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Original Article

Use of wogonin as a cooperative drug with praziquantel to better combat schistosomiasis

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KEYWORDS
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Abstract *Background:* Schistosomiasis is one of the most devastating tropical diseases in the world. Currently, praziquantel (PZQ) represents the best pharmacological option for the treatment of schistosomiasis as it effectively kills the worm. However, the inability to reverse established liver damages often makes treatment futile. In the current study, we investigate whether combining the use of wogonin, a compound that was found to be liver-protective, with PZQ can attribute to the greatest beneficial effect in *Schistosoma mansoni*-infected mice.
Methods: To determine the protective effect of PZQ-wogonin treatment on *S. manosni*-infected mice, histopathological analysis was done to evaluate the granuloma size and fibrotic areas in the liver. Western blotting was performed to analyze several injuries-related markers including fibrotic markers, inflammasomes, and apoptotic markers. Scanning electron microscopy was done to evaluate the effect of wogonin on the worms, and the worm and egg burden was calculated.
Results: Our results showed that PZQ-wogonin treatment significantly improved liver histopathology of *S. mansoni*-infected mice. Further analysis showed that PZQ-wogonin combinations are more effective in reducing fibrosis, inflammation, and apoptosis in the liver than that of

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individual drug use. Furthermore, our results revealed that wogonin is anthelmintic; and it works better with PZQ in reducing hepatic egg burden, further lessen the disease progression. *Conclusion:* In general, this combinatorial strategy may represent a new and effective approach to schistosomiasis treatment.
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Introduction

Schistosomiasis, also known as bilharzia, is a parasitic disease caused by *Schistosoma*. Schistosomiasis is one of the most devastating tropical diseases in the world, leading to more than 250 million infected cases.[1] Clinical manifestation of schistosomiasis includes itchy rash, anemia, hepatosplenomegaly, and other organ damages.[1] Chemotherapy with praziquantel (PZQ) has been shown effective in reducing the morbidity of schistosomiasis patients. Because PZQ is currently the only drug that has shown full effectivity against *Schistosoma* worms,[2] it represents the best pharmacological option for the treatment of schistosomiasis. Nevertheless, the inability of PZQ to alleviate schistosomiasis-associated organ damages often make treatment futile[3,4]; therefore, using PZQ in combination with other organ protective medication may be one way to address this problem.

Accumulating researches suggested that herbal plants have diverse physiological activities that are beneficial to humans and such a herbal intervention has only minimal adverse effects.[5,6] Wogonin, a flavonoid compound isolated from the dried root of *Scutellaria baicalensis* Georgi, has been shown to benefit inflammatory, fibrotic, and carcinogenic conditions in livers.[7–9] Moreover, wogonin was capable of reducing organ injuries in different diseases including osteoarthritis,[10,11] nephropathy,[12] acute lung injury,[13,14] neurodegenerative diseases,[15–17] and seizures.[18] Although many researches have attributed to the beneficial effect of wogonin, none have investigated their effect on schistosomiasis-associated liver fibrosis. In this current study, we aimed to investigate the therapeutic effect of wogonin on schistosomiasis; as well as its combined effect with PZQ.

Materials and methods

Parasites, mice, and animal ethics

Puerto Rico strain of *Schistosoma mansoni* (*S. mansoni*) was obtained from the Biomedical Research Institute (Rockville, MD, USA) and was maintained in our laboratory as previously described.[4] Eight-week-old male BABL/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and were maintained at the animal facilities of Tzu Chi University (Hualien, Taiwan). Animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) of Tzu Chi University (No. 109066).

Animal treatment

Forty-one mice were allocated into five groups—one age-matched, uninfected control (n = 5), one infection (n = 16), and three treatment group (n = 16 for each group). Each mouse of the infection and treatment group was percutaneously infected with 100 ± 10 cercariae via the tail; while mice from the control group were treated with sterile water. Mice from the treatment group were orally gavaged with a single dose of 300 mg/kg praziquantel (PZQ; Sigma–Aldrich, St. Louis, USA)[19–21] at week 8 post-infection, followed by 40 mg/kg wogonin (Chengdu Alfa Biotechnology, Chengdu City, Sichuan, China)[9,22] for 14 consecutive days. All groups were euthanized at week ten post-infection. Seven mice from each of the infection and treatment groups were used for worm reduction analysis and hepatic eggs count; while the rest of the mice were dissected with the liver's left lateral lobes[23,24] for histology and western blot investigation.

Histopathological examination

Tissues were harvested and immediately fixed with 10% formalin. Fixed tissues were immersed in a series of graded dilutions of alcohols, xylene, and paraffin. Tissues were then paraffin-embedded and sectioned into thin slices. Tissue sections were finally stained with hematoxylin & eosin (H&E), Masson's trichome, and Sirius red as previously described.[4] Histological images were captured on an Olympus BX51 microscope (Olympus; Tokyo, Japan) with a digital microscope camera (Ruishikeni, Shenzhen, China) under a 100 × magnification. Quantification of the granuloma's areas or positive-stained areas was done by Image J (Version 1.46, National Institute of Health, Bethesda, MD, USA). Fifteen random microscopic fields were examined on each slide.

Serum biochemical analysis

Whole blood was obtained by cardiac puncture and was centrifuged at 600×*g* for 10 min to obtain the serum. Serum was then analyzed for alanine transaminase (ALT) and aspartate transaminase (AST) using a Hitachi 7080 Chemistry Analyzer (Hitachi Ltd., Tokyo, Japan).

Western blot analysis

Protein samples were extracted from tissues and separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membranes (EMD Millipore,

Burlington, MA, USA). The membranes were blocked with 5% non-fat milk and then with the following primary antibodies (at a dilution of 1:1000) at 4 °C overnight: α-tubulin (monoclonal; Cat#: GTX628802; GeneTex, Irvine, CA, USA), fibronectin (polyclonal; Cat#: A12932; ABclonal, Woburn, MA, USA), collagen AI (polyclonal; Cat#: GTX112731; GeneTex), α-SMA (polyclonal; Cat#: 14391-1-AP; Proteintech, Chicago, IL, USA), TGF-β (polyclonal; Cat#: 3711; Cell signaling technology, Danvers, MA, USA), IL-1β (polyclonal; Cat#: 16806-1-AP; Proteintech), IL-18 (polyclonal; Cat#: 10663-1-AP; Proteintech), caspase-3 (polyclonal; Cat#: GTX110543; GeneTex), and BCL-2 (polyclonal; Cat#: GTX100064; GeneTex). Membranes were then incubated with HRP-conjugated secondary antibodies (at a dilution of 1:5000; EMD Millipore) for 1 h and developed using ECL detection reagent (EMD Millipore). Relative protein levels were quantified using Image J (Version 1.46, National Institute of Health, Bethesda, MD, USA).

Worm burden, hepatic eggs count, and scanning electron microscopy (SEM)

Adult worms were isolated from the portal vein and mesenteric veins by portal perfusion method[25]; hepatic eggs were counted on weighted liver fractions after digesting with 4% KOH for 4 h.[26] Isolated adult worms were collected, washed several times with PBS, fixed in 2.5% glutaraldehyde for 60 min at 4 °C, and rinsed with 5% sucrose. After that, the worms were incubated with 1% osmium tetroxide for 60 min and increasing concentrations of ethanol (50%, 70%, 80%, 90%, and 100%; 10 min each). The worms were critically point-dried and sputter-coated

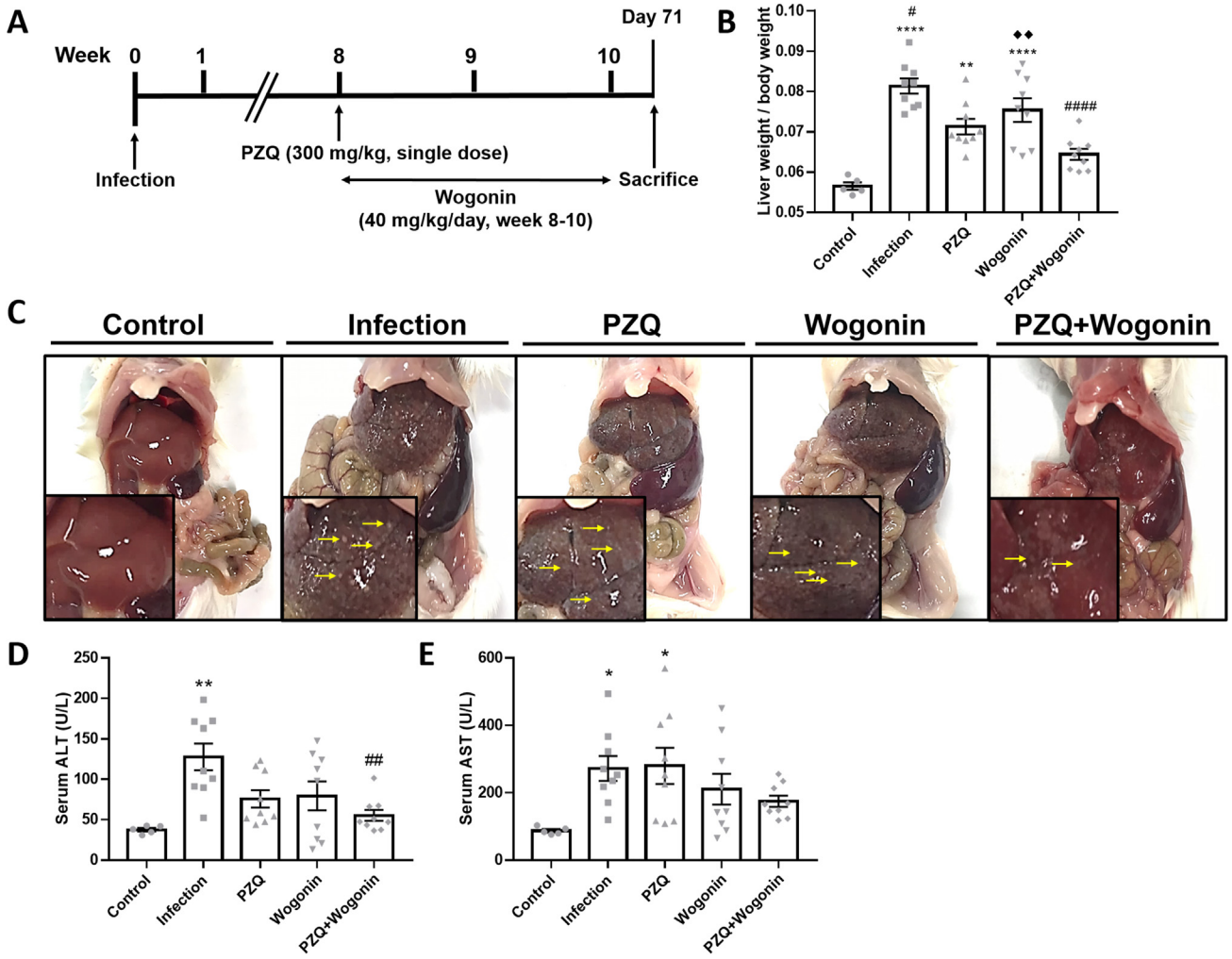

Figure 1. Praziquantel-wogonin treatment attenuates schistosomiasis-associated liver injuries. (A) Experimental design scheme. (B) Liver weight of the mice, relative to their corresponding body weight. (C) Representative images showing gross pathology of control, infected, PZQ-treated, Wogonin-treated, or PZQ and Wogonin-treated mice. White spots (yellow arrows) seen on the surface of liver indicate granuloma nodules. Serum level of (D) ALT and (E) AST. Results are shown as mean ± s.e.m. (n = 5 control mice; n = 9 infected and treated mice). * p-value < 0.05, ** p-value < 0.01, and **** p-value <0.0001 compared with control group; # p-value < 0.05, ## p-value < 0.01, and #### p-value <0.0001 compared with infection group; ◆◆ p-value < 0.01 compared with PZQ + Wogonin group.

with gold. A HITACHI S-4700 field emission scanning electron microscope (Hitachi Ltd, Tokyo, Japan) was used to visualize the worms.

Statistical analysis

All experimental data were analyzed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA). Data are represented as the mean ± s.e.m. One-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test was used to determine differences between groups. A p-value < 0.05 indicates a significant difference.

Results

Wogonin plays a collaborative role with praziquantel in reducing schistosomiasis-associated liver fibrosis

To evaluate the therapeutic role of using wogonin with PZQ, *S. manosni*-infected mice received a single praziquantel therapy at week 8 post-infection, followed by 40 mg/kg wogonin for 14 consecutive days (Fig. 1A). Infection of *S. mansoni* significantly induced hepatomegaly in BALB/c mice (Fig. 1B—C). The livers of these mice showed numerous white nodules, indicating the presence of granulomas (Fig. 1C). An increased level of serum ALT and AST were also observed, suggesting liver injuries (Fig. 1D—E). Upon ministration of PZQ or wogonin, the livers at biopsy showed better recovery and reduced granuloma nodules. When the two drugs were used in combination, the therapeutic effects were further improved; and these were accompanied by decreased, although not statistically significant, ALT and AST levels (Fig. 1B—E). Histological analysis revealed that the sole use of PZQ or wogonin yielded a similar reduction in fibrotic areas, and this reduction was further improved by using the two drugs together (Fig. 2A—F). To some degree, these data suggested that the combined use of wogonin and PZQ can be better in treating schistosomiasis.

Wogonin-praziquantel combination produces better therapeutic effect than monotherapy in schistosomiasis-associated liver fibrosis

To explore the therapeutic effect of PZQ-wogonin combination, total protein from liver tissues was extracted and was used to detect the expression of several indicators. Western blotting showed that PZQ-wogonin combination effectively inhibited the expression of fibrotic markers including fibronectin, collagen AI, α-SMA, and TGF-β, to a greater extent than that of individual drugs (Fig. 3A—B). Similarly, the PZQ-wogonin combination resulted in a stronger inhibition of inflammation markers, IL-1β and IL-18 (Fig. 3C—D). Although the PZQ-wogonin combination only slightly reduced the expression of apoptotic caspase-3, compared with individual drug treatment; there was a significant increase in the expression of anti-apoptotic protein, BCL2 (Fig. 3E—F).

Wogonin-praziquantel combination promotes better worm and eggs clearance

As expected, PZQ treatment killed all the worms in the mouse, leading to decreased eggs production and lesser hepatic egg burden (Fig. 4A—B). Interestingly, we also observed a decreased number of isolated worms in wogonin-treated mice (Fig. 4A; Supp Fig. 1); therefore, we want to know whether wogonin itself has an anthelmintic effect on the worms. By using scanning electron microscopy (SEM), we observed that the teguments of the male worms were damaged by wogonin (Fig. 4E), but not the female

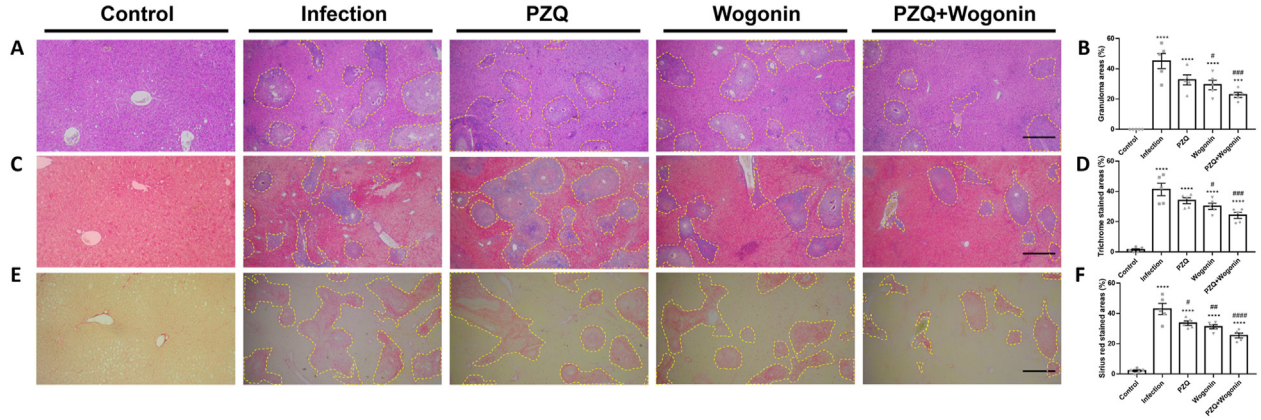

Figure 2. Praziquantel-wogonin combination is better than monotherapy in resolving liver fibrosis. (A) Representative images showing H&E staining of liver sections of the mice and (B) quantification of granuloma areas. (C) Representative images of Masson's trichrome staining on liver sections of the mice and (D) quantification of trichrome-stained areas. (E) Representative images of Sirius red staining on liver sections of the mice and (F) quantification of sirius red-stained areas. Each granuloma areas or positive-stained areas were encircled by a yellow dotted line. Quantification was performed on five slides in each group, with 15 random microscopic fields examined on each slide. Scale bars = 200 μm. Results are shown as mean ± s.e.m. (n = 5). *** p-value $<$ 0.001 and **** p-value $<$ 0.0001 compared with control group; # p-value $<$ 0.05, ## p-value $<$ 0.01, ### p-value $<$ 0.001, and #### p-value $<$ 0.0001 compared with infection group.

worms. This result therefore corroborated the difference in the number of isolated male and female worms (Supp Fig. 1). Although sole wogonin treatment did not result in a significant reduction of hepatic eggs, PZQ-wogonin combination yielded an effective reduction in hepatic egg burdens (Fig. 4B). These results also corroborated with histological and Western blot results that showed better improvement of liver injuries.

Discussion

Schistosomiasis is affecting many people worldwide and its major clinical feature is liver fibrosis. While PZQ effectively kills the worm, it cannot resolve the trapped eggs and the established fibrosis.[27,28] Therefore, in the current study we aimed to evaluate the combined use of PZQ with wogonin, a compound that has been found protective to livers,[7–9] in treating *S. mansoni* infection.

Improvement of liver function markers and liver histopathology in *S. mansoni*-infected mice after PZQ-wogonin treatment suggested that the two drugs may work in a synergistic fashion (Figs. 1 and 2). It has been suggested that the inability to stop or revert the progression of schistosomiasis-associated liver fibrosis may be due to the persistent inflammation induced by the trapped schistosome eggs.[29] NLRP3 activation has been incriminated in the pathogenesis of liver inflammation and fibrosis caused by *Schistosoma*.[30] After the hepatic stellate cells (HSCs) being stimulated by the eggs, they activate the NLRP3, leading to the release of IL-18 and IL-1β. Subsequently, these inflammatory cytokines can lead to further activation of HSCs

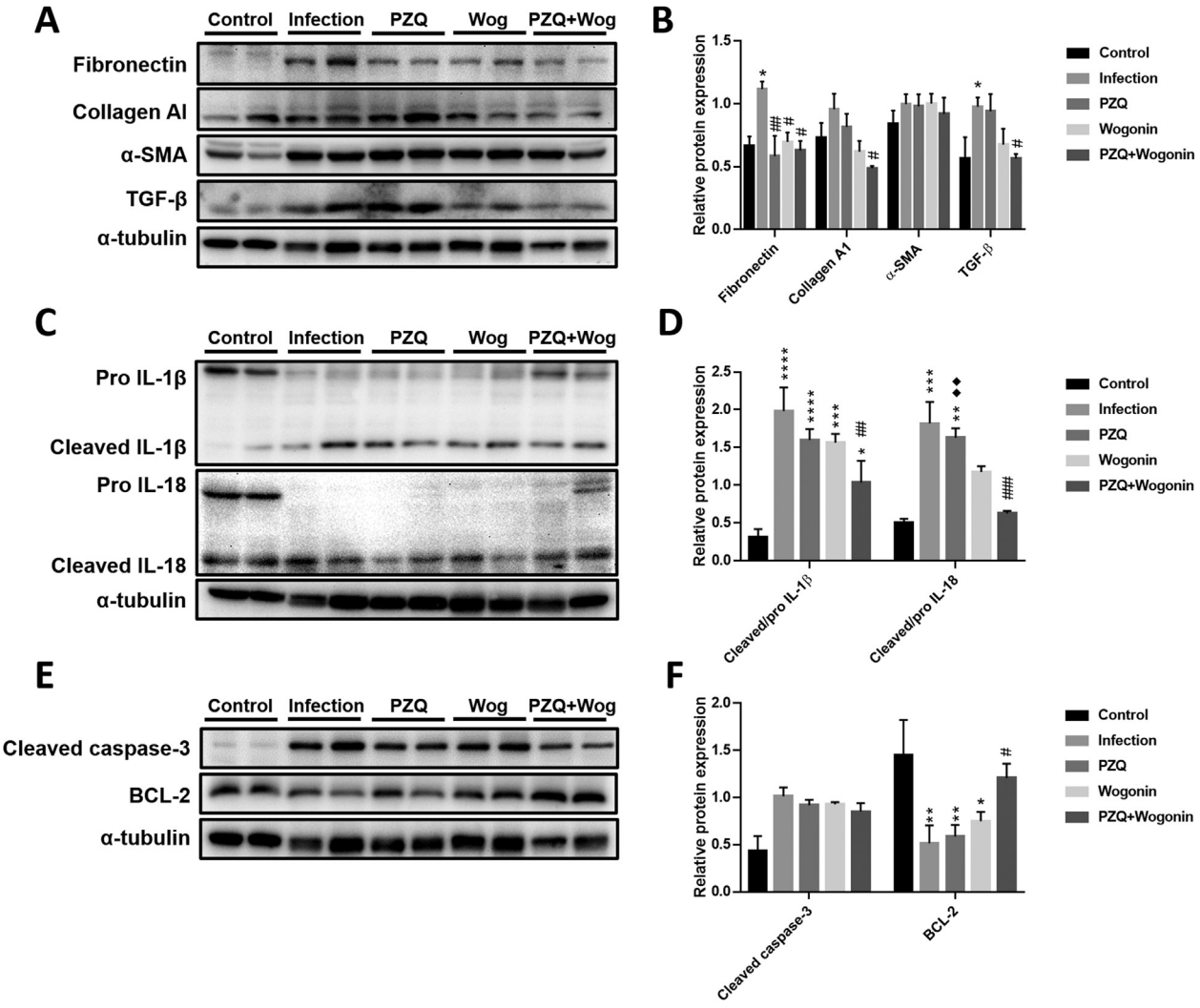

Figure 3. Praziquantel-wogonin significantly reduces expression of injury markers in livers. (A) Representative Western blot images showing protein levels of fibrotic markers. (B) Protein expression levels of fibrotic markers, relative to that of α-tubulin. (C) Representative Western blot images showing protein levels of inflammatory markers. (D) Protein expression levels of inflammatory markers, relative to that of α-tubulin. (E) Representative Western blot images showing protein levels of apoptotic markers. (F) Protein expression levels of apoptotic markers, relative to that of α-tubulin. Results are shown as mean ± s.e.m. (n = 4). * *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001, and **** *p*-value < 0.0001 compared with control group; # *p*-value < 0.05, ## *p*-value < 0.01 and ### *p*-value < 0.001 compared with infection group; ◆◆ *p*-value < 0.01 compared with PZQ + Wogonin group.

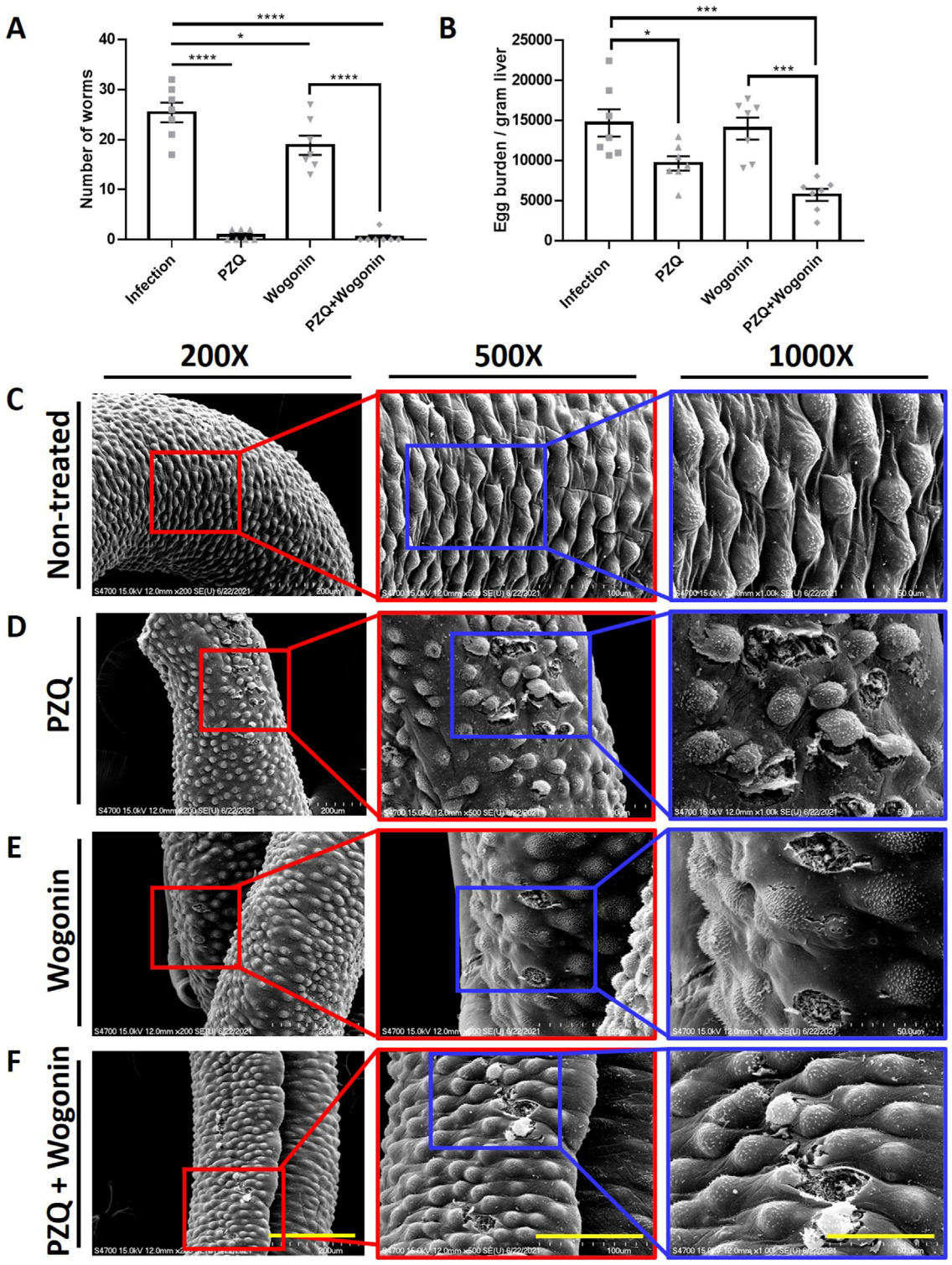

Figure 4. Wogonin plays a cooperative role with praziquantel in promoting worm and eggs clearance. (A) Number of worms isolated from the mice. (N) Number of eggs recovered per gram of mouse liver. Representative SEM images showing the ultra-structural surface of male adult worms isolated from the (C) infected, non-treated mice, (D) PZQ-treated mice, (E) Wogonin-treated mice, and (F) PZQ and Wogonin co-treated mice. Scale bars = 200 μm at 200 × magnification; 100 μm at 500 × magnification; 50 μm at 1000 × magnification. For (A) and (B), results are shown as mean ± s.e.m. (n = 7). * p-value < 0.05, *** p-value < 0.001, and **** p-value < 0.0001 compared between groups.

and fibroblasts; as well as inhibiting apoptosis of the HSCs.[31—33] Additionally, TGF-β is another inflammatory cytokine that aids in the activation of HSCs and fibroblasts.[34] Over-activated HSCs produce a large amount of collagen I and fibronectin, leading to a fibrogenic outcome. While trapped eggs also induce hepatocytes apoptosis, HSCs will migrate to the site to engulf the apoptotic bodies; and this engulfment again aggrandizes activation of the HSCs and fibrosis.[35] Therefore, as persistent inflammation and apoptosis lead to fibrosis, resolution of them may arrest fibrosis at their formation process. In the current study, we showed that wogonin is better than PZQ in the alleviation of fibrosis, inflammation, and apoptosis; and as expected, PZQ-wogonin leads to a more significant reduction in the expression of these markers (Fig. 3).

Interestingly, wogonin is able to damage male worms, but not females (Fig. 4C). This finding is in line with the decreased number of isolated male worms (Supp Fig. 1). During mating and egg production, the females reside in the gynecophoral canal of the males[36,37]; therefore, females are having a lower probability to be exposed to wogonin. Even so, the damaged males make them unsuccessful in mating and producing eggs[37,38]; leading to a lesser hepatic burden (Fig. 4B).

As the resistant strain of *Schistosoma* has been discovered after many years of excessive use of PZQ,[3] finding an alternative drug is needed. Although the therapeutic effect of wogonin was comparable, or even better than that of PZQ, the inability to effectively kill the worms makes wogonin not a suitable candidate as an alternative drug for PZQ. Nevertheless, wogonin itself showed a limited effect on worm killing (Fig. 4A and C); and it potentiates the reduction of hepatic eggs by PZQ (Fig. 4B). These results suggest that the PZQ-wogonin combination may provide benefits to the problem of PZQ resistance, and further investigation is required to confirm this hypothesis. Many studies have suggested that flavonoid compound such as casticin and artemisinin exhibits anti-parasitic effects.[39—42] While wogonin is also a flavonoid, it may process a similar mechanism on worm-killing; and research into solving this mechanism may further drug development for schistosomiasis. This also raises another fascinating question of whether a flavonoid-rich food diet could reduce disease severity or even prevent *Schistosoma* infection.

Previously, many scientists have searched for new and effective herbal and botanical substances to treat schistosomiasis. Among many herbal substances that have proved useful in alleviating schistosomiasis-associated liver fibrosis,[43,44] most of them are crude plant extracts that may contain potentially toxic compounds.[45—47] Therefore, more research is needed on these compounds individually. In previous studies, Schisandrin B (Sch B), a compound isolated from *Schisandra chinensis*, has shown promise when used alone or in combination with PZQ in attenuating *S. mansoni*-induced liver fibrosis.[4,21] However, fibronectin expression, instead of decreased in infected livers, significantly increased following Sch B treatment on *S. mansoni*-infected mice.[4] While many bacteria bind to the host cells through bacterial cell-wall-associated fibronectin-binding proteins A (FnBPA) and B (FnBPB),[48—50] whether the use of Sch B to treat liver fibrosis will increase the risk of bacterial infection remained an unsettled question, especially if treatment takes place in developing countries where schistosome, as well as bacterial infection, are still a very serious problem. Wogonin, on the other hand, did not increase fibronectin expression; and is therefore more suitable to use in developing countries without posing risks to secondary bacterial infection. Despite that, we do not wish to imply that Sch B is not a suitable drug for treating schistosomiasis. Given that PZQ-Sch B treatment ameliorates injuries in the lungs and intestine and improves *S. mansoni*-induced neurofunction defects, Sch B has its benefit in treating schistosomiasis.[21] Although the effect of wogonin on other organ injuries was not investigated in this study, we can reasonably hypothesize that PZQ-wogonin treatment can also benefit *S. mansoni*-induced injuries in the lungs and intestines as observed in other disease models.[13,14,51,52] Yet, more studies are required to evaluate the compound before they can successfully enter clinical trials, especially after the commercial drug Mirazid (a myrrh-derivative) has proven ineffective in its maximum recommended dose in human uses.[53,54] This also raises awareness that more new drugs may be needed.

Taken together, although the effects were not always statistically significant, our cumulative data are convincing that wogonin may act interactively with PZQ to potentiate the therapeutic effect against schistosomiasis. Therefore, this combinatorial strategy may represent a new and effective approach to treat schistosomiasis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.04.013.