Pentoxifylline decreases post-operative intra-abdominal adhesion formation in an animal model

Ya-Lin Yang [1], Meng-Tse Gabriel Lee [1], Chien-Chang Lee [1], Pei-I Su [1], Chien-Yu Chi [1], Cheng-Heng Liu [1], Meng-Che Wu [1], Zui-Shen Yen [1], Shyr-Chyr Chen [Corresp. 1]

[1] Department of Emergency Medicine, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan

Corresponding Author: Shyr-Chyr Chen
Email address: scchen@ntu.edu.tw

Background Intra-abdominal adhesions develop after nearly every abdominal surgery, commonly causing female infertility, chronic pelvic pain, and small bowel obstruction. Pentoxifylline (PTX) is a methylxanthine compound with immunomodulatory and antifibrotic properties. The aim of this study was to investigate whether PTX can reduce post-operative intra-abdominal adhesion formation via collagen deposition, tissue plasminogen activator (tPA) level, inflammation, angiogenesis, and fibrosis.

Methods Seventy male BALB/c mice were randomized into one of three groups: (1) sham group without peritoneal adhesion model; (2) peritoneal adhesion model (PA group); (3) peritoneal adhesion model with PTX (100 mg/kg/day i.p.) administration was started on preoperative day 2 and continued daily (PA+PTX group). On postoperative day 3 and day 7, adhesions were assessed using the Lauder scoring system. Parietal peritoneum was obtained for histological evaluation with hematoxylin and eosin (HE) and picrosirius red staining. Fibrinolysis was analyzed by tPA protein levels in the peritoneum by ELISA. Immunohistological analysis was also conducted using markers for angiogenesis (ki67+/CD31+), inflammation (F4/80+) and fibrosis (FSP-1+ and α-SMA+). All the comparisons were made by comparing the PA group with the PTX treated PA group, and $p < 0.05$ was considered statistically significant.

Results Intra-abdominal adhesions were markedly reduced by PTX treatment. Compared with the PA group, PTX treatment had lower adhesion scores than the PA group on both day 3 and day 7 ($p < 0.05$). Histological evaluations found that PTX treatment reduced collagen deposition and adhesion thickening. ELISA analysis showed that PTX treatment significantly increased the level of tPA in the peritoneum. In addition, in the immunohistological analysis, PTX treatment was found to significantly decreased the number of ki67+/CD31+ cells at the site of adhesion. Finally, we also observed that in the PTX treated group, there was a reduction in the expression of F4/80+, FSP-1+, and α-SMA+ cells at the site of adhesion.

Conclusion PTX may decrease intra-abdominal adhesion formation via increasing peritoneal fibrinolytic activity, suppressing angiogenesis, decreasing collagen synthesis, and reducing peritoneal fibrosis. Our findings suggest that PTX can be used to decrease post-operative intra-abdominal adhesion formation.

1 **Pentoxifylline decreases post-operative intra-abdominal adhesion formation in an animal**
2 **model**

4 Ya-Lin Yang[1‡], Meng-Tse Gabriel Lee[1‡], Chien-Chang Lee[1], Pei-I Su[1], Chien-Yu Chi[1], Cheng-
5 Heng Liu[1], Meng-Che Wu[1], Zui-Shen Yen[1], Shyr-Chyr Chen[1*]
6 1. Department of Emergency Medicine, National Taiwan University Hospital and College of
7   Medicine, National Taiwan University, Taipei, Taiwan.
8
9 ‡ The two authors contribute equally to this work
10 *** Correspondence and address reprint request to:** Dr. Shyr-Chyr Chen
11 Department of Emergency Medicine, National Taiwan University Hospital and College of
12 Medicine, National Taiwan University, Taipei, Taiwan.
13 * scchen@ntu.edu.tw

15   **Abstract**

16   **Background**

17   Intra-abdominal adhesions develop after nearly every abdominal surgery, commonly causing

18   female infertility, chronic pelvic pain, and small bowel obstruction. Pentoxifylline (PTX) is a

19   methylxanthine compound with immunomodulatory and antifibrotic properties. The aim of this

20   study was to investigate whether PTX can reduce post-operative intra-abdominal adhesion

21   formation via collagen deposition, tissue plasminogen activator (tPA) level, inflammation,

22   angiogenesis, and fibrosis.

23   **Methods**

24   Seventy male BALB/c mice were randomized into one of three groups: (1) sham group without

25   peritoneal adhesion model; (2) peritoneal adhesion model (PA group); (3) peritoneal adhesion

26   model with PTX (100 mg/kg/day i.p.) administration was started on preoperative day 2 and

27   continued daily (PA+PTX group). On postoperative day 3 and day 7, adhesions were assessed

28   using the Lauder scoring system. Parietal peritoneum was obtained for histological evaluation

29   with hematoxylin and eosin (HE) and picrosirius red staining. Fibrinolysis was analyzed by tPA

30   protein levels in the peritoneum by ELISA. Immunohistological analysis was also conducted

31   using markers for angiogenesis (ki67^{+}/CD31^{+}), inflammation (F4/80^{+}) and fibrosis (FSP-1^{+} and

32   α-SMA^{+}). All the comparisons were made by comparing the PA group with the PTX treated

33   PA group, and $p < 0.05$ was considered statistically significant.

34   **Results**

35   Intra-abdominal adhesions were markedly reduced by PTX treatment. Compared with the

36   PA group, PTX treatment had lower adhesion scores than the PA group on both day 3 and

37   day 7 ($p < 0.05$). Histological evaluations found that PTX treatment reduced collagen

38   deposition and adhesion thickening. ELISA analysis showed that PTX treatment

39   significantly increased the level of tPA in the peritoneum. In addition, in the

40   immunohistological analysis, PTX treatment was found to significantly decreased the number

41   of ki67^{+}/CD31^{+} cells at the site of adhesion. Finally, we also observed that in the PTX treated

42   group, there was a reduction in the expression of F4/80^{+}, FSP-1^{+}, and α-SMA^{+} cells at the site

43   of adhesion.

44   **Conclusion**

45   PTX may decrease intra-abdominal adhesion formation via increasing peritoneal fibrinolytic

46   activity, suppressing angiogenesis, decreasing collagen synthesis, and reducing peritoneal

47   fibrosis. Our findings suggest that PTX can be used to decrease post-operative intra-abdominal

48   adhesion formation.

49

Introduction

51

52   Post-operative intra-abdominal adhesion after laparotomy is a source of considerable morbidity.
53   It is estimated that more than 90% of patients develop primary intra-abdominal adhesion after
54   laparotomy. Post-operative adhesions affect the quality of life in millions of people worldwide,
55   causing many different types of complications, including chronic pelvic or abdominal pain, small
56   bowel obstructions (SBO), and even infertility (Arung et al. 2011; Liakakos et al. 2001). SBO is
57   the most common complication of adhesion and is observed in up to 70% of patients undergoing
58   laparotomy (Ellis 1997; Menzies & Ellis 1990; ten Broek et al. 2013). Although less commonly
59   observed, up to 20% of female infertility has been associated with post-operative adhesions
60   (Luijendijk et al. 1996).

61   The pathogenesis of post-operative intra-abdominal adhesion is a complex process that involves
62   inflammation, collagen related clot formation, angiogenesis, fibrinolysis, and tissue repairs
63   which include epithelial-mesenchymal transition (EMT)/endothelial-mesenchymal transition
64   (EndMT) or mesothelial -mesenchymal transition (MMT) (diZerega 1997; Hellebrekers &
65   Kooistra 2011; Homdahl & Ivarsson 1999). The key area in adhesion formation is the surface
66   lining of the peritoneum. Injury of the peritoneum leads to activation of coagulation cascade and
67   an inflammatory response consisting of hyperemia, fluid exudation, recruitment of floating
68   mesothelial cells, and release of white blood cells and platelets into the peritoneal
69   cavity(diZerega 1997; diZeregal & Campeau 2001). Normal fibrinolytic activity usually prevents
70   fibrinous attachments for 3-4 day, and mesothelial repair occurs in 5-6 day after surgery
71   (diZeregal & Campeau 2001). Therefore, previous studies focused on the cellular events 3-6 day
72   after the peritoneal injury.

73   Pentoxifylline (PTX), a non-specific phosphodiesterase inhibitors, has been used to improve the
74   walking ability in patients with intermittent claudication (Ernst 1994; Hood et al. 1996; Rossner
75   & Muller 1987). Previous animal studies have also demonstrated that PTX can reduce post-
76   operative adhesion, but the biological mechanisms that were responsible have not been fully
77   clarified (Durmus et al. 2011; Hung et al. 2008; Jafari-Sabet et al. 2015; Tarhan et al. 2006). Five
78   separate mechanisms on how PTX can alter the essential components in adhesion formation have
79   been proposed: (1) reduction of inflammation (Durmus et al. 2011; Pollice et al. 2001); (2)
80   reduction of collagen synthesis (Chen et al. 1999); (3) reduction of angiogenesis (Amirkhosravi
81   et al. 1998) (4) increased fibrinolysis by up-regulation of tissue plasminogen activator (tPA)
82   expression (Tarhan et al. 2006); (5) reduced fibrosis (Durmus et al. 2011; Wen et al. 2017).
83   However, it is unclear if PTX can decrease post-operative intra-abdominal adhesion formation
84   by simultaneously altering all of the five proposed mechanisms. Therefore, we aimed to

85 investigate the effects of PTX on peritoneum collagen expression, peritoneal tPA expression,
86 peritoneum angiogenesis, inflammation, and peritoneal fibrosis.

87

Methods

89 **Animals**

90 Male BALB/c mice weighing 25-30 g (Charles River Laboratories, BioLasco, Taiwan) were
91 maintained in a temperature and light-controlled room (12-hour light/dark cycle) and allowed
92 free access to water and food. The experimental protocols were approved by the Institutional
93 Animal Care and Use Committee (IACUCs) of the National Taiwan University Hospital
94 (approval ID: 20120285). All the protocols are in adherence to the guidelines established in the
95 Guide for the Care and Use of Laboratory Animals of the National Health Research Institutes.

96 **Experimental design**

97 70 BALB/c mice were randomly divided into three different groups: (1) sham group without
98 peritoneal adhesion model (sham group, n = 8); (2) peritoneal adhesion model (PA group, n =
99 31); (3) peritoneal adhesion model with PTX (Trental) (100 mg/kg/day i.p., once daily)
100 administration was started on preoperative day 2 and continued daily (PA+PTX group, n = 31).
101 We used a slightly modified standard adhesion model (Oncel et al. 2001). The details for
102 peritoneal adhesion model had been reported before (Lee et al. 2016).

103 Briefly, mice were anesthetized using 2% isoflurane in oxygen. The abdomen was then shaved
104 and disinfected with povidone iodine. A 4 cm median laparotomy was performed to gain access
105 to the abdominal cavity. In peritoneal adhesion model (PA and PA+PTX group), were pooled
106 and randomly underwent surgery. The cecum was gently removed and abraded with 20 vertically
107 reciprocal movements of dry gauze. After, the right abdominal sidewall be more aggressive with
108 the abraded than cecum and until punctate bleeding was seen. The injury sites were cleaned with
109 physiological salt solution and covered the gauze, making sure that was no active bleeding. The
110 cecum was then placed back into the abdominal cavity and surgical wound was sutured. For the
111 sham group, only open laparotomy and closure was conducted and there is no abrading of cecum
112 and abdominal wall.

113 The PTX group received 100 mg/kg of PTX from left abdominal cavity, whereas the other two
114 group (group sham and PA) received 0.125 ml of physiological saline solution. For preventing
115 postoperative pain, buprenorphine (0.05 mg/kg s.c., twice daily) was administered during the two
116 postoperative days. Mice were placed under a warming lamp and observed until they recovered

117   fully from anesthesia. Mice were monitored daily for signs of wound infection and general health
118   condition periodically until 3 or 7 day after surgery.

119   **Adhesion score**

120   Mice were euthanized on postoperative day 3 and day 7. The abdominal cavity was opened via a
121   U-shaped incision. The adhesion score was evaluated was performed by an observer blinded
122   treatment, using the Lauder scoring system (Lauder et al. 2011). The adhesion were graded in a
123   blinded fashion using the classification system described (Table 1).

124   **Histology staining**

125   Tissue samples from the parietal peritoneum, liver, and mesentery were collected after
126   euthanasia. For histological staining, tissue were fixed in 10% neutral-buffered formalin (NBF),
127   paraffin embedded, thinly sectioned. Tissue sections of 4-5μm thickness were prepared for
128   staining. After deparaffinization and rehydration, sections were counterstained in Gill's
129   hematoxylin (Sigma, St Louis, MO) and for 5 min, cleared in 0.1% acid alcohol for 30 sec, and
130   rinsed in tap water, then stained in eosin (Sigma) for 2 min, cleared in 95% alcohol , and rinsed
131   70% alcohol to remove the staining solution, dehydrated, and mounted, for histologic assessment.
132

133   **Picrosirius red staining**

134   Picrosirius red staining was used to compare collagen and fibrosis in tissue between different
135   groups. Peritoneal sections (4-5 μm) were deparaffinized, rehydrated and then subjection to
136   counterstaining in Gill's hematoxylin (Sigma) for 5 min, cleared in 0.1% acid alcohol 30 sec, and
137   rinsed in running tap water. Then stained in picrosirius red stain kit (polysciences, Warrington,
138   PA). Subsequently, sections were dehydrated and mounted for assessment.

139   **Tissue plasminogen activator**

140   Peritoneal tissue were prepared by grinding on ice in radioimmunoprecipation assay buffer
141   (RIPA buffer) with protease inhibitor cocktail (Sigma). After the samples were centrifuged at
142   12,000g for 15 min at 4°C, supernatants were aspirated and placed in new tubes. Samples were
143   analyzed for total antigen concentration of tPA, using commercially available ELISA kits from
144   Molecular Innovations (Molecular Innovations, Novi, MI). Total protein content was determined
145   by Bradford assay (Sigma).

146   **Immunohistochemistry**

147   Formalin-fixed and paraffin-embedded peritoneal tissue was sectioned at 4-5 μm and then
148   subjected to double immunostaining. Briefly, sections were deparaffinized, rehydrated and
149   endogenous peroxidase activity was quenched by 3% hydrogen peroxide (H_2O_2) for 10 min.
150   Sections were subjected to antigen retrieval was performed in pH 6.0 citrate buffer using a
151   microwave oven for 15 min. Blocking of non-specific binding was done by incubation with 2.5%
152   horse serum at room temperature for 30 min. Sections were incubated with primary antibodies,
153   rabbite anti-ki67 (1:200, Abcam, Cambridge, MA), rat anti-F4/80 (1:200, Abcam) or rabbit anti-
154   FSP-1 (1:200, Abcam) overnight at 4°C. After washing with Tris-buffered saline (TBS, pH7.4),
155   sections were incubated using the ImmPRESS AP anti-rabbit polymer reagent (Vector
156   Laboratories, Burlingame, CA) for 30 min at room temperature. Positive signal resulted in blue
157   nuclear staining with the VECTOR Blue kit (Vector Laboratories). After washing and blocking
158   again, the sections were incubated with goat anti-CD31 (1:500, R&D System, Minneapolis, MN)
159   for 1 hour at room temperature. After washing, ImmPRESS HRP anti-goat polymer reagent
160   (Vector Laboratories) was used for 30 min at room temperature. Positive reactions for
161   endothelial cells resulted in brown red staining with the NovaRed substrate kit (Vector
162   Laboratories). Sections were examined by light microscopy (Nikon Instruments, Nikon
163   Corporation, Tokyo, Japan).

164   **Immunofluorescence staining**

165   Peritoneal tissue sections (4-5 μm) were performed for double immunofluorescence staining.
166   Briefly, sections were deparaffinized, rehydrated and were treated with 0.3% H_2O_2 for 10 min to
167   block endogenous peroxide activity and boiled in pH 6.0 citrate buffer using a microwave oven
168   for 15 min. Sections were subsequently incubated with 5% Donkey serum for 20 min at room
169   temperature. Sections were incubated with rabbit anti-cytokeratin 18 (CK18) (1:200, Enogene,
170   New York, NY) overnight at 4°C, washed in phosphate-buffered saline (PBS, pH7.4) and
171   incubated using donkey anti-rabbit DyLight 488 antibody (Thermo Scientific, Rockford, IL) and
172   Cy3-conjugated mouse anti-α-smooth muscle actin (α-SMA) (Sigma) and then mounted and
173   subjected to fluorescence microscopy (Leica DMRA, Leica Microsystems, Wetzlar, Germany).
174   Images were recorded at x100, x200 and x400 magnification of light microscopy, which were
175   then digitalized and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville,
176   MD).

177   **Statistical Analysis**

178   Normal distributed continuous data were expressed as mean ± standard error (SE). For non-
179   parametric data, results were expressed as median ± interquartile range (IQR). The difference
180   between continuous variables were evaluated using one-way ANOVA when data distribution

181   was normal and a Mann-Whitney test was used for non-normal distributed continuous data. A p

182   value of less than 0.05 was considered statistically significant. The statistical analyses were

183   performed with GraphPad Prism (version 6.0, GraphPad Software, Inc., La Jolla).

184

185   **Results**

186   **Deaths of animal**

187   Surgical procedures were successfully completed on 69 animals, except for one mice in the sham

188   group, which died due to anesthesia-related complication before the commencement of surgery.

189   One mice in the PA+PTX group died during recovery from anesthesia. Four mice died within 48

190   hour of surgery and were excluded from the study (PA and PA+PTX group, n = 2/group). Two

191   mice in the PA group were excluded due to severe distress, according to three criteria in Health

192   Evaluation of Experimental Laboratory Mice: very rough hair coat, hunched, and not eating or

193   drinking. No mice in the PA+PTX treated group incurred any life-threatening side effects or

194   deaths at 48 hours after surgery, which lead to exclusion from the study. Therefore, a total of 8

195   animals were excluded from the study.

196   Mice were euthanatized for the planned experiments on postoperative day 3 (PA group, n = 14;

197   PA+PTX group, n = 13) and day 7 (sham group, n = 7; PA group, n = 13; PA+PTX group, n =

198   15).

199   **Pentoxifylline Treatment Reduces Adhesion score**

200   Total adhesion scores data was examined and plotted for post-operative day 3, and 7 (Figure 1).

201   We observed that the sham group, which had not undergone the adhesion model, had

202   significantly lower adhesion score than the animals that had undergone adhesion model, as

203   expected. PA+PTX group (median, 1.00; IQR, 0.50-2.00) had significantly lower adhesion score

204   than the PA group (median, 2.00; IQR, 2.00-3.00) on day 3 ($p< 0.05$). On day 7, mice treated

205   with PTX (median, 3.00; IQR, 0.00-3.00) still had lower adhesion score than the PA group

206   (median, 3.00; IQR, 3.00-4.50) ($p< 0.05$).

207   **Pentoxifylline Treatment Inhibits Collagen Deposition**

208   We used the HE staining to compare changes in peritoneal structure (Figure 2A). In general,

209   there were increased thickness of submesothelial layer on day 3, and the adhesion score also

210   increased. We observed that the sham group, had the thin submesothelial layer as demonstrated

211 in (Figure 2A). The severe adhesion and thick submesothelial layer was observed, as well as
212 increased cellularity in the PA group. In contrast, PA+PTX group had less peritoneal
213 submesothelial thickness and adhesion severity as compared with the PA group.

214 We further used picrosirius red staining to assess the quality of collagen fiber in peritoneal
215 adhesion (Figure 2B). Compared with PA group, PA+PTX group had less collagen deposition
216 and the thickness of the abdominal adhesions. Our data suggested that PTX could decrease
217 collagen deposition during adhesion formation.

218 **Pentoxifylline Treatment Increased tPA level**

219 The tPA protein levels in the peritoneum of mice were perceived to be measured and plotted on
220 post-operative day 3 and 7 (Figure 3). We observed that the sham group of mice had the lowest
221 tPA protein level throughout the study period ($p < 0.001$). Those mice were treated with PTX had
222 higher tPA protein level than those untreated mice (sham and PA group). There was significant
223 difference between PA+PTX group and PA group on day 7 (0.365 ± 0.024 vs. 0.193 ± 0.03, $p <$
224 0.001).

225

226 **Pentoxifylline Treatment Reduced Angiogenesis**

227 We performed immunohistochemical staining assay to analyze the status of angiogenesis during
228 peritoneal repair. Proliferating endothelial cells were identified as those cells with cytoplasmic
229 CD31 staining and nuclear Ki67 staining (Figure 4A, arrows). Cells that staining positive for
230 CD31 but without nuclear staining for Ki67 were scored as nonproliferating endothelial cells.
231 We examined the effect of endothelial cell proliferation in peritoneum was quantified by
232 measuring the number of ki67$^+$ CD31$^+$ cell at the site of adhesion (Figure 4B). Our results
233 showed that PA group had significantly higher proliferating endothelial cells compared to both
234 sham and PA+PTX group on day 3 and day 7(all $p < 0.001$). We also observed that number of
235 ki67$^+$ CD31$^+$ proliferating endothelial cell count decrease substantially over time ($p < 0.001$) in
236 the PA group. Otherwise, we examined the angiogenic effect in peritoneum by measuring the
237 area of CD31$^+$ microvessel at the site of adhesion (Figure 4C). We also observed that the PA
238 group, which had vessel coverage (percentage of area covered by CD31$^+$ per field) of 1.63% at
239 day 3, and 3.63% at day 7, respectively. On post-operative day 7, PA group demonstrated an
240 increase in CD31 expression compared to the day 3 and indicated that blood vessel formation
241 was significantly more prominent in the PA group compared to PA+PTX groups($p < 0.001$). Thus,
242 PTX can significantly suppress angiogenesis during peritoneal repair.

243 **Pentoxifylline Treatment Reduced Inflammation**

244 Inflammation, an important component both in normal and pathological healing, is a protective
245 response of the tissue injury, designed for removal of the causative agent and restoration of tissue
246 structure and function. We performed immunohistochemical staining assay to analyze the
247 infiltration of macrophage during peritoneal repair. F4/80 is a macrophage-specific marker in the
248 mice. As shown in figure 5A, a large number of F4/80$^+$-expressed cells were observed in the PA
249 group. Quantification analysis of IHC image revealed significantly increased expression of
250 F4/80$^+$ cells on day 3 (0.80% ± 0.10%), and highest expression on day 7 (2.56% ± 0.22%) in the
251 PA group as compared to the sham group ($p < 0.01$ for both day 3 and day7) or PA+PTX group
252 (1.37% ± 0.28%, $p < 0.01$ on day 7, Figure 5B). PA+PTX group had significantly lower
253 expression of F4/80$^+$ as compared with PA group.

254 **Pentoxifylline Treatment Reduced the Expression of Fibrosis Marker FSP-1**

255 FSP-1, also known as fibroblast-specific protein 1 (FSP1), belongs to the S100 superfamily of
256 cytoplasmic calcium-binding proteins and can be expressed by cell of mesenchymal origin or
257 fibroblastic phenotype. This protein is reported to be specific for fibroblasts and to play a causal
258 role in EMT. As shown in figure 6A and 6B, a large number of FSP-1$^+$-expressed cells were
259 observed in the PA group. Quantification analysis of IHC image revealed significantly increased
260 expression of FSP-1$^+$ on day 3 (5.37% ± 1.03%), and highest expression on day 7 (11.26% ±
261 1.66%) in the PA group compared to the PA+PTX group ($p < 0.05$ for both day 3 and day7,
262 Figure 6C). Consistently, we found the mice were treated with PTX had significantly reduced
263 expression of FSP$^+$ as compared with PA group.

264 **Pentoxifylline Treatment Reduced the Expression of Fibrosis Marker α-SMA**

265 As a response to injury, mesothelium is undergoing change to mesothelial-to-mesenchymal
266 transition (MMT). Thus, we further performed double immunofluorescence staining for CK18
267 and α-SMA for peritoneal injury (Figure 6, A and B). Many studies have demonstrated that
268 mesenchymal cell markers, including α-SMA, is proposed as indicator of EMT (Margetts et al.
269 2005). Cytokeratin (CK) are structural marker proteins specific for epithelial cell, and CK18 is
270 highly expressed in mesothelial cells. α-SMA has become the most reliable marker of
271 myofibroblasts. Figure 7A are consistent with previous studies, in the PA group observed a few
272 CK18$^+$α-SMA$^+$ double-positive cells appear first in the mesothelial monolayer and later in the
273 reorganized submesothelial matrix. We examined the extent of accumulation of myofibroblasts
274 in peritoneum was quantified by assessing the percentage α-SMA$^+$ cells (Figure 7C). Our result
275 showed that PA group had significantly increased α-SMA$^+$ expression at day 3 (3.48% ± 1.28%),
276 and highest expression at day 7 (13.71% ± 1.40%) compared with the PA+PTX group ($p < 0.05$
277 for day 3 and $p < 0.01$ for day 7, respectively). PTX significantly attenuated thickening of fibrotic

278   peritoneum, and accumulation of α SMA$^+$ myofibroblasts in peritoneum after injury.

279   **Discussion**

280   In this study, we demonstrated that PTX treatment could effectively reduce post-operative intra-
281   abdominal adhesion formation. PTX could prevent peritoneum adhesion formation via 5 related
282   biological processes: increasing fibrinolysis; reducing inflammation; reducing angiogenesis;
283   reducing collagen deposition; and reducing fibrosis.

284   Post-operative intra-abdominal adhesion formation is considered to be an inevitable result of
285   peritoneum injury after abdominal surgery. Peritoneum injury initiates an inflammatory response,
286   which increase in vascular permeability leading to fibrin release and adhesion formation
287   (diZeregal & Campeau 2001). Under normal conditions, the majority of fibrin is degraded within
288   a few days by locally released proteases of the fibrinolytic system (Harris et al. 1995; Sulaiman
289   et al. 2002). In a pathological state, fibrinolysis does not occur within 5-7 days of the peritoneal
290   injury, the provisional fibrin matrix persists and more gradually becomes organized as the
291   collagen-secreting myofibroblasts and other repairing cell infiltrate the matrix (Homdahl &
292   Ivarsson 1999). This process leads to peritoneal adhesion and new blood vessel formation
293   (angiogenesis) (Saltzman et al. 1996).

294   We hypothesized that there are at least four mechanisms that PTX treatment might result in
295   reduce post-operative adhesion. First, PTX has been reported to alter rheological properties of
296   blood such as: decreasing blood viscosity by stimulating fibrinolysis to reduce plasma fibrinogen
297   concentrations, increasing erythrocyte flexibility and platelet deaggregation, and inhibiting
298   neutrophil activity to reduce the tissue damage (McCarty et al. 2016). The alteration in the
299   rheological properties of blood may be the reason why we observed that the tPA level was
300   significantly higher in the PTX treated group than those without PTX treatment group. In fact,
301   we have previously found that mice treated with therapeutic hypothermia, have increased tPA
302   levels and reduced post-operative adhesion (Lee et al. 2016).

303   Second, the anti-inflammation property of PTX has been well established by several previous
304   studies, and has been found to attenuate the cardiopulmonary bypass (CPB)-induced systemic
305   inflammatory response syndrome and postoperative mortality (Barkhordari et al. 2011; Heinze et
306   al. 2007; Otani et al. 2008). PTX has been found to affect inflammation by reducing the plasma
307   levels of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Otani et al. 2008; Pollice et

308 al. 2001). The reduction of cytokines at the site of injury may explain why we observed a

309 reduction in the infiltration of macrophage in the PTX treated group.

310 Third, PTX also has been reported by previous studies to inhibit endothelial cell proliferation and

311 angiogenesis (Gude et al. 2001; Hasebe et al. 2000). Vlahos *et al.* reported that PTX might

312 cause suppression of endometriotic lesions by suppressing angiogenesis through VEGF-C and

313 flk-1 expression (Vlahos et al. 2010). Recent evidence also found that PTX inhibits PKC-

314 dependent activation of NFκB and prevent hypoxia-induced expression of VEGF (Amirkhosravi

315 et al. 1998). Our results on reduction in angiogenesis in PTX treated groups correspond with the

316 above findings.

317 Fourth, PTX was reported by previous studies to down regulate the intracellular signaling of

318 TGF-β; which can affect collagen synthesis and fibrosis through the cAMP–PKA pathway (Fang

319 et al. 2000; Kucich et al. 2000). Through PKA, PTX has been found to reduce TGF-β-induced

320 collagen synthesis in vascular smooth muscle cells and human peritoneal mesothelial cells (Chen

321 et al. 1999; Hung et al. 2003). This might explain why we observed lower amount of collagen

322 deposition in PTX treated mice. Moreover, TGF-β1 has been reported to be the key initiating

323 factor of fibrosis, and is also known to strongly induce EMT or EndMT (Lamouille et al. 2014;

324 Piera-Velazquez et al. 2011). EMT is defined as a cellular and molecular changes that are usually

325 characterized by loss of cell–cell adhesion, the down-regulation of E-cadherin and other

326 epithelial genes, accompanied by the acquisition of mesenchymal cell morphology, increased

327 contractility and actin stress fibers. This might explain why we observed reduction in markers of

328 fibrosis with PTX treatment.

329 In this study, we found that PTX treatment decreased intra-abdominal adhesion formation by

330 reducing fibrosis, but it is not in our initial objective to confirm whether the reduction in fibrosis

331 might affect general wound healing. The main reason is because several studies have already

332 found that PTX can instead improve general wound healing. Parra-Membrives et al. (2007)

333 showed that PTX improved healing of experimental ischemic colorectal anastomoses by

334 reducing wound and intra-abdominal infections, adhesion formation, and leaks. Comert et al.

335 (2000) showed PTX has positive effect of the obstructive jaundice on healing of intestinal

336 anastomosis healing by suppressing endotoxin-induced TNF-α release from macrophages and

337 monocyte and stabilizing effect on the neutrophils. Therefore, future studies may need to clarify

338 the time-frame on how PTX treatment can reduce fibrosis and yet improve wound healing,
339 before clinical trial of PTX can be recommend on post-operative patients. In addition, future
340 studies can also clarify the mechanism on how streptokinase interact synergistically with PTX to
341 reduce post-operative adhesion (Jafari-Sabet et al. 2015).

342 **Conclusion**

343 In conclusion, our study showed that PTX may decrease intra-abdominal adhesion formation via
344 increasing peritoneal fibrinolytic activity, reducing inflammation, suppressing angiogenesis,
345 decreasing collagen synthesis, fibroblast producing and peritoneal fibrosis. We believe that
346 future studies should take into the account that PTX can reduce intra-abdominal adhesion
347 formation through multiple pathways.

348 **Acknowledgments**

349 We thank the staff of the Core Labs, the Department of Medical Research, and National Taiwan
350 University Hospital for technical support

351

352 **References**
353 Amirkhosravi A, Meyer T, Warnes G, Amaya M, Malik Z, Biggerstaff JP, Siddiqui FA, Sherman
354   P, and Francis JL. 1998. Pentoxifylline inhibits hypoxia-induced upregulation of tumor
355   cell tissue factor and vascular endothelial growth factor. *Thrombosis and Haemostasis*
356   80:598-602.
357 Arung W, Meurisse M, and Detry O. 2011. Pathophysiology and prevention of postoperative
358   peritoneal adhesions. *World Journal of Gastroenterology* 17:4545-4553.
359   10.3748/wjg.v17.i41.4545
360 Barkhordari K, Karimi A, Shafiee A, Soltaninia H, Khatami MR, Abbasi K, Yousefshahi F,
361   Haghighat B, and Brown V. 2011. Effect of pentoxifylline on preventing acute kidney
362   injury after cardiac surgery by measuring urinary neutrophil gelatinase - associated
363   lipocalin. *Journal of Cardiothoracic Surgery* 6. Artn 8
364 10.1186/1749-8090-6-8
365 Chen YM, Wu KD, Tsai TJ, and Hsieh BS. 1999. Pentoxifylline inhibits PDGF-induced
366   proliferation of and TGF-beta-stimulated collagen synthesis by vascular smooth muscle
367   cells. *Journal of Molecular and Cellular Cardiology* 31:773-783. DOI
368   10.1006/jmcc.1998.0910
369 Comert M, Taneri F, Tekin E, Ersoy E, Oktemer S, Onuk E, Duzgun E, and Ayoglu F. 2000. The

370   effect of pentoxifylline on the healing of intestinal anastomosis in rats with experimental

371   obstructive jaundice. *Surgery Today-the Japanese Journal of Surgery* 30:896-902.

372   diZerega GS. 1997. Biochemical events in peritoneal tissue repair. *European Journal of Surgery*

373   163:10-16.

374   diZeregal GS, and Campeau JD. 2001. Peritoneal repair and post-surgical adhesion formation.

375   *Hum Reprod Update* 7:547-555. DOI 10.1093/humupd/7.6.547

376   Durmus AS, Yildiz H, Yaman M, and Simsek H. 2011. The effects of heparin and pentoxifylline

377   on prevention of intra-abdominal adhesions in rat uterine horn models: histopathological

378   and biochemical evaluations. *Revue Med Vet* 162:198-203.

379   Ellis H. 1997. The clinical significance of adhesions: Focus on intestinal obstruction. *European*

380   *Journal of Surgery* 163:5-9.

381   Ernst E. 1994. Pentoxifylline for Intermittent Claudication a Critical-Review. *Angiology* 45:339-

382   345. Doi 10.1177/000331979404500502

383   Fang CC, Yen CJ, Chen YM, Shyu RS, Tsai TJ, Lee PH, and Hsieh BS. 2000. Pentoxifylline

384   inhibits human peritoneal mesothelial cell growth and collagen synthesis: effects on

385   TGF-beta. *Kidney Int* 57:2626-2633. 10.1046/j.1523-1755.2000.00123.x

386   Gude RP, Binda MM, Boquete AL, and Bonfil RD. 2001. Inhibition of endothelial cell

387   proliferation and tumor-induced angiogenesis by pentoxifylline. *Journal of Cancer*

388   *Research and Clinical Oncology* 127:625-630. DOI 10.1007/s004320100262

389   Harris ES, Morgan RF, and Rodeheaver GT. 1995. Analysis of the Kinetics of Peritoneal

390   Adhesion Formation in the Rat and Evaluation of Potential Antiadhesive Agents. *Surgery*

391   117:663-669. Doi 10.1016/S0039-6060(95)80010-7

392   Hasebe Y, Thomson LR, and Dorey CK. 2000. Pentoxifylline inhibition of vasculogenesis in the

393   neonatal rat retina. *Investigative Ophthalmology & Visual Science* 41:2774-2778.

394   Heinze H, Rosemann C, Weber C, Heinrichs G, Bahlmann L, Misfeld M, Heringlake M, and

395   Eichler W. 2007. A single prophylactic dose of pentoxifylline reduces high dependency

396   unit time in cardiac surgery - a prospective randomized and controlled study. *European*

397   *Journal of Cardio-Thoracic Surgery* 32:83-89. 10.1016/j.ejcts.2007.04.01

398   Hellebrekers BWJ, and Kooistra T. 2011. Pathogenesis of postoperative adhesion formation.

399   *British Journal of Surgery* 98:1503-1516. 10.1002/bjs.7657

400   Homdahl L, and Ivarsson ML. 1999. The role of cytokines, coagulation, and fibrinolysis in

401   peritoneal tissue repair. *European Journal of Surgery* 165:1012-1019.

402   Hood SC, Moher D, and Barber CG. 1996. Management of intermittent claudication with

403   pentoxifylline: Meta-analysis of randomized controlled trials. *Canadian Medical*

404   *Association Journal* 155:1053-1059.

405   Hung KY, Huang JW, Chen CT, Lee PH, and Tsai TJ. 2003. Pentoxifylline modulates

intracellular signalling of TGF-beta in cultured human peritoneal mesothelial cells: implications for prevention of encapsulating peritoneal sclerosis. *Nephrol Dial Transplant* 18:670-676.

Hung KY, Huang JW, Chiang CK, and Tsai TJ. 2008. Preservation of peritoneal morphology and function by pentoxifylline in a rat model of peritoneal dialysis: molecular studies. *Nephrology Dialysis Transplantation* 23:3831-3840. 10.1093/ndt/gfn369

Jafari-Sabet M, Shishegar A, Saeedi AR, and Ghahari S. 2015. Pentoxifylline Increases Antiadhesion Effect of Streptokinase on Postoperative Adhesion Formation: Involvement of Fibrinolytic Pathway. *Indian J Surg* 77:837-842. 10.1007/s12262-013-1025-y

Kucich U, Rosenbloom JC, Shen G, Abrams WR, Hamilton AD, Sebti SM, and Rosenbloom J. 2000. TGF-beta1 stimulation of fibronectin transcription in cultured human lung fibroblasts requires active geranylgeranyl transferase I, phosphatidylcholine-specific phospholipase C, protein kinase C-delta, and p38, but not erk1/erk2. *Arch Biochem Biophys* 374:313-324. 10.1006/abbi.1999.1625

Lamouille S, Xu J, and Derynck R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. *Nature Reviews Molecular Cell Biology* 15:178-196. 10.1038/nrm3758

Lauder CIW, Garcea G, Strickland A, and Maddern GJ. 2011. Use of a Modified Chitosan-Dextran Gel to Prevent Peritoneal Adhesions in a Rat Model. *Journal of Surgical Research* 171:877-882. 10.1016/j.jss.2010.06.028

Lee MTG, Lee CC, Wang HM, Chou TH, Wu MC, Hsueh KL, and Chen SC. 2016. Hypothermia Increases Tissue Plasminogen Activator Expression and Decreases Post-Operative Intra-Abdominal Adhesion. *Plos One* 11. ARTN e0160627
10.1371/journal.pone.0160627

Liakakos T, Thomakos N, Fine PM, Dervenis C, and Young RL. 2001. Peritoneal adhesions: Etiology, pathophysiology, and clinical significance - Recent advances in prevention and management. *Digestive Surgery* 18:260-273. Doi 10.1159/000050149

Luijendijk RW, deLange DCD, Wauters CCAP, Hop WCJ, Duron JJ, Pailer JL, Camprodon BR, Holmdahl L, vanGeldorp HJ, and Jeekel J. 1996. Foreign material in postoperative adhesions. *Annals of Surgery* 223:242-248. Doi 10.1097/00000658-199603000-00003

Margetts PJ, Bonniaud P, Liu LM, Hoff CM, Holmes CJ, West-Mays JA, and Kelly MM. 2005. Transient overexpression of TGF-beta 1 induces epithelial mesenchymal transition in the rodent peritoneum. *Journal of the American Society of Nephrology* 16:425-436. Doi 10.1681/Asn.2004060436

McCarty MF, O'Keefe JH, and DiNicolantonio JJ. 2016. Pentoxifylline for vascular health: a brief review of the literature. *Open Heart* 3:e000365. 10.1136/openhrt-2015-000365

Menzies D, and Ellis H. 1990. Intestinal-Obstruction from Adhesions - How Big Is the Problem.

442   *Annals of the Royal College of Surgeons of England* 72:60-63.

443   Oncel M, Kurt N, Remzi FH, Sensu SS, Vural S, Gezen CF, Cincin TG, and Olcay E. 2001. The

444   effectiveness of systemic antibiotics in preventing postoperative, intraabdominal

445   adhesions in an animal model. *Journal of Surgical Research* 101:52-55.

446   10.1006/jsre.2001.6245

447   Otani S, Kuinose M, Murakami T, Saito S, Iwagaki H, Tanaka N, and Tanemoto K. 2008.

448   Preoperative oral administration of pentoxifylline ameliorates respiratory index after

449   cardiopulmonary bypass through decreased production of IL-6. *Acta Medica Okayama*

450   62:69-74.

451   Parra-Membrives P, Ruiz-Luque V, Escudero-Severin C, Aguilar-Luque J, and Mendez-Garcia

452   V. 2007. Effect of pentoxifylline on the healing of ischemic colorectal anastomoses.

453   *Diseases of the Colon & Rectum* 50:369-375. 10.1007/s10350-006-0803-z

454   Piera-Velazquez S, Li ZD, and Jimenez SA. 2011. Role of Endothelial-Mesenchymal Transition

455   (EndoMT) in the Pathogenesis of Fibrotic Disorders. *American Journal of Pathology*

456   179:1074-1080. 10.1016/j.ajpath.2011.06.001

457   Pollice PE, Rosier RN, Looney RJ, Puzas JE, Schwarz EM, and O'Keefe RJ. 2001. Oral

458   pentoxifylline inhibits release of tumor necrosis factor-alpha from human peripheral

459   blood monocytes - A potential treatment for aseptic loosening of total joint components.

460   *Journal of Bone and Joint Surgery-American Volume* 83a:1057-1061.

461   Rossner M, and Muller R. 1987. On the Assessment of the Efficacy of Pentoxifylline (Trental).

462   *Journal of Medicine* 18:1-15.

463   Saltzman AK, Olson TA, Mohanraj D, Carson LF, and Ramakrishnan S. 1996. Prevention of

464   postoperative adhesions by an antibody to vascular permeability factor/vascular

465   endothelial growth factor in a murine model. *American Journal of Obstetrics and*

466   *Gynecology* 174:1502-1506. Doi 10.1016/S0002-9378(96)70596-3

467   Sulaiman H, Dawson L, Laurent GJ, Bellingan GJ, and Herrick SE. 2002. Role of plasminogen

468   activators in peritoneal adhesion formation. *Biochemical Society Transactions* 30:126-

469   131.

470   Tarhan OR, Barut I, Sutcu R, Akdeniz Y, and Akturk O. 2006. Pentoxifylline, a methyl xanthine

471   derivative, reduces peritoneal adhesions and increases peritoneal fibrinolysis in rats.

472   *Tohoku Journal of Experimental Medicine* 209:249-255. DOI 10.1620/tjem.209.249

473   ten Broek RPG, Issa Y, van Santbrink EJP, Bouvy ND, Kruitwagen RFPM, Jeekel J, Bakkum

474   EA, Rovers MM, and van Goor H. 2013. Burden of adhesions in abdominal and pelvic

475   surgery: systematic review and met-analysis. *Bmj-British Medical Journal* 347. ARTN

476   f5588

477   10.1136/bmj.f5588

478 Vlahos NE, Gregoriou O, Deliveliotou A, Perrea D, Vlachos A, Zhao YL, Lai J, and Creatsas G.
479   2010. Effect of pentoxifylline on vascular endothelial growth factor C and flk-1
480   expression on endometrial implants in the rat endometriosis model. *Fertility and Sterility*
481   93:1316-1323. 10.1016/j.fertnstert.2008.10.056
482 Wen WX, Lee SY, Siang R, and Koh RY. 2017. Repurposing Pentoxifylline for the Treatment of
483   Fibrosis: An Overview. *Adv Ther* 34:1245-1269. 10.1007/s12325-017-0547-2
484

Scoring system for intra-abdominal adhesion.

Score	Adhesion grading scale
0	No adhesion
1	Thin filmy adhesion
2	More than one thin adhesion
3	Thick adhesion with focal point
4	Thick adhesion with planar attachment
5	Very thick vascular adhesion or more than one planar adhesion

Figure 1

Intra-abdominal adhesion score.

The PA+PTX group had lower adhesion score. Data are expressed as the median ± IQR. $^{*}P<$ 0.05, $^{**}P<$ 0.01, $^{***}P<$ 0.001, respectively.
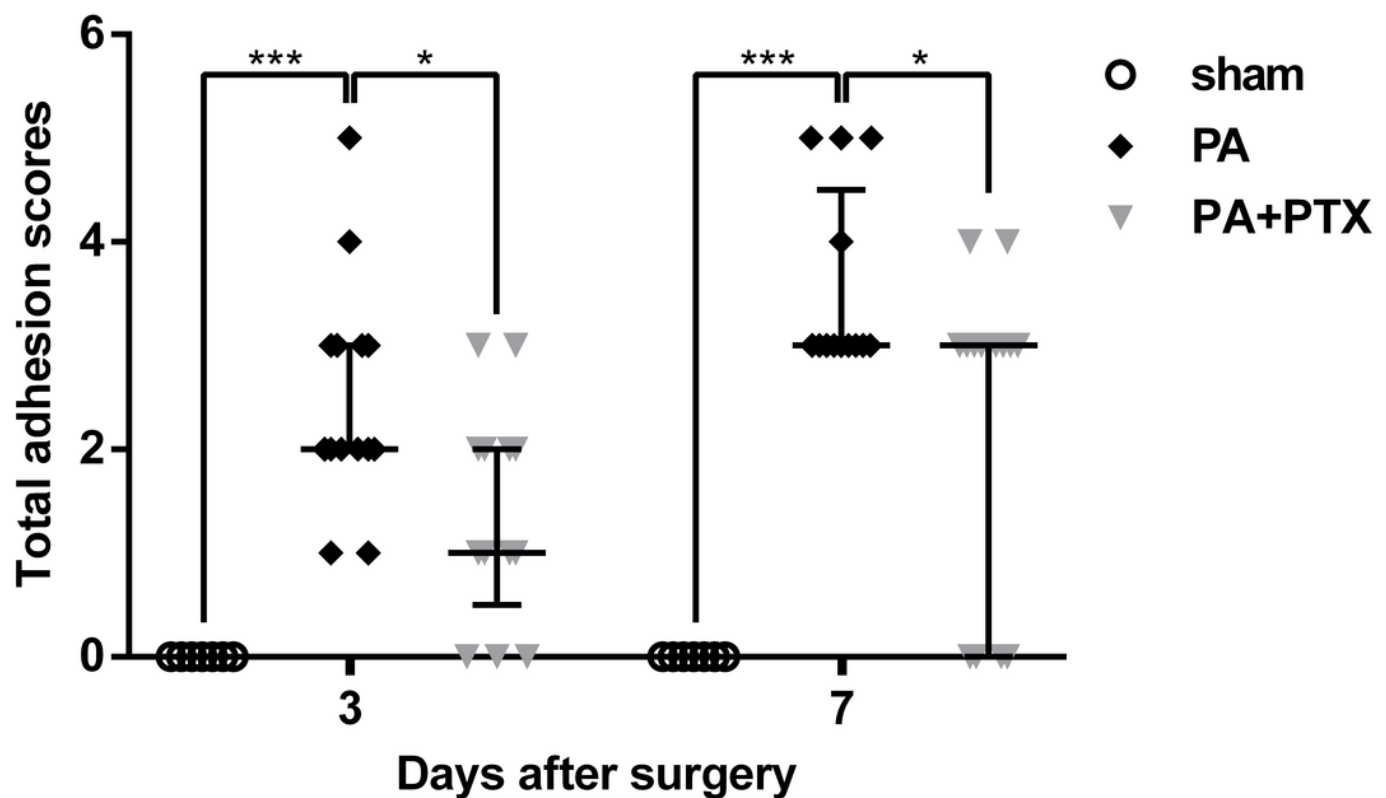
Figure 2

Pentoxifylline Treatment Inhibits Collagen *Deposition*

(A) Representative images of HE staining. No adhesion was observed in the sham group. Severe Liver or bowel adhesion and was observed in the PA group, whereas PTX treated group decreased adhesion severity. (B) Representative images of picrosirius red staining. The thickness of the collagen deposition was increased in PA group, whereas PTX treated group has less collagen deposition. (Original magnification, x200, bar = 100 μm).
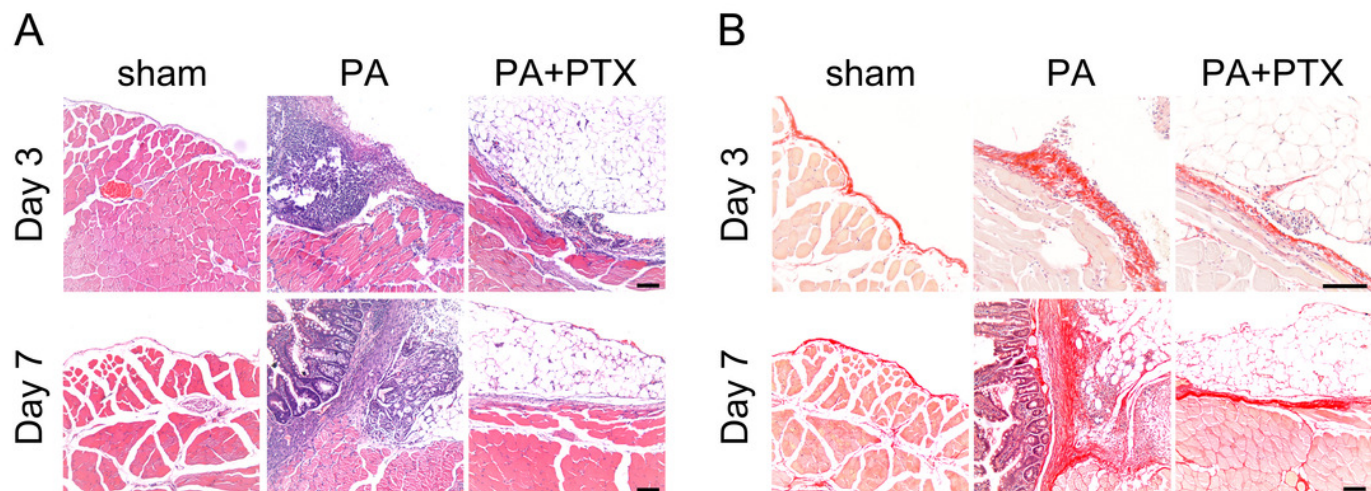
Figure 3

The levels of tPA protein in peritoneum .

tPA protein level increased in both PA group and PA+PTX group on postoperative day 3 and day7. PTX-treated mice increased tPA protein level at 7 day postoperative compared with 3 day postoperative. Data are expressed as the mean ± SE. *P< 0.05,**P< 0.01,***P< 0.001, respectively.
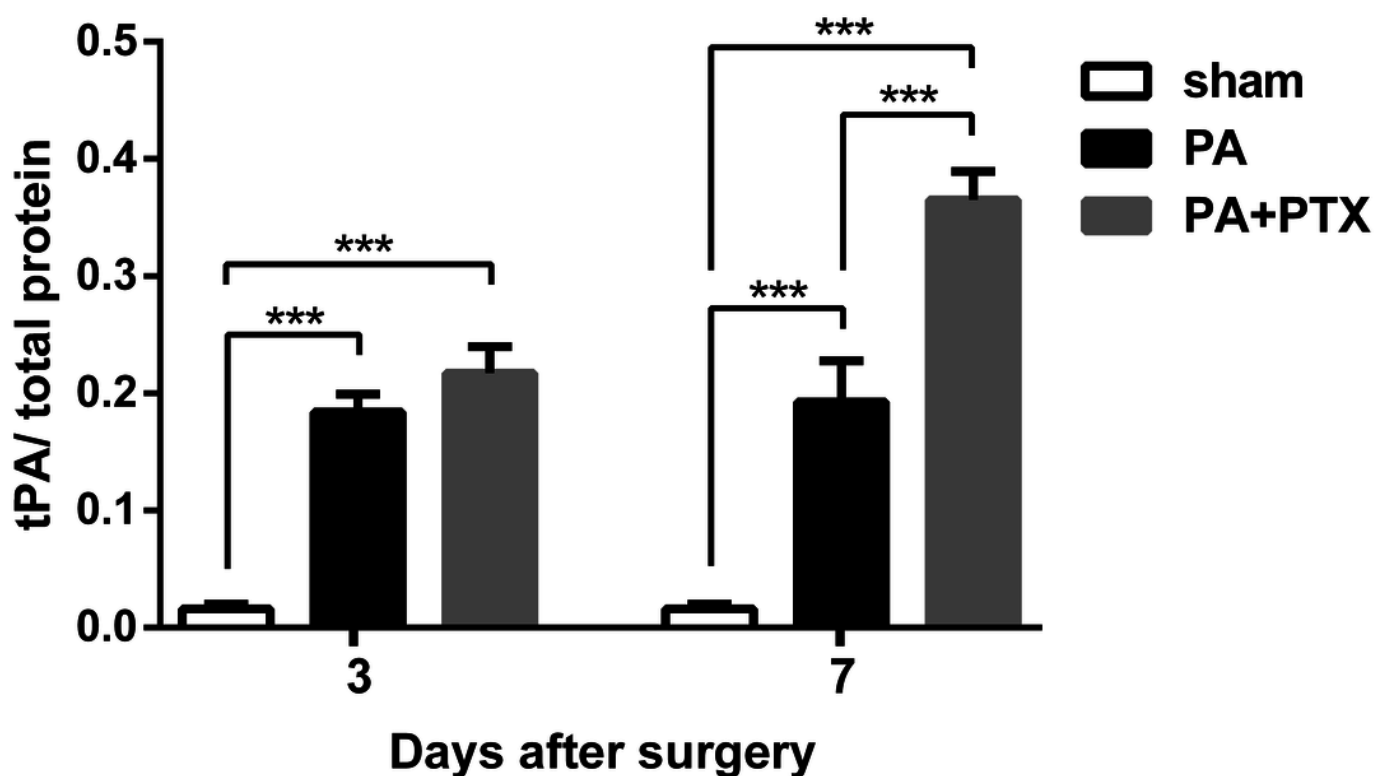
Figure 4

Pentoxifylline Treatment Reduced Angiogenesis.

(A) Representative examples of double immunohistochemistry staining of ki67 (blue) and CD31 (brown-red) (arrows) in peritoneum on day 3 and day 7. (Original magnification, x200, bar = 100 μm). (B) The graph shows numbers of cells expressing Ki67^{+} and CD31^{+} (proliferating endothelial cells) on day 3 and day 7. (C) The graph shows the percentage of CD31^{+} vessel area per field at x200 magnification on day 3 and day 7. Data are expressed as the mean ± SE. [*]$P< 0.05,$[**]$P< 0.01,$[***]$P< 0.001,$ respectively.
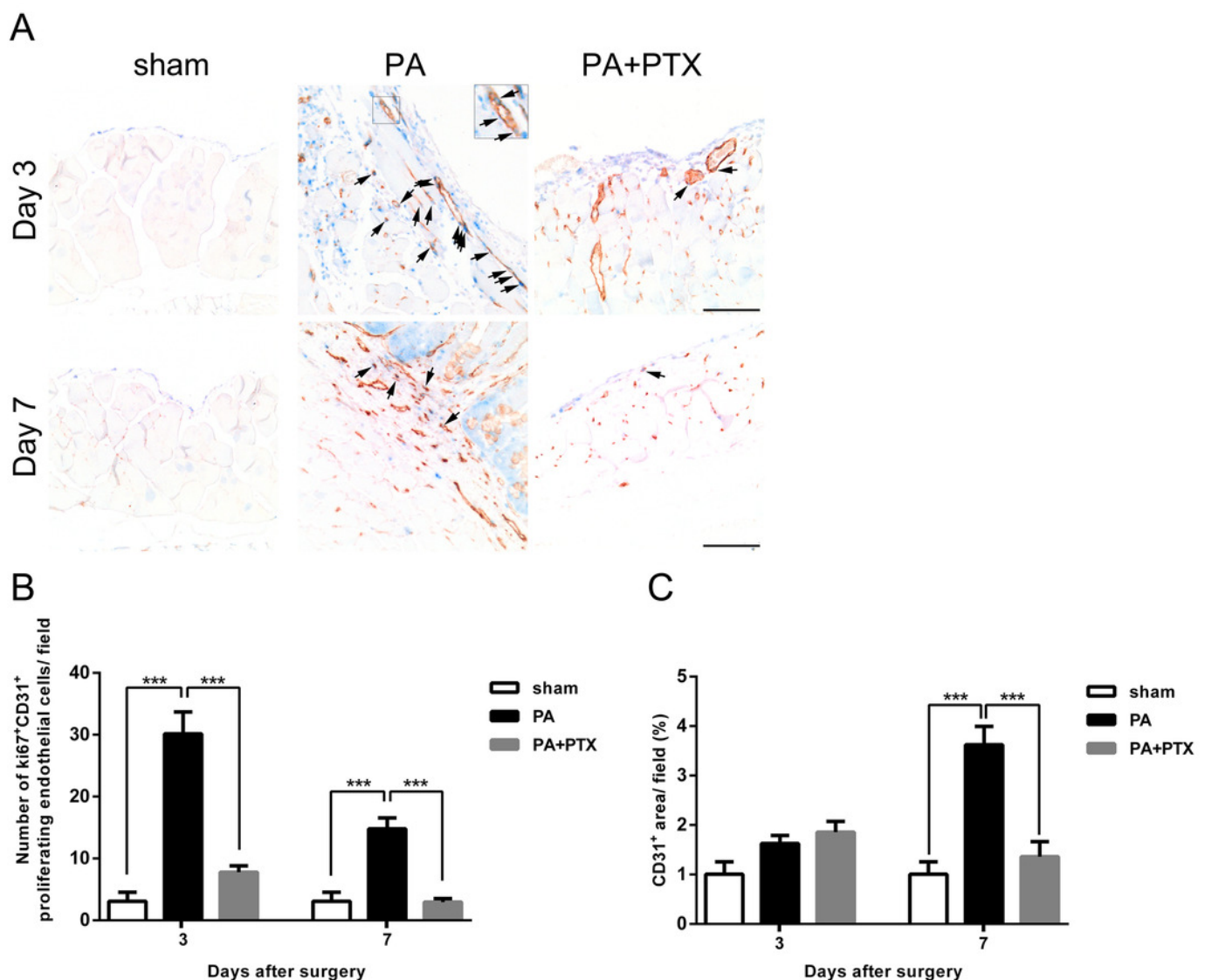
Figure 5

Pentoxifylline Treatment Reduced Inflammation

(A) Immunohistochemistry for F4/80 was performed on mice peritoneal tissue in the different groups at day 3 and day 7. The F4/80 expression was increased in PA group. Representative images of the sham group, PA group, and PA+PTX group are shown (Original magnification, x200, bar = 100 μm). (B) Quantification of F4/80$^+$ cells (%) in high-powered field (HPF) at x400 magnification. Data are expressed as the mean± SE. $^*P< 0.05,$$^{**}P< 0.01,$$^{***}P< 0.001,$ respectively.
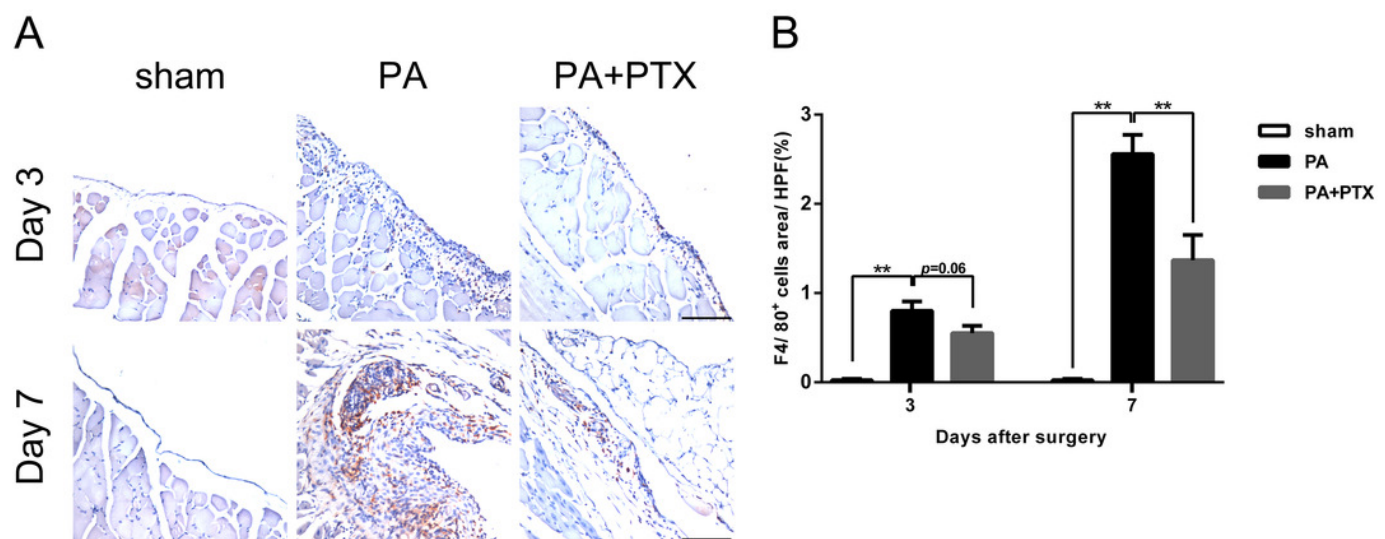
Figure 6

Pentoxifylline Treatment Reduced the Expression of Fibrosis Marker FSP1

(A-B) Immunohistochemistry for FSP-1 was performed on mice peritoneal tissue in the different groups at day 3 and day 7. The FSP-1 expression was increased in PA group. Representative images of the sham group, PA group, and PA+PTX group are shown. (Original magnification, x100, bar = 100 μm). (C) Quantification of FSP-1$^+$ cells (%) in high-powered field (HPF) at x400 magnification. Data are expressed as the mean± SE. $^*P< 0.05, ^{**}P< 0.01, ^{***}P< 0.001$, respectively.
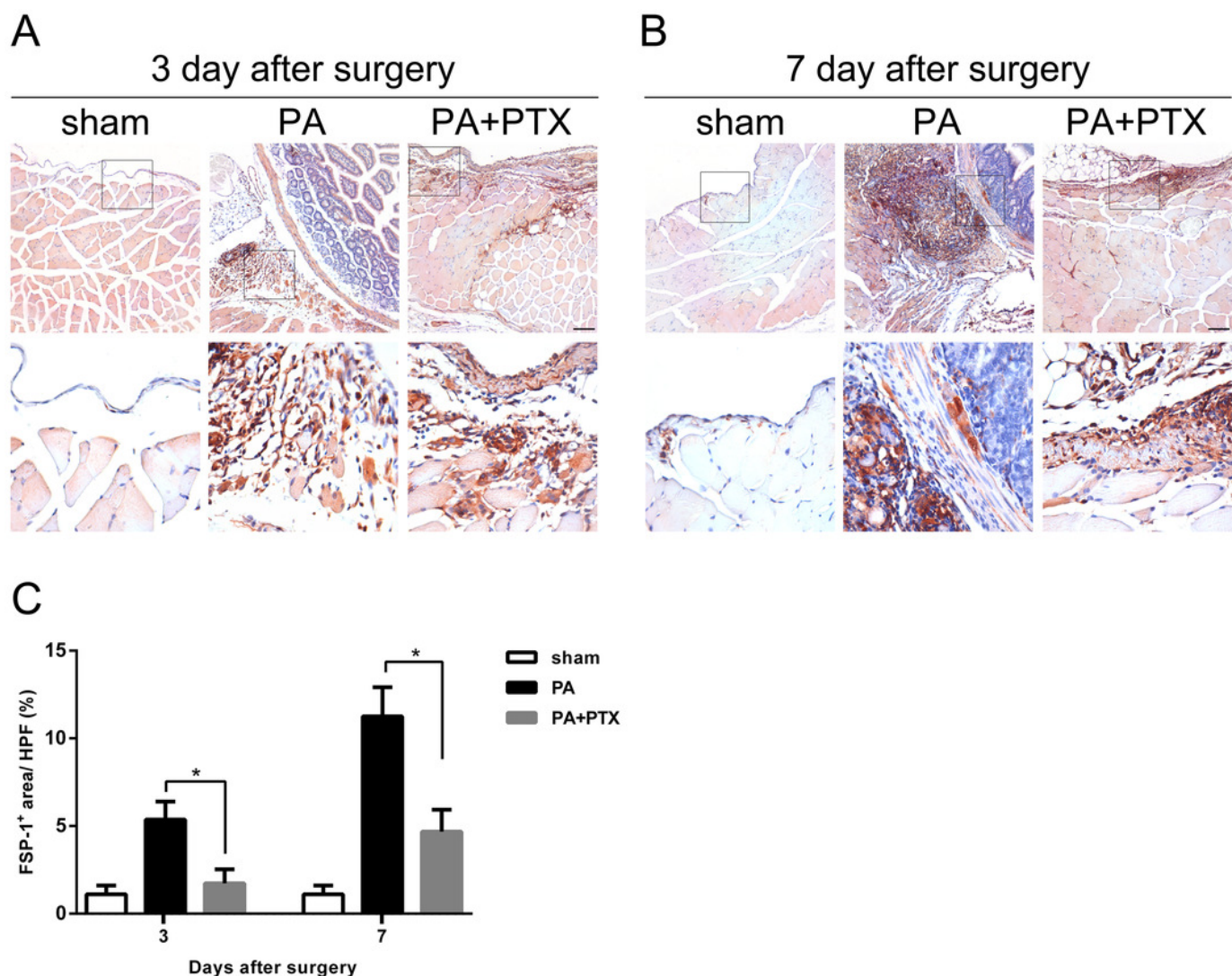
Figure 7

Pentoxifylline Treatment Reduced the Expression of Fibrosis Marker α-SMA

(A-B) Double immunofluorescence was performed with CK18 and α-SMA in mice peritoneum in the different groups at day 3 and day 7. Immunofluorescence shows the staining of mesothelial cells by CK18 was express ed in green color, and myofibroblast by α-SMA was expressed in red color. In PA group, we observed a few CK18$^+$ cells was co-localization with α-SMA in the mesothelial layer. (Original magnification, x400, bar = 100 μm). (C) Quantification of α-SMA$^+$ cells (%) in high-powered field (HPF) at x400 magnification. Data are expressed as the mean± SE. $^*P< 0.05,$$^{**}P< 0.01,$$^{***}P< 0.001$, respectively.

A

	Merge	α-SMA DAPI	CK18 DAPI

3 day after surgery

sham

PA

PA+PTX

B

	Merge	α-SMA DAPI	CK18 DAPI

7 day after surgery

sham

PA

PA+PTX

C

Day ☐ sham ■ PA ▨ PA+PTX

α-SMA⁺ cells area/ HPF (%)

$\alpha\text{-SMA}^+$ cells area/ HPF (%)

0   5   10   15   20