# High-mobility-group box protein 1 A box reduces development of sodium laurate-induced thromboangiitis obliterans in rats

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***Objective:*** **High-mobility-group box protein 1 (HMGB1), as a late mediator of inflammation, plays a key role in inflammatory responses by inducing and extending the production of proinflammatory cytokines. The effect of HGMB1 in the inflammatory disease thromboangiitis obliterans (TAO) is unknown. We aimed to investigate the role of HMGB1 in sodium laurate-induced TAO in rats.**

***Methods:*** **Male Wistar rats were randomly divided into five groups (n = 8 each) for treatment: normal, sham-operated, TAO model, and low-dose (15 mg/kg) or high-dose (30 mg/kg) recombinant A box (rA box) infection (administered intraperitoneally once daily for 15 days). The TAO model was induced by sodium laurate and graded by gross appearance on day 15 after femoral artery injection. Histologic changes were measured by histopathology in rat femoral arteries. Plasma levels of HMGB1, thromboxane B2, 6-keto-prostaglandin F1-α, and blood cell counts and blood coagulation levels were measured. Expression of HMGB1, receptor for advanced glycation end-products (RAGE), interleukin-6, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 was assessed by immunohistochemistry and immunofluorescence, Western blot analysis, and quantitative reverse-transcription polymerase chain reaction.**

***Results:*** **The typical signs and symptoms of TAO were observed on day 15 after sodium laurate injection. The expression of HMGB1, RAGE, interleukin-6, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 was markedly increased in rat femoral arteries. Plasma levels of HMGB1 and thromboxane B2 were elevated, but the level of 6-keto-prostaglandin F1-α was decreased. Blood was in a hypercoagulable state, and prothrombin, thrombin, and activated partial thromboplastin times were all significantly shortened, whereas fibrinogen level was increased in TAO rats compared with sham-operated rats. These effects were terminated by the HMGB1 antagonist rA box.**

***Conclusions:*** **HMGB1 is involved in the inflammatory state in a model of TAO induced by sodium laurate in rats, probably via its receptor RAGE. As the antagonist of HMGB1, rA box can attenuate the development of TAO, which may be a potential therapeutic target for the treatment of TAO. (J Vasc Surg 2013;57:194-204.)**

***Clinical Relevance:*** **Thromboangiitis obliterans (TAO), or Buerger disease, is a segmental nonatherosclerotic inflammatory disorder. Patients with Buerger disease have a lower quality of life because of intermittent claudication, rest pain, ulcers, and superficial thrombophlebitis. The specific etiology and pathologic mechanisms remain not elucidated. High-mobility-group box protein 1, as a late mediator of inflammation, plays a key role in inflammatory responses to tissue injury and infection by inducing and extending the production of proinflammatory cytokines. Here, we explored the role of high-mobility-group box protein 1 in rat model of TAO, discovering a new damage marker in TAO. We also investigated the unique role of recombinant A box in the prevention and treatment of TAO.**

Thromboangiitis obliterans (TAO), also known as Buerger disease, is a segmental nonatherosclerotic inflammatory disorder that involves primarily small and medium arteries and veins of the extremities.[1] Patients with this disease have a lower quality of life because of intermittent claudication, rest pain, ulcers, and superficial thrombophlebitis.[2] Recent studies showed the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and tumor necrosis factor (TNF)-α was increased in endothelium and inflammatory cells in the thickened intima in all TAO patients.[3] The acute inflammation involving all layers of the vessel wall and occlusive inflammatory thrombosis in acute-phase lesions led TAO to be classified as an inflammatory disease.[4] However, the specific etiology and pathologic mechanisms for TAO remain unclear. In the sodium laurate–induced arterial occlusive disease model in rats, the injected sodium laurate is postulated to cause endothelial cell damage that may lead to the aggregation of platelets in peripheral vascular beds.[5] The progression in this model resembles that reported in TAO patients.[6]

High-mobility-group box 1 (HMGB1), a highly conserved nonhistone protein, presents in the nuclei. During

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cell activation and cell death, HMGB1 can translocate to the cytoplasm and extracellular space for proinflammatory effects via interaction with two types of receptors on the cell surface: Toll-like receptor (TLR) and receptor for advanced glycation end-products (RAGE).[7] RAGE, a transmembrane, pattern-recognizing receptor expressed in most tissues and present on a wide range of cells, has a high affinity with HMGB1.[8,9] HMGB1 interaction with these receptors transduces intracellular signals and mediates cellular responses, including chemotactic cell movement and release of proinflammatory cytokines (eg, TNF-α and interleukin [IL]-6), adhesion molecules (eg, ICAM-1 and VCAM-1) in vitro, and causes fever, epithelia barrier dysfunction, endothelial dysfunction, and acute inflammation in vivo.[10] Elevated levels of HMGB1 as a proinflammatory cytokine in serum and tissues occurs during sterile tissue injury, such as in arthritis, and ischemia-reperfusion injury,[11,12] and during infection, such as lethal endotoxemia and sepsis.[13,14]

HMGB1 has two DNA-binding motifs: A and B box. Structure–function analyses demonstrated that the active cytokine domain of HMGB1 is localized to the DNA-binding B box, whereas the A box competes with HMGB1 for binding sites and attenuates the biologic function of the full-length HMGB1. Recently, recombinant A (rA) box, as a specific antagonist of HMGB1, was found protective in multi-established preclinical inflammatory disease models.[15-17] Nevertheless, rA box has not been administered in humans, and its safety still needs to be evaluated in clinical trials.

We therefore hypothesized that HMGB1 might be a potent proinflammatory factor in TAO and that rA box would inhibit the progression of the disease by attenuating the release of inflammatory factors and inflammation-induced thrombotic tendencies. In this study, we explored the role of HMGB1 in sodium laurate-induced TAO rats. We also investigated whether rA box plays a unique role in the treatment of TAO.

## METHODS

**Animals.** All animal care and procedures in this investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, National Academy Press, Washington DC, revised 1996). Experimental protocols were approved by the Ethical Review Board of Shandong University. Male Wistar rats (200-250 g) were provided by Shandong University Experimental Animal Center. Water and food were given ad libitum.

**Preparation for rA box protein.** The rA box protein was prepared as described.[18] Briefly, recombinant prokaryon expression vector pQE-80L/DHFR/HMGB1 A box (Third Military Medical University, Chongqing, China) was transformed into *Escherichia coli* bacillus M15 and incubated in Luria-Bertani culture media for 5 to 7 hours at 37°C. Fusion protein expression was induced by the addition of 1 mM isopropyl thiogalactose at optical density 600 between 0.6 and 0.8. Bacteria were collected by centrifuging at 4000 g at 4°C for 15 minutes and ultrasonically shattered to obtain supernatant fluid. Then, 50% nickel-nitrilotriacetic acid slurry (1 mL) and phenylmethyl sulfonyl fluoride were added. The mixture was intensively centrifuged at 200 rpm at 4°C for 1 hour and then purified by chromatography separation and dialysis desalination. Proteins were further purified in a polymyxin B column (Pierce, Rockford, Ill).

**Experimental groups and protocol.** Male Wistar rats were randomly divided into five groups (n = 8 each) for treatment: normal, sham-operated, TAO model, and rA box infection at 15 mg/kg (low dose) and 30 mg/kg (high dose). Sodium laurate-induced TAO was prepared as described.[5] Briefly, rats were anesthetized with 10% (w/v) chloral hydrate (3.5 mL/kg intraperitoneally). The left hind leg was shaved, and the femoral artery was exposed by surgical incision. Sodium laurate (0.1 mL; Sigma-Aldrich, St. Louis, Mo) solution (10 mg/mL in normal saline, pH 8.0) was injected into the left femoral artery. The sham-operated group was treated with normal saline as a vehicle. All groups underwent the surgical procedure except the normal group. rA box (15 or 30 mg/kg) or phosphate-buffered saline (PBS) vehicle was administered intraperitoneally 2 hours after the sodium laurate injection and once a day for the next 14 days. Buprenorphine (0.05 mg/kg/d intraperitoneally) was used as analgesia. At the end of the experiment, a blood sample was drawn from the common carotid artery for biochemical analysis, and femoral arteries were collected for homogenate and histologic examination.

**Main pathologic signs.** The gross appearance in rat hind legs was checked daily after the operation. The degree of the disease on day 15 was graded as described[19]: 0, normal appearance; I, change in nail color; II, change in digit color; III, gangrene of digit; IV, loss or mummification of digit.

**Hematology.** Blood cell counts (XE-2100) and blood coagulation (CA-7000; Sysmex Medical Electronics, Kobe, Japan) were measured by the clinical laboratory in the Provincial Hospital affiliated to Shandong University.

**Plasma analysis.** Plasma levels of HMGB1 (Shino-Test Corp, Tokyo, Japan), thromboxane B2 (TXB2; Cayman Chemical, Ann Arbor, Mich), and 6-keto-prostaglandin F1-α (6-K-PGF1-α) (Enzo Life Sciences Inc, Farmingdale, NY) were measured by enzyme-linked immunosorbent assay (ELISA) kits.

**Histologic evaluation, immunohistochemistry, and immunofluorescence.** Rat femoral arteries were fixed in 10% neutral formalin for at least 24 hours, paraffin-embedded, cut into 5-μm slices, and deparaffinized. Hematoxylin and eosin staining was performed by a standard protocol. Sections were incubated overnight with 50 μL of primary antibody for HMGB1 (3935; Cell Signaling Technology, Danvers, Mass), IL-6 (sc-57315; Santa Cruz Biotechnology, Santa Cruz, Calif), ICAM-1 (sc-8439), and VCAM-1 (sc-1504) after blocking, then washed and incubated with the appropriate secondary antibody for 1 hour.

**Table I.** Specific sense and antisense primers for real-time quantitative reverse-transcription polymerase chain reaction analysis

| *Primer* | *Sense sequence* | *Antisense sequence* | *Size (bp)* | *Annealing temp (°C)* |
|---|---|---|---|---|
| HMGB-1 | GAGATCCTAAGAAGCCGAGA | CTTCCTCATCCTCTTCATCC | 595 | 52 |
| RAGE | ACCCTTAGCTGGCACTTGGATG | ATCGGAGAGCCACTTATGCT | 391 | 64 |
| ICAM-1 | AGAAGGACTGCTTGG GGA A | CCTCTGGCGGTAATA GGTG | 331 | 58 |
| VCAM-1 | CTGACCTGCTCAAGTGATGG | GTGTCTCCCTCTTTG ACGCT | 260 | 60 |
| IL-6 | AATCTGCTCTGGTCTTCTGGAG | GTTGGATGGTCTTGGTCCTTAG | 363 | 62 |
| GAPDH | CTCAAGATTGTCAGCAATGC | CAGGATGCCCTTTAGTGGGC | 394 | 58 |

*GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase; *HMGB1,* high-mobility-group box 1; *ICAM-1,* intercellular adhesion molecule-1; *IL-6,* interleukin-6; *RAGE,* receptor for advanced glycation end-products; *VCAM-1,* vascular cell adhesion molecule.

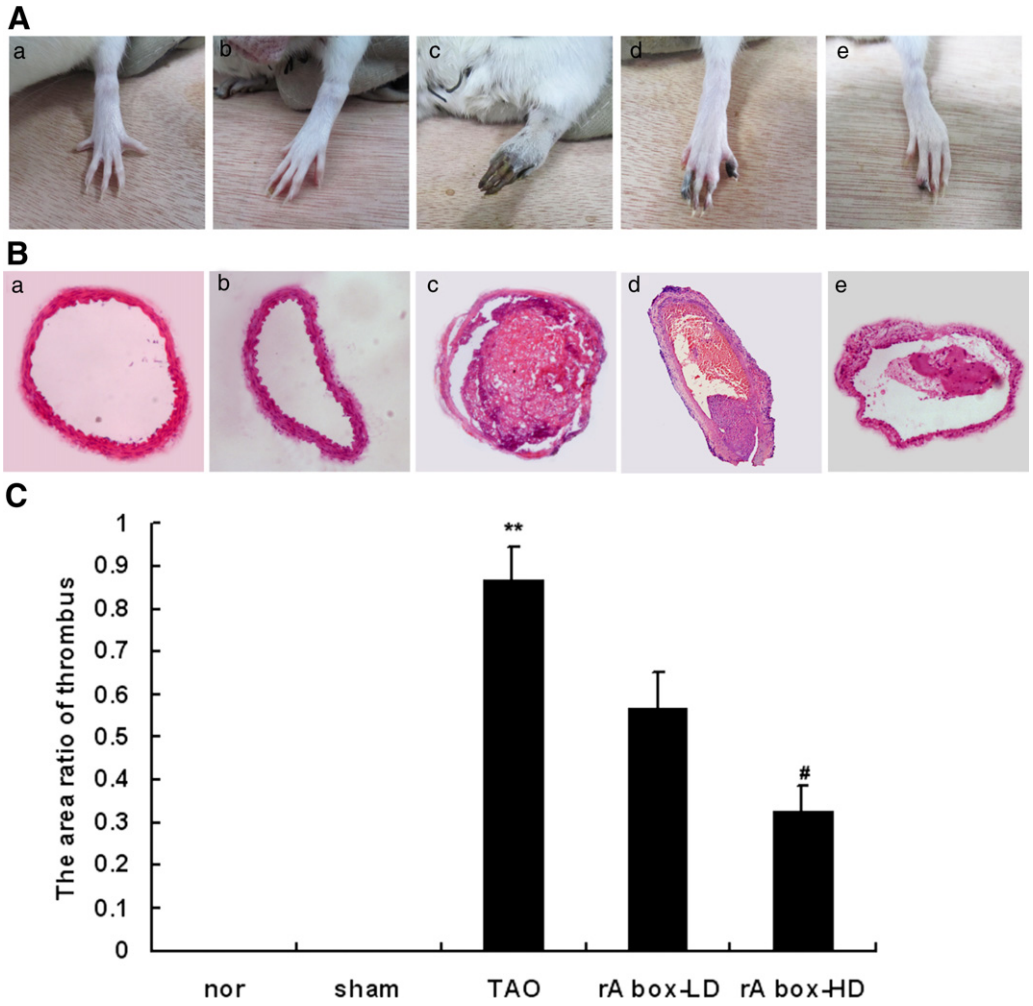


**Fig 1. A,** Gross appearance of rat paws; **(B)** hematoxylin and eosin staining of femoral arteries (original magnification, ×100) in **(a)** normal (*nor*), **(b)** sham-operated (*sham*), **(c)** thromboangiitis obliterans (*TAO*) model, and **(d)** low-dose (*rA box-LD*) and **(e)** high-dose (*rA box-LD*) recombinant A (*rA*) box-treated groups; and **(C)** data for the area ratio of thrombus of femoral arteries on day 15 after sodium laurate injection. Data are shown as mean ± standard error of the mean (*error bars*). $^{**}P < .01$ vs sham-operated group. $\#P < .05$ vs TAO model group.

Slides were stained with 3,3-diaminobenzidine. Sections were counterstained with hematoxylin. The remaining sections were used for immunofluorescence with the following antibodies: RAGE (sc-5563) and cluster of differentiation 31 (sc-31045). After incubation with the appropriate Alexa Fluor 488/546-conjugated secondary antibodies (Invitrogen, Carlsbad, Calif), the sections were observed by confocal laser scanning microscopy (Leica, Solms, Germany).

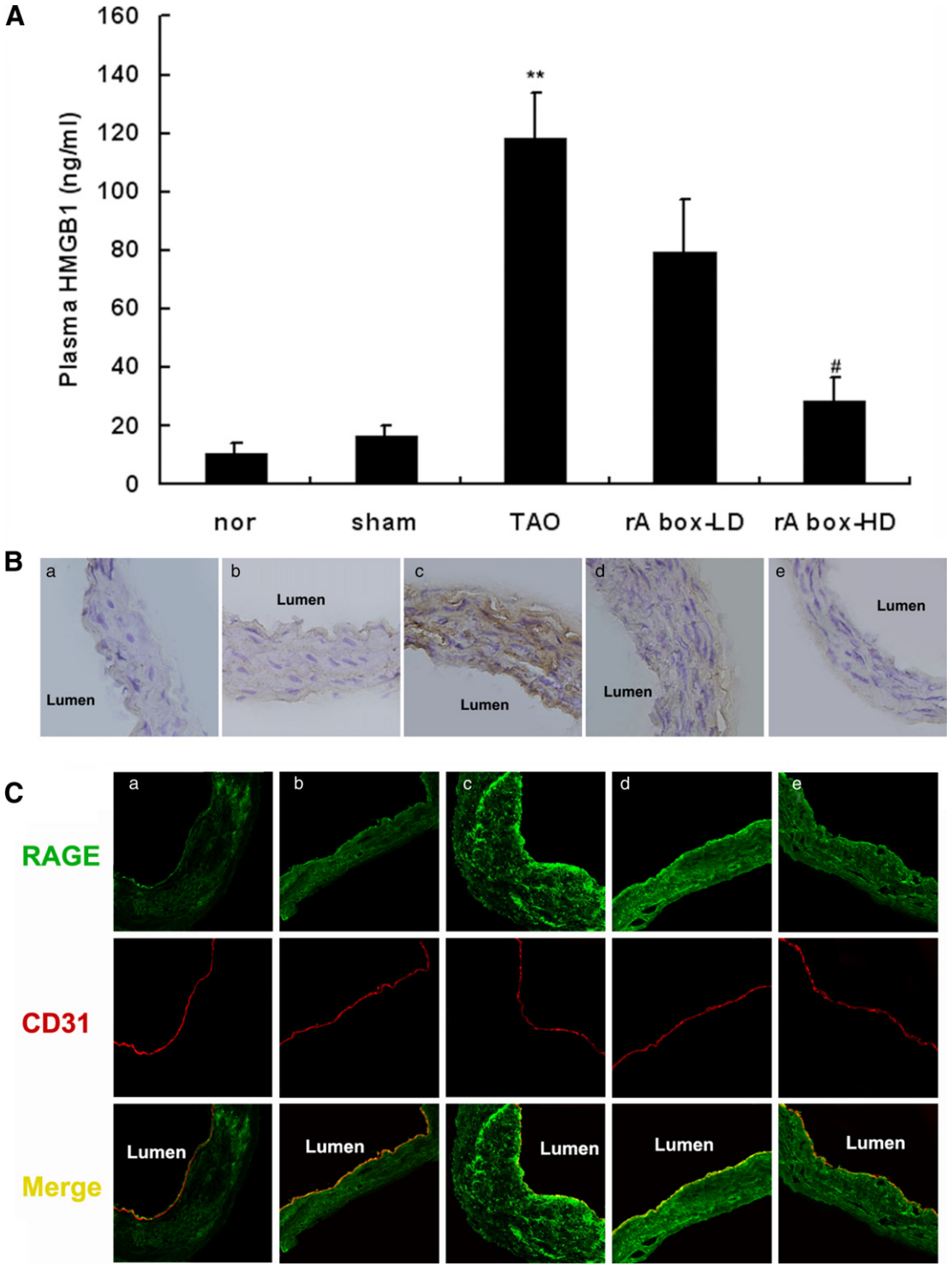


**Fig 2. A,** Plasma levels of high-mobility-group box protein 1 (*HMGB1*) are shown for normal (*nor*), sham-operated (*sham*), and thromboangiitis obliterans (*TAO*) model and the low-dose (*rA box-LD*) and high-dose (*rA box-HD*) recombinant A (*rA*) box-treated groups. Results are means ± standard error of the mean (*error bars*); n = 8. $^{**}P < .01$ vs sham-operated group. $\#P < .05$ vs TAO model group. **B,** Representative micrographs of HMGB1 in rat femoral arteries by 3,3-diaminobenzidine staining and **(C)** double immunofluorescent staining for receptor for advanced glycation end-products (*RAGE*) and cluster of differentiation 31 (*CD 31*) are shown for **(a)** nor, **(b)** sham, **(c)** TAO, and **(d)** rA box-LD and **(e)** rA box-HD groups.

**Western blot analysis.** Arterial tissue protein was extracted by the cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) after removal of thrombus. Protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred to polyvinylidene difluoride membranes, which were blocked with 5% nonfat dry milk in PBS-0.05% Tween 20 for 1 hour, then the primary antibody for HMGB1 (3935; Cell Signaling Technology), RAGE (sc-5563), IL-6 (sc-57315), ICAM-1 (sc-8439), VCAM-1 (sc-1504), and β-actin (sc-81178) was applied overnight at 4°C. The membranes were incubated with appropriate secondary antibodies for 1 hour at 37°C and visualized by enhanced chemiluminescence assay (Thermo Fisher Scientific, Rockford, Ill). Band concentration was calculated by quantification of optical density.

**Quantitative real-time reverse-transcription polymerase chain reaction.** Total RNA was extracted from femoral arteries by use of the RNeasy Mini kit (Qiagen, Hilden, Germany) after removal of thrombus. After purification, RNA was converted to complimentary (c)DNA by the use of polymerase chain reaction (PCR) (RNA PCR Core Kit, Applied Biosystems, Foster City, Calif). Real-time quantitative reverse-transcription PCR was performed on a LightCycler (Roche, Mannheim, Germany) as described,[20] with 2 μL of cDNA, 0.2 μmol of each primer, and 10 μL of SYBR Green PCR Master Mix in each PCR at a final volume of 20 μL. The primers are summarized in Table I. Data were analyzed with the comparative threshold cycle relative-quantification method, and the expression of target genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase as a constitutive control.

**Statistical analysis.** Data are expressed as means ± standard error of the mean and were analyzed by SPSS 13.0 software (SPSS Inc, Chicago, Ill). Significant differences after one-way analysis of variance tests were measured with Student–Newman-Keuls multiple comparison methods. The Kruskal-Wallis *H* test was used to compare the gross appearance among groups. $P < .05$ was considered statistically significant.

## RESULTS

**Rat TAO model induced by sodium laurate injection.** The rat model of TAO induced by sodium laurate is a widely accepted model.[5,21,22] In our study, the left hind paw of rats went pale 1 or 2 minutes after sodium laurate injection. On day 15, TAO rats (Fig 1, *Ac*) but not sham-operated or normal rats (Fig 1, *Aa* and *Ab*) showed typical signs and symptoms of TAO. Stained sections of TAO rats showed thrombi that resulted in narrowing or complete occlusion of the vessel lumen (Fig 1, *Bc*).

**Elevated HMGB1 and RAGE expression in TAO rats.** Compared with sham-treated animals, TAO rats showed increased plasma HMGB1 level ($P < .01$; Fig 2, *A*), with no difference between the normal and sham-operated group. HMGB1 was expressed in intima and media in TAO rats (Fig 2, *Bc*) and appeared frequently within the cytoplasm and was diffusely distributed within lesions, which probably reflected secreted HMGB1. Compared with sham-operated rats, TAO rats showed increased expression of RAGE mainly in the intima (Fig 2, *C*) and increased protein expression of HMGB1 and RAGE in femoral artery lysates ($P < .01$; Fig 3, *A* and *B*). The messenger (m)RNA levels of HMGB1 and RAGE were significantly increased in femoral arteries of TAO compared with normal and sham-operated rats (Fig 3, *C*).

**Effect of rA box on local pathologic signs in TAO rats.** TAO rats were administered rA box to determine whether it plays a protective role. TAO rats showed severe ischemia, which was markedly decreased with rA box injection (Fig 1, *Ad* and *Ae*). The gross appearance of the rat hind legs was evaluated on day 15. The lesion values were significantly higher in TAO rats than in sham-operated rats and were significantly attenuated dose-dependently in rats that received low-dose or high-dose rA box injection compared with TAO rats (Table II). Moreover, no thrombus occurred in femoral arteries of the sham group (Fig 1, *Bb*), whereas TAO rats showed tissue adhesion, mild lymphocyte infiltration, and various thrombi in femoral arteries (Fig 1, *Bc*). The low-dose rA box mildly and high-dose rA box significantly reduced the levels and area of thrombus (Fig 1, *Bd, Be,* and *C*).

**Effect of rA box on the expression of HMGB1 and RAGE in TAO rats.** To further confirm the protective mechanisms of rA box, ELISA showed that plasma HMGB1 level decreased with high-dose rA box injection compared with TAO rats ($P < .05$; Fig 2, *A*). Immunohistochemical and immunofluorescence staining showed a marked reduction of HMGB1 and RAGE expression in the vascular wall with high-dose rA box injection compared with TAO rats (Fig 2, *Be* and *Ce*). Low-dose injection decreased their expression slightly (Fig 2, *Bd* and *Cd*). These results were consistent with the protein and mRNA expression of HMGB1 and RAGE analyzed by Western bolt and quantitative reverse-transcription PCR (Fig 3). Thus, high-dose rA box attenuated the gross appearance of TAO by reducing the expression of HMGB1 and RAGE.

**Effect of rA box on the expression of IL-6, ICAM-1 and VCAM-1 in TAO rats.** Elevated IL-6, ICAM-1, and VCAM-1 levels are found in various inflammatory responses, including TAO.[3,22,23] HMGB1 can also activate macrophage and endothelial cells to release IL-6, ICAM-1, and VCAM-1.[24,25] Protein staining and levels of IL-6, ICAM-1, and VCAM-1 in femoral arteries were higher in TAO rats than in sham-operated rats ($P < .05$; Fig 4, *A-C*), which agreed with previous findings. Treatment with high-dose but not low-dose rA box reduced the levels of IL-6, ICAM-1, and VCAM-1 significantly ($P < .05$; Fig 4, *A-C*). mRNA levels were similar to levels for protein (Fig 4, *D*). Therefore, rA box inhibited HMGB1 to decrease the expression of inflammatory mediators in TAO rats.

**Effects of rA box on hematology of TAO rats.** To further analyze the effects of rA box on TAO rats, we investigated blood cell counts and blood coagulation. Blood platelet count was markedly higher in TAO rats than in sham-operated rats ($P < .05$; Table III, *A*). Blood

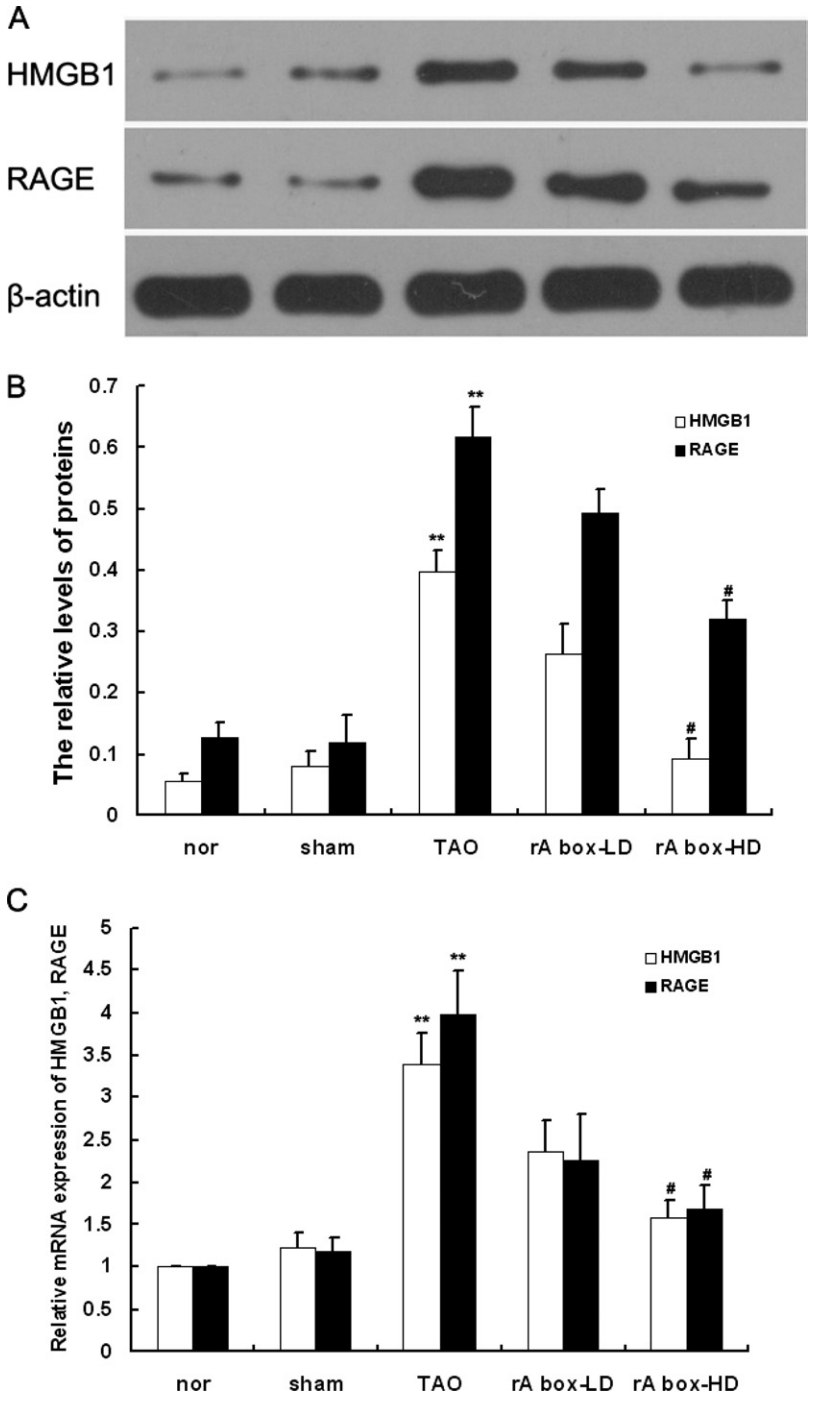


**Fig 3.** Western blot and reverse transcription polymerase chain reaction analysis of protein and messenger RNA levels of high-mobility-group box protein 1 (*HMGB1*) and receptor for advanced glycation end-products (*RAGE*) in femoral arteries from normal (*nor*), sham-operated (*sham*), thromboangiitis obliterans (*TAO*) model, and low-dose (*rA box-LD*) and high-dose (*rA box-HD*) recombinant A (*rA*) box-treated rats. **A**, Representative Western blot of protein levels of HMGB1 and RAGE (β-actin as a control). **B,** Summary data for the rat groups are presented as means ± standard error of the mean (*error bars*); n = 8. $^{**}P < .01$ vs sham-operated group. $\#P < .05$ vs TAO model group. **C,** The relative messenger RNA levels of HMGB1 and RAGE are presented as means ± standard error of the mean (*error bars*); n = 8. $^{**}P < .01$ vs sham-operated group. $\#P < .05$ vs TAO model group.

**Table II.** Grade of local pathologic signs in thromboangiitis obliterans (*TAO*) rats

| | *Grade of lesion* | | | | | |
|---|---|---|---|---|---|---|
| *Group* | *0* | *I* | *II* | *III* | *IV* | P |
| Normal | 8 | 0 | 0 | 0 | 0 | |
| Sham operated | 8 | 0 | 0 | 0 | 0 | |
| TAO model | 0 | 1 | 1 | 4 | 2 | <.01[a] |
| rA box treatment | | | | | | |
| Low dose | 0 | 2 | 2 | 3 | 1 | <.05[b] |
| High dose | 1 | 3 | 2 | 2 | 0 | <.01[b] |

*rA,* Recombinant A box.
[a]Vs sham-operated group.
[b]Vs TAO model group.

platelet count was slightly decreased with low-dose rA box ($P > .05$) and greatly decreased ($P < .05$) with high-dose treatment compared with TAO rats. However, counts of red blood cells, leukocytes, and neutrophils did not differ among the groups ($P > .05$). Prothrombin, thrombin, and activated partial thromboplastin times were all significantly shortened, and the fibrinogen level was increased in TAO rats compared with sham-operated rats ($P < .05$). The values reversed to almost normal with high-dose rA box treatment ($P < .05$), with the effect of low-dose rA box relatively weak (Table III, *B*).

**Effects of rA box on plasma levels of TXB2 and 6-K-PGF1-α and TXB2/6-K-PGF1-α ratio in TAO rats.** TXB2 and 6-K-PGF1-α are the stable metabolites of TXA2 and prostacyclin ($PGI_2$), which, respectively, induces and inhibits platelet aggregation. We found the plasma level of 6-K-PGF1α was lower and the levels of TXB2 and TXB2/6-K-PGF1-α ratio were markedly higher in TAO rats than in sham-operated or normal rats (Fig 5). However, rA box treatment increased the 6-K-PGF1-α level and decreased the TXB2 level and TXB2/6-K-PGF1-α ratio with low-dose and high-dose rA box treatment compared with TAO rats ($P < .05$).

## DISCUSSION

TAO is a nonatherosclerotic segmental inflammatory disease of small and medium distal limb vessels of predominantly young male smokers.[26] Although the disease was first described almost 130 years ago, the etiology, pathogenesis, and treatment of TAO are still unresolved.[27] HMGB1 has been identified as a proinflammatory mediator, and because of its proinflammatory and immunostimulatory properties, it may contribute to the pathogenesis of multiple chronic inflammatory and autoimmune diseases by binding its receptor RAGE.[17,28] HMGB1 is released in response to proinflammatory stimuli and induces the production of inflammatory mediators by macrophages and neutrophils. Moreover, HMGB1 is also an activator of endothelial cells, leading to the upregulation of the adhesion molecules[24,29] found in the thickened intima in TAO patients.[3] However, no report has demonstrated the potential role of HMGB1 in TAO.

In this study, sodium laurate-induced rats were used as a TAO model, which has been used to study human TAO disease because the injection of sodium laurate into the rat femoral artery imitates signs and symptoms in TAO patients.[5,6,21,22] Here, after injection of sodium laurate, most TAO rats showed typical symptoms of ischemia and vasculitis in the lower limb, and the femoral artery was structurally changed with thrombi, recanalization, and injury of endothelial cells. Results from our study agree with previous observations.[6,22,30] Meanwhile, the TAO rats were found to have significantly elevated levels of HMGB1 in plasma and tissues, and protein and mRNA levels of HMGB1 and RAGE in femoral arteries were increased in TAO rats compared with the sham-operated rats. Although the receptors of HMGB1 include RAGE and TLRs, receptor-binding studies revealed that HMGB1 has higher affinity with RAGE,[9] and recent studies suggest that HMGB1 as a regulator and inducer plays an essential role in RAGE-dependent inflammation.[31,32] Thus, HMGB1 takes part in TAO, which is mediated at least in part by RAGE.

Anti-HMGB1-based therapy using HMGB1 inhibitors has shown beneficial effects in HMGB1-involved diseases such as sepsis, ischemia-reperfusion injury, and rheumatoid arthritis.[12,15-17] The antagonists of HMGB1 include neutralizing anti-HMGB1 antibody, A box, and the anti-inflammatory agents ethyl pyruvate and sodium butyrate. A box, a specific blockade for endogenous HMGB1, has functional antagonism of extracellular HMGB1 cytokine activity and interaction with RAGE, so purified A box has a potential role in the clinical treatment of inflammatory diseases, where an excessive amount of HMGB1 is produced. As mentioned above, the treatment with rA box is safe, effective, and has no side effects.[15-17] We chose high and low doses of rA box in rats and grouped rats according to our previous study and other references to observe the effect of rA box in TAO rats but not in normal rats, in which the expression of HMGB1 was normal.[17,18,33]

As we expected, rA box significantly reduced the visible signs of ischemia and levels and area of thrombus in TAO rats. HMGB1 in plasma, the protein and mRNA levels of HMGB1, and levels of RAGE in the femoral artery wall reverted to almost normal levels with rA box treatment. The inhibitory effect of rA box on HMGB1 agrees with our previous findings and results of acute cardiac allograft rejection.[18,34] RAGE may be decreased with the reduction of its ligand. That high-dose rA box could almost completely reverse but low-dose rA box only partially reversed the effects of HMGB1 suggests that the protective role of rA box may be dose-dependent.

IL-6 acts as a proinflammatory cytokine, and ICAM-1 and VCAM-1 are two key inflammatory adhesion molecules that could be secreted by HMGB1-stimulating macrophage and endothelial cells.[24,25] HMGB1 antagonists can reduce their release.[35,36] In TAO patients, the expression of IL-6 and cell adhesion molecules in inflammatory and endothelial cells induced by proinflammatory cytokines allows cells interactions to occur, leading to inflammatory cell adhesion, activa-

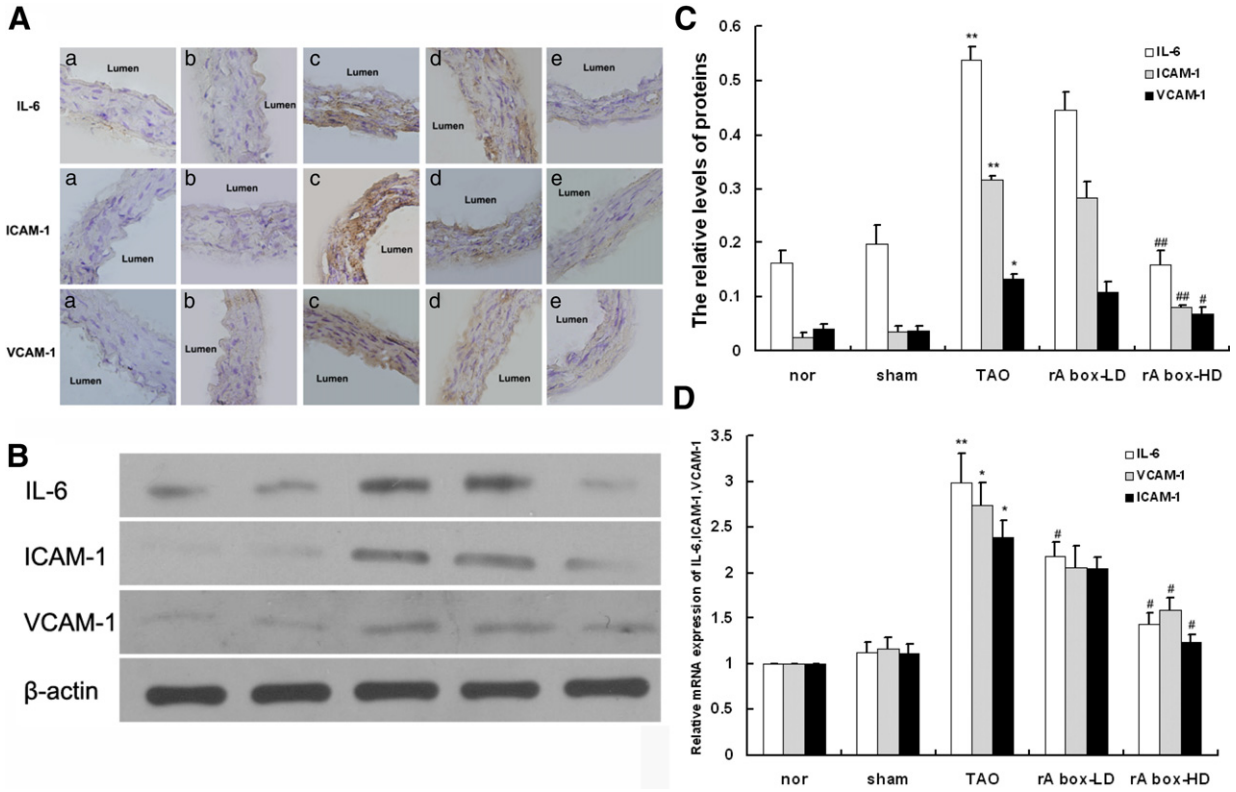


**Fig 4.** Immunohistochemistry, Western blot, and reverse transcription polymerase chain reaction analysis of protein and messenger RNA levels of interleukin-6 (*IL-6*), intercellular adhesion molecule-1 (*ICAM-1*), and vascular cell adhesion molecule-1 (*VCAM-1*) in femoral arteries from **(a)** normal (*nor*), **(b)** sham-operated (*sham*), **(c)** thromboangiitis obliterans (*TAO*) model, and **(d)** low-dose (*rA box-LD*) and **(e)** high-dose (*rA box-HD*) recombinant A (*rA*) box-treated rats. **A,** Representative photomicrographs of IL-6, ICAM-1, and VCAM-1 in rat femoral arteries by 3,3-diaminobenzidine staining. **B,** Representative Western blot of protein levels of IL-6, ICAM-1, and VCAM-1 (β-actin as a control). **C,** Summary data for rat groups presented as means ± standard error of the mean (*error bars*); n = 8. **P* < .05 vs sham-operated group. ***P* < .01 vs sham operated group. #*P* < .05 vs TAO model group. ##*P* < .01 vs TAO model group. **D**, The relative messenger RNA levels of IL-6, ICAM-1, and VCAM-1 for rat groups are presented as means ± standard error of the mean (*error bar*); n = 8. **P* < .05 vs sham operated group. ***P* < .01 vs sham operated group. #*P* < .05 vs TAO model group.

**Table III. A,** Effect of recombinant A (*rA*) box on blood cell counts in rats

| *Group*[a] | *Platelets* ($10^8$/mL) | *Red blood cells* ($10^9$/mL) | *Leukocytes* ($10^6$/mL) | *Neutrophils* ($10^6$/mL) | *Hemoglobin* (g/L) |
|---|---|---|---|---|---|
| Normal | 7.23 ± 0.46 | 6.32 ± 0.44 | 4.88 ± 0.23 | 1.06 ± 0.27 | 130.36 ± 5.89 |
| Sham operation | 7.46 ± 0.57 | 6.28 ± 0.32 | 5.12 ± 0.36 | 1.09 ± 0.15 | 128.72 ± 6.43 |
| TAO model | 10.54 ± 0.44[b] | 6.19 ± 0.76 | 5.09 ± 0.59 | 1.12 ± 0.18 | 130.08 ± 8.67 |
| rA box treatment | | | | | |
| Low dose | 9.11 ± 0.69 | 6.34 ± 0.55 | 5.01 ± 0.63 | 1.16 ± 0.19 | 131.37 ± 5.62 |
| High dose | 7.68 ± 0.54[c] | 6.53 ± 0.78 | 4.98 ± 0.24 | 1.11 ± 0.13 | 132.45 ± 6.24 |

*TAO,* Thromboangiitis obliterans.
[a]Values are presented as means ± standard error of the mean (n = 8).
[b]*P* < .05 vs sham-operated group.
[c]*P* < .05 vs model group.

tion, and tissue extravasation.[37] These effects are positive-feedback–amplified, probably due to HMGB1 and finally lead to endothelial cell damage. We found increased expression of IL-6, VCAM-1, and ICAM-1 in the femoral arteries of the TAO model, consistent with previous studies.[3,23,37] More importantly, rA box treatment downregulated the expression of proinflammatory cytokines to attenuate inflammatory responses and vascular injury induced by sodium laurate. Thus,

**Table III. B,** Effect of recombinant A (*rA*) box on blood coagulation in rats

| *Group*[a] | *Prothrombin time (seconds)* | *Activated PTT (seconds)* | *Thrombin time (seconds)* | *Fibrinogen level (g/L)* |
|---|---|---|---|---|
| Normal | 38.13 ± 1.72 | 39.01 ± 2.89 | 24.82 ± 1.79 | 3.02 ± 0.17 |
| Sham operation | 37.77 ± 1.31 | 38.76 ± 3.04 | 23.97 ± 2.03 | 3.16 ± 0.25 |
| TAO model | 27.43 ± 1.54[b] | 22.13 ± 3.12[b] | 12.67 ± 1.89[b] | 5.87 ± 0.48[b] |
| rA box treatment | | | | |
| Low dose | 30.07 ± 2.68 | 31.68 ± 3.58[c] | 18.72 ± 2.34 | 4.64 ± 0.31 |
| High dose | 36.89 ± 1.56[c] | 37.98 ± 4.65[c] | 24.23 ± 1.82[c] | 3.58 ± 0.39[c] |

*PTT,* Partial thromboplastin time*; TAO,* thromboangiitis obliterans.
[a]Values are presented as means ± standard error of the mean (n = 8).
[b]$P < .05$ vs sham operated group.
[c]$P < .05$ vs model group.

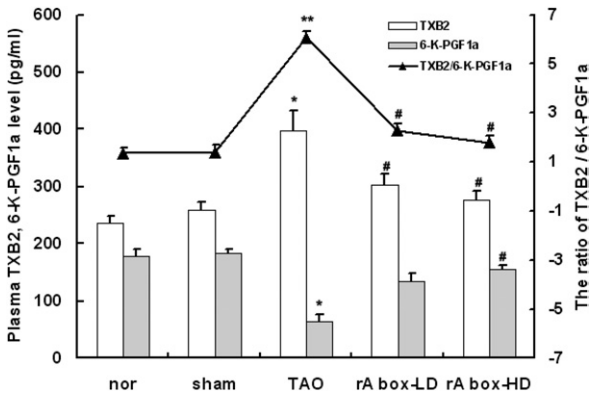


**Fig 5.** Plasma levels of thromboxane B2 (*TXB2*) and 6-keto-prostaglandin F1-α (*6-K-PGF1-α*) and the TXB2/6-K-PGF1α ratio are shown in normal (*nor*), sham-operated (*sham*), thromboangiitis obliterans (*TAO*) model, and low-dose (*rA box-LD*) and high-dose (*rA box-HD*) recombinant A (*rA*) box-treated group. Results are expressed in means ± standard error of the mean (*error bars*); n = 8. *$P < .05$ vs sham operated group. **$P < .01$ vs sham operated group. #$P < .05$, vs TAO model group.

rA box plays a protective role in reducing inflammatory mediators by inhibiting HMGB1. However, rA box did not completely ameliorate the inflammatory responses compared with controls, even with a high dose, which is probably because the network is complex and other mediators are also involved. This may explain why inflammatory-induced thrombosis was not completely attenuated with high-dose rA box.

The mechanism may be that macrophages and endothelial cells activated by sodium laurate can secrete HMGB1 initiatively, whereas HMGB1 stimulates macrophages and endothelial cells to secrete IL-6, VCAM-1, and ICAM-1, which, in turn, enhance HMGB1 expression by macrophages and endothelial cells, thus amplifying the inflammatory response. The function of HMGB1 was inhibited with rA box administration, which may explain the decreased production of inflammatory factors, and inhibition of HMGB1 positive feedback may reduce the expression level of HMGB1. However, we did not study the effect of RAGE antagonism on the progress of the disease and the role of other receptors of HMGB1, such as TLR, which need further investigation.

The TAO model rats presented with a hypercoagulable state of blood and inflammatory thrombus within the lumen of small vessels, as in a previous study.[38] Moreover, TXA2 is produced by activated platelets and stimulates activation and aggregation of new platelets. TXB2, its stable product, was used to reflect the level of TXA2. To further analyze the protective mechanism of rA box on the TAO rats, we explored the effect of rA box on TXB2 and 6-K-PGF1-α, the stable hydrolysis product of $PGI_2$, which inhibits platelet activation and dilate vessels. Normally, both are kept in homeostasis, but the state was broken in TAO. As in a previous study,[22] plasma TXB2 was obviously elevated in experimental TAO rats, but the plasma 6-K-PGF1-α level was reduced. Treatment with rA box reverses the increased TXB2 level and the decreased 6-K-PGF1-α level in a dose-dependent manner; the ratio of TXB2/6-K-PGF1-α was almost returned to normal. The blood platelet count and fibrinogen level were reduced, and prothrombin, thrombin, and activated partial thromboplastin times were close to normal compared with TAO rats; the change in the low-dose group was slight, which suggests that the effect of rA box on hematology and coagulation may be dose-dependent.

TAO is characterized by a highly cellular inflammatory thrombus, because of inflammation-induced thrombosis.[1,39] Here, we speculated that HMGB1 and its induced inflammatory factors further activate platelets and exacerbate the endothelial injury and endothelial cell dysfunction after the direct injury of sodium laurate, causing the loss of physiologic anticoagulant and vasodilatory properties of the normal endothelium. rA box may reverse this by inhibiting the inflammatory response. Thus, endothelial cells may restore anticoagulant functions to produce thrombomodulin, which activates protein C, inactivates thrombin and HMGB1, and accelerates antithrombin III.[40] The production of nitric oxide and $PGI_2$ may also be recovered. The downregulated activation of platelets may cause reduced expression of cyclooxygenase-2; then, TXA2, which is the major platelet-derived cyclooxygenase-2 product, decreased accordingly. Overall, rA box improved the hypercoagulable state of TAO rats by regulating endothelial cell dysfunction and inhibiting platelet aggregation.

## CONCLUSIONS

We have shown that HMGB1 and its receptor RAGE are involved in the sodium laurate-induced TAO model in rats and that rA box, as the antagonist of HMGB1, improves the pathologic condition by inhibiting the release and injury of inflammatory mediators and improving the hypercoagulable state of blood. The findings provide new experimental evidence for elucidating the pathogenesis of TAO and define a new potential therapeutic target for TAO. However, to explore the precise mechanism of HMGB1 and RAGE in the development of TAO needs further study at different times, with gene-knockdown animals and endothelial cells, as well as macrophages.

## AUTHOR CONTRIBUTIONS

Conception and design: XW, XJ
Analysis and interpretation: XK, JZ, HZ, MW, YL
Data collection: XK, JZ, HZ, MW, YL
Writing the article: XK
Critical revision of the article: XK, HY, XJ
Final approval of the article: HY, XW, XJ
Statistical analysis: XK, JZ, HZ, MW, YL
Obtained funding: HY
Overall responsibility: XW

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