Construction of multifunctional porcine acellular dermal matrix hydrogel blended with vancomycin for hemorrhage control, antibacterial action, and tissue repair in infected trauma wounds

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ABSTRACT

Prevention of bacterial infection and reduction of hemorrhage, the primary challenges posed by trauma before hospitalization, are essential steps in prolonging the patient's life until they have been transported to a trauma center. Extracellular matrix (ECM) hydrogel is a promising biocompatible material for accelerating wound closure. However, due to the lack of antibacterial properties, this hydrogel is difficult to be applied to acute contaminated wounds. This study formulates an injectable dermal extracellular matrix hydrogel (porcine acellular dermal matrix (ADM)) as a scaffold for skin defect repair. The hydrogel combines vancomycin, an antimicrobial agent for inducing hemostasis, expediting antimicrobial activity, and promoting tissue repair. The hydrogel possesses a porous structure beneficial for the adsorption of vancomycin. The antimicrobial agent can be timely released from the hydrogel within an hour, which is less than the time taken by bacteria to infest an injury, with a cumulative release rate of approximately 80%, and thus enables a relatively fast bactericidal effect. The cytotoxicity investigation demonstrates the biocompatibility of the ADM hydrogel. Dynamic coagulation experiments reveal accelerated blood coagulation by the hydrogel. In vivo antibacterial and hemostatic experiments on a rat model indicate the healing of infected tissue and effective control of hemorrhaging by the hydrogel. Therefore, the vancomycin-loaded ADM hydrogel will be a viable biomaterial for controlling hemorrhage and preventing bacterial infections in trauma patients.

1. Introduction

Uncontrolled bleeding is the leading cause of death among trauma patients, with approximately 33%–56% of the deaths occurring during the phase before hospitalization [1]. Without appropriate intervention, trauma-related bleeding can induce a series of systemic reactions and acute coagulopathy in the wound, which may prove fatal to the patient [2,3]. Timely and effective emergency hemostasis even before hospitalization can provide valuable time for the subsequent treatment, thereby reducing the chances of disability or mortality. Currently, HemCon and QuikClot are widely used as hemostatic materials in fields such as defense where trauma injuries are common [4–6]. However, these materials do not have sufficient durability to prevent uncontrolled bleeding from blood vessels. In addition, they often bring inevitable side-effects such as high rebleeding rates (HemCon) [4] and tissue thermal damage (Quikclot) [5], which severely limit their further application. Moreover, wounds exposed to a polluted environment are prone to bacterial infection [7–9], which can hinder the healing process and cause life-threatening complications including sepsis, severe sepsis, and septic shock [10,11]. Nevertheless, bacterial infection receives relatively less

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focus in the treatment stage prior to hospitalization than hemorrhage [11]. Thus, treatment before hospitalization faces the dual challenges of controlling bleeding and preventing microbial infection. Therefore, developing a novel biomaterial that can effectively control hemorrhage and rapidly prevent microbial growth in the emergency stage prior to hospitalization is important for trauma care.

Research on the development of antimicrobial and hemostatic materials is gaining prominence. For example, studies have demonstrated the use of silver (Ag) ions as a broad-spectrum antimicrobial agent [12–14] that can inhibit the growth of fungi, gram-positive bacteria, gram-negative bacteria, and viruses, and these have since become common in biomedical applications. Ong et al. [15] refined the chitosan dressing by combining a coagulant (polyphosphate) and Ag nanoparticles (NPs) to achieve hemostatic and antimicrobial effects. However, Ag NPs are cytotoxic [16–18] i.e. they cause cell membrane damage [19], DNA damage [20], oxidative stress [21], and the formation of reactive oxygen species (ROS) [22]. Staphylococcus aureus (S. aureus) is the most common type of bacterium that causes skin infection [23,24]. As the first generation of glycopeptide antibiotic, vancomycin has been demonstrated to possess remarkable bactericidal properties, especially against S. aureus. It interferes with the synthesis of the cell wall of the bacteria by disturbing the peptidoglycan molecules [25], which are key components in the cell wall structure, and then inhibit the generation of phospholipids and polysaccharides. Especially, the use of these antibiotics, alone or combined with other antibiotics, does not induce cross-resistance [9]. Therefore, vancomycin has become the preferred antimicrobial agent for the treatment of contaminated wounds. Recently, Hsu et al. [7] developed a biodegradable composite film loaded with thrombin and vancomycin and observed instant hemostasis and effective antibacterial effects. However, few surgical dressings are compatible with acute irregularity wounds [9].

Extracellular matrix (ECM) hydrogel is a promising regenerative material that can effectively promote the repair of tissue defects. As previously reported [51], the entire ECM contains proteoglycans, growth factors, and matricellular proteins, including collagen, elastin, laminin, fibronectin, hyaluronic, heparan, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and thrombospondin. Among them, a major component of ECM is collagen. ECM hydrogel formation is a collagen-based self-assembly process that is regulated in part by the presence of glycosaminoglycans, proteoglycans, and ECM proteins [52]. Furthermore, Kim, B young Sook et al. [55] formulated decellularized porcine skin as a printable bio-ink, and engineered a perfusable and vascularized 3D human skin equivalent composed of epidermis, dermis, and hypodermis with this bio-ink. The four compartments (epidermis, dermis, hypodermis, and blood vessels) were successfully matured under polycaprolactone (PCL)-based printed platform, indicating that extracellular matrix of skin can promote skin regeneration and wound healing. As the ECM hydrogels derived form porcine skin possesses good plasticity, injectability, biocompatibility and renewability, it can be applied to irregular skin tissue injuries and accelerate tissue healing [26–29]. Moreover, ECM is closely related to thrombosis [30]; under certain physiological conditions, the exposure of subcutaneous ECM and the subsequent platelet accumulation influence the control of hemorrhage [31–33]. Thus, ECM proteins (including fibrin, fibronectin, collagen, etc.) can effectively control bleeding. However, few studies have been applied to hemostasis. Recently, Hu et al. designed a nanocomposite hydrogel based on an acellular cardiac extracellular matrix (ECM) for vascular embolization, a wide range of miniature and standard clinical catheters are available. They can swiftly inject the hydrogel into the wound and cause hemostasis, which has been published in Adv. Mater. [34]. Therefore, ECM hydrogels is a relative new point to be applied in he-

2. Materials and methods

2.1. Materials

Vancomycin was purchased from Aladdin (Shanghai, China), Triton X100 and CH3(CH2)11SO4Na from Sinopharm Chemical Reagent Co., Ltd. (China), Pig glycosaminoglycan (GAG) ELISA Kit from Jianglai Biotech-
nology Co., Ltd. (Shanghai China), Hyp assay kit from Beijing solarbio science & technology Co., Ltd. (China), Agar from Sangon Biotech Co., Ltd. (Shanghai, China), TRYPOTONE from OXOID (Shanghai, China), and soya peptone from Sinopharm Chemical Reagent Co., Ltd. (China). DNA kit from CW Biotech (Beijing, China), Live/dead cell staining dye was purchased from BioVision (USA), and Cell Counting Kit-8 (CCK8) from APEXBio Technology LLC (USA).

2.2. Preparation of porcine acellular dermal matrix hydrogel

Fresh porcine skin was obtained from a local abattoir. After cleaning the cadaver, removing the subdermal fat tissue and hair, the tissues were rinsed three times with sterile water and subjected to freeze–thaw (−80 to 37°C) cycles for three consecutive times. At 120 rpm and constant temperature (25°C), the tissues were treated with 1% Triton X-100 solution (Sinopharm Chemical Reagent Co., Ltd., China) for 12 h and 1% CH3(CH2)11SO4Na (Sinopharm Chemical Reagent Co., Ltd., China) for 6 h. The resulting acellular dermal matrix (ADM) tissues were washed by phosphate-buffered saline (PBS) for several times and freeze-dried
overnight. Then those samples were milled to a powder (i.e. ADM) and digested by high-quality pepsin powder in a dilute HCl solution (pH 2–3) containing different concentrations of vancomycin for 6 h. The osmotic pressure was adjusted by dropping 40X PBS buffer, and the pH was adjusted to 7.2–7.4 by gradual dropwise addition of 10-M sodium hydroxide (NaOH) cooled beforehand. These steps were performed aseptically. Five samples of the skin were separately prepared in the following compositions: (1) Porcine ADM hydrogel; (2) Porcine ADM hydrogel containing 1 mg/mL vancomycin (represented as VADM1); (3) VADM2; (4) VADM4; and, (5) VADM6. The pH-adjusted formative gel can be stored at 4°C for up to 1 week.

2.3. Characterization of porcine acellular dermal matrix materials

We used hematoxylin and eosin (H&E) and 4',6-diamidino-2-phenylindole (DAPI) stains and a DNA assay to evaluate the cellular and nuclear removal efficacies. DNA was extracted with a Universal Genomic DNA kit and its concentration was determined from the absorbance at 260 nm on a microplate spectrophotometer. The GAG content of native and decellularized tissue was quantified using the Pig glycosaminoglycan (GAG) ELISA Kit and collagen content was measured with a hydroxyproline (HYP) assay kit.

The nanofibrous structures of the ADM and VADM hydrogels could be examined by scanning electron microscopy (SEM). After solidification, Hydrogels from each group were fixed in 2.5% (w/v) glutaraldehyde for an hour. Then, the samples were washed with PBS solution thrice (10 min per wash cycle). Subsequently, they were again washed thrice with PBS for 5 min each, and then dehydrated by sequential treatment with 50%, 70%, 80%, 90%, and 100% CH3CH2OH (10 min for each gradient). After the treatment, the samples were dehydrated at a critical point dryer with liquid CO2. Finally, they were sputter-coated with Au and investigated according to a previous study with slight modifications [37].

2.4. Diffusion of antibiotic (zone of inhibition)

The zone of inhibition (ZOI) is a circular area around the region of an antibiotic in which the bacteria colonies do not grow. The inhibitory effects of the five hydrogels were measured using the Oxford cup method. 

2.4.1. S. aureus and Enterococcus (control group) were cultured in Luria-Bertani (LB) medium containing tryptophan (15 g/L) and peptone from soya (5 g/L) at 37 °C overnight. Inhibition zone assay was performed using the agar diffusion method (the Oxford cup method). Briefly, 15 g/L of LB agar was used as the solid medium, sterilized, and heated until it melted. The melt was then poured into 90-mm-diameter Petri dishes, 15 mL per dish (lower layer), and allowed to solidify. Oxford cups with an inner diameter of 6 mm each were placed in the center of the agar plates. The molten LB agar medium was then cooled to approximately 50 °C and mixed with the test bacteria. Subsequently, 5 mL of the molten LB agar contain with bacteria was added to the solidified medium (lower layer) and allowed to coagulate and form the upper layer. After the coagulation process, the Oxford cup was removed, a well could be left in the solid medium, and then the hydrogel sample (ADM, VADM1, VADM2, VADM4, VADM6) was added to the well. After 16–18 h of incubation, the zone of inhibition (ZOI) was estimated by subtracting the diameter of the hydrogel cylinder from the diameter of the inhibition zone.

2.5. Antibacterial activity test

Considering that the experimental group was loaded with gradient concentrations of vancomycin as an intervention measure, we chose untreated acellular pig skin gel as the control group. The experimental design was based on previous reports [37]. To compare the bactericidal effect of the materials, we first studied the relationship between the bacterial dilution ratio and the optical density (OD) value (600 nm). After centrifugation, the samples at 5000 rpm for 5 min, we removed the supernatant and suspended S. aureus in normal saline (NS). The bacterial suspension (100 μL) was transferred to a 96 well UV-visible-transparent plate, and the absorbance was measured at an OD of 600 nm using a UV spectrophotometer. The OD value was measured after diluting the bacterial suspension with NS, and the standard curve of OD vs. dilution ratio was plotted.

The antibacterial activity of the hydrogels against S. aureus was investigated according to a previous study with slight modifications [37]. First, the ADM and VADM hydrogels (0.3 mL each) were placed in 2-mL Eppendorf tubes. 0.5 mL of the bacterial suspension was added, the OD value was measured, and then the mixture was incubated at 37°C for 2 h. After incubation for 12 h, the bacterial suspension was centrifuged at 5000 rpm for 5 min. After removing the supernatant, the bacteria were suspended in NS. The bacterial suspension (100 μL) was transferred to a 96 well UV-visible transparent plate, and the absorbance was measured at an OD of 600 nm using a UV spectrophotometer. The residual bacterial suspension was serially diluted, placed on agar plates, and measured after being incubated for 24 h. The antibacterial ratio of the planktonic bacteria (Rap) was calculated according to the equation: Rap (%) = [(A – B)/A] × 100%, where A and B represent the number of viable bacteria in the control group (ADM hydrogel) and experimental group (VADM hydrogel), respectively.

2.6. In vitro release kinetics of vancomycin from hydrogels

First, the absorbance of the solution at different vancomycin concentrations was measured at an OD of 281 nm, and the standard curve between the vancomycin concentration and absorbance was plotted. 100 μL of VADM1, VADM2, VADM4, and VADM6 were prepared in an Eppendorf tube (2 mL). 1 mL of phosphate buffer (pH 6) was added after solidifying the hydrogels at 37°C. Then, hydrogels with the added buffer were steadily stirred at 37°C. The buffer solution was collected and centrifuged at 3000 rpm for 5 min to homogenize it. 100 μL of the solution was transferred to a 96 well UV-visible transparent plate, and the absorbance was measured in duplicate at an OD of 281 nm using a UV spectrophotometer. The absorbance was measured for 120 min in 10-min intervals. The concentration of the released vancomycin was determined, and the vancomycin release rates of VADM1, VADM2, VADM4, and VADM6 were calculated according to the standard curve of vancomycin.

2.7. Hemolytic activity

Liu et al. was referred for information on the hemolytic activities of the antibiotic-loaded hydrogels [46]. The gels were prepared in 2-mL Eppendorf tubes. Red blood cells (RBCs) obtained from a healthy donor were isolated by centrifugation at 1000 rpm for 10 min, and diluted in NS (volume ratio, 4/5). Then, a 0.2-mL RBC suspension was mixed with 5 mL of the NS (negative group), 5 mL of distilled water (positive group), and hydrogels. The mixtures were incubated for 60 min at 37°C, and then centrifuged at 3000 rpm for 5 min. The supernatant was carefully removed and transferred to a 96 well plate. The OD was determined by spectroscopy at an OD of 540 nm. The hemolysis ratio was calculated by the hemolysis ratio % = ([(experiment group (OD) – negative group (OD))] / (positive group (OD) – negative group (OD)))) × 100%; where OD = 540 nm.

2.8. Cell viability determined by live/dead assay

Hydrogels were prepared in a six well plate with a volume of 500 μL and sterilized before use. The mouse embryonic cells (NIH3T3 cells) were seeded in the ADM and VADM hydrogel-coated wells at a density of 4 × 10^4 cells per well. Dulbecco’s Modified Eagle Medium (DMEM, Hyclone) with 10% (v/v) FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin were added. The cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. At time periods determined beforehand, the cells were stained using a live/dead cell stain kit and
observed under a fluorescence microscope.

2.9. Cytotoxicity of hydrogel against NIH3T3 cell line

Cytotoxicity was determined using the Cell Counting Kit-8 (CCK8) (APEXBIO Technology LLC, USA). The ADM and VADM hydrogels were immersed in DMEM (volume of hydrogel/volume of medium = 1:5) at 37 °C for 24 h. Collected the supernatant extract as the 100% leaching solution, then mixed with a certain proportion of DMEM medium to obtain 0%, 25%, 50%, 75%, 100% leaching solution for further experiments. The NIH3T3 cells were seeded in a 96 well plate at a density of 3000 cells per well. After incubation at 37 °C for 24 h, the supernatant medium was removed from each well and 100 μL graded concentrations of leaching solution were added (0%, 25%, 50%, 75%, 100%, n = 5 for each concentration per day) for 1, 3, and 5 days. The NIH3T3 cells cultured with DMEM (0% leaching solution) was the control group. Subsequently, 10 μL of the CCK8 reagent was added to each well and quantified the cell metabolism after incubating for 1 h at 37 °C. The absorbance at an OD of 450 nm was measured using a spectrophotometer.

2.10. Dynamic coagulation test

The ability of the hydrogels to clot whole blood was tested according to a method reported in the literature with slight modifications [47]. First, 300 μL of the hydrogel was added to a 24 well plate and incubated for 10 min at 37 °C to allow gel solidification. Ten microliters of a recalcified whole-blood solution (0.2 M calcium chloride [CaCl2], 1 μL in blood) was added to the hydrogels in the 24 well plate. The well was incubated at 37 °C for 30, 60, 90, 120, 150s and 180s. The gelatin sponge and gauze were used as control groups for comparison with the hydrogel. After the period set beforehand, 2 mL of de-ionized water was gradually added to release unbound blood without disturbing the clot. The absorbance of the supernatant was measured at an OD of 450 nm using a spectrophotometer. All experiments were repeated three times. The absorbance of 10 μL of recalcified whole blood in 2 mL of de-ionized water was used as the negative control. The blood-clotting index (BCI) was calculated according to the formula: BCl% = ([A – C] / [B – C]) × 100%, where A is the absorbance of the hydrogel, B the absorbance of the negative control, and C the absorbance of de-ionized water.

2.11. Rat liver and kidney laceration

Healthy male Sprague-Dawley (SD) rats purchased from the experimental animal center of Zhejiang Academy of Medical Sciences were fed in a controlled environment with constant temperature (22–25 °C) and humidity (50–60%) for 1 week with food and water available. Six SD rats (300–400 g, 8 weeks) were used as test subjects in a series of experiments. Those rats were anesthetized with 10% chloral hydrate for 2 min before surgery (intraperitoneal injection, chloral hydrate 10%/rat body weight = 0.5 mL/100 g). The abdominal cavity of the rat was gently cut open with surgical scissors to fully expose the abdominal aorta. After decellularization, a histological analysis was performed to assess the completeness of decellularization and the integrity of the extracellular matrix. According to the H&E stain results, cell nuclei were

Four SD rats weighing 300–400 g were chosen for the aortic hemostasis test. Those rats were anesthetized with 10% chloral hydrate for 2 min before surgery (intraperitoneal injection, chloral hydrate 10%/rat body weight = 0.5 mL/100 g). The abdominal cavity of the rat was gently cut open with surgical scissors to fully expose the abdominal aorta. Then the abdominal aorta was peeled from the surrounding tissues and clamped using hemostatic forceps, and subsequently clipped using surgical scissors. The hydrogels (0.2 mL) were applied to the hemorrhaging site. After 5 min of gelation, the forceps were removed, and the site was visually inspected to determine if the bleeding had stopped. The control group did not use the hydrogel, and the bleeding continued even after 5 min. The abdominal aorta treated with VADM hydrogel were collected for hematoxylin and eosin (H&E) analysis to further explore the mechanism of hemostasis.

2.12. Rat abdominal aorta hemostasis tests

The SD rat model was established based on a previously reported method [46]. Based on the application of vancomycin hydrogel with different concentrations, the healing rate of infected incisions in SD rats demonstrated different results. Ten Rats (weight ~ 300g) were randomly into two groups: one group was treated for 1 week, and other for 2 weeks. Those rats were anesthetized by intraperitoneal injection of chloral hydrate (10%, 0.5 mL per 100 g of body weight). Once the rats were fully anesthetized, their dorsal hair was shaved to form an area of approximately 8 × 8 cm2. After disinfecting it with 75% alcohol, a round full-thickness skin incision with a diameter of 1 cm was made on the dorsal skin. A S. aureus suspension (1 × 107 CFU/mL) was administered to the round skin wounds and then dried for 2 h. Subsequently, the ADM or VADM hydrogel was applied to the infected wounds, whereas the untreated wounds were used as negative controls. The area of the wound was measured at time periods set beforehand after treatment. Wound closure was calculated by the following equation: wound closure rate (%) = (W0 – Wn/W0) × 100%, where W0 and Wn represent the wound area at day 0 and the sampling time, respectively. The H&E stain was used to evaluate the wound-healing process. To distinguish infiltrated inflammatory cells from the infected wound, formalin-fixed wound was used for the CD68 stain and myeloperoxidase (MPO) stain.

2.14. Statistical analysis

Data are presented as the mean ± standard deviation. Data were analyzed by one way analysis of variance with graphpad prism7 (Graphpad Software Inc., USA). The sample size (n) for each statistical analysis has been reported in the corresponding ‘experimental section’ and ‘figure legends’ and significance was defined as p < 0.05.

2.15. Ethics approval

Animal experiments were carried out in the experimental animal center of Zhejiang Academy of Medical Sciences. All animal studies were conducted in accordance with the National Institutes of Health Guidelines and guidelines for the care and use of experimental animals of Zhejiang Academy of Medical Sciences.

3. Results and discussion

3.1. Hydrogel synthesis and characterization

After decellularization, a histological analysis was performed to assess the completeness of decellularization and the integrity of the extracellular matrix. According to the H&E stain results, cell nuclei were
absent, and the ECM remained intact after decellularization (Fig. 1a–b). The DAPI staining revealed no staining nuclei in the acellular tissues (Fig. 1c–d). These results prove that the entire extracellular matrix structure of the porcine dermal tissue is preserved after decellularization. DNA quantification also revealed a significant decrease in DNA content compared to that of native tissue: porcine dermal tissue (753.5 ± 143.6 ng/mg dry weight vs. 11.83 ± 3.74, p < 0.05, Fig. 1k). Collagen content quantification (Fig. 1k) revealed no significant change in the porcine dermal tissue (389.4 ± 32.39 ng/mg dry weight vs. 373 ± 26.79, p > 0.05, Fig. 1k). Pig glycosaminoglycan (GAG) quantification revealed a significant decrease in GAG content compared to that of native tissue: porcine dermal tissue (0.26 ± 0.01 μg/mg dry weight vs. 0.22 ± 0.01, p < 0.05, Fig. 1k). Liquid N2 and ADM, VADM1, VADM2, VADM4, and VADM6 were added to the decellularized porcine dermal tissue. The tissue was ground into powder using TissueLyser (Jingxin, Shanghai) and the hydrogels were made with different concentration vancomycin (Fig. 1e–g). As shown in Fig. 1h, the resulting gel solution was incubated at 37°C for 5 min and then transformed from the solution (liquid) state into a homogeneous, translucent gel (solid) state. The potential mechanism of gel forming process may be as described by Kadler Karl E [57]. The GAGs cause the collagen to gel faster and form larger fibers, while the proteoglycans cause the collagen to gel faster. Scanning electron microscopy (SEM) revealed nanofibrous networks in the hydrogel scaffolds (Fig. 1i), which is beneficial for the adsorption of antibiotics. The compositions of different elements present in the hydrogels were studied using the energy-dispersive spectrometry (EDS)
The ADM hydrogels dehydrated by an ethanol (CH₂OH) gradient no longer contained Cl element, whereas the VADM hydrogels did, which contain 0.11 ± 0.07%, albeit in small quantities (Fig. 1). The Cl element content comes from vancomycin hydrochloride and can strongly bond with the extracellular matrix hydrogel, making rinsing or dehydration ineffective in debonding it.

### 3.2. In vitro antibacterial activity

As *Staphylococcus aureus* is responsible for most skin infections, we tested the antibacterial performance of the vancomycin-loaded hydrogel against an *S. aureus* suspension. This suspension was diluted multiple times, and the optical density (OD) values at 600 nm were measured. The results demonstrate a power function relationship between the OD value of *S. aureus* and the dilution ratio (Fig. 2a), which was demonstrated by Lambert Beer’s law [58]. This, in turn, indicates that the antibacterial efficacy of hydrogels can be evaluated by comparing the OD values of *S. aureus* for the five hydrogel concentrations. The hydrogels (porcine acellular dermal matrix hydrogel [ADM], porcine ADM hydrogel with vancomycin group 1 [VADM1], VADM2, VADM4, and VADM6) were prepared in 2-mL Eppendorf tubes and then co-cultured with a 1-mL *S. aureus* suspension at 37°C for 12 h to measure the OD value. The results revealed a significant increase in the OD value of the ADM group, but the opposite trend in those of the VADM1, VADM2, VADM4, and VADM6 groups. A notable fact is that the downward trend of the OD value between the VADM2, VADM4, and VADM6 groups demonstrated no significant changes, which may be attributed to the following two reasons: one is that the concentration of bacteria in the solution exceeds the applicable range of Lambert Beer’s law, and the other is that the concentration of bacteria in the solution is indeed not of significant difference, because when vancomycin released from the material exceeds the minimum inhibitory concentration (MIC, 20 μg/mL [59]), all bacteria would be killed, indicating they exhibit similar antimicrobial effects (Fig. 2b).

To further evaluate the bactericidal behavior of the VADM hydrogels, we measured the antiplanktonic bacteria rate by a plate colony-formation assay. Numerous colonies of bacteria belonging to the ADM group had grown on the agar plate (Fig. 2cA1), whereas those belonging to VADM1, VADM2, VADM4, and VADM6 decreased significantly (Fig. 2cA2-5). These results indicate that the VADM groups can kill *S. aureus*. The antiplanktonic bacteria ratio (Rap) of VADM1, VADM2, VADM4, and VADM6 were 86.48 ± 0.38%, 95.86 ± 0.68%, 97.34 ± 0.79%, 98.09 ± 0.73%, respectively. The antiplanktonic bacteria rate of the VADM hydrogel groups increased steadily with an increase in the vancomycin concentration (Fig. 2d). However, when the concentration of vancomycin exceed 2 mg/mL, the Rap was not of statistical significance (p > 0.05). Therefore, high vancomycin antibacterial loads are not required to stop the spread of infection in traumatic injuries. This in turn may further reduce the cytotoxicity caused by high vancomycin concentrations [48,49].

### 3.3. Zone of inhibition experiment

The antibacterial activity effect was evaluated on both Gram-negative (*Enterococcus*) and Gram-positive (*S. aureus*) bacterium. As for infected wounds, the bacteria usually colonize the wound surface and easily invade deep tissues, causing a series of pathophysiological changes [50]. A zone of inhibition (ZOI) experiment was performed to determine the bactericidal effect of vancomycin ejected by the VADM hydrogels in deep tissues. The ADM hydrogel did not form any ZOI against *S. aureus* because it did not contain vancomycin (Fig. 3a). However, VADM1, VADM2, VADM4, and VADM6 formed significant ZOI against the *Enterococcus* and *S. aureus* lawns grown on the agar plate, thereby indicating the diffusion of the agent into the surroundings that deactivated bacteria in those areas (Fig. 3a). The VADM6 group possessed the largest ZOI because it had the highest content of vancomycin. The VADM1 group formed the smallest ZOI with the least amount of vancomycin (Fig. 3b). These results indicate a clear relationship between ZOI and vancomycin concentration, which is consistent with the results of previous studies [37]. However, the antimicrobial activity between *Enterococcus* and *S. aureus* were no significant difference (p > 0.05).

### 3.4. In vitro drug release study

Pre-hospital trauma wounds are vulnerable to a variety of bacterial infections, ranging from superficial skin infections to more severe invasive diseases including endocarditis, osteomyelitis, and septicemia. Therefore, this study used an extracellular matrix hydrogel to absorb...
significant amounts of vancomycin and enable rapid bactericide through explosive release, which is crucial in care before hospitalization. The amount of vancomycin released was determined by measuring the absorbance using a UV spectrophotometer. Four VADM hydrogels (100 μL) were prepared in 2-mL Eppendorf tubes. VADM1, VADM2, VADM4, and VADM6 contained 100, 200, 400, and 600 μg of vancomycin, respectively. Related studies have shown that the acidic environment of infected wounds originates from an insufficient supply of nutrients and oxygen, and increased glycolysis [35]. Meanwhile, in our previous study, the muscle tissue in infected rats was homogenized, and the pH value of the supernatant as measured by blood gas analysis was 6.1 ± 0.6, which confirmed the acidic microenvironment under the infected state [40]. Therefore, in this study, we investigated the release kinetics of vancomycin in a phosphate-buffered saline (PBS) solution at pH = 6. Fig. 3c indicates a linear relationship between the vancomycin concentration and the absorbance at 281 nm. Hence, the concentration of vancomycin can be estimated based on its OD value. As shown in Fig. 3d, at an hour, the amount of vancomycin released from VADM1, VADM2, VADM4, and VADM6 were 81.85 ± 9.45, 179.89 ± 17.64, 348.15 ± 17.58, 569.63 ± 36.35 μg, respectively and the release rate of vancomycin from VADM1, VADM2, VADM4, and VADM6 were 81.85 ± 9.45%, 89.44 ± 8.82%, 87.04 ± 4.40%, 94.94 ± 6.06%, respectively (Fig. 3e). The vancomycin release was detectable in the laboratory for up to 9 h. These results proved that the hydrogel can expeditiously eject vancomycin in acute contaminated wounds to create a relatively sterile environment, which is of great significance in the prevention of infection in emergency treatment before hospitalization.

3.5. Biocompatibility

Vancomycin has demonstrated a strong bactericidal effect on gram-positive bacteria, mainly by inhibiting the synthesis of bacterial cell walls. However, in high concentrations, vancomycin is cytotoxic [48,49]. Therefore, the compatibility of the ADM and VADM hydrogels in vitro with human RBCs (hRBCs) was investigated. The hRBCs were directly exposed to the surfaces of ADM and VADM hydrogels, and the hemolysis rate was calculated to evaluate the blood compatibility of the hydrogels. The vancomycin-loaded hydrogels (VADM1, VADM2, VADM4, and VADM6) exhibited less than 3% hemolysis, which is negligible, similar to the ADM (Fig. 4a). Thus, the hydrogels are compatible with human erythrocytes.

Subsequently, the cytocompatibility of the VADM hydrogels by live/dead staining was evaluated. The hydrogels were then co-cultured with NIH3T3 cells. After one week, the results of the live/dead staining revealed living cells on the ADM and VADM hydrogels (Fig. 4b) without hydrogel-induced cytotoxicity. Further, the cell toxicity of the hydrogels was investigated by CCK-8 assay to measure the cell metabolic activity. Fig. 4c–d reveal no significant decrease in cell metabolic activity was observed between the control group (NIH3T3 cells cultured with DMEM) and other group (NIH3T3 cells cultured with 25%, 50%, 75% and 100% extraction DMEM of ADM and VADM1) on days 1, 3, or 5 (all p > 0.05). However, Fig. 4e–g shown in VADM2, VADM4, and VADM6, the cell metabolic activity was decreased obviously between the control group (NIH3T3 cells cultured with DMEM) and other group (NIH3T3 cells cultured with 100% extraction DMEM) on day 5 (p < 0.0001). The
NIH3T3 cell growth became more restrained with the increase in vancomycin concentration, the incubation time stretched, and the inhibition effect became more obvious (Fig. 4h). Nevertheless, the metabolic activity of NIH3T3 cells in the VADM1 group distinctly increased. The vancomycin-loaded ADM hydrogels had a low hemolysis rate, which was not affected by vancomycin content. This demonstrates the blood compatibility of the VADM hydrogels. In terms of cytocompatibility, NIH3T3 cells survived on the VADM hydrogels by the live/dead staining assay, even in the VADM6 group (Fig. 4b). However, the CCK8 test indicated that the cytotoxicity gradually became severe with increasing vancomycin concentration. Therefore, VADM1 not only kills bacteria swiftly and effectively, but it is also more biocompatible than VADM2, VADM4, and VADM6.

3.6. Hemostasis experiments

A dynamic whole-blood clotting time test was performed to evaluate the coagulability of the VADM hydrogels. The higher the blood-clotting index of the hemoglobin solution, the slower the coagulation rate of the VADM hydrogels. The hemostatic gauze and gelatin sponge were used as the control groups. As shown in Fig. 5a, the blood-clotting index of the gelatin sponge, gauze, ADM, VADM1, VADM2, VADM4 and VADM6 were 17.68 ± 1.35%, 10.03 ± 1.65%, 8.97 ± 0.79%, 8.44 ± 0.46%, 6.07 ± 2.29%, 4.48 ± 1.83% and 6.86 ± 2.78% at 180s, respectively. This indicates the VADM hydrogels have good coagulability. Fig. 5a showed the coagulation ability of VADM1 hydrogel was significantly better than gelatin sponge (p < 0.05). After 90s, the...
Fig. 5. *In vitro* and *in vivo* hemostasis test. (a) *In vitro* dynamic whole-blood clotting evaluation (A1, A2 and A3) of the ADM, VADM1, VADM2, VADM4 and VADM6 hydrogels, with gelatin sponge and gauze used as the control. (b) Photographs captured during the liver laceration model and renal tissue-defect model. (B1) A wound with a length of 1 cm in the liver. (B2) VADM1 hydrogel formed *in situ* on the trauma injury. (B3) A small portion of the kidney tissue was removed with surgical scissors. (B4) VADM1 hydrogel formed *in situ* on the defective tissue. (c) Photographs captured during a hemostasis test on the abdominal aorta (highlighted by red circles and ellipses). (c1) Clipped abdominal aorta. (c2,c3) Arterial spurts can be observed after release of the artery clip. (c4) VADM1 hydrogel applied to the wound. (c5) Abdominal aorta surrounded by VADM1 hydrogel. (c6) Artery clip was removed after 5 min, and the bleeding was stopped completely. The abdominal aorta was clogged with hydrogel. (c7–c9) Arterial spurts still occur on the control group after 5 min, when the artery clip was released. (d) H&E stained micrographs, showing significant accumulation of red cells (yellow arrow) within the incision site. Scale bar = 100 μm. (e) Blood loss from liver and kidney incisions. Three replicates of each sample were tested and the data were shown as mean ± SD.
The coagulation ability of VADM1 hydrogel was no significant difference compared with gauze (p > 0.05). In Fig. 5aA3, with time prolonging, the coagulation ability of VADM1 and VADM6 was significantly enhanced. We speculated that vancomycin may enlarge the spatial structure of the extra-cellular matrix hydrogel. The net-like structure on the surface of VADM is looser than that of ADM, allowing more blood to penetrate into the structure and contact more collagen fiber, increasing the contact area of the blood and the hydrogel, thus leading to coagulation, which was similar to the report of Maas Coen’s [56].

To further investigate the actual in vitro hemostatic capacity of the hydrogel on mammalian tissues, we used rat livers and kidneys to simulate hemorrhages. These results demonstrate that the VADM1 hydrogel is not only an effective bactericide, but also biocompatible. Therefore, we selected VADM1 for the subsequent animal experiments. The liver was lacerated using a surgical scalpel to induce incompressible bleeding (Fig. 5bB1). Then, we patched VADM1 to the incision, which stopped blood flow, achieving complete hemostasis (Fig. 5bB2). In liver incisions without treatment, 0.83 ± 0.25g of blood flowed onto the filter papers. The amount of blood loss in incisions with gauze and VADM1 was 0.71 ± 0.18g and 0.13 ± 0.03g, respectively. In renal tissue-defect model, we dissected part of the tissue with surgical scissors and allowed blood to continue to flow out of the incision (Fig. 5bB3). On filling injecting the tissue with VADM1, the hemorrhage stopped and no fresh blood flow could be detected (Fig. 5bB4). In renal tissue-defect without treatment, 0.82 ± 0.24g of blood flowed onto the filter papers. The amount of blood loss in incisions with gauze and VADM1 was 0.71 ± 0.18g and 0.13 ± 0.03g, respectively. In renal tissue-defect model, we dissected part of the tissue with surgical scissors and allowed blood to continue to flow out of the incision (Fig. 5bB3). On filling injecting the tissue with VADM1, the hemorrhage stopped and no fresh blood flow could be detected (Fig. 5bB4).
increase with the temperature rise from 10°C. In our work, the hemostasis process of VADM hydrogel was evaluated post-trauma (Fig. 5d). It was then clipped using surgical scissors, revealing large regions of hemorrhage after the clip was released (Fig. 5c2-c3). Then clipped the ruptured artery, 2 mL of VADM1 was injected into the bleeding site (Fig. 5c4), and the ruptured artery was surrounded by the VADM1 hydrogel (Fig. 5c5). The artery clamp was removed after 5 min, and the hemorrhage stopped (Fig. 5c6). Interestingly, when the VADM1 hydrogels were removed, the broken end of the abdominal aorta continued to pulsate but did not bleed. This is because the hydrogel seals the ruptured blood vessels. Fig. 5c7-c9 shows the mass of blood spouting from the ruptured vessel without the intervention of the hydrogel. These results demonstrate the potential of the hydrogel in stopping the hemorrhaging of ruptured vessels, even in regions like the abdominal aorta with the strongest blood-flow velocity. It covers not only injury following the trauma but also occult vascular injury during surgery.

To explore the hemostatic mechanism of the hydrogels, the incision of liver, the tissue-defect of kidney and the broken end of the abdominal aorta treated with VADM1 were washed and stained using the HE method. The staining results showed significant cell accumulation in the trauma (Fig. 5d). In our work, the hemostasis process of VADM hydrogel contained two steps. First, when the hydrogel encounters the wound, it changes from liquid to solid to form a physical barrier to prevent further blood flow. Its potential mechanism may be as described by Nyambar Bartzaya [51]. Both the storage modulus (G') and the loss modulus (G'') increase with the temperature rise from 10°C to 37°C in hydrogels, promoting the transformation from liquid phase to solid phase. Subsequently, extra-cellular matrix components (include collagen, fibronectin, laminin and other ECM proteins) are important mediators for thrombosis [30] to promote blood coagulation together. So ECM hydrogel is an effective hematostatic material.

3.7. Staphylococcus aureus-infected skin wound healing by antibacterial hydrogel

Extracellular matrix hydrogels are rich in collagen, glycosaminoglycan, bone morphogenetic protein, growth factors, etc., which promote the repair of skin tissue defects [29]. Therefore, after being loaded with vancomycin, VADM hydrogel can not only promote skin tissue healing, but also play an anti-infection effect. A skin-wound model with S. aureus infection was established on the backs of the rat subjects. The wound was then sealed with each VADM hydrogel separately. The untreated incision and the ADM hydrogel were used as the control groups. Fig. 6a illustrates the healing process. On day 7, the edges of all the groups started to contract. The wounds treated with VADM1, VADM2, VADM4, and VADM6 became significantly smaller than those in the control group. On day 14, all the wounds in the six groups were closed, and VADM1, VADM2, VADM4, and VADM6 had the best healing performance compared with the control groups.

Wound closure rates based on the wound area were quantitatively calculated, as shown in Fig. 6c. On day 7, compared with the wound closure rates in the untreated and ADM-treated groups (51.37 ± 5.04% and 58.45 ± 8.30%, respectively), the wound closure rates in the VADM1, VADM2, VADM4, and VADM6 groups significantly increased to 87.29 ± 1.84%, 87.65 ± 3.45%, 86.97 ± 6.90%, and 87.56 ± 4.27%, respectively (p < 0.05). On day 14, the untreated and ADM-treated wounds displayed closure rates of 72.25 ± 1.92% and 85.13 ± 3.21%, respectively, VADM1, VADM2, VADM4, and VADM6 had higher closure rates of 97.96 ± 0.63%, 96.76 ± 1.06%, 96.6 ± 0.67%, and 96.34 ± 0.63%, respectively. The healing tissue was stained with H&E to further investigate the histopathological structure during wound healing between these groups. On day 7, numerous inflammatory cells appeared in the untreated wound and ADM groups, more than those in the VADM1, VADM2, VADM4, and VADM6 groups (Fig. 6b). Immunohistochemical staining for CD68+ macrophages and myeloperoxidase (MPO)-labeled neutrophils reveals the infiltration of neutrophils and macrophages into the trauma (Fig. 6d). On day 14, the untreated and ADM groups contained many inflammatory cells and a few myofibroblasts, whereas the vancomycin-loaded ADM groups had few inflammatory cells and lots of myofibroblasts. The hair follicle-like histopathological structures can be seen in the VADM1 and VADM2 groups.

4. Conclusion

We designed a porcine ADM hydrogel loaded with vancomycin as an antimicrobial agent. The hydrogel's physical adsorption property allows for the local rapid release of vancomycin. The cumulative release rate is approximately 80% within 1 h. This demonstrates a strong antibacterial effect in the early stage of trauma healing. In vivo hemostasis experiments on rats demonstrated the hydrogel's ability to effectively control hemorrhage and prevent blood vessel damage. Thus, vancomycin-loaded porcine ADM hydrogel has been demonstrated as a potential biomaterial for hemorrhage control and bacterial infection prevention in trauma and occult vascular injuries.

Credit author statement

Bin Fang, Lei Lu and Dan Zu guided all the experiments. Dan Cai, Sunfang Chen and Bing Wu prepared acellular porcine skin hydrogel and completed the characterization analysis. Jianjian Chen was responsible for bacteriological examination, Danhua Tao and Zhiqing Li completed the pathological examination of the tissues, and the animal experiments were completed by Qidong and Yubin Zou. Yating Chen and ChENCHEN Bi performed all the cytology related experiments.

Declaration of competing interest

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

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