitoneal injection of AG490, suggesting that JAK2 may be used as a target for the treatment of 5-FU-induced intestinal mucositis. Further results revealed that puerarin targeted JAK2, with a high binding affinity, indicating that puerarin may act as a potential JAK inhibitor by impairing the phosphorylation of JAK2. Puerarin exerted protective effects was partially influenced by AG490, however, no significant difference was observed. So, we speculated that puerarin may be not a specific JAK2 inhibitor.

JAK1, JAK3, TYK2 have been described as crucial effectors for the modulation of immune function and barrier function in intestinal disease (Danese et al., 2019). Our results suggested that puerarin could serve as pan-JAK inhibitor, which has binding affinity with JAK1, JAK2, JAK3, and TYK2. The inhibitory rate of JAK2 by puerarin was higher than that of other JAKs in 5-FU induced intestinal mucositis. Puerarin has been widely used in clinic with good efficacy and reliable safety. Our results showed no obvious toxic side effects at doses from 0 to 320 mg/kg in vivo and from 0 to 320 μM in vitro, which is consistent with previous studies (Qin et al., 2016; Liu et al., 2019). In order to improve biological activities and absorption, while diminish toxicity, current JAK2 inhibitors research and development mainly focus on modifying the structure of AG490 or screening natural products (Yang et al., 2011). Currently, there remain gaps in the field of clinical treatment of 5-FU-induced

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intestinal mucositis. Tofacitinib is one of the main JAK inhibitors approved by FDA/EMA for the treatment of ulcerative colitis (Rogler, 2020). Given the critical role of JAK in the pathology of intestinal mucositis, tofacitinib, which has a broad JAK inhibitory effect, is theoretically efficacious for the treatment of 5-FU-induced intestinal mucositis. However, its clinical efficacy and safety remain to be studied.

The over-activation of the JAKs plays an important role in promoting colorectal cancer metastasis (Zhang et al., 2018). Puerarin inhibits the proliferation and metastasis of colon cancer cells (Deng et al., 2019). Here, puerarin had protective effects against 5-FU-induced intestinal mucositis in IEC-6 cells, but was cytotoxic to Caco-2 cells under the same in vitro cultivation conditions (Supplemental Figure 2A-C). Notably, puerarin enhanced the toxicity of 5-FU against Caco-2 cells, and improved hematological side effects compared with 5-FU, which caused significant decreases in several hemogram indexes, including white-blood-cell counts, lymphocyte counts, and monocyte counts, suggesting that puerarin inhibited tumor cells with a high drug safety score and limited side effects (Supplemental Figure 2D-F). These results provide a basis for subsequent relevant mechanism studies.

In conclusion, this study found that puerarin improved 5-FU-induced intestinal mucositis-related symptoms, and it play a therapeutic role by reducing the levels of inflammatory mediators and restoring intestinal barrier functions by inhibiting the activation of JAK. The findings increase our understanding of the pathological mechanisms of 5-FU-induced intestinal mucositis and may lead to the development of puerarin as a new drug in the treatment of 5-FU-induced intestinal mucositis. Moreover, the results suggest that puerarin may serve as a potential natural JAK inhibitor and provide new options for the treatment of many diseases.

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Authorship Contributions

Participated in research design: L. Wang, B.H. Song, Y. Hu, J.Y. Wang, D.P. Chen

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Contributed new reagents or analytic tools: L. Wang, B.H. Song, Y. Hu

Performed data analysis: L. Wang, B.H. Song, Y. Hu

Wrote or contributed to writing of the manuscript: L. Wang, B.H. Song, Y. Hu, J.Y. Wang, D.P. Chen

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Footnotes

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Conflict of interests

The authors declare no financial conflict of interest.

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Compliance and ethics

All the studies involving animals were conducted in accordance with the Animal Protection Council of Canada (CCAC) guidelines or guidelines for the Care and Use of Laboratory Animals (8th edition, National Academy of Sciences press).

Figure legends

Figure 1. Puerarin alleviated 5-FU-induced intestinal mucositis symptoms. Intestinal mucositis symptoms were examined after 7 days of puerarin treatment. Effects of puerarin on the body weight (A), food intake (B), diarrheal score (C), colon mucosal damage index (D), and colon weight-to-length ratio (E). Hematoxylin–eosin staining of mouse colonic tissues (F, G). Scale bars, 100 μm (upper panel) and 50 μm (lower panel). The green ring indicates damage to the villous structure; the black arrow indicates the infiltration of inflammatory cells. **$P < 0.01$ *vs.* sham group; ##$P < 0.01$ *vs.* 5-FU control group; $n = 3$.

Figure 2. Effects of puerarin on inflammation in a 5-FU-induced intestinal mucositis model. Western blot analysis of the inflammation-related proteins COX2 (A), and iNOS (B). The levels of the cytokines IL-1β (C), IL-6 (D), and TNF-α (E) were determined by ELISA. The levels of GSH (F), MDA (G), SOD (H) were also examined. **$P < 0.01$ *vs.* sham group; ##$P < 0.01$ *vs.* 5-FU control group; $n = 3$.

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Figure 3. Puerarin restored barrier functions by decreasing intestinal epithelial cell apoptosis and intestinal permeability. Effects of puerarin on the serum recovery of FD-4 (A). Effects of puerarin on the expression of the tight junction protein MLCK (B) and the apoptosis-related protein Bax-to-Bcl-2 ratio (C). Apoptosis in intestinal mucositis and drug-treated groups measured by flow cytometry (D). **P < 0.01 *vs.* sham group; ##P < 0.01 *vs.* 5-FU control group; n = 3.

Figure 4. Effects of puerarin on the JAK2-STAT3 signaling pathway in vivo. Molecular docking models of puerarin with JAK2 (A). Binding analysis of puerarin to JAK2 as assessed by SPR(B). Expression of JAK2 mRNA as assessed by qRT-PCR (C). Western blot analyses of JAK2 and p-JAK2, calculated as p-JAK2-JAK2 (D), STAT3 and p-STAT3, calculated as p-STAT3-STAT3 (E), and SOCS3 (F). Immunohistochemical staining of SOCS3 in the colonic epithelium (G). Scale bar = 100 μm. Data are presented as the means ± SDs. **P < 0.01 *vs.* sham group; ##P < 0.01 *vs.* 5-FU control group; n = 3.

Figure 5. Effects of puerarin on the JAK2–STAT3 signaling pathway in vitro. Effects of puerarin on the JAK2–STAT3 signaling pathway in the absence and presence of a JAK2 inhibitor. Western blot analyses of JAK2 and p-JAK2, calculated as p-JAK2-JAK2 (A), p-STAT3 and STAT3, calculated as p-STAT3-STAT3 (B), SOCS3 (C), apoptosis-related proteins Bcl-2 and Bax, calculated as Bax-Bcl-2 (D), and inflammation-related proteins COX-2 (E) and iNOS (F). Data are presented as the means ± SDs. **P < 0.01 *vs.* sham group; ##P < 0.01 *vs.* 5-FU control group; n = 3.

Figure 6. Effects of puerarin on the JAK1, JAK3, TYK2 in vivo.

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Molecular docking models of puerarin with JAK1(A), JAK3 (B), TYK2 (C). Western blot analyses of JAK1 and p-JAK1, calculated as p-JAK1-JAK1 (D), JAK3 and p-JAK3, calculated as p-JAK3-JAK3 (E), TYK2 and p-TYK2, calculated as p-TYK2-TYK2 (F). The inhibition rate of AG490 and puerarin on JAKs (G). Data are presented as the means ± SDs. $^{**}P < 0.01$ *vs.* sham group; $^{\#\#}P < 0.01$ *vs.* 5-FU control group; n = 3.

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Figure 1

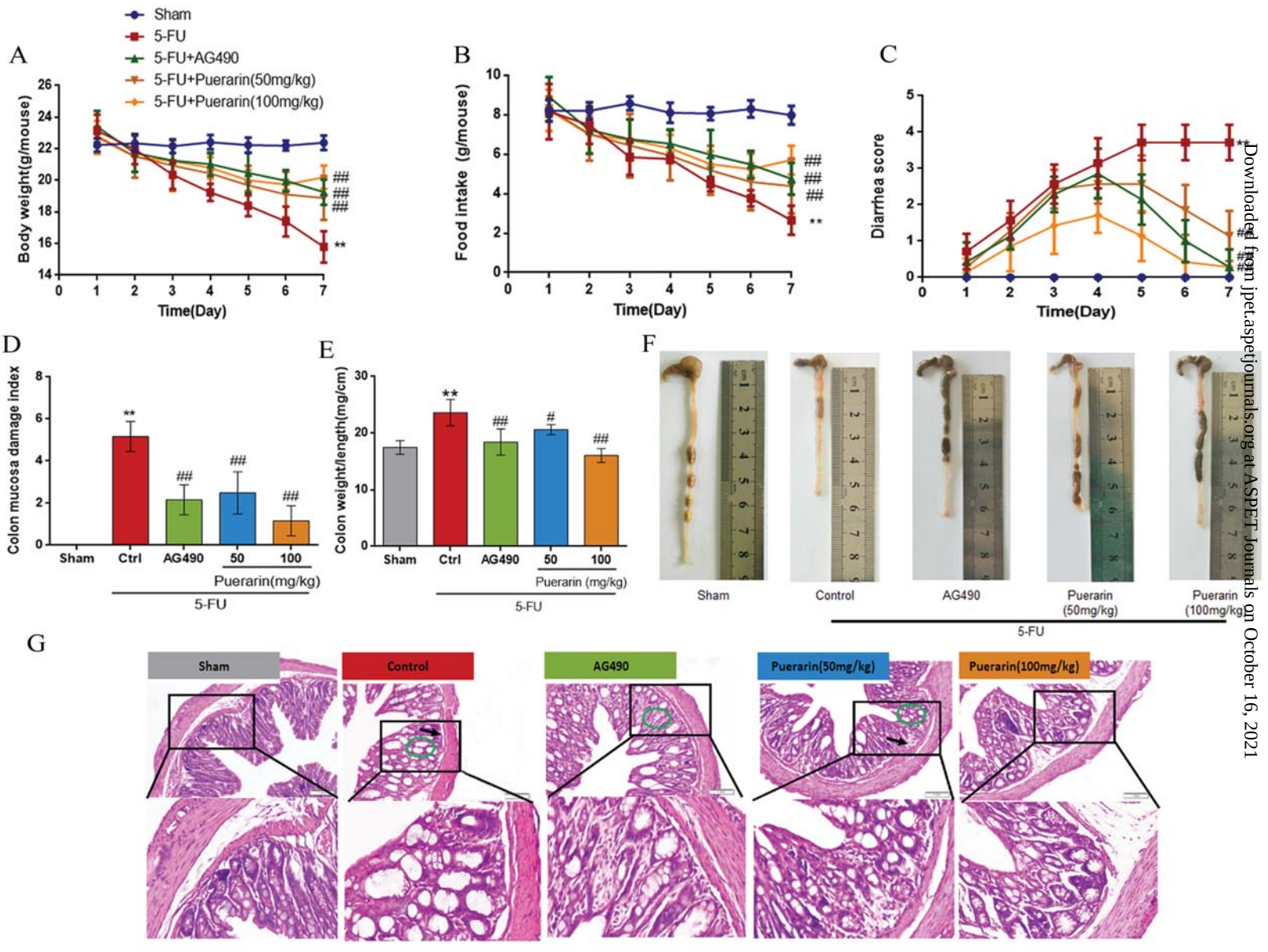

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Figure 2

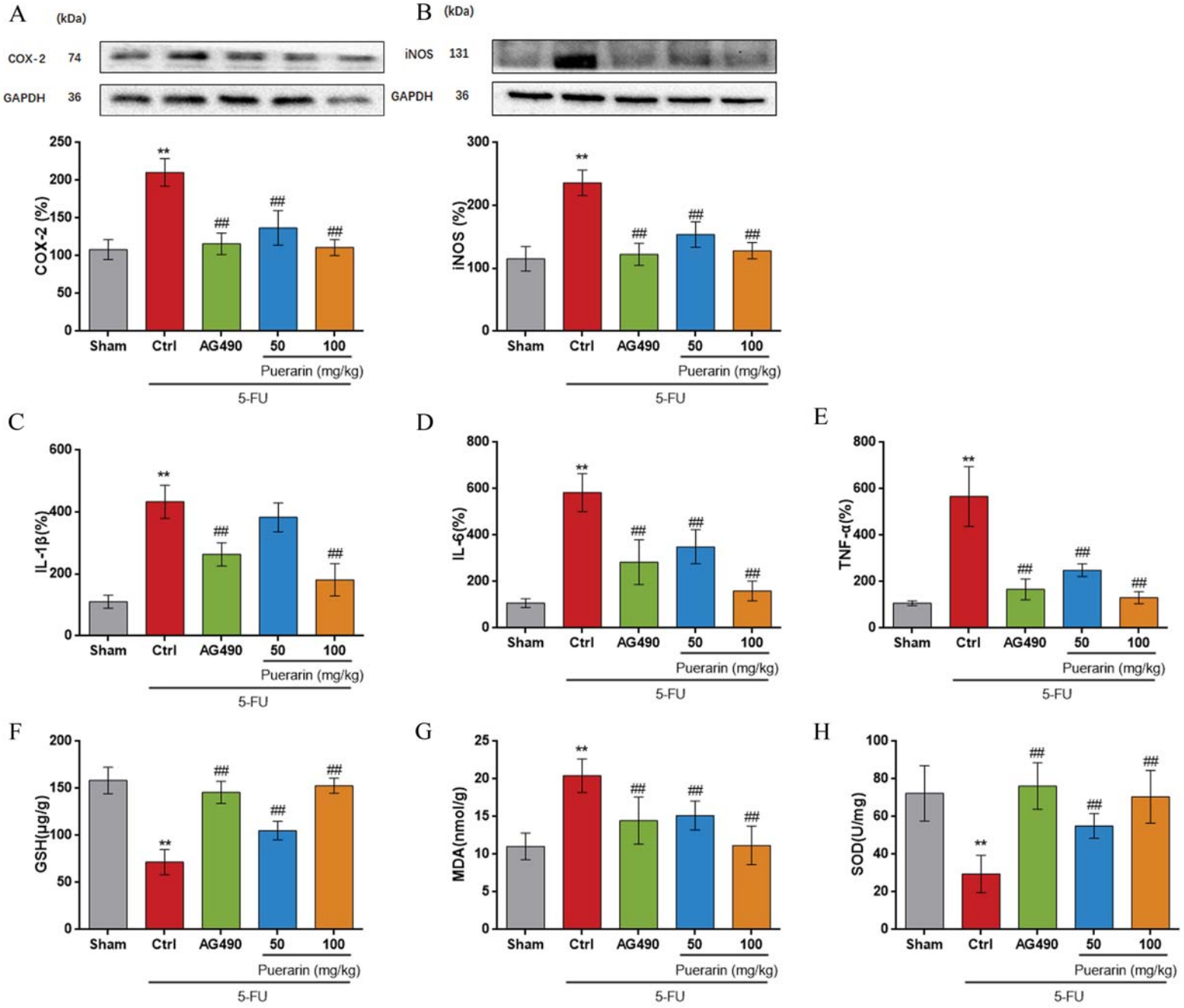

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Figure 3

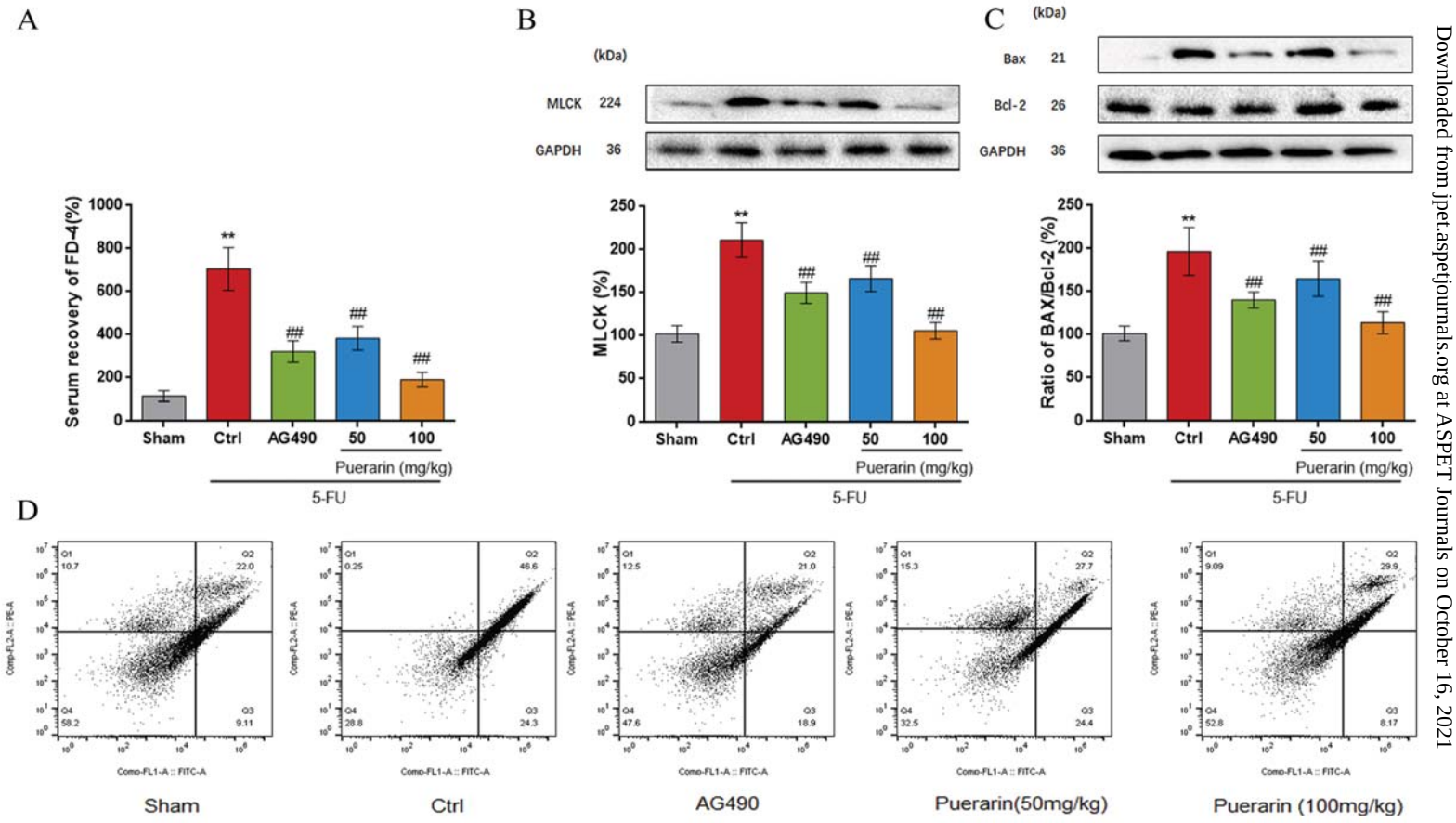

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Figure 4

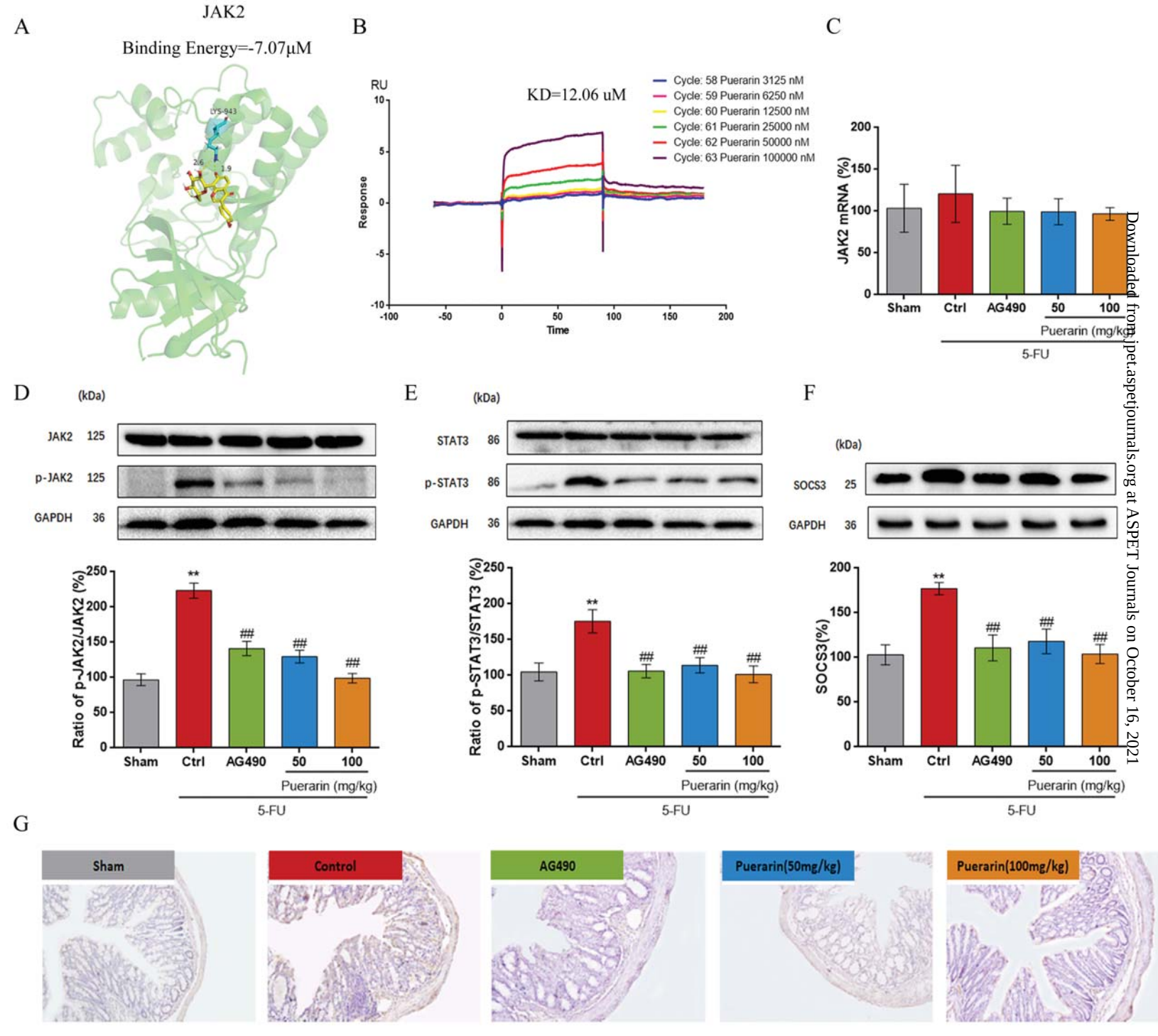

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Figure 5

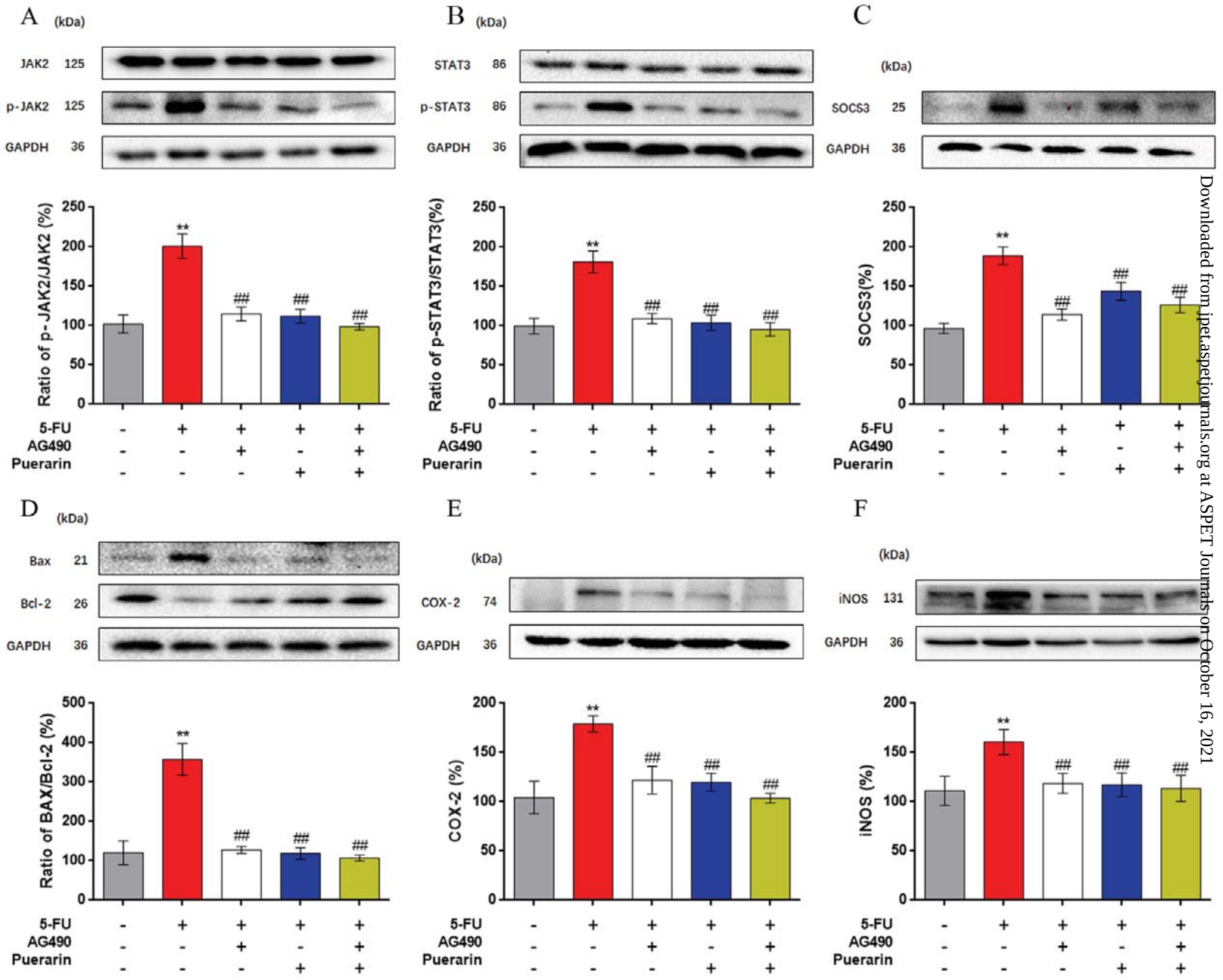

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Figure 6