Abstract. The protective effect of FPS-ZM1 via regulating the receptor for advanced glycation end products/nuclear factor-κB (RAGE/NF-κB) signal pathway on thromboangiitis obliterans (TAO) was investigated. A total of 40 Sprague-Dawley (SD) rats were randomly divided into 4 groups, the control group (n=10), the FPS-ZM1 group (n=10), the RAGE group (n=10) and the FPS-ZM1+RAGE group (n=10). One month later, ultrasonic examination was carried out to detect the inner diameter of vessel in dorsum of foot, peak flow velocity and blood flow volume. Analysis of biochemical indicators was performed using the rat blood samples, and we collected the samples of femoral artery of rats in each group. Real-time polymerase chain reaction (RT-PCR) and western blot assay were carried out to detect the mRNA and protein expression of RAGE, NF-κB, interleukin-6 (IL-6) and IL-10 in samples of femoral artery, while differences in the tissue structures of femoral artery in rats were detected via hematoxylin and eosin (H&E) staining. Ultrasonic examination showed that the inner diameter and the volume and velocity of the blood flow of rats in the RAGE group were significantly lower than those in the control group (P<0.05), while the ultrasonic parameters in dorsal artery of foot in the RAGE+FPS-ZM1 group were increased compared to those in the RAGE group (P<0.05); biochemistry examination revealed that in comparison with the control group, the prothrombin time and thrombin time in the RAGE group were obviously shortened (P<0.05) and the level of fibrinogen was significantly elevated (P<0.05), but these indicators in the RAGE+FPS-ZM1 group were significantly elevated compared to those in the RAGE group (P<0.05). Results of the RT-PCR and western blot assay also indicated that the mRNA and protein expressions of RAGE, NF-κB, IL-6 and IL-10 in the RAGE group were significantly higher than those in the control group (P<0.05). However, compared with the RAGE group, the mRNA expression of RAGE, NF-κB, IL-6 and IL-10 in the RAGE+FPS-ZM1 group was significantly decreased (P<0.05). H&E staining results showed a widespread thrombotic region in the aorta of rats (P<0.05), which was significantly reversed in the FPS-ZM1+RAGE group (P<0.05). Thus, FPS-ZM1 shows a significant protective effect on TAO, which is expected to provide new ideas and a theoretical basis for clinical treatment of TAO.

Introduction

Thromboangiitis obliterans, also known as TAO, is a disease mainly characterized by thrombosis and acute inflammatory responses, and frequently involves the median and small arteries, veins, as well as the nerves in arms and legs (1). Different from vasculitis in other forms, TAO is mainly manifested pathologically as severe cellular, inflammatory thrombosis and vascular occlusion with direct decrease in effect of blood on tissues, thereby causing pain and damage to tissues. In the early stage of disease, patients usually have intermittent claudication, while after the disease progresses into the advanced stage, symptoms such as skin ulcer or gangrene in fingers or toes emerge (2,3). Although its pathogenesis remains unclear, TAO has a strong correlation with smoking status, which is considered as a significant feature of TAO (4,5). Additionally, literature reports that in type I-III diabetes mellitus (DM) patients, a high incidence rate of necrotic lesion in peripheral vessels, not only prolongs the length of stay (LOS) in hospital of patients, but also gives rise to an increase in patients requiring a large-scale amputation surgery (6). Thus, discovering the potential molecular mechanism of TAO is of great significance.

The protective effect of FPS-ZM1 via regulating the receptor for advanced glycation end products/nuclear factor-κB (RAGE/NF-κB) signal pathway on thromboangiitis obliterans (TAO) was investigated.

Keywords: FPS-ZM1, receptor for advanced glycation end products, nuclear factor-κB, thromboangiitis obliterans
Materials and methods

**Major reagents.** FPS-ZM1 was purchased from Selleck (batch no. S8185; Shanghai, China); exogenous receptor for advanced glycation end products (RAGE) factor was purchased from MultiSciences (Lianke) Biotech Co., Ltd. (batch no. H6-FAB10521G; LiankeBio, Hangzhou, China); primary antibodies of RAGE, nuclear factor-xB (NF-xB), interleukin-6 (IL-6) and IL-8 were purchased from Abcam (Cambridge, UK) and anti-rabbit secondary antibodies of these factors were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA).

**Treatment of animals.** A total of 40 male Sprague-Dawley rats weighing 200±20 g were purchased from the Experimental Animal Center (Jiangsu, China). Under specific pathogen-free (SPF) environment at 25±2°C, the rats were fed with food and water ad libitum. The intraperitoneal injection of FPS-ZM1 and RAGE was performed in accordance with manufacturer's protocol (FPS-ZM1, 25 mg/kg/time, three times a week; RAGE, 10 mg/kg/time, three times a week). One month later, the rats were sacrificed by cervical dislocation, and a 5 ml blood sample was collected from the eyeballs and delivered to the Department of Clinical Laboratory of the Provincial Hospital for the measurement of biochemical indicators. The femoral artery of rats was dissected for sample preparation which was later divided into 3 sections: one for paraffin embedding, one for RNA extraction and one for extraction of total protein.

Ethics approval was received from the Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China).

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from SD rats, and the extracts were used for detection of the concentration of RNA in a spectrometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Therefore, cDNA was synthesized using 1 μg RNA with the reverse transcription kit (batch no. 639505; Takara, Otsu, Japan) via the One-step method. The ReverTra Ace qPCR RT kit (batch no. FSQ-101; Toyobo Co., Ltd., Osaka, Japan) was applied for the measurement of RNA expression of each indicator. The reaction conditions were set as follows: 95°C for 10 min, 95°C for 10 sec, 60°C for 20 sec and 70°C for 20 sec; these steps were repeated for 40 cycles. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the relative expression of each indicator was calculated as: \[ \Delta \Delta Cq = Cq \text{(target gene)} - Cq \text{(GAPDH)} \]. Primers were designed and produced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and the sequences are shown in Table I.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences (5'-3')</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>RAGE</td>
<td>F: ACCCTTAGCTGGCACTTTGATG R: AATCGGAGAGCCACTTATGCT</td>
<td>391</td>
</tr>
<tr>
<td>NF-xB</td>
<td>F: AACAGAGAGAGTTCTGTTCCG R: TTTGACCTGGAGGTAAAGACTTCT</td>
<td>321</td>
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<tr>
<td>IL-10</td>
<td>F: GACTTTAAGGGTTACCTGGGTTG R: TCACATGCGCCCTGTAAGTCTG</td>
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<tr>
<td>IL-6</td>
<td>F: AATCTGCTCTGCTCTTCTGGAG R: GTTGAGTTGCTTGGTTTAGTC</td>
<td>363</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: AGGTCGGTGTTAAGCGGATTG R: TGTAGACCATGTAGTTGAGTTCA</td>
<td>394</td>
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</table>

RAGE, receptor for advanced glycation end products; NF-xB, nuclear factor-xB; IL-10, interleukin-10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Western blot assay.** A total of 500 mg of sample from the femoral artery of SD rat was cut into sections, which was placed into the mixture consisting of 300 μl radio-immunoprecipitation assay reagent (RIPA; Beyotime, Guangzhouh, China) and 1% cocktail (protease inhibitor; Proteintech, Chicago, IL, USA) for homogenization followed by centrifugation at 4°C and 13,000 x g for 30 min. Supernatant was extracted to measure the concentration of protein using an automatic microplate reader (PerkinElmer, New York, NY, USA) and 40 μg protein was used for isolation via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gel were then transferred onto the nitrocellulose (NC) membrane, and the bands were incubated with the rabbit anti-RAGE, NF-xB p65, IL-6 and IL-10 polyclonal primary antibodies (dilution, 1:8,000; cat. nos. ab37647, ab66299, ab208113 and ab9969) and goat anti-rabbit IgG H&L secondary polyclonal antibody (dilution: 1:1,000; cat. no. ab205718) (all from Abcam, Cambridge, MA, USA). Then, the abundance of target proteins was detected using the highly sensitive luminescent reagent in enhanced chemiluminescent system (both from Millipore, Mahopac, NY, USA).

**Hematoxylin and eosin (H&E) staining.** The dissected femoral artery of SD rats was fixed in 50 ml 4% paraformaldehyde (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) overnight followed by dehydration and embedding. The paraffin-embedded samples were sliced into sections of 0.4 μm and heated in 65°C oven for 3 h. The sections received sequential treatment of dewaxing, rehydration of gradient ethanol, H&E staining, and rehydration of gradient ethanol again, and were then dried, sliced and sealed using neutral balsam. Under the microscope (DM-5000B; Leica, Wetzlar, Germany), we observed and photographed the histological morphology of femoral artery in SD rats, and each image was required to cover at least three regions for statistics of the area of thrombosis.

**Statistical analysis.** Experimental data were analyzed using the GraphPad Prism software (version 5.01; GraphPad Software, La Jolla, CA, USA). Measurement data are presented as mean ± SD. Indicator differences of enumeration data between two groups were tested using the Chi-square test. For the differences of indicators among groups, one-way analysis of variance was carried out. SNK test was used as post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Table I. Primer sequences of RT-PCR.**

- RAGE: F: ACCCTTAGCTGGCACTTTGATG R: AATCGGAGAGCCACTTATGCT
- NF-xB: F: AACAGAGAGAGTTCTGTTCCG R: TTTGACCTGGAGGTAAAGACTTCT
- IL-10: F: GACTTTAAGGGTTACCTGGGTTG R: TCACATGCGCCCTGTAAGTCTG
- IL-6: F: AATCTGCTCTGCTCTTCTGGAG R: GTTGAGTTGCTTGGTTTAGTC
- GAPDH: F: AGGTCGGTGTTAAGCGGATTG R: TGTAGACCATGTAGTTGAGTTCA

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<td>F: AGGTCGGTGTTAAGCGGATTG R: TGTAGACCATGTAGTTGAGTTCA</td>
<td>394</td>
</tr>
</tbody>
</table>
Results

Effects of FPS-ZM1 on ultrasonic parameters of dorsal artery in the foot of SD rat. Comparison between the FPS-ZM1 group and the control group showed no statistically significant difference (P>0.05). In the RAGE group, the inner diameter of vessel and velocity, and volume of blood flow were all significantly lower than those in the control group (P<0.05), while the ultrasonic parameters of dorsal artery in SD rats in the RAGE+FPS-ZM1 group were elevated compared to those in the RAGE group (P<0.05) (Table II).

Effects of FPS-ZM1 on coagulation function of SD rats. As shown in Table III, there was no statistically significant difference in comparison of the coagulation indicators between the FPS-ZM1 group and the control group (P>0.05). In comparison with the control group, the prothrombin time and thrombin time in the RAGE group were obviously shortened (P<0.05) and the level of fibrinogen was significantly elevated (P<0.05). By contrast, prothrombin and thrombin time in the RAGE+FPS-ZM1 group were significantly elevated compared to those in the RAGE group (P<0.05).

Pathological grades of SD rats in all the groups. As described in Table IV, there were 2 cases of rats in grade I, 2 in grade II, 4 in grade III, 1 in grade IV, and 1 in grade V in the RAGE group, while in the RAGE+FPS-ZM1 group, only 3 rats were found in grade I and the remaining rats were normal, suggesting the protective effect of FPS-ZM1 on vasculitis induced by RAGE.

Detection of mRNA expression of RAGE and NF-κB in femoral artery of SD rats by RT-PCR. As shown in Fig. 1, changes in the expression of RAGE, NF-κB, IL-6 and IL-10 in the FPS-ZM1 group were similar to those in the control group (P>0.05). In the RAGE group, the mRNA expressions of RAGE, NF-κB, IL-6 and IL-10 were significantly elevated in comparison with the control group (P<0.05). Compared with the RAGE group, the mRNA expressions of RAGE, NF-κB, IL-6 and IL-10 in the RAGE+FPS-ZM1 group were significantly decreased (P<0.05).

Detection of the protein expression of RAGE and NF-κB in femoral artery of SD rats by western blot assay. In agreement with changes in the results of RT-PCR, results of the western blot assay (Fig. 2) showed that alterations in the expression of indicators in the FPS-ZM1 group conformed to the control.
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Table IV. Pathological grades of SD rats in all groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>0</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>P-value</th>
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<tbody>
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<td>Control</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<td>FPS-ZM1</td>
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<td>10</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RAGE</td>
<td>10</td>
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<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
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<tr>
<td>RAGE+FPS-ZM1</td>
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<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0241b</td>
</tr>
</tbody>
</table>

*a*P<0.05 compared with the control group; *b*P<0.05 compared with the RAGE group. RAGE, receptor for advanced glycation end products; SD, Sprague-Dawley.

Figure 2. Detection of the protein expression of receptor for advanced glycation end products (RAGE), nuclear factor-κB (NF-κB), interleukin-6 (IL-6) and IL-10 via western blot assay. *P<0.05 and **P<0.01 compared with the control group; *P<0.05 compared with the RAGE group.

Figure 3. Observation of changes in histological morphology of rats in each group via H&E staining (x100). ***P<0.001 compared with the control group; *P<0.05 compared with the RAGE group.

group (P>0.05). In the RAGE group, the protein expression of RAGE, NF-κB, IL-6 and IL-10 was significantly elevated in comparison with the control group (P<0.05). Compared with the RAGE group, the protein expression of RAGE, NF-κB,
IL-6 and IL-10 in the RAGE+FPS-ZM1 group was significantly decreased (P<0.05).

**Examination of thrombosis in femoral artery of SD rats via H&E staining.** H&E staining was applied to detect the changes in the histological morphology of femoral artery of SD rats in each group, and the results (Fig. 3) showed that the variations in femoral artery in the FPS-ZM1 group were consistent with those in the control group revealing no thrombosis and pathological injuries. However, a large area of thrombosis was identified in the aorta of rats in the RAGE group (P<0.05), which was significantly reversed by the administration of RAGE combined with FPS-ZM1 (P<0.05).

**Discussion**

RAGE, the receptor for advanced glycation end products, plays a vital role in pathological damage to cells, such as inflammatory responses, neurological degeneration, excitatory toxicity, and oxidative stress (7,8). RAGE is also a cell surface marker in the immunoglobulin superfamily, and is frequently rich in lung tissues, but less found in other tissues of normal adults (9). A study has shown that RAGE at a low level is associated with the incidence of lung cancer, and interestingly, the activation and increase of RAGE in other tissues can affect the widespread pathological status (10). In addition, RAGE, as a diversified receptor, can be upregulated through long-term activation by inducing the signal pathway of cell damage, thereby leading to cellular dysfunction and tissue injuries, which has been confirmed in diabetes mellitus (DM), pro-inflammatory condition and neurological degeneration (11-13).

Literature has shown the molecular mechanism involved in this process, in which the combination of RAGE and its ligand can facilitate the oxidative stress and activation of the NF-κB signaling pathway, thus increasing the expression of inflammatory cytokines, such as IL-6 and IL-10 (10). In diseases involving inflammation in bronchial epithelial cells, the exogenous high mobility group box 1 protein (HMGB1) can activate the RAGE/NF-κB signaling pathway in normal human bronchial epithelial (NHBE) cells, inducing the chemotactic effect of leukocytes and acute effect of damage to vascular barrier (14). Bekircan-Kurt et al (15) believed that RAGE is vital to the incidence of complications in DM and DM-associated neurological lesions, wherein NF-κB signal pathway is significantly activated (15). Additionally, RAGE is also involved in many pathological processes, involving the progression in cancer, DM, arteriosclerosis, and Alzheimer's disease (16-19). A recent study also revealed that RAGE acts as the major pro-inflammatory receptor in vasculitis (20). The RAGE signaling pathway plays an important role in the pathogenesis of many inflammatory neurological lesions. Thus, RAGE may be a therapeutic target in inflammatory diseases.

FPS-ZM1, a specific inhibitor of RAGE, can block the combination of amyloid-β (Aβ) and V-region of RAGE. In cells expressing RAGE, FPS-ZM1 can decrease the cellular stress induced by Aβ40, Aβ42, human soluble protein-100B (S100B), AGE and HMGB1 (21). The in vivo study also showed that FPS-ZM1 can pass through the blood-brain barrier of mice without evident toxicity. FPS-ZM1 can suppress the activity of RAGE in brain and blood-brain barrier, which can realize the effective management of progression in brain diseases, related neurovascular diseases and cognitive dysfunction (22).

In this study, we found that exogenous RAGE protein can promote the incidence of TAO through regulation of the NF-κB signaling pathway with molecular manifestations of increases in mRNA and protein expression of IL-6 and IL-10. However, FPS-ZM1, a specific antagonist of RAGE, could significantly narrow the thrombosis area, alleviate the coagulation function and decrease the pathological grade in rats. In conclusion, we have found that RAGE is involved in the development and progression of TAO, while the administration of FPS-ZM1 can effectively ameliorate the symptoms of TAO.

**Acknowledgements**

Not applicable.

**Funding**

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

CL and XK contributed equally to writing the manuscript and RT-PCR. LW and KS performed and analyzed western blot assay. YS and YH helped with Hematoxylin and eosin staining. XJ and HY helped in the conception of the study and statistical analysis. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Ethics approval was received from Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China).

**Consent for publication**

Not applicable.

**Competing interests**

The authors have no competing interests to declare.

**References**


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