Tropical Journal of Pharmaceutical Research January 2023; 22 (1): 135-141
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i1.19

Original Research Article

Salvianolic acid A exerts a protective effect against sodium laurate-induced thromboangiitis obliterans in rats

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Sent for review: 4 August 2022 *Revised accepted: 19 December 2022*

Abstract

Purpose: *To study the potential therapeutic effects of salvianolic acid A (Sal A) on thromboangiitis obliterans (TAO).*
Methods: *An in vitro model of TAO-mimicking endothelial cell damage was established by incubating ECV304 cells with H_2O_2. An in vivo model of TAO rats was developed via injection of sodium laurate. After treatment with varying doses of Sal A, TAO symptoms were monitored over time and compared. The effects of Sal A on oxidative stress, pyroptosis, inflammation, and thrombosis were assessed using biochemical assays, enzyme-linked immunosorbent assay (ELISA), western blot, and histopathology.*
Results: *Sal A significantly alleviated the inhibition of cell viability induced by H_2O_2 ($p < 0.05$). The H_2O_2-induced increases in levels of ROS, IL-1β, and IL-18 in ECV304 cells were significantly decreased by Sal A ($p < 0.05$). Moreover, Sal A alleviated TAO symptoms in rats. The enhanced levels of IL-1β, IL-18, NLRP3, and active Caspase-1 observed in TAO rats were reduced by Sal A in vivo ($p < 0.05$). Moreover, Sal A significantly reduced the size of thrombus in TAO rats. In addition, upregulated levels of ROS were significantly inhibited by Sal A ($p < 0.05$).*
Conclusion: *Sal A exerts significant therapeutic effects on TAO. This provides mechanistic insights into the clinical effects of RSMA on TAO patients, which might be beneficial in the development of effective drugs against TAO in the future.*

Keywords: *Inflammation, Salvianolic acid A, Thromboangiitis obliterans, Thrombosis*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Thromboangiitis obliterans (TAO), also known as Buerger's disease, is a non-atherosclerotic inflammatory disease that causes blood clot formation (thrombosis) in blood vessels mainly in the extremities, especially in the feet [1]. The pathogenesis of TAO originates from abnormalities in the patient's immunoreactivity, which trigger inflammation in the blood vessels. Inflammation attracts the accumulation of large amounts of innate leukocytes (primarily neutrophils and giant cells) onto the vessel wall. The leucocytes crosslink platelets and red blood cells in the bloodstream and eventually form a thrombus that partially or completely occludes the blood vessels [2]. To date, no cure has been discovered for TAO. Management measures, such as tobacco discontinuation and the use of vasodilators, only suppress disease progression.

At the molecular level, TAO has been found to increase the expression of many proinflammatory markers in endothelial cells and inflammatory cells [3]. This suggests potential molecular targets and corresponding strategies for TAO treatment.

In traditional Chinese medicine, *S. miltiorrhiza* roots (RSMA) have been adopted in treating TAO, and they produce considerable effects. A study demonstrated that an RSMA extract containing a mixture of phenolic acids successfully alleviated disease symptoms, inflammatory response, as well as the extent of thrombosis in TAO rats [4]. Salvianolic acid B, a component of RSMA extract, suppressed TAO in rats by inhibiting the expression of proinflammatory factors [3]. In this study, *in vitro* and *in vivo* models of TAO were used to investigate the biological role of another main component of RSMA, salvianolic acid A (Sal A).

EXPERIMENTAL

Cell culture

ECV304 cells were maintained in DMEM containing FBS (10 %) and 1 % antibiotic mix. The cells were kept at 37 °C in a 5 % CO_2 incubator.

Animal model of TAO

Sprague-Dawley (SD) rats were bought from the Experimental Animal Center of Bengbu. This study received approval from the Animal Ethical Authority of the First Affiliated Hospital of Bengbu Medical College *vide* approval no. BBLD2017-092. All procedures were in line with the principles of the use and care of laboratory animal [5]. The SD rats were allowed to undergo acclimatization for a week. The TAO model was developed by injection of rats with sodium laurate (2 mg) for 7 days, in line with a previous paper [6]. Then, the animals were assigned to five groups, each having 6 rats. Pentobarbital Na was employed for anesthesia which was intraperitoneally injected at a dose of 40 mg/kg. To establish an *in vivo* model of TAO, femoral arterial blood circulation in rat hind limb (right) was blocked using an artery clamp after surgical exposure of the artery, followed by injection of sodium laurate solution (10 mg/mL) in study group at a dose of 0.2 mL per animal. Rats in the sham-operation (control) received saline injection in place of sodium laurate. The arterial block was relieved 15 min post-drug administration. After the surgical incision was closed, each rat was treated with penicillin to prevent infection.

Following 7 days of disease establishment, rats were treated with different concentrations (10, 20, and 40 mg/kg) of Sal A via daily intravenous injections for three weeks, while the vehicle group was injected with saline only. After 7, 14, and 21 days of Sal A treatment, pathological grading of rat hind legs was performed. Grading was based on the pathological signs of TAO, including temperature and color change in the skin, limb swelling, and gangrene development: level I: pathological signs only observable in the nails; level II: pathological signs only observable in the toes; level III: pathological signs only observable in the feet; level IV: pathological signs only observable below the knees; and level V: pathological signs observable above the knees.

CCK-8 test

The CCK-8 test was carried out with PCA Kits (SAB, CP002; College Park, MD, USA). Basically, ECV304 cells were seeded in 96-well plates at a density of 2×10^3 cells/well; 100 mL) and incubated for 12 h. Then, the ECV304 cells were assigned to 6 groups stimulated with 300 µmol/L H_2O_2, followed by exposure to gradient concentrations of Sal A (2, 5, 10, 20, 40 or 80 µM). Finally, 10 mL of CCK-8 solution was added to each well, and cell viability was measured in terms of absorbance at 450 nm.

Immunoblot assay

Total protein was extracted by lysing the cells with Radio-Immunoprecipitation Assay (RIPA) buffer. For artery specimens, the specimens were ground and lysed using RIPA buffer. Protein was quantified in lysates using BCA procedure, followed by SDS-PAGE and transferred to PVDF membranes which were then incubated with 5 % non-fat milk to block non-specific binding of the blot. The membranes were incubated with 1^o immunoglobulins at 4 °C overnight, followed by incubation with 2^o immunoglobulins for 1 h at room temperature. Then, the blots were visualized using enhanced chemiluminescence (ECL, Millipore) and the protein expression levels were measured using a chemiluminescent imaging system (Tanon 5200, Shanghai, China), with GAPDH as internal control. The antibodies used were anti-NLRP3 (Ab263899), anti-Pro-Casp-1 (Ab179515) (both from Abcam), anti-Casp-1 (22915-1-AP, Proteintech), and anti-GAPDH (#5174, CST).

Reactive oxygen species (ROS) assay

The ROS levels were determined using an Active Oxygen Detection Kit (S0033, Beyotime). A

probe staining working solution was prepared *via* 1000-fold dilution of 10 mM DCFH-DA probe solution in serum-free medium. This was incubated with cell or tissue samples away from light for 0.33 h at 37 °C under constant mixing. Then, the samples were washed thrice using serum-free medium, followed by examination using flow cytometry or fluorescence microscopy (ECLIPSE Ni, NIKON).

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β, IL-18, TXB_2, and 6-K-$PGF_{1\alpha}$ in supernatants or plasma were measured using Human or Rat IL-1β, IL-18, TXB_2, and 6-K-$PGF_{1\alpha}$ ELISA Kits according to the manufacturers' instructions.

Biochemical assays

The levels of SOD, MDA and GSH-Px in cells or tissues were measured using SOD (A001-1), MDA (A003-2) and GSH-Px (A005) Kits (Nanjing, Jiancheng Biotechnology Research Institute, Jiangsu, China) in line with the kit manufacturer's protocols.

Histopathological examination

After 7, 14 and 21 days of Sal A treatment, artery specimens were dehydrated using alcohol and xylene, embedded in paraffin, sliced to 4-µm sections, and stained with H & E. Then, the sections were examined under a light microscope (BX51, Olympus, Japan) and imaged at x 200 magnification.

Statistics

GraphPad Prism 7.0 (San Diego, CA, USA) was used for statistical analysis. At least three repetitive experimental results were derived for each data set. Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's post hoc tests was used for the comparison of three and more values, with $p < 0.05$ regarded as statistically significant.

RESULTS

Sal A alleviated H_2O_2-induced endothelial cell injury *in vitro*

Oxidative stress-induced pro-inflammatory phenotype of endothelial cells is a typical sign of TAO. In order to establish an *in vitro* model to mimic this pathological condition, human umbilical vein endothelial cells (ECV304) were cultured with 300 µmol/L H_2O_2 for 3 h, which created considerable oxidative-stress damage [7]. As shown in Figure 1 A, H_2O_2 treatment significantly reduced the viability of ECV304 cells. Interestingly, incubation of damaged cells with Sal A resulted in the dose-dependent recovery of cell viability. Specifically, cell viability was increased to > 50 % by as low as 10 µM Sal A, whereas a concentration of 40 µM or higher further restored the viability to about 60 % (Figure 1 A). These results indicate that Sal A was effective in alleviating oxidative stress-induced damage in ECV304 cells, and in restoring the proliferative capacity of these cells.

To further understand the therapeutic effects of Sal A, flow cytometry was employed for measuring cellular levels of ROS, the direct indicators of oxidative stress. As shown in Figure 1 A, ROS levels were significantly enhanced in H_2O_2-treated cells when compared to untreated cells. However, ROS levels in H_2O_2-treated cells were effectively reduced by Sal A (Figure 1 B). Consistent with this, a biochemical assay showed that the cellular level of malondialdehyde (MDA), another key clinical marker of oxidative stress, was greatly increased by H_2O_2 treatment, but it was dose-dependently reduced by Sal A treatment (Figure 1 C). On the other hand, the cellular expressions of SOD and GPx which were decreased by H_2O_2 treatment were increased by Sal A treatment (Figure 1 D and E). Considering the fact that SOD and GPx protect cells against oxidative stress [8], these results indicate that Sal A exerted protective effects by upregulating the expression of antioxidants, thereby accelerating the consumption of ROS.

Oxidative stress is known to induce pyroptosis in endothelial cells, which explains the observed decrease in cell viability (Figure 1 A). One mechanism underlying this effect involves the NLR-family pyrin domain-containing protein 3 (NLRP3) inflammasome which is activated by oxidative stress. In turn, it activates caspase-1 and facilitates the secretion of inflammatory cytokines and induction of pyroptosis. In H_2O_2-treated ECV304 cells, the expressions of major pro-inflammatory factors, and pro-pyroptosis signaling molecules, NLRP3 and Active-caspase-1, were significantly increased, but they were downregulated by Sal A treatment (Figure 1 F and H). Altogether, the *in vitro* results demonstrated that Sal A effectively alleviated oxidative stress in H_2O_2-treated ECV304 cells and inhibited downstream activation of the NLRP3 caspase-1 signaling pathway, leading to suppression of cell inflammation and pyroptosis.

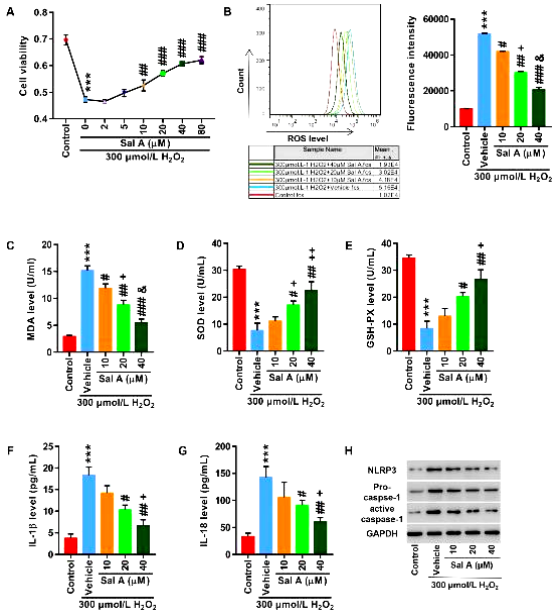

Figure 1: Sal A alleviated H_2O_2-induced endothelial cell injury *in vitro*. ECV304 cells were stimulated with H_2O_2 (300 μmol/L) for 3 h and treated with 2, 5, 10, 20, 40, or 80 μM Sal A for 24 h. No H_2O_2 or Sal A treatment in control. (**A**) Cell viability, as evaluated using CCK-8 assay and compared among the groups. ***P < 0.001, vs control; $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. 0 Sal A. (**B**) Flow cytometry results showing ROS levels in different cell groups. (**C-E**) MDA (C), SOD (D) and GPx (E) levels in different cell groups, as measured biochemically and compared. (**F-G**) ELISA results for levels of IL-1β (F) and IL-18 (G) in supernatants. (H) Western blot results for NLRP3, pro-caspase1 and Active-caspase-1 protein levels. ***P < 0.001, vs control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001, vs vehicle; ^{+}p < 0.05, ^{++}p < 0.01 vs. 10 μM Sal A; $^{\$}p$ < 0.05 vs. 20 μM Sal A

Sal A alleviated TAO symptoms *in vivo*

Intrigued by the *in vivo* therapeutic potential of Sal A against TAO, a rat TAO disease model was established via sodium laurate injection [6]. Without sodium laurate injection, none of the control group rats developed any pathological signs during the 21 days of observation. However, with sodium laurate injection, the TAO group started to develop signs of disease on day 7, with 4 rats at level II and 2 at level III. Over time, the rats gradually manifested more severe symptoms. On day 21, 4 out of the 6 rats had level V pathological signs, while the other 2 were at level IV, as shown in Table 1. Thus, Sal A treatment in TAO rats showed significant therapeutic effects. At lower doses of 10 and 20 mg/kg, a moderate alleviation was observed, mainly after 21 days. On the other hand, at the highest dose of 40 mg/kg, Sal A treatment started to produce effects on day 14, and on day 21, it effectively eliminated disease progression towards level V, with only one rat at level IV and most at level III. Collectively, these results showed that Sal A alleviated TAO *in vivo*.

Sal A alleviated TAO injury-induced inflammation and thrombosis *in vivo*

To further understand whether the therapeutic effects of Sal A were due to the mechanism characterized *in vitro*, the rats were sacrificed 24 h after the last Sal A injection. The levels of IL-1β and IL-18 in the TAO group were greatly enhanced, when compared to control, but Sal A treatment reduced this effect in a dose-dependent manner (Figures 2 A and B). Furthermore, as shown using Western blot, pyroptotic signaling molecules NLRP3 and active-caspase-1 in muscle tissues were significantly upregulated in TAO rats, but were downregulated by Sal A treatment (Figure 2 E). Moreover, TXB2, an important indicator of thrombogenic dysfunction, was elevated in TAO rats, but was strongly inhibited by Sal A (Figure 2 C). Consistently, 6-K-$PGF_{1\alpha}$, another important metabolite that stimulates thrombosis [9], also showed the same pattern (Figure 2 D). More importantly, histopathological examination of diseased artery specimens indicated that TAO rats developed severe thrombosis in their hind limbs, but treatments with Sal A (20 and 40 mg/kg) significantly reduced the size of the thrombus (Figure 2 F).

Sal A alleviated TAO injury-induced ROS *in vivo*

In agreement with the results in ECV304 cells, biochemical assays revealed greatly reduced muscle concentrations of SOD and GPx in TAO

Table 1: Effect of Sal A on the local pathological signs in TAO rats (n = 6)

Group			Lesion grade														
			0	7 days					14 days					21 days				
				I	*II*	*III*	*IV*	*V*	*I*	*II*	*III*	*IV*	*V*	*I*	*II*	*III*	*IV*	*V*
Control			6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Vehicle		0	0	4	2	0	0	0	0	3	3	0	0	0	0	2	4
TAO	Sal A (mg/kg)	10	0	0	2	4	0	0	0	0	4	2	0	0	0	0	4	2
		20	0	0	3	3	0	0	0	0	3	3	0	0	0	2	3	1
		40	0	0	4	2	0	0	0	2	4	0	0	0	1	4	1	0

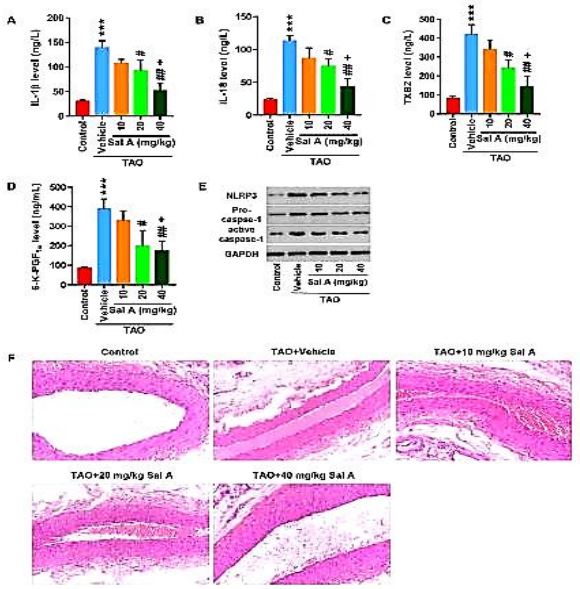

Figure 2: Sal A alleviated TAO injury-induced inflammation and thrombosis *in vivo*. A series of concentrations of Sal A were injected into different groups of TAO model rats once per day for three weeks. Control: healthy mice with no Sal A treatment. 24 h after the last injection, rats were sacrificed. Blood serum and diseased muscle tissues were isolated for analysis and comparison. (**A-D**) ELISA results for serum levels of IL-1β (A), IL-18 (B), TXB2 (C), and 6-K-PGF$_{1\alpha}$ (D) in different groups. (**E**) Immunoblot results for protein levels of NLRP3, Pro-Caspase-1, and active Caspase-1 in muscle tissues. (**F**) Histopathological examination of artery specimens. ***P < 0.001 vs. control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 vs vehicle; ^{+}p < 0.05 vs 10 mg/kg Sal A dose

rats, but these were restored by Sal A treatment, whereas the level of MDA was enhanced in TAO rats but decreased by Sal A (Figures 3 A and C). Furthermore, DCFH-DA molecular probe was used to directly determine ROS levels in muscle tissues, which showed increased ROS levels in TAO rat muscles. Taken together, ROS upregulation was significantly inhibited by Sal A, thereby confirming the role of Sal A in suppressing oxidative stress (Figure 3 D).

DISCUSSION

Oxidative stress, inflammation, and vessel occlusion which are closely interlinked pathological conditions in many life-threatening diseases like atherosclerosis and deep vein thrombosis [10], play critical roles in TAO. Since the three factors reinforce each other, an ideal treatment approach should simultaneously address all three. This often requires cocktail therapy. For instance, for the treatment of atherosclerosis, anti-platelet medications are specifically used for preventing vessel occlusion, whereas anti-inflammatory drugs are used for suppressing inflammation [11]. Similarly, in severe cases of TAO, patients need to be administered a combination of anti-coagulant (e.g., heparin), anti-platelet (e.g., aspirin), and anti-inflammatory (e.g., corticosteroid) drugs and other medications to address the various symptoms [12]. The uptake of multiple medications will increase patients' health burden and cause several side effects.

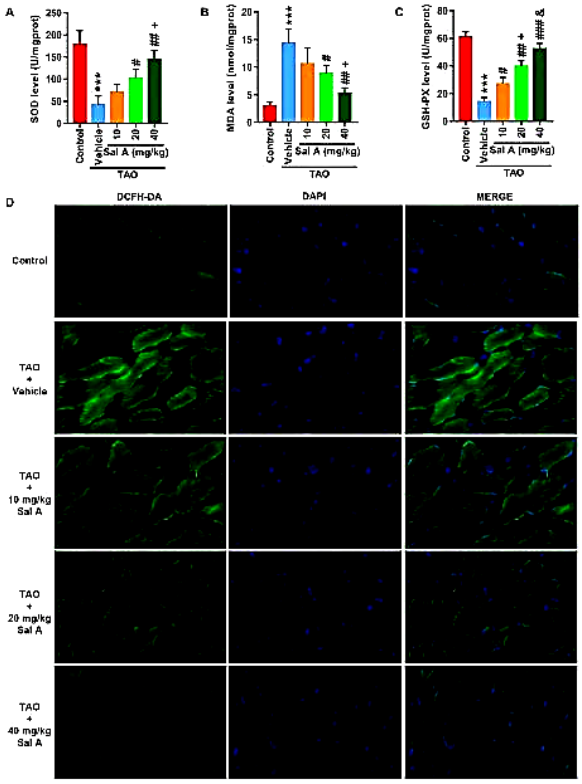

Figure 3: Sal A alleviated TAO injury-induced ROS *in vivo.* (A-C) The amounts of SOD (A), MDA (B), and GPx (C) in muscle tissues of different rat groups, as biochemically measured and compared. (**D**) The ROS levels in muscle tissues of different rat groups, as determined using a DCFH-DA probe. ***P < 0.001 vs control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs vehicle; ^{+}p < 0.05 vs Sal A (10 mg/kg); $^{\$}p$ < 0.05 vs Sal A (20 mg/kg)

The therapeutic function of salvianolic acid has been explored widely in many diseases [13]. In particular, Sal A has been found to reduce pyroptosis in rats with hepatic fibrosis [13]. Moreover, it exhibited anti-inflammatory effects in acute kidney injury, inhibited arterial thrombosis by reducing platelet activation, and displayed antioxidant activities [14]. In this study, Sal A was found to simultaneously inhibit oxidative stress, inflammation, and vessel occlusion in response to the complicated pathological situation of TAO. Therefore, with further investigations on its underlying molecular mechanisms, Sal A may

serve as a promising prototype for developing the next-generation therapeutics for TAO.

Although the pathogenesis of TAO is not fully understood, several lines of evidence have indicated its close correlation with dysfunction in vascular endothelium, with pathological signs of excessive oxidation, inflammation, and cell damage [1,15]. In turn, oxidation and inflammation facilitate thrombosis, which is consistent with the primary symptom of TAO, i.e., vessel occlusion. In this study, elevated oxidative stress in cells caused the activation of the NLRP3/caspase-1 signaling pathway, leading to inflammation and pyroptosis. However, Sal A provided protective effects by inhibiting the NLRP3/caspase-1 signaling pathway. Based on this mechanism, Sal A was shown to prevent thrombogenesis in the blood vessel of TAO rats and alleviate pathological symptoms like limb swelling and gangrene development. Altogether, these findings not only partially unravel the molecular mechanism underlying the pathogenesis of TAO, but also provide mechanistic insights into the therapeutic effects of Sal A. Consistent with these data, the NLRP3/caspase-1 inflammasome complex acts downstream of ROS to facilitate oxidative stress-associated diseases, and also in thrombosis where it activates integrins and potentiates occlusive platelet aggregation [16]. Together with these published findings, the data in this study provide insights into the non-inflammatory actions of NLRP3/caspase-1 and suggest that this molecular complex is a promising target for designing not only anti-inflammatory medications but also anti-thrombotic and anti-oxidant drugs [17]. It is unclear whether Sal A inhibits NLRP3 activation via direct interaction. If it does, then, finding and characterizing the binding interface between the two molecules will provide new hints for the design of more precise therapeutic inhibitors. On the other hand, the dynamic balance between TXB2 and 6-K-$PGF_{1\alpha}$ is important for vascular homeostasis, whereas their imbalance increases the risk of thrombosis by stimulating platelet aggregation. It has been reported that TAO disrupts the balance by elevating the plasma level of TXB2 while suppressing that of 6-K-$PGF_{1\alpha}$ [18].

Treating TAO mice with exogenous urocortin enhanced the imbalance and intensified the signs of ischemia and vasculitis, whereas inhibiting the receptors of urocortin or administering the antagonist of high-mobility-group box protein 1 restored the balance between the two molecules and alleviated the pathological signs in the mice [18]. In this study, Sal A also restored the balance between TXB2 and 6-K-$PGF_{1\alpha}$, which may serve as the mechanism of its inhibitory effects on TAO-induced thrombosis. Nonetheless, it is important to note that these results do not exclude the possibility that other factors may also serve as major mediators of thrombosis in TAO. An example is enhanced platelet uptake of serotonin. It remains unclear whether some other factors contribute to the inhibitory function of Sal A in thrombosis.

Still, the current work has limitations in understanding the biochemical mechanisms of the functional effects of Sal A. For instance, the target molecules are unknown. Moreover, it is not clear whether Sal A inhibits multiple signaling pathways related to oxidative stress, inflammation, and thrombosis, or whether it addresses only one of them, but causes downstream effects on the other two. Addressing these questions would provide clear directions for the development of drugs against TAO. Nonetheless, the current work serves as another successful example of deriving benefits from traditional Chinese medicine to stimulate drug discovery.

CONCLUSION

The findings of this study show that Sal A, a major component of RSMA, exerts significant therapeutic effects on TAO. It simultaneously alleviates oxidative stress, inflammation, and thrombosis. This discovery provides a mechanistic basis for the use of RSMA administration for treating TAO, a therapy that has been frequently adopted in traditional Chinese Medicine. More importantly, the multi-faceted function of Sal A shown in this work, is considered potentially useful for the development of a more comprehensive therapeutic strategy for TAO.

DECLARATIONS

Acknowledgements

This work was supported by Natural Science Research Projects of the University in Anhui Province (KJ2018A0234), and Key Research and Development Plan Projects in Anhui Province (no. 201904a07020020).

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chaowen Yu and Yong Gao conceived and designed the study. Chaowen Yu, Wenbo Tang, Yuan Tao, Tiancai Ren and Qiwei Chen performed the experiments. Chaowen Yu and Wenbo Tang wrote the manuscript. All authors read and approved the final manuscript.

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