J Appl Biomed
DOI: 10.32725/jab.2023.018
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

Shenhuang plaster enhances intestinal anastomotic healing in rabbits through activation of the TGF-β and Hippo/YAP signaling pathways

Fengchun Xiao [a], Chenchen Zhu [a], Xing Wei, Guiping Chen *, Xiaohong Xu *

The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang Provincial Hospital of Chinese Medicine), Hangzhou 310006, Zhejiang, China

Abstract
Although many efforts have been made to improve management strategies and diagnostic methods in the past several decades, the prevention of anastomotic complications, such as anastomotic leaks and strictures, remain a major clinical challenge. Therefore, new molecular pathways need to be identified that regulate anastomotic healing, and to design new treatments for patients after anastomosis to reduce the occurrence of complications. Rabbits were treated with a MST1/2 inhibitor XMU-XP-1, a Chinese medicine formula Shenhuang plaster (SHP) or a control vehicle immediately after surgery. The anastomotic burst pressure, collagen deposition, and hydroxyproline concentration were evaluated at 3 and 7 days after the surgery, and qRT-PCR and western-blot analyses were used to characterize mRNA and protein expression levels. Both XMU-XP-1 and SHP significantly increased anastomotic burst pressure, collagen deposition, and the concentration of hydroxyproline in intestinal anastomotic tissue at postoperative day 7 (POD 7). Importantly, SHP could induce TGF-β1 expression, which activated its downstream target Smad-2 to activate the TGF-β1 signaling pathway. Moreover, SHP reduced the phosphorylation level of YAP and increased its active form, and treatment with verteporfin, a YAP-TEAD complex inhibitor, significantly suppressed the effects induced by SHP during anastomotic tissue healing. This study demonstrated that activation of the Hippo-YAP pathway enhances anastomotic healing, and that SHP enhances both the TGF-β1/Smad and YAP signaling pathways to promote rabbit anastomotic healing after surgery. These results suggest that SHP could be used to treat patients who underwent anastomosis to prevent the occurrence of anastomotic complications.

Keywords: Anastomosis; Anastomotic healing; MST1/2 inhibitor; Shenhuang plaster (SHP); TGF-β1; YAP

Highlights:
- Chinese medicine Shenhuang plaster (SHP) promotes anastomotic healing in rabbits after surgery.
- SHP activates both TGF-β1/Smad and YAP signaling pathways in rabbits after surgery.
- SHP promotes anastomotic healing through regulation of the TGF-β1 and Hippo/YAP signaling pathways.

Abbreviations:
ABP: Anastomotic burst pressure; HPC: hydroxyproline concentration; PDGF: platelet-derived growth factor; POD: postoperative day; RT-PCR: reverse transcriptase-polymerase chain reaction; SHP: Shenhuang plaster; TAZ: PDZ-binding motif; TGF-β: transforming growth factor-β; VEGF: vascular endothelial growth factor; XMU-MP-1: 4-((5,10-dimethyl-6-oxo-6,10-dihydro-5H-pyrimido[5,4-b] thieno[3,2-e][1,4]diazepin-2-yl)amino) benzenesulfonamide; YAP: Yes associated protein

Introduction

Over the past several decades, significant progress has been made in the perioperative management and the availability of improved diagnostic adjuncts, such as imaging techniques and biochemical markers. However, anastomotic complications, such as anastomotic leaks and strictures, which are often associated with high morbidity and mortality, continue to occur at a high rate (Guyton et al., 2016; Morgan and Shogan, 2022). One of the main challenges is that the exact mechanisms impacting anastomotic healing are not yet fully understood (Morgan and Shogan, 2022). Previous efforts have mainly focused on using engineering principles to promote device improvement and compound development (*e.g.*, glues or seam bindings) to prevent the incidence of anastomotic complications. However, the iterative improvements in technology have had little impact on the incidence rates of anastomotic

* **Corresponding authors:** Guiping Chen and Xiaohong Xu, The First Affiliated Hospital of Zhejiang Chinese Medical University (Zhejiang Provincial Hospital of Chinese Medicine), Hangzhou 310006, Zhejiang, China; e-mail: G. Chen: 1941206479@qq.com and X. Xu: 20163329@zcmu.edu.cn; http://doi.org/10.32725/jab.2023.018
[a] These authors contributed equally.

Submitted: 2023-06-08 • Accepted: 2023-10-30 • Prepublished online: 2023-11-15
J Appl Biomed 21/4: 208–217 • EISSN 1214-0287 • ISSN 1214-021X

leaks and strictures (Guyton et al., 2016). In recent years, an increasing number of studies have focused on the pathobiology of anastomotic complications at the cellular and molecular level (Guyton et al., 2016; Morgan and Shogan, 2022; Türlü et al., 2021).

Similar to other types of wound healing, inflammation, proliferation and remodeling are the three main phases of the intestinal healing process (Gonzalez et al., 2016; Lam et al., 2020). The strength in the anastomosis is gradually enhanced as fibroblasts and smooth muscle cells in the healing tissue synthesize new collagen while the wound is remodeled. Any disturbance of that process may contribute to the development of anastomotic complications. More and more evidence is showing that cell-to-cell signaling cross-talk plays an essential role in the regulation of this process; mainly through cytokines and growth factors, including transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), which are released from the healing tissues (Morgan and Shogan, 2022). Enhancement of these signaling pathways has been shown to improve anastomotic strength in animals, and in recent years the identification of new pathways to improve anastomotic healing has attracted the interest of researchers.

It is well known that the Hippo pathway and its downstream effectors, the transcriptional coactivator Yes associated protein (YAP) – associated with its transcriptional coactivator with PDZ-binding motif (TAZ) – play essential roles in regulating the self-renewal and regeneration of different tissues, including the intestines, through the regulation of stem cell fate and cell proliferation and differentiation (Felley-Bosco and Stahel, 2014; Deng et al., 2022; Hong et al., 2016; Moroishi et al., 2015). The activation of YAP/TAZ, the core components of the Hippo pathway, is controlled by two kinases, MST1 and MST2 (MST1/2), the mammalian Hippo orthologs. Therefore, chemical compounds that selectively target MST1/2 could induce tissue regeneration and enhance healing after injury. For example, 4-((5,10-dimethyl-6-oxo-6,10-dihydro-5H-pyrimido [5,4-b]thieno[3,2-e][1,4] diazepin-2-yl)amino) benzenesulfonamide (XMU-MP-1), a selective MST1/2 inhibitor, has been shown to promote liver repair and regeneration after injury and to augment mouse intestinal repair as well (Fan et al., 2016). Although the crucial function of the Hippo pathway in tissue regeneration has been intensively studied, little is known about the role of the Hippo pathway in intestinal anastomotic healing and regeneration to prevent anastomotic complications.

Shenhuang plaster (SHP), a traditional Chinese medicine formula, consists of seven herbs that have been well documented since the earliest Chinese pharmaceutical monograph "ShenNong Ben Cao Jing", including Sun-dried *Panax ginseng*, Salviae Miltiorrhizae Radix et Rhizoma, Rhubarb, Fructus Aurantii Immaturus, Cortex Magnoliae Officinalis, Flos Caryophyllata, and Frutcus Evodiae (Liu et al., 2022; Shi et al., 2020; Xu et al., 2016). The HPLC profile of SHP revealed that it contained hesperidin, quercetin, ginsenoside-Rb1, aloeemodin, ginsenoside-Rg1, magnolol and chrysophanol. SHP has been widely used to treat abdominal obstructions and inflammatory bowel diseases with a prominent efficacy in clinics for more than two decades (Chen et al., 2012; Wei et al., 2014a; Yu et al., 2013). In clinical observation, our group found that the external application of SHP on Shenque (CV8), a method that has been used for more than 2,000 years (Yang and Xu, 2008), can shorten the first exhaust and defecation time, promote early rehabilitation of gastrointestinal dysfunction and intestinal peristalsis, and relieve symptoms of gastrointestinal dysfunction in patients after abdominal surgery. For instance, SHP application in CV8 could significantly enhance bowel movement and improve constipation in breast cancer patients after surgery (Chen et al., 2012; Wei et al., 2014a, b; Yu et al., 2013). To further understand the detailed effect of SHP application on gastrointestinal function in patients after abdominal surgery, we and Dr. Sun's group have investigated the role of SHP in gastrointestinal function for more than a decade, by using different animal models of postoperative ileus (POI) – a gastrointestinal function complication that arises from surgery. In the studies of POI rat models, we found transdermal application of SHP could improve gastrointestinal motility and significantly increase gut hormone ghrelin concentration and the expression of its receptor, but reduced obestatin concentration and the expression of GPR39 receptors in blood (Chen et al., 2012; Shi et al., 2020; Wei et al., 2014a, b; Yang and Xu, 2008; Yu et al., 2013; Zheng et al., 2018). Our study also showed that transdermal administration of SHP suppressed the intestinal inflammatory response and improved the impaired motility of the gastrointestine (Xu et al., 2016). Further study of a rat model of POI showed that SHP application suppressed the expression of inflammatory mediators, including IL-10, TNF-α, IL-1β, IL-6, iNOS and ICAM-1 (Xu et al., 2015, 2016). The studies from a mouse POI model showed SHP could enhance gastrointestinal motility by effectively regulating intestinal flora (Shi et al., 2022), and SHP could significantly decrease the expressions of interleukin (IL)-1β and tumor necrosis factor TNF-α in the intestine with promotion of gastrointestinal motility, and suppressing the inflammatory cytokine expression of POI by SHP treatment likely through inhibition of the PI3K/AKT/NF-κB pathways (Liu et al., 2022). In a tumor-bearing mouse model, we showed SHP could alleviate paclitaxel (PTX)-induced constipation and intestinal morphological damage through regulation of the expression of cytokines of TLR4 pathway and IL-1β (Shi et al., 2020). In summary, the previous studies demonstrated that transdermal administration of SHP could improve gastrointestinal function and suppress inflammatory response in *in vivo* animal model of POI.

Based on the above *in vivo* studies, we proposed that SHP might potentially benefit intestinal healing, which could prevent the occurrence of anastomotic complications. Therefore, the aims of the study were to investigate whether SHP could enhance anastomotic healing, explore the potential role of the Hippo pathway in intestinal anastomotic healing using an MST1/2 inhibitor XMU-MP-1, and investigate the potential molecular mechanisms of SHP in the regulation of anastomotic healing.

Materials and methods

The ingredients of Shenhuang plaster

The ingredients of Shenhuang plaster are listed in Table 1 with their scientific species names.

Rabbit model of intestinal anastomosis

All animal care and experimental procedures complied with the Guide for Animal Use and Care published by the Zhejiang Chinese Medical University and were approved by the Institutional Review Board of the Zhejiang Chinese Medical University (approval code P10-482). All animals were housed in wire cages supplied with wood shavings and hay. They were housed under conditions of constant temperature and relative humidity at 18–20 °C and 40–60%, respectively, and a 12-hour light/dark cycle. The animals were allowed free access to food and

Table 1. The ingredients of SHP formula

Name in Chinese	Name in English	Name of species	Dry weight of crude drugs in SHP (g)
人参 (Renshen)	Ginseng	*Panax ginseng* C. A. Mey.	300
大黄 (Dahuang)	Rhubarb	*Rheum palmatum* L.	300
丹参 (Danshen)	Dan-Shen Root	*Salvia miltiorrhiza* Bge.	300
枳实 (Zhishi)	Immature Bitter Orange	*Citrus aurantium* L.	200
厚朴 (Houpo)	Magnolia Bark	*Magnolia officinalis*	250
丁香 (Dingxiang)	Clove	*Eugenia caryophyllata* Thunb.	125
吴茱萸 (Wuzhuyu)	Medicinal Evodia Fruit	*Euodia rutaecarpa* (Juss.) Benth.	125

drinking water, and were acclimatized for seven days before surgery.

Male New Zealand rabbits, each weighing 3.0–3.6 kg (SCXK 2015-0004, Zhejiang, China), were used for the establishment of the rabbit intestinal anastomosis model, which followed the protocol previously described (Nakamura et al., 2016). Briefly, the animals, fasted for 24 h before the operation, were fully anesthetized with 3% isoflurane inhalation, and the skin was shaved with electric clippers and cleaned with 10% povidone-iodine for disinfection. A laparotomy with a midline incision measuring approximately 4 cm in length was performed. The small intestine was exteriorized and cut with a scalpel at the site about 25 cm proximal to the ileocecal junction. Then, the end-to-end anastomosis was performed with one layer of 12–16 full-thickness interrupted sutures with 5-0 PDS II® (Ethicon Inc, USA). After this, the the small bowel was placed back into the abdominal cavity, and the incision was closed with a continuous 4-0 suture intracutaneously (Yangzhou Fuda Medical Devices CO., Ltd,China). All rabbits wore an Elizabethan collar after surgery to prevent them from biting or licking the wound. After the operation, the rabbits were treated with the following conditions: a negative control group treated with saline, a MST1/2 inhibitor XMU-MP-1 (Cat. HY-100526, MedChemExpress) group, a SHP treated group, and a SHP combined with a YAP inhibitor Verteporfin (Cat. SML0534, Sigma-Aldrich, St. Louis, USA) treated group. 12 rabbits were used in each group. The stock solution of XMU-MP-1 or Verteporfin was dissolved in DMSO, then was diluted with PBS to make working solution for the treatment. 1 mg/kg XMU-MP-1 or 100 mg/kg, or the same volume of DMSO diluted in PBS as a corresponding control vehicle, was administered via intraperitoneal injection immediately after surgery; for the SHP and control groups, the rabbits were transdermally treated with SHP or saline as control immediately after surgery. For testing the role of YAP pathway, the rabbits were pretreated with Verteporfin together with SHP before the intestinal anastomotic surgery. Six rabbits in each group were sacrificed at postoperative day 3 (POD 3), and the other 6 rabbits were sacrificed at postoperative day 7 (POD 7) for all experiments.

Measuring Anastomotic Burst Pressure (ABP)

The mechanical strength of intestinal anastomoses was assessed under anesthesia by measuring the burst pressure before the animals were sacrificed. A laparotomy was again performed under anesthesia as described above. After identifying the ileal anastomosis, the ileum was cut at 5 cm proximal to the anastomotic site. After the faeces were carefully washed out, a catheter (inside diameter = 2.2 mm, outside diameter = 3.5 mm) was inserted 1 cm toward the anastomotic segment from the cut end of the ileum. The cut end of the ileum was ligated around the catheter with a 3-0 silk suture. A site 5-cm distal to the anastomotic site was then clamped with forceps. The catheter was connected to an infusion pump (LSP01-1C, Longer, England) and a pressure transducer. Normal saline was then continuously injected at a flow rate of 2.5 ml/min. The burst pressure was recorded in millimeters of mercury for each animal when there was a sudden loss of pressure or when the anastomosis started to leak. Furthermore, the specific area of leakage or rupture, *i.e.*, at the anastomotic site or far from it, was also recorded. After the anastomotic tract segments were resected, one-third of this tissue was fixed in 10% formalin solution and subjected to a histological examination. The remainder of the resected anastomotic tissue was placed in sterile tubes, frozen in liquid nitrogen, and stored at –80 °C. After all procedures were completed, the anesthetized rabbits were euthanized with an intravenous injection of 1 ml sodium thiopental.

Hydroxyproline concentration in anastomotic tissue

For analysis of hydroxyproline concentration (HPC), a 2 × 2 cm piece of anastomotic tissue was resected from each rabbit (n = 6 for each group) and stored at –20 °C. Determination of HPC was performed as described previously with some modifications (Reddy and Enwemeka, 1996). Briefly, soluble collagen was extracted from the tissue sample by overnight incubation in acetic acid 0.5 N at 4 °C. About 70 ml of each standard or test sample was hydrolyzed in 30 ml NaOH 10.125 N for 25 min at 120 °C using an autoclave. The hydrolyzed sample was then mixed with a buffered chloramine-T reagent (0.056 M) and the oxidation was allowed to proceed for 25 min at room temperature. The chromophore was then developed with the addition of Ehrlich reagent (1 M) for 20 min at 65 °C, after which the absorbance was measured at 550 nm using a Thermo Scientific Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Absorbance values were plotted against the concentration of standard hydroxyproline, and the concentration of hydroxyproline in unknown tissue extracts was determined from the standard curve. The results are expressed as μg/g of tissue.

Masson trichrome staining

Masson's trichrome staining was carried out according to a protocol provided by the manufacturer (Cat. ab150686, Abcam, Waltham, MA, USA). Briefly, the healing anastomotic tissues collected at POD 3 and POD 7 from each group were fixed with neutral formalin, embedded in paraffin, sectioned, deparaffinized in water, then stained with Masson composite staining solution for 5 min. The sections were then washed with 0.2% acetic acid solution, washed with 5% phosphotungstic acid for

5–10 min, washed twice with 0.2% acetic acid solution, and stained with bright green staining solution for 5 min. The sections were then washed twice with 0.2% acetic acid solution, dehydrated in absolute alcohol, put in xylene for transparency and mounted with neutral gum. Collagen fibers were stained blue. To quantify the percentage of blue stained collagen fibers, microscopic analysis was conducted by a pathologist in a blinded fashion. A DMR-Q550 pathological picture analyzer (Lei, Germany) analyzed the Masson-stained sections, and 10 optical areas in the microscope (magnification, ×100) were selected from each area and Leica Qwin analysis software was used for analysis. The collagen area/total area × 100% was used to calculate the relative density and distribution of collagen in the anastomotic tissue.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from anastomotic tissues using TRIZOL reagent (Invitrogen, Waltham, MA, USA). After DNase I treatment, the RNA was purified using a RNeasy kit (Qiagen, Hilden, Germany). One ug of each total RNA sample was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Waltham, MA, USA). Gene-specific primers for CTGF, Smad-2, and TGF-β were designed according to published sequences and GenBank accession numbers using Primer Express soft-ware (PE Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in Table 2 and each sample was analyzed in duplicate. Amplification and real-time detection were performed using a Thermal Cycler Dice Real-Time System and SYBR Premix Ex Taq (Takara, Shiga, Japan), according to the manufacturer's instructions. The expression levels of the target genes were normalized to GAPDH as an internal control. The data were analyzed using the Thermal Cycler analysis software and the 2-ΔΔCt method to analyze gene expression.

Table 2. Oligo sequences of primers

Gene	Forward primer	Reverse primer
CTGF	5'-GAAATGCTGCGAGGAGTG-3'	5'-CATCATGGTTGGGTCTGG-3'
TGFβ1	5'-CTGTCTCCGTTTCTTTCGT-3'	5'-CCGGGCGTCAACACTA-3'
Smad2	5'-TGTCCAATGTGAACCGAAAC-3'	5'-AGGGTGCCAGCCGTAT-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

Western-blot analysis

The healing anastomotic tissues collected at POD3 and POD7 were weighed, immediately placed in liquid nitrogen, and kept at –80 °C. The frozen tissues were ground and dissolved in ice cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 and PMSF). After vortexing and incubation on ice for 15 min, the lysates were centrifuged at 10,000 rpm for 15 min to remove debris. The supernatants (15 μl containing ~40 μg protein) were boiled for 5 min in Laemmli sample buffer, electrophoresed through 10% SDS-polyacrylamide gels and then transferred onto PVDF membranes (Millipore, Burlington, MA, USA). The membranes were blocked in Tris-buffered saline containing Tween 20 (TBST) (pH 7.4) containing 4% non-fat dry milk (Lab scientific, Highlands, NJ, USA) for 1 h and incubated with the following primary antibodies overnight at 4 °C: Rabbit anti-YAP (Cat. 4912), rabbit anti-phospho-YAP (Cat. 4911), rabbit anti-MOB1 (Cat. 3863), rabbit anti-phospho-MOB1 (Cat. 8699), rabbit anti-TGF-β1 (Cat. 3711), rabbit anti-Smad-2 (Cat. 5339), rabbit anti-phospho-Smad-2 (Cat. 18338), and rabbit anti-GAPDH (Cat. 5174) (all from Cell Signaling Technology, Danvers, MA, USA). The following day, after washing 3 times with TBST buffer for 5 min each, the membranes were treated with secondary antibodies for 1 h at room temperature. Signal detection of protein bands was visualized using enhanced chemiluminescence reagents and then analyzed using ImageJ.

Statistical analysis

Statistical analysis was performed with Prism software (GraphPad, San Diego, CA, USA) using an unpaired *t*-test when comparing two groups. The data are presented as means ± SEM or SD as indicated in the figure legends, and a P value < 0.05 is considered statistically significant.

Results

SHP improves the anastomotic burst pressure

All animals recovered normally after the surgical procedure, no deaths occurred, and the anastomoses remained intact until POD 7. There was no evidence of intraabdominal abscesses or anastomotic leakage in any of the animals. To examine the effects of SMU-MP-1 and SHP on anastomotic healing, we measured the anastomotic burst pressure, which is a standard criteria for assessing anastomotic strength and integrity during healing, at POD 3 and POD 7. The anastomotic burst pressure was not different among the three groups at POD 3: the control group, the XMU-MP-1 group, and the SHP group (Fig. 1). However, at POD 7, the anastomotic burst pressure in the XMU-MP-1 and SHP groups was significantly higher than in the control group. In addition, we noticed that the anastomotic burst pressure in the SHP group was higher than in the XMU-MP-1 group, although the difference was not statistically significant. These results show that both the XMU-XP-1 and SHP treatments significantly increased the anastomotic burst pressure, indicating that SHP enhances anastomotic healing.

SHP treatment enhances collagen deposition in healing anastomotic tissues

The mechanical strength development of anastomotic tissue mainly depends on the synthesis of collagen by proliferating fibroblasts during the healing process, and a considerable deposition of collagen is essential for the healing. To further evaluate the effects of XMU-MP-1 and SHP treatments on anastomotic healing after injury, we performed Masson's trichrome staining to analyze the relative deposition of collagen

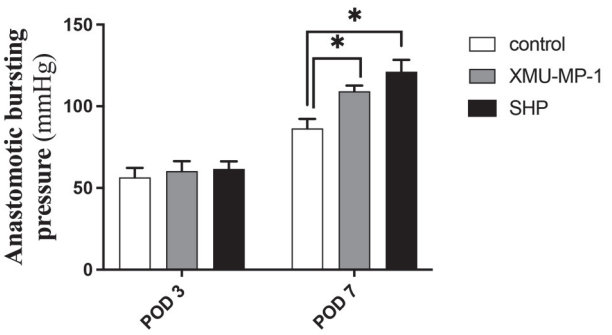

Fig. 1. SHP treatment increases the anastomotic burst pressure at POD 7. The anastomotic burst pressure (in mmHg) was measured in the different groups at POD 3 and POD 7. Error bars represent means ± SEM; *P* values are indicated with "*", * indicates $P < 0.05$, when comparing the two indicated groups.

in healing tissues. There was strong blue staining in the SHP group at POD 3, but there was no clear difference among the three groups (Fig. 2A). However, at POD 7, the blue staining was significantly stronger in all groups than at POD 3, and the strongest blue staining appeared in the SHP group, where the size of the collagen bundles was also larger than the control group. The relative quantification data of collagen deposition for each group at POD 3 and POD 7 (Fig. 2B) shows that the average collagen deposition at POD 7 in the XMU-MP-1 and SHP groups was significantly higher than in the control group. Although the average collagen deposition of the SHP group was higher than the XMU-MP-1 group, the difference was not statistically significant. These data suggest that SHP treatment, as well as XMU-MP-1, can increase collagen deposition in healing anastomotic tissue after injury.

SHP treatment increases the HPC in healing anastomotic tissue

To further investigate the effect of SHP treatment on collagen function during healing after injury, we analyzed the intestinal HPC, a major component of collagen that plays essential roles in collagen stability and is crucial for anastomotic strength. We found that the HPC in both the XMU-MP-1 and SHP groups was significantly increased at POD 3 compared with the control group, and the HPC increased further at POD 7 (Fig. 3). There was no statistical difference between the SHP group and the XMU-MP-1 group at POD 3 or POD 7 (Fig. 3). These data further demonstrate that XMU-MP-1 and SHP treatment enhances collagen deposition and the function of healing anastomotic tissue.

SHP treatment enhances activation of the TGF-β1 signaling pathway

Next, we explored how the SHP treatment enhances anastomotic wound healing. It has been shown that transforming

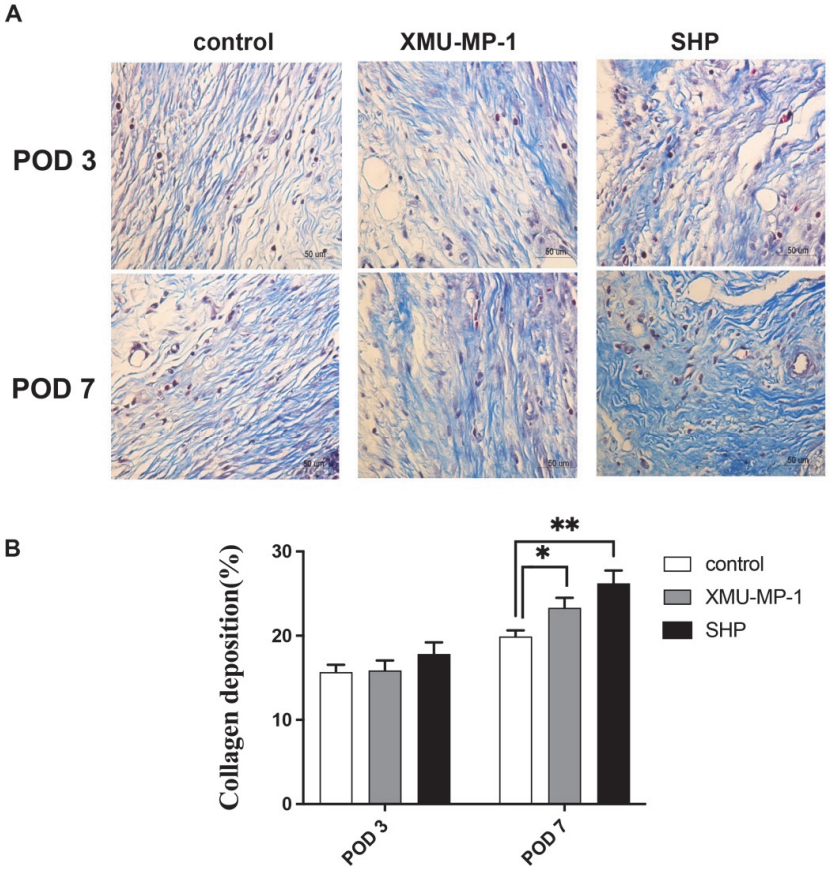

Fig. 2. SHP treatment increases collagen deposition in anastomotic tissues at POD 7. (**A**) Representative images of Masson's trichrome staining carried with intestinal anastomotic tissues in the different groups at POD 3 and POD 7; the collagen fibers are stained blue. (**B**) The experiment was repeated 3 times, and the quantification of the collagen staining in A was calculated. Error bars represent means ± SEM; *P* values are indicated with "*", * indicates $P < 0.05$, ** indicates $P < 0.01$ when comparing the two indicated groups.

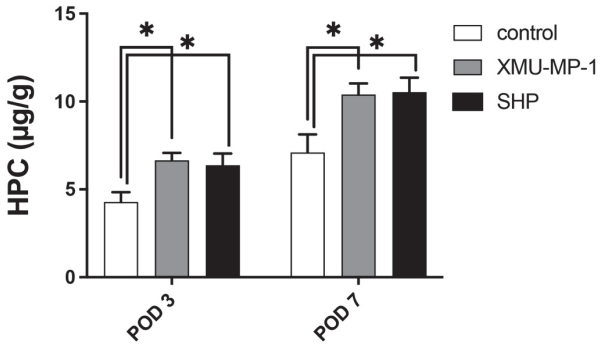

Fig. 3. SHP treatment increases the HPC of anastomotic tissues after injury. The HPC was measured in the different groups at POD 3 and POD 7. Error bars represent means ± SEM; *P* values are indicated with "*", * indicates $P < 0.05$, when comparing the two indicated groups.

growth factor (TGF-β1) is one of the key mediators of collagen biosynthesis during anastomotic wound healing. Therefore we investigated whether SHP treatment enhances the TGF-β1 signaling pathway (Morgan abd Shogan, 2022). Firstly, we performed RT-PCR analyses of TGF-β1 mRNA levels, and found that both SHP and XMU-XP-1 significantly increased the mRNA level of TGF-β1 at POD 7 compared to the control (Fig. 4A). The increased TGF-β1 mRNA level was verified by western-blot analysis of the TGF-β1 protein level (Fig. 4B). Secondly, we examined the activation of Smad-2, which is a downstream target and is phosphorylated when TGF-β1 is activated. Although SHP or XMU-XP-1 treatment did not change the mRNA level of Smad-2, it significantly increased the phosphorylated level of Smad-2 compared to the control, indicating that SHP and XMU-MP-1 treatment activated the TGF-β1 signaling pathway. Finally, we examined the expression level of connective tissue growth factor (CTGF), which has been shown to function as an important downstream mediator of TGF-β1 activation in connective tissue cells (Song et al., 2007). We found that both SHP and XMU-MP-1 significantly increased the mRNA levels of CTGF (Fig. 4A). Taken together, these data suggest that both SHP and XMU-MP-1 can enhance activation of the TGF-β1 signaling pathway during anastomotic healing.

SHP treatment induces YAP activity in intestinal anastomotic tissue

Several studies have reported the essential involvement of crosstalk between Hippo/YAP-signaling and TGF-β1 for intestinal regeneration after injury. Importantly, XMU-MP-1, which directly inhibits Mst1/2 to activate the YAP pathway, was shown to significantly enhance intestinal anastomotic

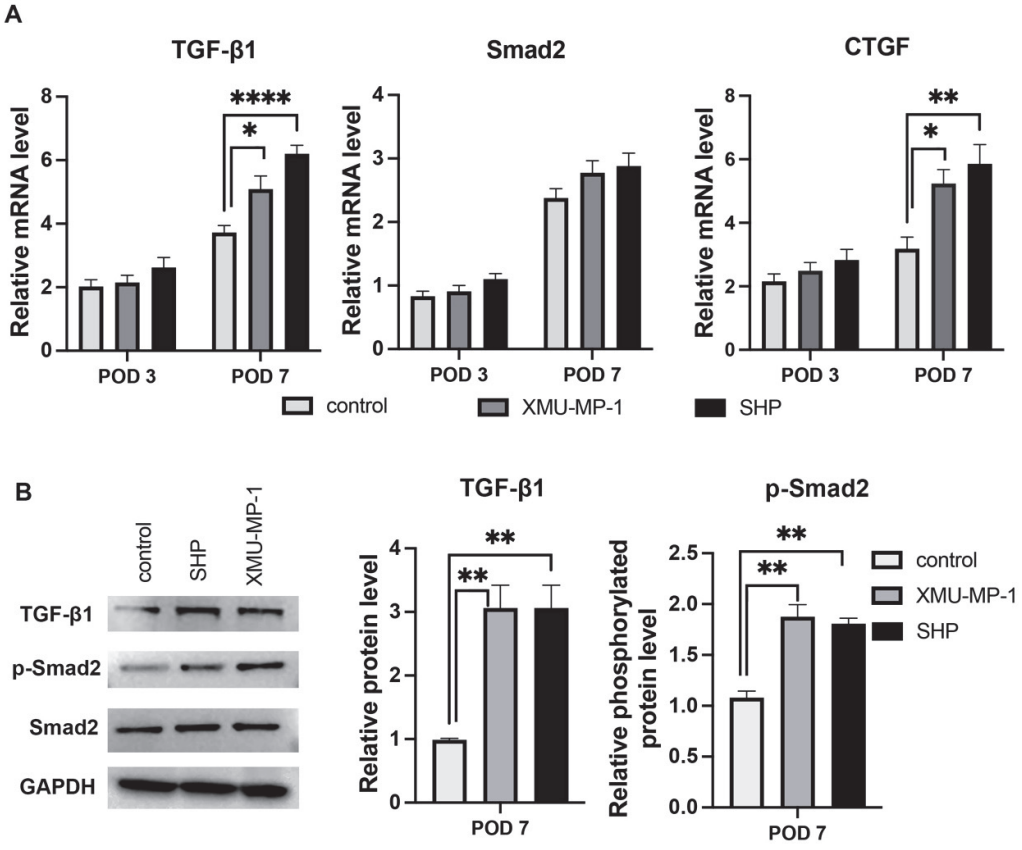

Fig. 4. SHP treatment elevates the TGF-β1 signaling pathway in intestinal anastomotic tissues after injury. (**A**) Total mRNAs isolated from intestinal anastomotic tissues in the different groups at POD 3 and POD 7 were analyzed by qRT-PCR for the expression of TGF-β1, Smad-2 and CTGF; the results were normalized to mRNA levels of the housekeeping gene GAPDH. (**B**) The healing intestinal anastomotic tissues were subjected to western-blot analysis of protein expression levels of TGF-β1, p-Smad-2, Smad-2 and GAPDH; quantification of protein expression levels is shown on the right, and expression of the TGF-β1 protein level was relative to the band density of the loading control GAPDH. The phosphorylation level of Smad-2 (p-Smad-2) was normalized to the band density of total Smad-2 protein. Error bars represent means ± SD; *P* values are indicated with "*", * indicates $P < 0.05$, ** indicates $P < 0.005$ when comparing the two indicated groups.

healing – as shown above. Therefore, we investigated whether SHP treatment also affected the YAP pathway. We performed western-blot analysis of the phosphorylation level of MOB1, which is downstream of Mst1/2, and of YAP, which is a target of MOB1. As expected, the Mst1/2 inhibitor XMU-MP-1 markedly suppressed the phosphorylation level of MOB1 associated with a reduction of the phosphorylation level of YAP, which results in the induction of the active form of YAP at POD 7 (Fig. 5). Similar to the effect of XMU-MP-1, SHP also reduced the phosphorylation level of both MOB1 and YAP, and led to an increase in the active form of YAP (Fig. 5). This result suggests that SHP treatment, similar to SMU-MP-1, can induce activation of the YAP pathway in healing anastomotic tissues.

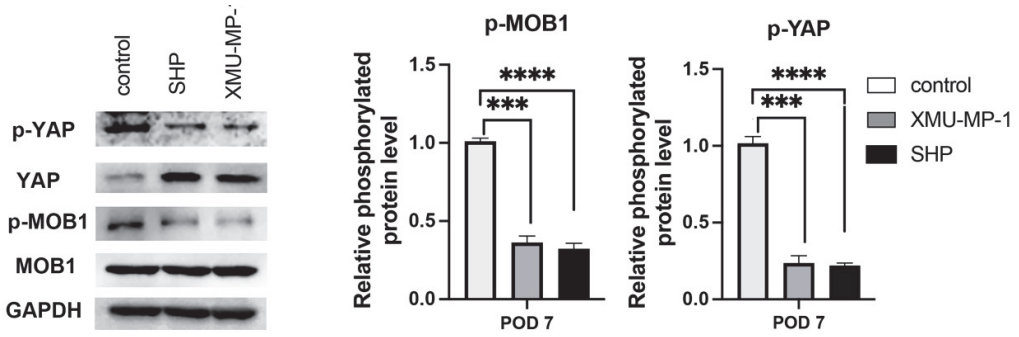

Fig. 5. SHP treatment activates the YAP signaling pathway in intestinal anastomotic tissues after injury. The healing intestinal anastomotic tissues collected at POD 7 were subjected to western-blot analysis to determine protein expression levels of p-YAP, YAP, pMOB1, MOB1, and GAPDH. Quantification of protein expression levels are shown on the right, and the expression levels of the phosphorylation of MOB1 (p-MOB1) and YAP (p-YAP) were normalized to the band density of the total protein expression level of MOB1 and YAP, respectively. Error bars represent means ± SD; *P* values are indicated with "*", *** indicates $P < 0.005$, **** indicates $P < 0.001$ when comparing the two indicated groups.

SHP treatment enhances anastomotic healing depending on activation of the YAP pathway

To further confirm that SHP treatment could activate the YAP pathway to play an essential role in the promotion of anastomotic healing, we pre-treated the rabbits with Verteporfin, a specific YAP inhibitor (Wei et al., 2017), together with SHP before the intestinal anastomotic surgery. We found that induction of the anastomotic burst pressure and the HPC by SHP treatment at POD 7 was significantly abolished by the addition of Verteporfin (Fig. 6A, B). This result suggests that SHP treatment promotes intestinal anastomotic healing through activation of the YAP pathway.

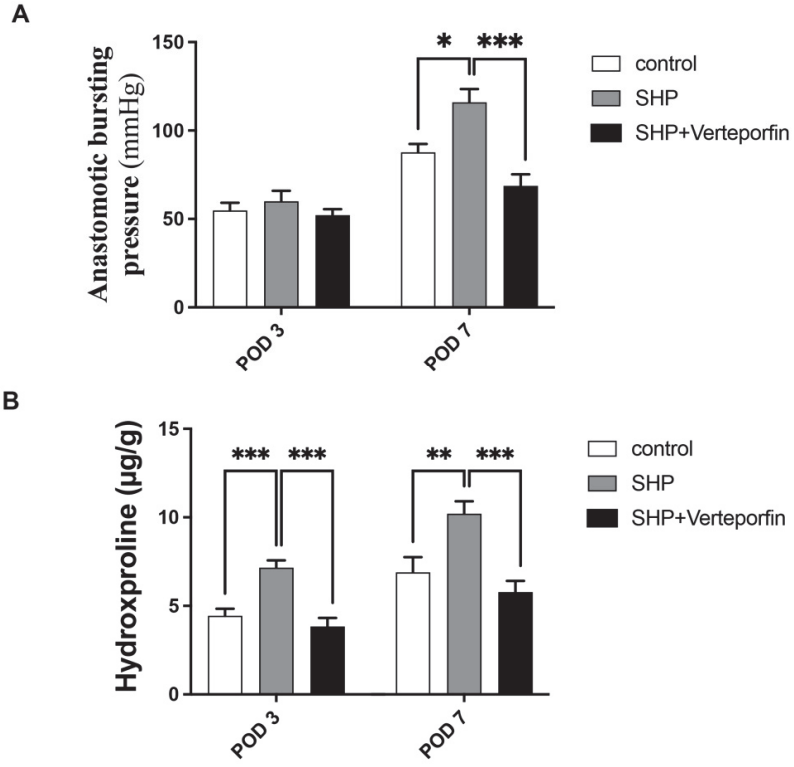

Fig. 6. SHP treatment enhances anastomotic healing through regulation of the YAP pathway. (**A, B**) The anastomotic burst pressure (in mmHg, in A) and the HPC (μg/g, in B) of healing anastomotic tissues were measured in the different groups at POD 3 and POD 7. Error bars represent means ± SEM; *P* values are indicated with "*", * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.005$ when comparing the two indicated groups.

Discussion

The occurrence of anastomotic complications in patients not only prolongs the length of their hospital stays and increases their medical expenses but can also potentially result in severe electrolyte disorders and malnutrition, even septic shock and multiple organ failure (Guyton et al., 2016). According to statistics, the perioperative mortality of anastomotic fistula after colorectal cancer surgery is as high as 6–22% (Di Cristofaro et al., 2014). The process of anastomotic healing is affected by many factors, including the blood supply, tension, infection, distal obstruction, and associated diseases (Morgan and Shogan, 2022). Enhancing the healing of anastomotic tissues is essential for the prevention of anastomotic complications (Guyton et al., 2016; Morgan and Shogan, 2022). Currently, the traditional anti-inflammatory fluid replacement and gastrointestinal decompression in the clinic have produced few effects to avoid anastomotic complications. Therefore, it is important to identify a new signaling pathway that controls anastomotic healing and to explore new management strategies to prevent the occurrence of severe complications of anastomosis after surgery.

The first aim of the present study was to examine whether the Hippo pathway plays an important role in anastomotic healing. The Hippo pathway is a signaling cascade conserved from *Drosophila melanogaster* to mammals (Felley-Bosco and Stahel, 2014). The mammalian core kinase components are comprised of MST1, MST2, SAV1, LATS1, LATS2, MOB1A, and MOB1B (Deng et al., 2022). The transcriptional co-activators YAP1 and TAZ are downstream effectors of the Hippo pathway and regulate target gene expression (Deng et al., 2022). Hippo signaling has crucial roles in controlling organ size, tissue homeostasis, and regeneration. Dysregulation of the Hippo pathway can lead to uncontrolled cell growth and malignant transformation (Moroishi et al., 2015). Several studies have established the important role of the Hippo pathway in those processes (Felley-Bosco and Stahel, 2014; Hong et al., 2016; Moriushi et al., 2015). In addition, crosstalk between the Hippo pathway and other signaling pathways provides tight yet versatile regulation of tissue homeostasis (Futakuchi et al., 2018; Piersma et al., 2015). By using XMU-XP-1, a specific MST1/2 inhibitor (Fan et al., 2016), this study demonstrated that XMU-XP-1 could significantly increase the anastomotic burst pressure, collagen deposition, and the HPC of intestinal anastomotic tissue at POD 7 (Figs 1–3). Those results indicate that the Hippo pathway plays an important role during intestinal anastomotic healing and that activation of the Hippo pathway can promote the healing process of anastomotic tissues after surgery.

The second aim of this study was to test whether Shenhuang powder (SHP), a traditional Chinese medicine formula that has been widely used in clinics for the treatment of different abdominal obstructions and inflammatory bowel diseases with profound efficacy, could enhance the intestinal anastomotic healing. As we expected, similar to the effect of XMU-XP-1, SHP treatment enhanced intestinal anastomotic healing associated with significant increases of anastomotic burst pressure, collagen deposition, and the HPC (Figs 1–3). Our previous study reported that SHP treatment reduces the intestinal inflammatory response associated with the down-regulation of inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12), likely through inhibition of the PI3K/AKT/NF-κB pathway in the mouse postoperative ileus (POI) model (Liu et al., 2022; Xu et al., 2016). The anti-inflammation function of SHP might shorten the inflammation phase to accelerate the healing process of anastomotic tissue after surgery. Here, we found that SHP treatment increases the expression of TGF-β1, which activates its downstream target Smad-2 to activate the TGF-β1 signaling pathway, a well-known core pathway that regulates different phases of anastomotic tissue healing (Morgan and Shogan, 2022). A previous study reported that elevated levels of TGF-β1 signaling through adenoviral gene transfer with healing colonic anastomoses can promote the burst pressure at POD 6 in rats (Türlü et al., 2021). Notably, along with this study of the YAP signaling activator XMU-XP-1, we also found that SHP could reduce the phosphorylation level of YAP and increase the active form of YAP which enters into nuclei to activate the transcription of YAP downstream factors. Importantly, the treatment with verteporfin, a suppressor of the YAP-TEAD complex (Wei et al., 2017), significantly blocked the effects induced by SHP during anastomotic tissue healing. These results suggest that SHP treatment enhances anastomotic healing, likely through activation of the YAP pathway.

The next step of this study will be interesting in order to identify a bioactive component of SHP to regulate TGF-β1 expression and activate the YAP pathway. The HPLC analysis, performed in our previous study, revealed that the prepared SHP enriched the following bioactive constituents: hesperidin, quercetin, ginsenoside-Rb1, aloeemodin, ginsenoside-Rg1, magnolol and chrysophanol, and these bioactive constituents have already been reported to have anti-inflammatory and anti-oxidative functions (Xu et al., 2016). For example, enrichment of hesperidin in Fructus Aurantii Immaturus has been shown to have anti-inflammatory activity and prokinetic actions on GI tract (Fang et al., 2009; Guardia et al., 2001). Cortex Magnoliae Officinalis enriched with honokiol and magnolol exhibited antioxidative and anti-inflammatory effects (Miao et al., 2013). Importantly, our previous study showed these bioactive constituents could be absorbed after SHP topic application on Shenque. For example, we detected both rhein and magnolol in the plasma after SHP was applied at Shenque in New Zealand rabbits (Ding et al., 2018). Both rhein and magnolol have been reported to be involved in the regulation of TGF-β signaling pathway (Chen et al., 2020; Gao et al., 2010; Liu et al., 2001). Therefore, we expect that these bioactive constituents in SHP are likely to have synergistic effects in promoting anastomotic healing. In our group, the identification of major bioactive constituents of SHP responsible for activation of the YAP pathway to enhance the anastomotic healing is still ongoing.

Conclusion

The present study demonstrated that activation of the Hippo-YAP pathway enhances anastomotic healing, and that the Chinese medicine SHP – which is widely used in clinics to treat gastrointestinal disorders – can increase both the TGF-β1/Smad and YAP signaling pathways to promote anastomotic healing in rabbits after surgery. This suggests that SHP can be used for the prevention of anastomotic complications in patients who have undergone anastomosis in the clinic.

Funding
This work was supported by a grant from the National Natural Science Foundation of China (81973756), the Experimental Animal Science and Technology Foundation of Zhejiang Province, China (2018C37133), the TCM Science and Technology Program of Zhejiang Province, China (2020ZA054), and the

Science and Technology Project of Zunyi [Zunyi City Kehe Support NS (2020) No. 18].

Acknowledgements
We would like to thank Xiaohong Xu's lab members for the discussion and technical help.

Ethics approval
The animal care and experimental procedures complied with the Guidelines for Animal Use and Care (published by Zhejiang Chinese Medical University) and were approved by the Institutional Review Board of Zhejiang Chinese Medical University (approval code P10-482). All animal experiments also complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest
The authors have no conflict of interest to declare.

Authors' contributions
FX and CZ performed most of the experiments and analyzed the data. XW carried out the rabbit model and SHP treatments. GC and XX designed the study and supervised the project. XX also had a major role in writing the manuscript. All the authors read and approved the final manuscript.

References

Chen J, Wei X, Zhou J, Chen Y, Zhang Z, Zhang Q, et al. (2012). Shen-huang-san Promoting Rehabilitation of Postoperative after Gastrointestinal Tumour Radical Operation Dysfunction. Journal of Zhejiang Chinese Medical University 36(4): 372–374.

Chen S, Shen J, Zhao J, Wang J, Shan T, Li J, et al. (2020). Magnolol Suppresses Pancreatic Cancer Development *In Vivo* and *In Vitro* via Negatively Regulating TGF-beta/Smad Signaling. Front Oncol 10: 597672. DOI: 10.3389/fonc.2020.597672.

Deng F, Wu Z, Zou F, Wang S (2022). The Hippo-YAP/TAZ Signaling Pathway in Intestinal Self-Renewal and Regeneration After Injury. Front Cell Dev Biol 10: 894737. DOI: 10.3389/fcell.2022.894737.

Di Cristofaro L, Ruffolo C, Pinto E, Massa M, Antoniutti M, Cagol M, et al. (2014). Complications after surgery for colorectal cancer affect quality of life and surgeon-patient relationship. Colorectal Dis 16(12): O407–419. DOI: 10.1111/codi.12752.

Ding MWY, Wan H, Mei Z, Xu X, Shi S, Sun Q (2018). Pharmacokinetic comparison of Shenque (CV8) point application and non-acupoint application with Shenhuang Powder in rabbit plasma. Chin J Tradit Chin Med Pharm 33(6): 2321–2326.

Fan F, He Z, Kong LL, Chen Q, Zhang S, Ye J, et al. (2016). Pharmacological targeting of kinases MST1 and MST2 augments tissue repair and regeneration. Sci Transl Med 8(352): 352ra108. DOI: 10.1126/scitranslmed.aaf2304.

Fang YS, Shan DM, Liu JW, Xu W, Li CL, Wu HZ, Ji G (2009). Effect of constituents from Fructus Aurantii Immaturus and Radix Paeoniae Alba on gastrointestinal movement. Planta Med 75(1): 24–31. DOI: 10.1055/s-0028-1088342.

Felley-Bosco E. Stahel R (2014). Hippo/YAP pathway for targeted therapy. Transl Lung Cancer Res 3(2): 75-83.

Futakuchi A, Inoue T, Wei FY, Inoue-Mochita M, Fujimoto T, Tomizawa K, Tanihara H (2018). YAP/TAZ Are Essential for TGF-beta2-Mediated Conjunctival Fibrosis. Invest Ophthalmol Vis Sci 59(7): 3069–3078. DOI: 10.1167/iovs.18-24258.

Gao Q, Quin WS, Jia ZH, Zheng JM, Zeng CH, Li LS, Liu ZH (2010). Rhein improves renal lesion and ameliorates dyslipidemia in db/db mice with diabetic nephropathy. Planta Med 76(1): 27–33. DOI: 10.1055/s-0029-1185948.

Gonzalez AC, Costa TF, Andrade ZA, Medrado AR (2016). Wound healing – A literature review. An Bras Dermatol 91(5): 614–620. DOI: 10.1590/abd1806-4841.20164741.

Guardia T, Rotelli AE, Juarez AO, Pelzer LE (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Farmaco 56(9): 683–687. DOI: 10.1016/s0014-827x(01)01111-9.

Guyton KL, Hyman NH, Alverdy JC (2016). Prevention of Perioperative Anastomotic Healing Complications: Anastomotic Stricture and Anastomotic Leak. Adv Surg 50(1): 129–141. DOI: 10.1016/j.yasu.2016.03.011.

Hong AW, Meng Z, Guan KL (2016). The Hippo pathway in intestinal regeneration and disease. Nat Rev Gastroenterol Hepatol 13(6): 324–337. DOI: 10.1038/nrgastro.2016.59.

Lam A, Fleischer B, Alverdy J (2020). The Biology of Anastomotic Healing-the Unknown Overwhelms the Known. J Gastrointest Surg 24(9): 2160–2166. DOI: 10.1007/s11605-020-04680-w.

Liu T, Xu M, Shi Z, Li M, Wang R, Shi Y, et al. (2022). Shenhuang plaster ameliorates the Inflammation of postoperative ileus through inhibiting PI3K/Akt/NF-kappaB pathway. Biomed Pharmacother 156: 113922. DOI: 10.1016/j.biopha.2022.113922.

Liu ZH, Li YJ, Chen ZH, Liu D, Li LS (2001). Glucose transporter in human glomerular mesangial cells modulated by transforming growth factor-beta and rhein. Acta Pharmacol Sin 22(2): 169–175.

Miao B, Zhang S, Wang H, Yang T, Zhou D, Wang BE (2013). Magnolol pretreatment prevents sepsis-induced intestinal dysmotility by maintaining functional interstitial cells of Cajal. Inflammation 36(4): 897–906. DOI: 10.1007/s10753-013-9617-z.

Morgan RB, Shogan BD (2022). The Science of Anastomotic Healing. Semin Colon Rectal Surg 33(2): 100879. DOI: 10.1016/j.scrs.2022.100879.

Moroishi T, Hansen CG, Guan KL (2015). The emerging roles of YAP and TAZ in cancer. Nat Rev Cancer 15(2): 73–79. DOI: 10.1038/nrc3876.

Nakamura H, Yokoyama Y, Uehara K, Kokuryo T, Yamaguchi J, Tsuzuki T, Nagino M (2016). The effects of bevacizumab on intestinal anastomotic healing in rabbits. Surg Today 46(12): 1456–1463. DOI: 10.1007/s00595-016-1342-4.

Piersma B, Bank RA, Boersema M (2015). Signaling in Fibrosis: TGF-beta, WNT, and YAP/TAZ Converge. Front Med (Lausanne) 2: 59. DOI: 10.3389/fmed.2015.00059.

Reddy GK, Enwemeka CS (1996). A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 29(3): 225–229. DOI: 10.1016/0009-9120(96)00003-6.

Shi Y, Xu J, Ding B, Chen G, Jin L, Ke L, et al. (2020). Gastrointestinal Motility and Improvement Efficacy of Shenhuang Plaster Application on Shenque: Identification, Evaluation, and Mechanism. J Immunol Res 2020: 2383970. DOI: 10.1155/2020/2383970.

Shi Y, Xu X, Liu T, Wang R, Xu J, Wu Y, et al. (2022). Shenhuang Plaster Application Improves Gastrointestinal Motility in Mice with Postoperative Ileus through Intestinal Microbiota. Evid Based Complement Alternat Med 2022: 2823315. DOI: 10.1155/2022/2823315.

Song JJ, Aswad R, Kanaan RA, Rico MC, Owen TA, Barbe MF, et al. (2007). Connective tissue growth factor (CTGF) acts as a downstream mediator of TGF-beta1 to induce mesenchymal cell condensation. J Cell Physiol 210(2): 398–410. DOI: 10.1002/jcp.20850.

Türlü C, Willumsen N, Marando D, Schjerling P, Biskup E, Hannibal J, et al. (2021). A Human Cellular Model for Colorectal Anastomotic Repair: The Effect of Localization and Transforming Growth Factor-beta1 Treatment on Collagen Deposition and Biomarkers. Int J Mol Sci 22(4): 1616. DOI: 10.3390/ijms22041616.

Wei X, Qiu H, Zhang Q, Li D, Sun Y, Li G, et al. (2014a). Shenhuang Powder Paste for 110 Patients With Gastrointestinal Dysfunction After Abdominal Surgery: A Prospective Multi-Center Randomized Controlled Clinical Study. J Tradit Chin Med 55(5): 386–390.

Wei X, Qiu H, Zhang Q, Li D, Sun Y, Li G, et al. (2014b). Prospective, Multi-centered, Randomized and Controlled Trial on Effect of Shenhuang Ointment in Promoting Rehabilitation of Postoperative Gastrointestinal Dysfunction with Syndrome of Stagnation of Qi and Blood Stasis. Chinese Archives of Traditional Chinese Medicine 32(8): 1831–1834.

Wei H, Wang F, Wang Y, Li T, Xiu P, Zhong J, et al. (2017). Verteporfin suppresses cell survival, angiogenesis and vasculogenic mimicry of pancreatic ductal adenocarcinoma via disrupting the YAP-TEAD complex. Cancer Sci 108(3): 478–487. DOI: 10.1111/cas.13138.

Xu X, Chen Y, Wei X, Kong J, Qiu H, Sun Q (2015). Efect of Senhuang Powder on Gastrointestinal Motility and Expression of ICAM-1, iNOS, IL-I~and IL-10 in the Rat ModeI of Postoperative lleus. Journal of Zhejiang Chinese Medical University 39(11): 777–781.

Xu XH, Chen YL, Wei X, Sun QH, Qiu HS (2016). Shenhuang Powder reduces the intestinal inflammatory response and ameliorates impaired gastrointestinal motility in a rat model of postoperative ileus. Int J Clin Exp Med 9(6): 10747–10756.

Yang J, Xu H (2008). External application of herbal medicine to acupoints. J Tradit Chin Med 28(1): 21–23. DOI: 10.1016/s0254-6272(08)60008-4.

Yu H, Ying X, Qiu H, Wei X (2013). Clinical study of Shenhuang Powder at Shenque (CV 8) on early rehabilitation in gastrointestinal dysfunction after operation for gastric carcinoma. J Tradit Chin Med Pharm 28(10): 3131–3133.

Zheng C, Xu X, Sun Q, Wang B (2018). The effect of external application of Shenhuang powder on colonic transport and plasma gastrointestinal hormone levels in rats with postoperative intestinal dysfunction. Zhejiang Journal of Clinical Medicine 20(3): 400–402.