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Original Article

Antibacterial Effects of Vascular Grafts Treated with Rifampicin, Colistin, Vancomycin, or Daptomycin

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ABSTRACT

Introduction: Some reports suggest that rifampicin treatment of vascular grafts is useful when surgically treating vascular infections and mycotic aneurysms. However, the experimental evidence is limited, and the antibacterial effects of other antibiotic-treated vascular grafts are unknown.

Methods: Vascular grafts were immersed in several antibiotic solutions (rifampicin, colistin, vancomycin, or daptomycin). After *Pseudomonas aeruginosa* PAO1 or *Staphylococcus aureus* N315 were inoculated into the graft exterior, the number of colony-forming units (CFU) inside and outside the grafts was measured over time. Moreover, bonding of colistin and vancomycin to the grafts was quantified.

Results: For grafts immersed in rifampicin, the CFU values outside the grafts decreased over time and those inside the grafts did not increase when the grafts were exposed to a 10^6 CFU/mL concentration of PAO1 or N315. However, at 10^8 CFU/mL, the CFU values increased over time outside and inside the grafts. In PAO1 testing, grafts treated with colistin resisted pathogens in the tested area, and grafts treated with both colistin and rifampicin had even greater resistance. In N315 testing, vancomycin-treated grafts had some resistance to infection, but daptomycin-treated grafts did not. The bonding of colistin and vancomycin to the grafts depended on the immersion time.

Conclusions: Rifampicin-bonded grafts have a strong antibacterial effect when the concentrations of pathogens are low. Colistin-bonded grafts are effective against *P. aeruginosa* infection, and vancomycin-bonded grafts are effective against *S. aureus* infection.

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KEYWORDS: rifampicin-bonded vascular graft, colistin, vancomycin, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

Introduction

The incidence of infections after using prosthetic grafts in vascular surgery is about 1-6%. Such infections can be life-threatening, and interventions are sometimes unable to save the affected patients. Even when an infection is successfully treated, recurrence accompanied by severe complications develops in 50-90% of patients.[1, 2)] The patho-

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genic bacteria most often responsible for infections are Gram-positive cocci, such as *Staphylococcus aureus*, but Gram-negative bacteria such as *Pseudomonas aeruginosa* have also been reported. In some cases, the pathogen is unknown.[3] Antibacterial treatment and lavage and drainage of the infection source are the standard treatments for such infections. Removal of any infected vascular grafts, extra-anatomical reconstruction, and packing of the greater omentum or muscles and reconstruction using autogenous veins or allografts may also be necessary.[2–9]

If vascular grafts near the source of the initial infection are replaced, the risk of reinfection is high. Extra-anatomical reconstruction has been attempted to avoid this risk, but in situations in which extra-anatomical reconstruction is challenging (e.g., in the region of the thoracic aorta), the surgeon may have no option but to replace vascular grafts in the area of the previous infection. In such cases, grafts are usually first immersed in rifampicin. The graft surface is coated in gelatin so that the rifampicin ionically bonds with it.[10] Although some reports suggest that rifampicin treatment of vascular grafts is useful when surgically treating vascular infections and mycotic aneurysms, the experimental evidence is limited.

This study used a previously described *in vitro* vascular graft infection model[11] to evaluate infection resistance in vascular grafts treated with rifampicin. In addition, because wounds are often washed with normal saline during surgery, thus potentially limiting rifampicin binding to the graft, we evaluated the ability of rifampicin-treated grafts to resist infections after saline irrigation. Furthermore, we assessed infection resistance in vascular grafts immersed in colistin, vancomycin, and daptomycin. Finally, colistin and vancomycin bonding to vascular grafts was evaluated quantitatively.

Methods

Tested bacterial strains and preculture preparation

P. aeruginosa PAO1 was used as the representative Gram-negative pathogen, and *S. aureus* N315 was used as the representative Gram-positive pathogen. The N315 strain was provided by Dr. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan). The use of pathogens was approved by Toho University Safety Committee for Pathogen (No. 14-52-58). For routine culture, PAO1 or N315 was grown for one day at 35℃ in Luria-Bertani (LB) or Brain Heart Infusion (BHI) agar under oxic conditions. A single colony was then inoculated into 10 mL of LB or BHI broth. After aerobic growth at 160 rpm for one day at 35℃, cell numbers (colony counts) of the shaking cultures were determined. As precultures used for the *in vitro* vascular graft infection model in this study, shaking cultures were diluted with LB or BHI broth, and colony counts were adjusted to 10^6 or 10^8 colony-forming units (CFU)/mL.

Vascular grafts and immersion treatments

Straight, 8 mm diameter, gelatin-coated woven vascular grafts (Gelweave; Vascutek, a Terumo company, Tokyo, Japan) were used in this study. Before immersion, the grafts were cut into 6 cm segments, and then immersed in 30 mL of the antibiotic solutions for 5 min. Normal saline was used for the untreated control group. For washing after immersion, antibiotic-bonded vascular grafts were transferred into 30 mL of normal saline and kept for one day at 35℃. At that time, the normal saline was not changed.

Antibiotics used for immersion treatment of vascular grafts

Rifampicin (RIF) 0.6% dissolved in normal saline was prepared at the Department of Pharmacy, Toho University Omori Medical Center. Colistin sulfate (CST; Sigma-Aldrich, St. Louis, MO, USA) and vancomycin (VAN; Wako, Osaka, Japan) were purchased. Daptomycin (DAP) was provided by MSD Co. Inc. U.S.A. CST, VAN, and DAP were dissolved in normal saline, and concentrations were adjusted to 600 μg/mL.

In vitro vascular graft infection model

The antibacterial effects of the antibiotic-bonded vascular grafts were evaluated using a previously developed model.[11] A representative picture of the *in vitro* infection experiments using this model is shown in Fig. 1. Briefly, antibiotic-bonded vascular grafts were placed in six-well plates, and 3 mL of precultures of the tested strains was added to the outer area of the vascular grafts, whereas 2 mL of normal saline was added to the inner area. After incubation at 37℃ and 5% CO_2, specimens were collected from the inside or outside area and spread on LB or BHI agar, after which the colony count was determined.

Measurement of the binding activities of colistin and vancomycin to vascular grafts

Gelatin-coated vascular grafts were cut into 3 cm segments and then immersed in 30 mL of 100 μg/mL CST or 5 μg/mL VAN solutions. After immersion for 5, 10, or 30 min, the vascular grafts were recovered and the antibiotic concentrations in the solutions were measured using previously described methods: high-performance liquid chro-

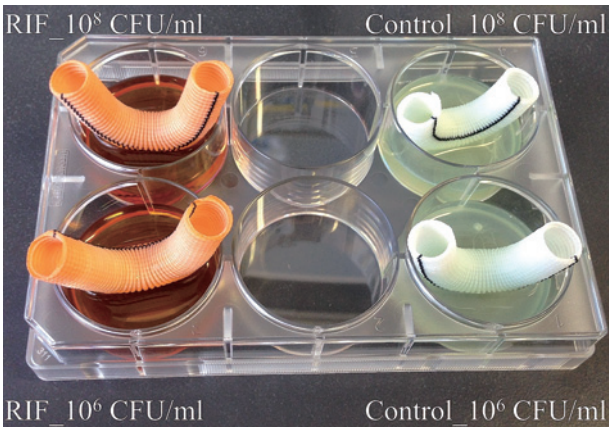

Fig. 1 *In vitro* vascular graft infection model for evaluating the antibacterial effects of antibiotic-bonded vascular grafts
A representative picture of infection experiments using this *in vitro* model is shown. Rifampicin- (RIF-) bonded vascular grafts were placed in six-well plates, and 3 mL of precultures of the tested strain (*P. aeruginosa* PAO1) was added to the outer area of the vascular grafts, whereas 2 mL of normal saline was added to the inner area. In the control group, vascular grafts were immersed in normal saline. The initial colony count was 10^6 or 10^8 colony-forming units (CFU) /mL. After incubation at 37℃ and 5% CO_2 for 24 h, this picture was taken and specimens were collected from the inside or outside area and spread on Luria-Bertani (LB) agar, after which the colony count was determined.

matography for CST[12] and the ARCHITECT Vancomycin (Abbott Japan, Tokyo, Japan) chemiluminescent immunoassay for VAN. To calculate the antibiotic binding values, the initial antibiotic concentration before immersion was measured. The value for antibiotic binding was calculated as C = V (B − A), where C is the antibiotic binding, V is the solution volume, B is the antibiotic concentration before immersion, and A is the antibiotic concentration after immersion.

Results

Study 1: Antibacterial effects of rifampicin-bonded vascular grafts without normal saline washing

For the *in vitro* vascular graft infection model, various grafts were first immersed in an antibacterial agent for 5 min, after which infection resistance was evaluated. When untreated control grafts were placed in initial pathogen concentrations of 10^6 CFU/mL of PAO1, bacterial growth was clearly visible outside and inside the graft (Fig. 2A). However, when grafts were first immersed in a 0.6% RIF solution and then placed in the same pathogen solution, the number of CFU on the outside of the grafts was reduced for 24 h (Fig. 2B). In addition, no bacteria were detected inside the grafts for 24 h. In contrast, when the initial pathogen concentration was 10^8 CFU/mL, the number of CFU did not increase on the exterior of grafts treated with 0.6% RIF but gradually increased in the interior, as was the case for the untreated grafts (Fig. 2C, 2D). When untreated grafts were exposed to an initial concentration of 10^6 CFU/mL of N315, the CFU values increased in the graft exterior and interior (Fig. 2E), as described for PAO1, above. When grafts were first immersed in 0.6% RIF, however, no pathogens were detected in the graft interior and exterior after 6 h (Fig. 2F). In contrast, when the initial N 315 concentration was 10^8 CFU/mL, the exterior of grafts immersed in 0.6% RIF had fewer CFU as compared with the exterior of untreated grafts; however, there was almost no difference after 6 h (Fig. 2G, 2H). A few CFU were visible in the graft interior.

Study 2: Antibacterial effects of rifampicin-, colistin-, vancomycin-, and daptomycin-bonded vascular grafts washed with normal saline

In all of the following cases, the treated vascular grafts were first immersed in the respective antibacterial solution for 5 min and then kept in a normal saline solution for 24 h, after which antibacterial effectiveness was evaluated. The initial pathogen concentration was 10^6 CFU/mL in all experiments. When untreated grafts were exposed to PAO1, bacterial growth was visible at the graft exterior and interior (Fig. 3A). Bacterial growth was not seen at the exterior of grafts immersed in 0.6% RIF, but some growth was visible at the graft interior (Fig. 3B). In contrast, for up to 12 h, CFU values were lower for the exterior of grafts immersed in 600 μg/mL CST (Fig. 3C). After 12 h, bacterial growth slowly resumed at the graft exterior, but no bacteria were seen at the interior after the initial exposure, even at 24 h. After 6 h, no bacteria were seen at the exterior and interior of vascular grafts that had been treated with both RIF and CST (Fig. 3D). When N315 was used, bacterial growth was seen at the exterior and interior of untreated grafts (Fig. 3E). For grafts treated with 0.6% RIF, no bacteria were detected at the graft exterior and interior at 6 h after the initial exposure (Fig. 3F), and treatment with 600 μg/mL VAN reduced exterior bacteria for up to 18 h after exposure (Fig. 3G), at which point bacteria were no longer detected. No bacteria were detected at the graft interior even at 24 h after the initial exposure. In contrast, treatment with 600 μg/mL DAP did

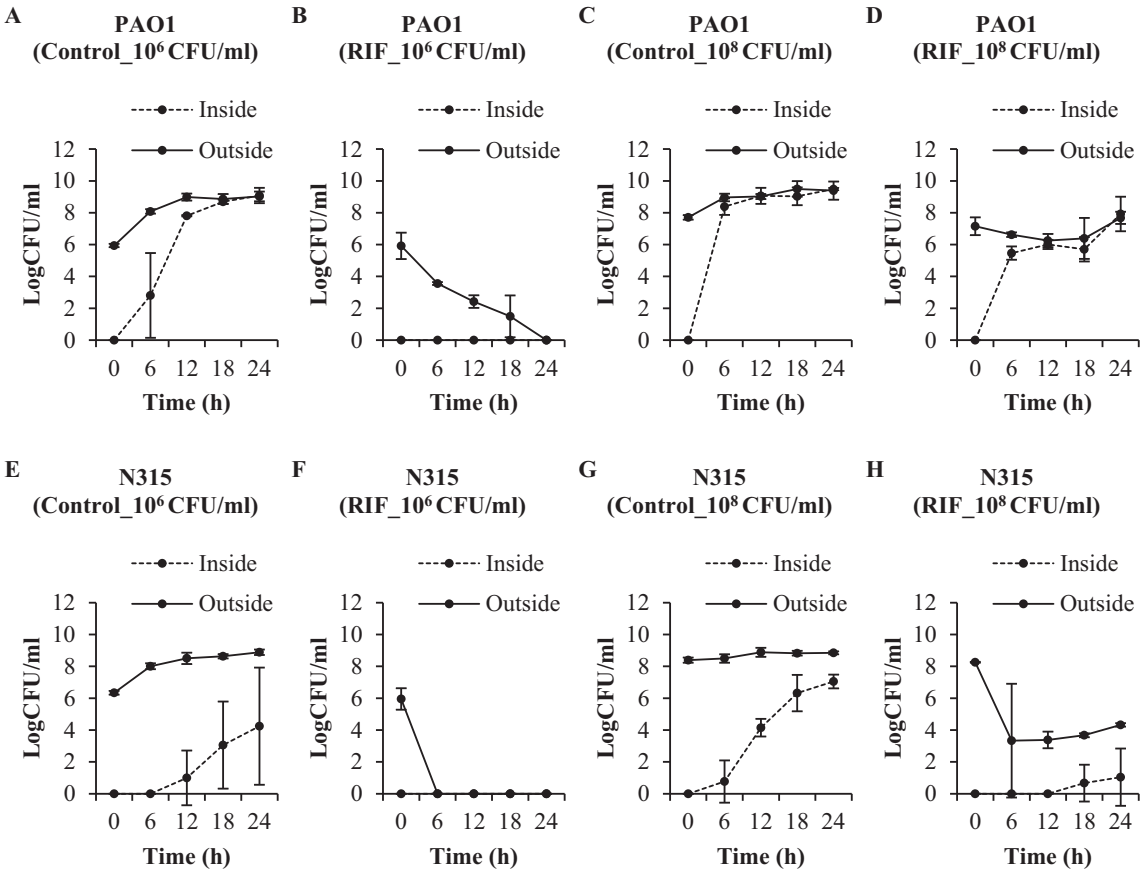

Fig. 2 Antibacterial effects of rifampicin- (RIF-) bonded vascular grafts, without normal saline washing
Gelatin-coated vascular grafts were immersed in a 0.6% RIF solution for 5 min and then placed in six-well plates. In the control group, vascular grafts were immersed in normal saline. The tested strains (*P. aeruginosa* PAO1 or *S. aureus* N315) were inoculated in the nutrient medium outside the vascular grafts, and normal saline was added to the inside of the grafts. The colony counts of PAO1 (A-D) and N315 (E-H) in the inside and outside areas were monitored for 24 h. The initial colony count was 10^6 or 10^8 colony-forming units (CFU) / mL. Values are represented as the mean ± standard deviation from three independent experiments.

not seem to stop or reverse bacterial growth at the graft exterior or interior (Fig. 3H).

Study 3: Binding of colistin and vancomycin to vascular grafts

Study 2 examined the infection resistance of grafts treated with CST or VAN. The results indicated that the antibacterial agents had bonded with the grafts. To evaluate bond strength, the grafts were first immersed in CST or VAN for 5, 10, or 30 min and then removed, after which the various concentrations of antibacterial agents were measured in the solvents. The amount of binding was calculated using the formula C = V (B − A). As shown in Fig. 4, bond strength, C, increased over time. However, after 10 min, the increase in C slowed greatly. Thus, binding reached a plateau at around the 10 min mark.

Discussion

The primary aim of this study was to evaluate infection resistance in vascular grafts treated with various antibacterial agents. For grafts immersed in RIF, the CFU values outside the grafts decreased over time and those inside the grafts did not increase when the grafts were exposed to a 10^6 CFU/mL concentration of PAO1 or N315. However, at 10^8 CFU/mL, the CFU values increased over time outside and inside the grafts. In PAO1 testing, grafts treated with CST resisted pathogens in the tested area, and grafts treated with both CST and RIF had even greater resistance. In N315 testing, VAN-treated grafts had some resistance to infections, but DAP-treated grafts did not. The bonding of CST and VAN to the grafts depended on the immersion time.

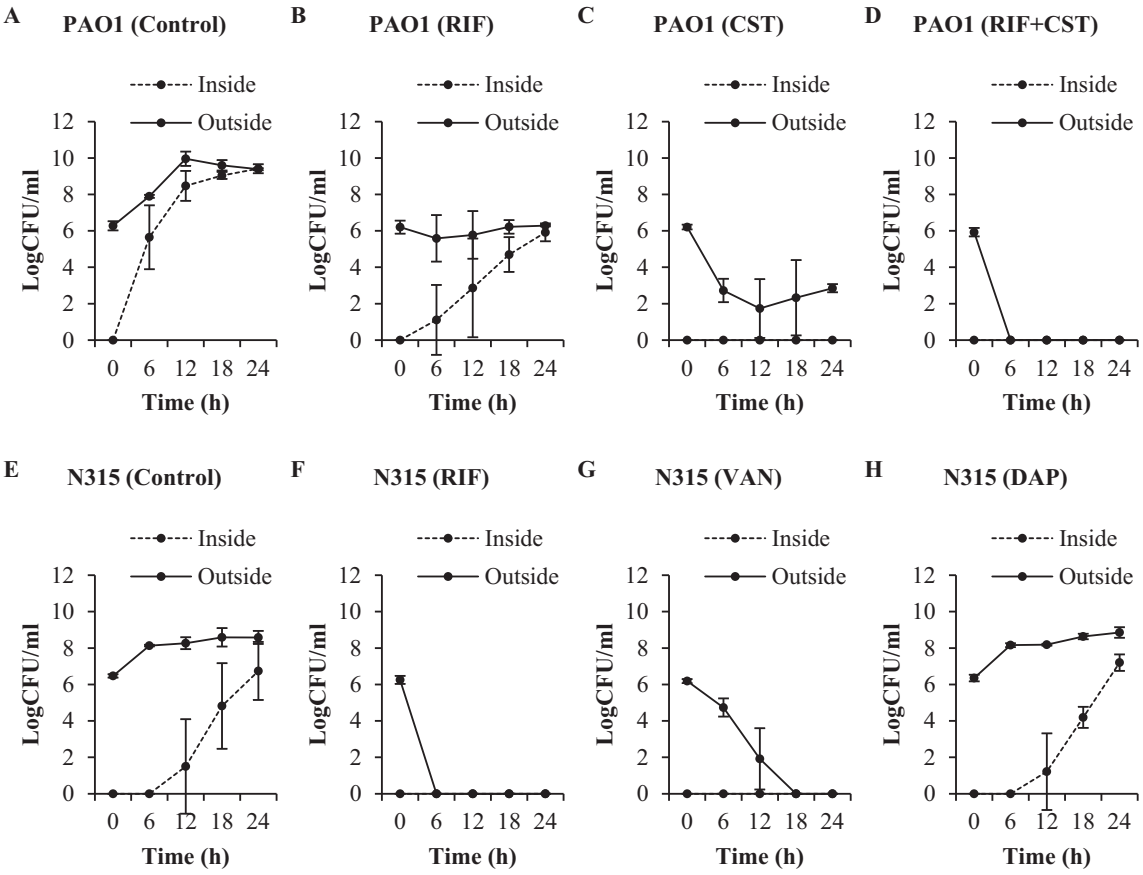

Fig. 3 Antibacterial effects of rifampicin- (RIF-), colistin- (CST-), vancomycin- (VAN-), and daptomycin- (DAP-) bonded vascular grafts, with normal saline washing

Gelatin-coated vascular grafts were immersed in one of several antibiotic solutions for 5 min and then placed in six-well plates after washing with normal saline. A 0.6% RIF solution, 600 μg/mL CST, and their combination were used to treat samples for *P. aeruginosa* PAO1 infection, whereas 0.6% RIF, 600 μg/mL VAN, and 600 μg/mL DAP were used to treat samples for *S. aureus* N315 infection. In the control group, vascular grafts were immersed in normal saline. The tested strains (*P. aeruginosa* PAO1 or *S. aureus* N315) were inoculated in the nutrient medium outside the vascular grafts, and normal saline was added to the inside of the grafts. The colony counts of PAO1 (A-D) and N315 (E-H) in the inside and outside areas were monitored for 24 h. The initial colony count was 10^6 colony-forming units (CFU) /mL. Values are represented as the mean ± standard deviation from three independent experiments.

Few experimental studies have evaluated infection resistance in RIF-treated vascular grafts. Although a few studies used animal models to investigate infection resistance,[13,14] *in vivo* studies of antibacterial ability are challenging. Bisdas et al. used vascular grafts treated with antibacterial agents to evaluate their antibacterial effects in pathogen-containing solutions.[15] However, because their method involved a simple measurement of the number of bacteria that had attached to grafts, it was not suitable for evaluating effectiveness over time, as measurement required the collection of bacteria attached to the grafts. In contrast, the present *in vitro* model of vascular graft infection was easy to perform experimentally and permitted us to measure the number of bacteria on the graft exterior and interior over time. This procedure could be used to investigate the effectiveness of other antibacterial agents against other pathogens.

In the present *in vitro* model, for washing, the vascular grafts were immersed in normal saline at 35℃ for 24 h. At that time, the normal saline was not changed. In clinical practice, brief washing with a large amount of normal saline is performed. As it was expected that the concentration of antibacterial drugs would vary with short-term cleaning, an experimental system of normal saline soaking for 24 h was constructed.

An *in vitro* vascular graft infection model is also applica-

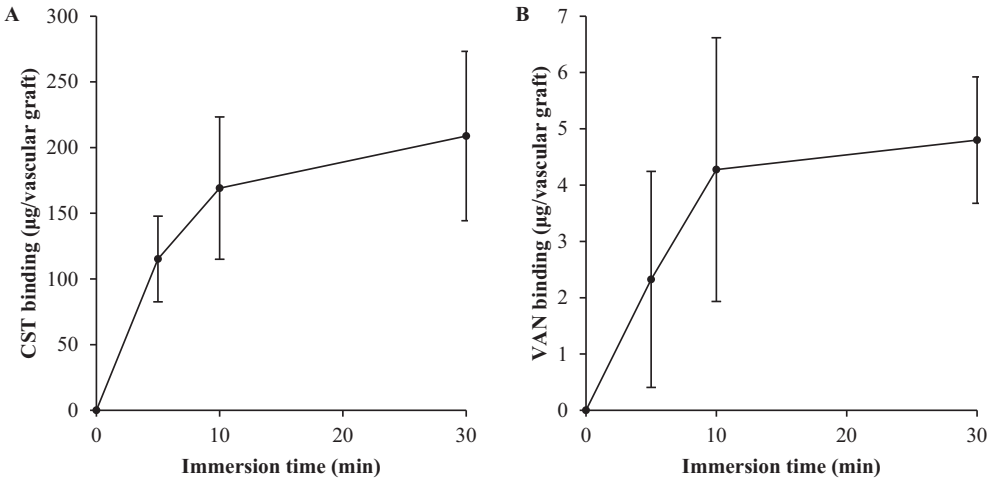

Fig. 4 Binding of colistin (CST) and vancomycin (VAN) to vascular grafts
Gelatin-coated vascular grafts were immersed in 30 mL of 100 μg/mL CST or 5 μg/mL VAN solutions for 5, 10, or 30 min. The antibiotic concentrations before and after immersion were measured using high-performance liquid chromatography for CST (A) or a chemiluminescent immunoassay for VAN (B). The antibiotic binding values were calculated as C = V (B − A), where C is the antibiotic binding, V is the solution volume, B is the antibiotic concentration before immersion, and A is the antibiotic concentration after immersion. Values are represented as the mean ± standard deviation ($n = 4$).

ble to evaluate infections inside vascular grafts (bacteremia). This study focused on the saline washing from the outer area of the vascular grafts. Bacteremia should be controlled with intravenous antibiotics. It is also better to use an animal model for the examination. Further studies will be required to identify effective strategies for bacterial infections.

When treating vascular infections and mycotic aneurysms, lavage and debridement are first performed. RIF-treated vascular grafts are then used to replace the infected vessels, and the site is thoroughly irrigated with saline before closing the wound. Although this final saline irrigation is useful in removing foreign matter and infectious bacteria that may have entered the site during the operation, it is unclear how much RIF binds to the graft after irrigation. The difference in the results shown in Fig. 2B, 3 B strongly suggests that saline irrigation reduces the amount of RIF that binds to the grafts and that the clinical antibacterial effect would therefore also be diminished. Fig. 2 shows that a high concentration of RIF has a strong antibacterial activity against pathogen concentrations of 10^6 CFU/mL. Scattering the remaining RIF solution used for graft immersion likely removes pathogens remaining at the infection site after irrigation.

Resistance to *P. aeruginosa* was strongest after combination treatment with CST and RIF. CST was approved for antibacterial use in 2015 and was shown to be effective against Gram-negative pathogens.[16] The combination with RIF appears to increase the effectiveness.[16] The results for this combination, shown in Fig. 3A-D, are consistent with those from previous studies. An immersion time of 10 min was sufficient to achieve near-maximal antibacterial effectiveness (Fig. 4A). RIF does not dissolve easily in water, and surfactants and filters are necessary to create a surgically useful RIF solution, which is time-consuming. In contrast, the preparation of CST solutions is fast and easy and, in emergency procedures, may yield results similar to those for RIF.

The results of testing with *S. aureus* indicate that VAN treatment of grafts was effective and that VAN bonded with grafts (Fig. 3E, 3G, 4B). As was the case for CST, 10 min of immersion was sufficient to achieve near-maximal effectiveness. Thus, VAN might be better than RIF for emergency graft treatment when *S. aureus* is known to be the pathogen. DAP showed little effectiveness in suppressing pathogenic activity (Fig. 3E, 3H), which contradicts the results of a previous study.[15] In that study, grafts were immersed in a DAP solution for 15 min and were not later washed with saline. The present grafts were immersed in DAP for only 5 min and then soaked in saline for one day. An immersion period of 5 min may have been insufficient for DAP to bind with the graft. In addition, saline soaking

may have removed most of the DAP from the graft surface. Future studies should examine the clinical effectiveness of DAP under varied experimental conditions.

In conclusion, our study demonstrated that RIF-bonded grafts have a strong antibacterial effect when the concentrations of pathogens are low and that CST-bonded grafts are effective against *P. aeruginosa* infection and VAN-bonded grafts are effective against *S. aureus* infection. The pathogen responsible is not always identifiable in cases of vascular infections and mycotic aneurysms. In such emergency cases, vascular grafts should be treated with a combination of antibacterial agents known to be effective against both Gram-negative and Gram-positive pathogens (e.g., CST and VAN). In this paper, gelatin-coated vascular grafts, which are widely used for RIF immersion, were used with other antibacterial drugs, assuming an emergency. Therefore, no other vascular grafts were used. Antibiotic binding to vascular grafts depends on the properties of the vascular grafts and the antibiotics. Future studies should attempt to identify other antibacterial agents that are effective in treating vascular grafts.

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Author contribution: S.O. and M.H. contributed equally to this article.

Conflicts of interest: None declared.

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