



Chitosan-Based Haemostatic Systems (Axiostat): An Integrated Approach to Post-operative Wound Management in Vascular Surgery

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Abstract

The effective management of perioperative and post-traumatic bleeding is critical to preventing severe complications, shock, and fatal outcomes. In this context, chitosan emerges as a premier polymeric biomaterial due to its exceptional biochemical properties and inherent hemostatic nature. Among commercially available solutions, the Axiostat sponge stands out as a promising device for promoting hemostasis at surgical and endovascular access sites. This study aimed to conduct a comprehensive chemical-physical characterization of the Axiostat device. The investigation sought to quantify its antioxidant capacity, antibacterial activity, and swelling ratio upon contact with biological fluids—all critical parameters for clot stability and infection prevention. The evaluation was conducted through a multi-scale approach: *in vitro* Cytotoxicity tests on human adult epidermal keratinocytes (HaCaT) cell lines confirmed the material's biocompatibility. Results demonstrated significant antibacterial activity, with particularly marked efficacy against the pathogen *Klebsiella pneumoniae*. *In vivo* application on patients undergoing endovascular procedures validated the device's effectiveness in the secure closure of arterial access sites. The analysis demonstrates that the Axiostat sponge does not merely act as a mechanical barrier but as a multifunctional medical device. The combination of excellent hemostatic properties, biocompatibility, and antibacterial capacity renders it a high-value clinical tool for optimizing post-operative recovery and reducing infectious complications.

Keywords Axiostat · Chitosan · Dressing · Antioxidant · Antibacterial · Bleeding wounds · Chronic peripheral arterial disease (PAO)

Introduction

Rapid and effective control of bleeding is essential in many medical fields, from emergency surgery to pre-hospital medicine. Currently commercially available topical hemostatic devices used in clinical practice are unfavorable

for the wound healing process [1]. In fact, they present numerous disadvantages including suboptimal hemostasis time, reduced efficacy in conditions of very flowing blood, dependence on physiological conditions, traumatic removal, adverse and immunogenic reactions, risk of infection, poor biodegradability, limited applicability on irregular or internal surfaces and finally they are not indicated for major arterial hemorrhages [2]. In this context, innovative hemostatic devices offer numerous advantages over conventional ones, thanks to the use of advanced materials, bio-engineered technologies and intelligent release and activation strategies [3]. These devices are designed to address the most complex clinical challenges, reducing time, complications and costs [4]. Chitosan is considered as one of the ideal materials to produce devices intended for wound management. Being a polymer of natural origin, it is biocompatible and biodegradable as it is metabolized by some human enzymes including lysozyme; it stimulates

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hemostasis and accelerates tissue regeneration; it is a powerful antimicrobial agent thanks to its cationic nature and also has anti-inflammatory activity [5, 6]. It is a polymer that can take various forms, such as membranes, hydrogels, fibers and sponges [7]. The latter, in particular, are able to absorb large quantities of fluids (over 10/20 times their dry weight) thanks to their microporosity; they offer good cellular interaction and can absorb wound exudates, promoting the haemostatic process and at the same time helping tissue regeneration [8]. Axiostat is an innovative haemostatic consisting of a highly porous chitosan matrix, which comes in the form of a flexible adhesive dressing, similar to a tampon, approved by the FDA as a medical device 93/42/EEC, 50. This dressing has a microporous structure attributable to the manufacturing process which consists of freeze-drying the polymer (2% w/v), previously solubilized in diluted acetic acid, to induce phase separation of the solvent crystals from the chitosan suspension [9]. Subsequently, during the decrease in temperature, a porous polymer structure is generated by the removal of frozen solvent crystals [10]. Using a highly deacetylated chitosan with a high molecular weight and a narrow polydispersity precisely regulates the solubility of the polymer at a given temperature and allows them to obtain a highly integrated structure based on the intertwining of long polymer chains. This process is called uncontrolled partial crystallization of the polymer, resulting in the formation of pores of various sizes capable of adsorbing large amounts of fluids. This allows Axiostat to trap red blood cells and exploit the cationic nature of chitosan to accelerate the hemostasis process. In fact, under natural physiological conditions, the aggregation of red blood cells is slowed by the electrostatic repulsion between negatively charged glycolipids on the surface of red blood cells [11]. However, the positive charge ($-NH_3^+$ groups formed by the protonation of amino groups) on the chitosan chain can interact electrostatically with negatively charged red blood cells, resulting in increased aggregation of the same around the bleeding point for the formation of clots. As for platelets, under natural physiological conditions, they remain in a quiescent state and do not adhere to endothelial cells. Once Axiostat touches the blood, platelets are activated at the bleeding site by electrostatic interactions between positive charge (protonated amino groups of CS) and negative charge (negatively charged phospholipid surface of platelets) [12, 13]. The formation of the protein membrane was closely related to the degree of deacetylation and the level of protonation of chitosan. In the study by Huang et al., it was found that protonated chitosan can recruit clotting factors and assemble plasma proteins from platelet-poor plasma, as well as adhere to and activate platelets, stimulating the secretion of intracellular clotting substances. To evaluate the polymer's ability to induce protein clot formation, the

researchers incubated it with platelet-rich plasma (PRP) and added $CaCl_2$ to initiate the clotting cascade reaction. At the end of the reaction, it was observed that the control group had produced the smallest protein clot, while the protonated chitosan group had produced the largest clot with the least amount of supernatant remaining, suggesting superior hemostatic performance [14, 15]. The main objective of this study was to investigate some properties of the chitosan-based dressing not reported in the literature, including antioxidant and antibacterial activity and swelling capacity in the presence of fluids. We also investigated, through the collaboration with the vascular surgery of the hospital of Cosenza, the in vivo application and efficacy of Axiostat in the management of bleeding wounds at the inguinal level, resulting from endovascular procedures for the treatment of chronic peripheral arterial disease (PAO).

Materials and Instruments

This study is not prospectively designed, initiating observational study. It consists of a retrospective analysis of completely anonymized clinical data collected during routine clinical practice. The data derives from the use of a CE-marked medical device, applied strictly according to its intended purpose, without any modifications, additional procedures, patient enrollment, or deviations from standard clinical care. No prospective study protocol was established, and no experimental intervention was performed. Therefore, this analysis does not fall within the scope of observational studies requiring prior approval from the Institutional Ethics Committee. The cell strains of *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615) were provided by Thermo Fisher Scientific, Inc, Monza, Italy. The cells were grown in Müller-Hinton broth II (MHB; Difco, Detroit, MI, USA) containing 2 g/l beef infusion solids, 17.5 g/l casein hydrolysate, 1.5 g/l starch. The final pH was adjusted to 7.4. Axiostat chitosan dressings were purchased from AxioBiosolutions Pvt. 2,2-difenil-1-picrylidrazyl (DPPH) and PBS phosphate buffer saline were purchased from Sigma-Aldrich (SGFma Chemical Co., St Louis, MO, USA). The spectra UV–Vis were recorded with a Jasco V-530 UV/Vis spectrophotometer (Thermo Fisher Scientific, Monza, Italy).

Evaluation of Antibacterial Activity

The experiment was conducted following the Kirby-Bauer method. The initial bacterial suspension was diluted to a standardized density of 0.5 McFarland. The diluted bacterial suspension was evenly spread across a Mueller–Hinton

agar (Fisher Scientific Italia, Segrate, MI) plate, onto which the testing device was directly applied. The plate was then incubated under aerobic conditions at 35–37 °C for 16–18 h. After incubation, the diameter of the zone of inhibition (ZOI) surrounding each patch was measured. The experiment was conducted in triplicate. We investigated the in vitro anti-bacterial activity against two Gram+ve (*Staphylococcus aureus* and *Streptococcus pyogenes*) and two Gram–ve bacterial strains (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

DPPH Test

A concentration of 0.25 mM DPPH ethanol solution 1.5 mL of DPPH solution and 3 mg of the sample were mixed. This was shaken vigorously and left at room temperature for 30 min. The bleaching of DPPH was determined by measuring the absorbance at 517 nm with a UV–Vis spectrophotometer. The radical scavenging activity of DPPH was calculated according to Eq. (1):

$$\text{Scavenging activity\%} = (A_0 - A_1) / A_0 \times 100 \quad (1)$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the dressing.

Swelling Studies

The affinity of Axiostat towards the aqueous environment was determined by its degree of swelling (%WR) in phosphate buffered saline (PBS). Several dry samples were placed in contact with 5 ml PBS solution at pH 7.4 (to simulate the physiological environment) at 37 °C for 2.5, 5, 10 min. Subsequently, the wet weight (wet W) of the swollen gauze was measured and then the gauze was lyophilized and weighed (dry W). The water content of the gauze was calculated using the following Eq. (2):

$$\text{WC\%} = (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{wet}} \times 100 \quad (2)$$

where WC is the water content of Axiostat, W_{wet} is the weight of the wet dressing and W_{dry} is the weight of the gauze after freeze-drying.

Gauze Characterization

The Axiostat chitosan dressing was characterized by optical microscopy using a Motic BA300POL binocular microscope and by high-resolution scanning electron microscopy (SEM; FEI Quanta 200) to evaluate its structure and morphology.

Cytotoxicity Evaluation

Cell Line

Human Adult Epidermal Keratinocytes (HaCaT) was obtained from ATCC (American Type Culture Collection; Manassas, VA, USA). Cells were cultured at 37 °C with 5% of CO₂. Experiments were conducted in full media: DMEM high glucose for HaCaT with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin.

MTT

Cells were seeded into 96-well plate at an appropriate density (4×10^3 or 6×10^3 , depending on the cell type) in complete growth medium. The following day, cells were starved with red phenol-free and serum-free medium. After 24 h of starvation, cells were treated with different concentrations of Chitosan Hemostatic Pad (ranging from 0.01 to 1 mg/ml) for 24, 48 and 72 h. After the incubation time with the treatments, a solution of 5 mg/mL of MTT (yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added in each well. The plate was left in the incubator for 3 h at 37 °C with 5% of CO₂, allowing metabolically active cells reduced MTT to purple formazan crystals. 100 µL of DMSO (Dimethyl Sulfoxide) was added to solubilize the formazan crystals. The plate was gently agitated to ensure complete solubilization. Absorbance was measured at 570 nm using a microplate reader. The cytotoxic effect of various concentration was calculated by comparing the absorbance of treated wells to untreated control group and normalizing the values to the absorbance at time zero (T₀). The EC₅₀ value (the concentration of the treatment that induced 50% of the maximum effect) was determined using GraphPad Prism software.

ROS Detection and Cell Viability Test

Cells were seeded in 6-well plate in full medium at an appropriate density, then were starved in -S phenol-free medium for 24 h. Following the starvation period, cells were treated with Chitosan Hemostatic Pad at concentrations of 0.05 and 0.5 mg/ml for 24 and 48 h. After incubation, cells were trypsinized, pellets were collected by centrifugation and washed with PBS to remove any residual medium. The cells were subsequently resuspended in appropriate buffer for ROS measurement.

Scratch Assay

HaCaT cells were seeded at an appropriate density in 6-well plate, to achieve approximately 80% of confluence the next

day. After 24 h of starvation in red phenol-free, serum-free medium, a scratch was created, using a 200µL pipette tip. The cells were then treated with Chitosan Hemostatic Pad at concentration of 0.05 and 0.5 mg/mL. Cell migration was monitored at 7 h and 24 h, taking pictures. Wound closure was quantified using ImageJ, measuring the width of the scratch at the initial time point (T₀) and at subsequent time points. The wound closure percentage was calculated using the following formula:

$$\text{Wound closure (\%)} = [(\text{Wound width } T_0 - \text{Wound width } T_x) / \text{Wound width } T_0] \times 100$$

In Vivo Application

Study Design

This study is a single-centre analysis conducted at the Annunziata Hospital in Cosenza, in collaboration with the Department of Pharmacy of the University of Calabria. It is based on a retrospective data collection, carried out between January 2023–December 2023, of patients who underwent femoral arterial access site closure, after endovascular technique for the treatment of Chronic Obstructive Peripheral Arteriopathy (AOCp), using the Axiostat hemostatic patch. The inclusion criteria were:

- Endovascular treatment with introductive sheath placement in the common femoral artery (CFA);
- Introductive sheath size between 4 Fr, and 8 Fr;
- Age 18 years or older;
- Left femoral artery size greater than 6 mm;
- Chronic symptoms (>4 weeks);
- Presence or absence of fibrocalcific plaques.
- Presence or absence of course abnormalities at the femoral bifurcation;
- Surgeon's decision to perform manual compressive haemostasis of the vascular access site;

Exclusion criteria included:

- Age under 18 years;

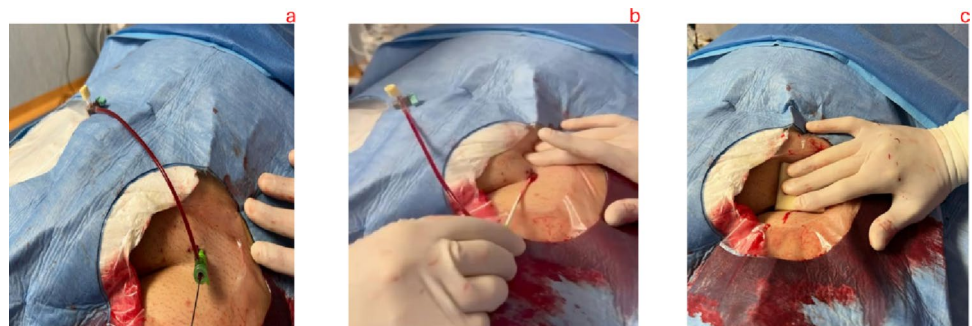
- Previous surgery in the access area or presence of inguinal scarring;
- Previous endovascular treatments or vascular accesses using VCD at the same access site;
- Risk of bleeding above the CIRSE standard of practice for anticoagulant and peri-operative management [16];
- Pregnancy.

Treatment Data Collection

Patients undergoing an endovascular angioplasty (THA) procedure, in which femoral access is preferred for treatment of AOCp, undergo local anaesthesia with lidocaine by injection through the skin and subcutaneous tissue. Subsequently, an echo-guided puncture is performed on the common femoral artery for the placement of the 8–12Fr introducer using ultrasound investigation (B-Mode ultrasound, LINEAR 7mHz probe, SONOSITE M-TURBO ultrasound) as a diagnostic means to reveal right or left iliac stenosis (Fig. 1a). The BOSTON INNOVA 8 × 40 mm stent type is then delivered, and angiographic control is performed to assess vessel patency.

At the end of the procedure, the skin around the vascular access site and the operator's gloves are cleaned. The operator applies proximal pressure to the arterial access site and removes the introducer sheath (Fig. 1b) and with the other hand modulate proximal pressure to check for the absence of bleeding from the access site and then initiate three-finger manual compression by placing the Axiostat in contact with the access site (Fig. 1c). Manual compression lasts 7 min for 4–6 Fr sheaths and 10 min for 8–10 Fr sheaths. At the end, the access site is inspected: if there is an expanding haematoma or significant bleeding, compression continues until appropriate haemostasis is achieved, as defined by Schulz-Schüpke et al. Subsequently, a secondary adhesive compression dressing (Tensoplast Sport 10 cm × 2.5mt) is applied at maintained pressure for 6 h (genuine of 4–6 Fr) or 12 h (genuine of 8–10 Fr). After removal of the patch, the site is checked and, in the absence of complications, the patient can resume walking. The Axiostat patch is removed by irrigating it with saline solution and gently peeling it off. Prior to discharge, the patient undergoes a duplex ultrasound

Fig. 1 10Fr introducer placement at the level of the right common femoral artery (a); Removal of 10Fr introducer from vascular access site (b); application of Axiostat by manual compression using the three-finger technique (c)



and after 30 days is recontacted to rule out complications. Any abnormalities require further clinical and ultrasound follow-up.

Results and Definitions

The primary objective of this study was to evaluate the efficacy and safety of the Axiostat haemostatic dressing in facilitating manual compression closure of the access site at the femoral artery in patients undergoing endovascular procedures for the treatment of AOCP and Leriche syndrome. The primary efficacy endpoint was the technical success rate of femoral artery haemostasis, defined as single removal of the compression dressing within 7 min of placement without bleeding requiring immediate recompression. The secondary objective of the study was to evaluate and compare the efficacy, safety and performance of Axiostat at 24 h and 30 days after the procedure. Primary technical success was defined as adequate haemostasis achieved within 7 min of manual compression assisted by the Axiostat haemostatic dressing. Secondary technical success was defined as adequate haemostasis after Axiostat-assisted manual compression and successful intraoperative arterial closure without evidence of stenosis, occlusion or persistent haemorrhage requiring further intervention. Clinical success was defined as the absence of bleeding and follow-up complications. Clinical evaluation was defined as the success rate of femoral artery haemostasis, the incidence of femoral artery thrombosis, the rate of haematomas and false aneurysms at 24 h and 30 days postoperatively. Complications were classified according to the SVS (Society for Vascular Surgery clinical practice guidelines) reporting standards. Haemostasis time was defined as the elapsed time (in minutes) between removal of the introducer sheath and observation of adequate haemostasis. Ambulation time was defined as the elapsed time (in minutes) between the application of the compression dressing and the time when the patient can ambulate and resume activities without the adhesive compression dressing.

Assessment of Haemostasis

After removal of the Axiostat dressing, each wound was qualitatively assessed for haemostasis at 3, 5 and 7 min after application using the validated bleeding scale. After assessment of haemostasis at 7 min, the lesions were irrigated with saline solution to remove the dressing without affecting the blood clot.

Statistical Analysis

Statistical analyses were conducted on all patients recruited for this study. The collected data were analysed using one-way ANOVA. Specifically, SPSS for Analytics 25 software (IBM, New York, NY, USA) was used for this analysis. Values of $p \leq 0.001$ were considered statistically significant for the above-mentioned tests.

Results and Discussion

Antibacterial Activity Evaluation

Axiostat sponges were subjected to antibacterial tests, by evaluating the zone of inhibition (ZOI) on plate-based assays, in order to appreciate their potential health applications. The obtained results (Fig. 2) showed a significant antibacterial activity of Axiostat sponges against the Gram-negative bacterium *Klebsiella Pneumoniae*, as well as an effective antibacterial profile against *Pseudomonas Aeruginosa*, *Staphylococcus Aureus* and *Streptococcus Pyogenes*. These results showed a halo of inhibition with a diameter of almost 3 cm for *Klebsiella pneumoniae* and almost 1 cm of inhibiot for the other bacteria strains tested. Overall, Axiostat sponges demonstrated to have an excellent antibacterial activity against gram negative bacteria, in particular against *Klebsiella Pneumoniae* that is a major cause of hospital-acquired pneumonia, especially in patients under surgery or on ventilators. It can occur in various parts of the body, including the lungs (causing pneumonia), urinary tract, bloodstream, surgical wounds or other wounds, and even the brain. Base on that, Axiostat sponges minimize the infection generated by *Klebsiella Pneumoniae*, reducing risk of a severe healthy problems, potentially leading to sepsis and septic shock.

The antimicrobial assessment was designed as a screening and functional evaluation of the antibacterial performance of the dressing material, rather than as a comprehensive pharmacological characterization of minimum inhibitory/bactericidal concentrations. Consequently, we used diffusion plate-based methods to demonstrate antibacterial activity under the tested conditions and to support the intended application of the material.

DPPH Test

The DPPH test is designed for the estimation of antioxidant properties and is based on measuring the scavenging capacity of antioxidants towards 2,2-diphenyl-1-picrylhydrazyl radicals. After 30 min of interaction of the gauze (3 mg) with DPPH, the scavenging efficiency of DPPH reached 82.5%.

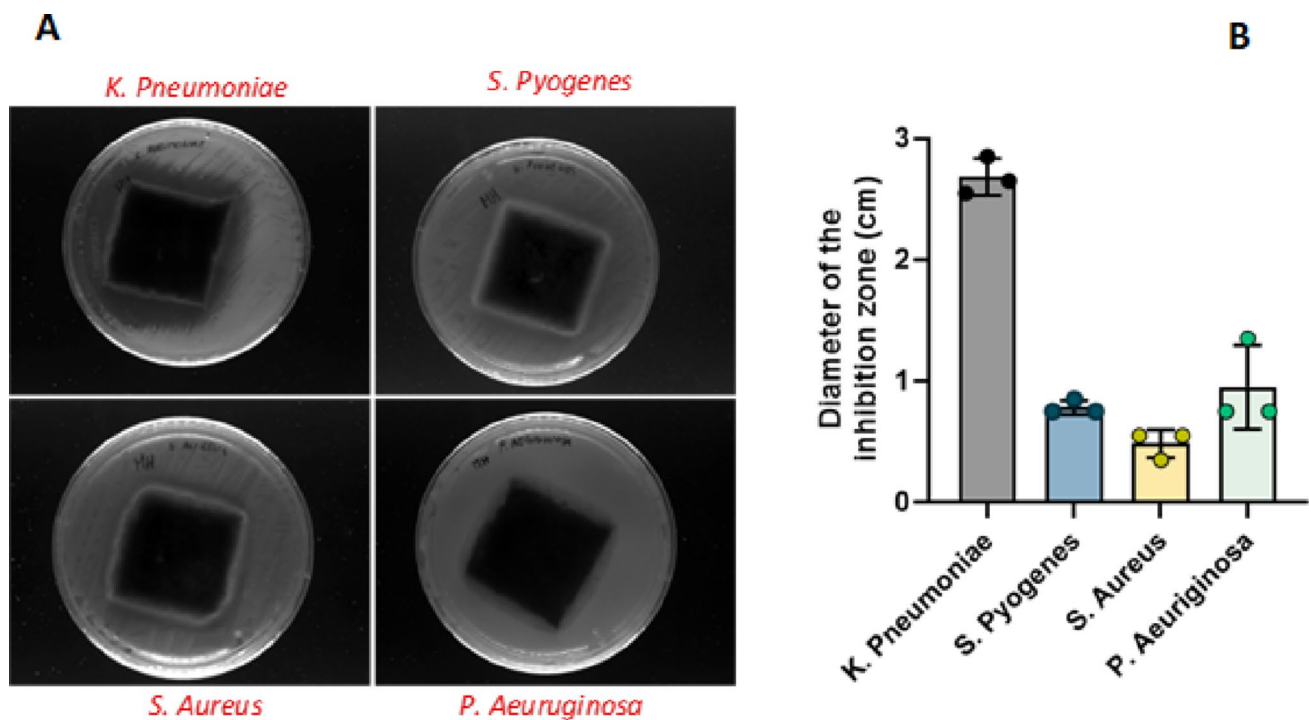


Fig. 2 A Antibacterial activity of chitosan patches. B Diameter of the inhibition area after patches activity evaluation at 24 h

This high antioxidant potential (82.5%) is not merely an isolated chemical figure, but plays a crucial biological role in the wound microenvironment. During acute trauma or vascular access, tissue disruption and local hypoxia trigger a massive production of reactive oxygen species (ROS), which perpetuate oxidative stress, damage cell membranes and delay the early stages of healing. The matrix's marked ability to capture free radicals suggests that the device, in addition to its primary haemostatic effect, exerts a cytoprotective action. By neutralizing excess ROS, the material attenuates the secondary inflammatory cascade and preserves the integrity of the perivascular tissues, laying the biochemical foundations for faster and less inflammatory tissue regeneration compared to traditional cotton dressings.

Swelling Studies

The Axiostat dressing had a significant water content of 96.3% when placed in contact with aqueous medium for 2.5 min; a water content of 97.07% after 5 min exposure with aqueous medium; and finally, a water content of 96.12% after 10 min contact with aqueous medium. A high degree of swelling should correspond to a high capacity of the dressing to absorb blood components and promote acceleration of coagulation at the affected site. The swelling kinetics show extremely rapid hydration, reaching a plateau within the first 150 s. From a haemodynamic and clinical perspective, this behavior

is crucial: a high and sudden degree of hydrophilic swelling translates directly into an extraordinary ability to absorb exudate and cellular blood components (erythrocytes and platelets) directly from the arteriotomy lumen. However, unlike traditional gelatin-based haemostatic sponges, which expand macroscopically in volume, introducing the risk of deleterious mechanical compression on adjacent nerve or vascular structures, the stability of Axiostat's swelling between 5 and 10 min (96–97%) indicates that the material transforms into a cohesive hydrogel without uncontrolled volumetric expansion. This phenomenon allows for the local concentration of blood cellular factors and maximizes the electrostatic interaction between the positive charges of chitosan and the negative charges of red blood cells, accelerating the formation of the haemostatic plug without compromising the surrounding anatomical space.

Optical Microscopy

From optical microscopy observations, cross-sectional and frontal images (Fig. 3a and b) showed a highly porous dressing with evidence of pores of various sizes. In addition, Fig. 3c showed a very uniform porous structure with homogenous distribution of cavity sizes in the dressing.

Fig. 3 Axiostat patch cross-section (a); front section (b); centre section of Axiostat patch (c)

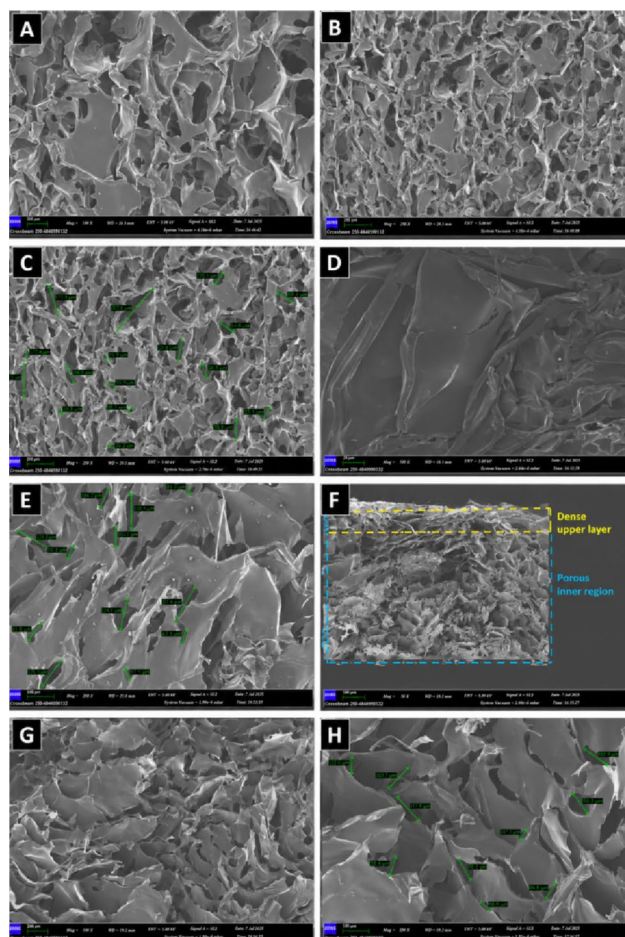
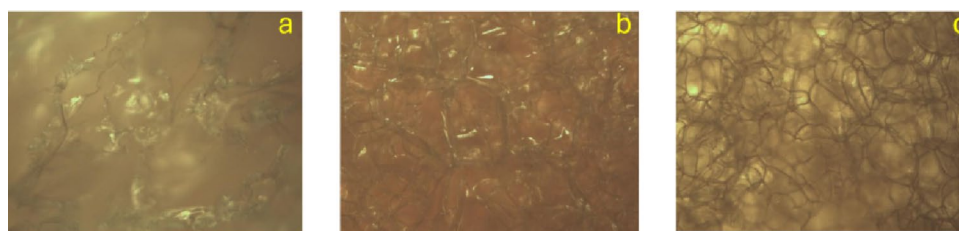
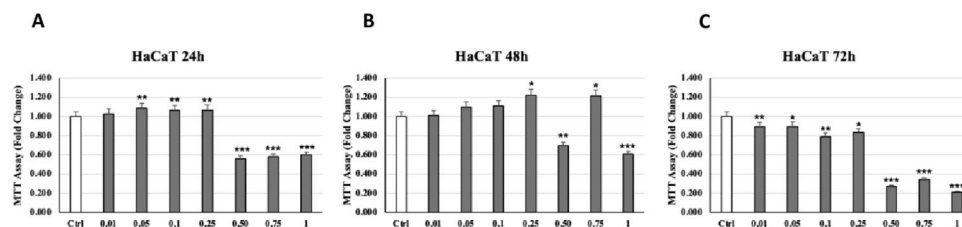


Fig. 4 Scanning electron microscopy (SEM) images of the gauze at different magnifications. **A, B** Low- and medium-magnification views showing the highly interconnected porous structure. **C, E, H** Representative pore size measurements, indicating pore diameters ranging from approximately 30 to 210 μm , with most pores between 50 and 130 μm . **D** High-magnification image of the upper surface showing a compact and dense morphology. **F** Cross-sectional SEM image revealing a bilayer architecture composed of a dense superficial layer and a porous inner region. **G** Overview of the internal porous network, highlighting the interconnected three-dimensional architecture throughout the scaffold

Fig. 5 Cytotoxicity of the extract after different time points: 24 h (A), 48 h (B) and 72 h (C). Control groups are untreated cells (1-Fold Change). Each result was obtained in triplicates. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ (Student's T test)



SEM Morphology

SEM analysis demonstrated (Fig. 4) that the gauze exhibits a highly porous and interconnected three-dimensional architecture with irregularly shaped pores (Fig. 4A–C). Pore size measurements (Fig. 4C, E, H) showed a broad distribution ranging from approximately 30 to 210 μm , with the majority of pores between 50 and 130 μm . Higher magnification of the surface (Fig. 4D) revealed a compact morphology with limited surface openings. Cross-sectional observation (Fig. 4F) confirmed the presence of a bilayer structure consisting of a dense upper layer covering a highly porous inner network, which extended throughout the scaffold thickness (Fig. 4G). This hierarchical architecture combines a protective superficial layer with an interconnected porous core, which is expected to favor fluid absorption and cell infiltration, supporting its potential application as a wound dressing.

Treatment with Haemostatic Patch Promotes Cell Proliferation and Migration In Vitro

Considering the haemostatic properties of the patch we wanted to evaluate its biocompatibility, through MTT assay. HaCaT cells were exposed to varying concentrations of the extract (ranging from 0.01 to 1 mg/ml) for 24, 48 and 72 h, and the resulting cytotoxic effect was measured. The results showed that the patch significantly improved cell proliferation by increasing concentration from 0.01 to 0.25 mg/mL (Fig. 5A–C). However, with further increase of concentration, this effect was no longer noticeable. The calculation of the extract's median effective concentration (EC50) was then used to guide the design of subsequent experiments. Furthermore, the results of the cell migration experiment demonstrated that the haemostatic patch treatment significantly promoted the migration of HaCaT cells (Fig. 6A, B).

Fig. 6 Wound scratch assay images (A) and quantitative analysis (B) for evaluation of the migration in monolayer HaCaT, after 24 h of treatment with different concentrations of patch extract. * $p < 0.05$; ** $p < 0.01$, *** $p > 0.001$ (Student's T test). Experiments were conducted in triplicates, six fields per well were analyzed. (Scale bar=30 micron)

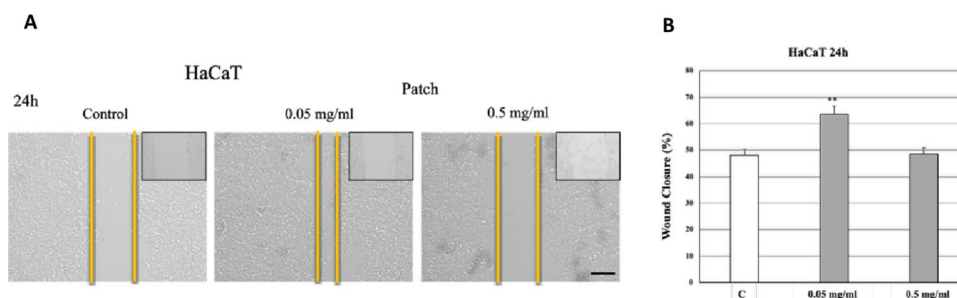
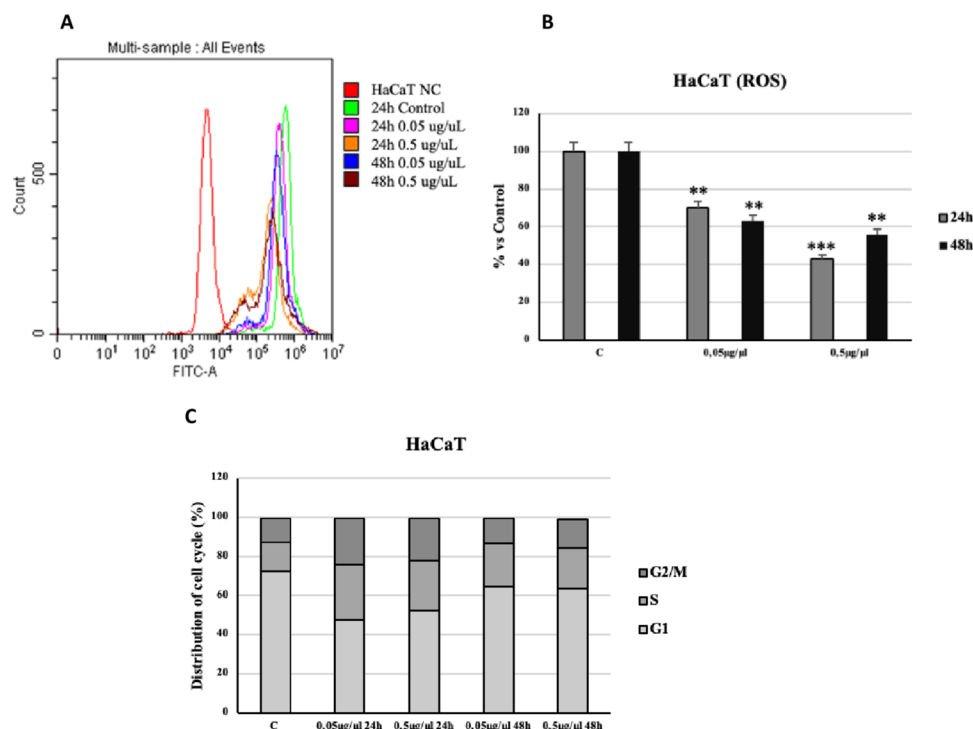


Fig. 7 Flow cytometry analysis of ROS levels (A) and its quantification (B) ** $p < 0.01$, *** $p < 0.001$ (Student's T test). Graph of cell cycle distribution from HaCaT cells after treatment with different concentration of patch extract (C)



Direct-contact cytotoxicity testing with the intact sponge was not performed due to its three-dimensional and highly porous structure, which could cause non-specific mechanical effects (e.g., cell compression or detachment) and poor standardization of the cell–material interface. Instead, we performed an extract-based cytotoxicity assay. Briefly, a defined amount of the Chitosan Hemostatic Pad was incubated in complete cell culture medium for 24 h (37 °C) at a final extraction ratio corresponding to 0.2 g/mL. The extract was then filtered to remove residual particles before being applied to the cells. This procedure follows ISO 10993–12 guidelines for sample preparation and is consistent with ISO 10993–5 recommendations for in vitro cytotoxicity testing, which advise the use of material extracts to evaluate the potential release of cytotoxic substances under standardized and reproducible conditions.

The stock extract (0.2 g/mL) was prepared to ensure adequate extraction of soluble components. Serial dilutions were subsequently generated to obtain concentrations ranging from 0.01 to 1 mg/mL for the MTT assay. This

concentration range was selected to cover a broad dose interval, including low, intermediate, and relatively high concentrations, and to comply with standard in vitro biocompatibility testing practices, which recommend testing multiple dilutions to identify possible cytotoxic thresholds.

Antioxidant Properties of the Haemostatic Patch Extract

Our results demonstrated that the haemostatic patch has antioxidant properties, as revealed by ROS measurements. By decreasing ROS levels, these enzymes protect epidermal cells from oxidative injury, promoting cell survival and tissue repair. This hypothesis is further supported by the observed enhancement in cell cycle, as evidenced by the improved S phase population in treated cells, specially after 24 h (28.72% and 25.2%, respectively) compared with the control group (15.03%) (Fig. 7C).

Table 1 Population study

Variables	AOCP Patients (n=110)
Age	68 ±3.6(68.4)
Sex (%M/%F)	60% M/ 40% F
aPTT ** (S)	33 ±0.89(33.12)
Plastrin Count (× 10 ⁶ /mL)	330 ±7.33 (330.4)
Diabetes	66 (60%)
Hypertension	44 (40%)
Hyperlipidemia	55 (50%)
Smoker	49 (45%)
Ex-smoker	16 (15%)

Table 2 Population variables

Variables	Stage				
	1/2	3	4	5	6
Rutherford classification (N° patient)	0	38	22	59	0
Antihypertensive therapy (N° patient)	0	28	18	53	0
Statin therapy (N° patient)	0	20	10	30	0
Mono-antiaggregant therapy (N° patient)	0	22	0	51	0
Duo-antiaggregant therapy (N° patient)	0	0	0	8	0
Anticoagulant therapy (N° patient)	0	0	16	0	0

Population Study

During the study interval (January 2023–December 2023), 110 patients underwent manual compression closure of the left femoral arterial access site with the aid of the Axiostat haemostatic dressing. The average age was 68 years with a small proportion of patients with the condition before the age of 65 years. 60% of the patients had diabetes mellitus, hyperlipidemia, hypertension and a previous history of smoking; all risk factors that significantly influence the onset of AOCP (Table 1).

The following pre-operative clinical instrumental data were collected to define the stage of peripheral arteriopathy:

- Rutherford clinical classification
- Previous revascularization surgery on the limb in question

According to Rutherford's classification, of the 110 limbs that underwent surgery, none were in stage 1 or 2, respectively

mild and moderate claudication; 38 cases (34%) had stage 3 with severe claudication; 22 (20%) had stage 4 with pain at rest; 59 (45%) had stage 5 with minor trophic lesions and finally none had major trophic lesions characteristic of stage 6. Examination of previous operations showed that 88% had never been treated surgically, 10.5% had undergone previous endovascular revascularization and 0.9% had undergone open surgery. Finally, the drug therapy taken by the patients at the time of admission was investigated, which showed that 83.18% of the treated cases were taking antihypertensive drugs, 59.29% were taking statins, 64.60% were taking antiplatelet drugs (of which 8 were dual antiplatelet) and 16 were on anticoagulant therapy (warfarin or oral anti-coagulants) Table 2.

Procedure Data

The most used size of the vascular introducer sheath was 8–12 Fr with a mean number of vascular access site punctures per patient of 1.3 (±0.8). The number of punctures was calculated considering each time the needle was removed from the skin after its insertion. Patients undergoing the vascular procedure continued the drug regimen they were already following before the procedure. Specifically, for patients diagnosed with AOCP, 20% of them maintained therapy with an antithrombotic agent.

Results and Follow-Up

The primary technical success with Axiostat was 99.9% of patients with stage 2 AOCP. In other disease stages shown in Table 3, the primary success was approximately 98.5%. Access site complications were mainly related to hematoma formation at 24 h (15%) and 7 days (10%) in patients with AOCP who were on antiplatelet and/or anticoagulant therapy during the anamnesis (Rutherford's stage 3, 4, or 5), pseudoaneurysm formation (6%), and excessive bleeding requiring re-surgical intervention with closure of the femoral access site by suture (4%). Another outcome measure in patients treated with Axiostat was the return to ambulation at 24 h after the endovascular procedure with values

Table 3 Outcome of evaluation and follow-up in patients with different AOCP stages treated with Axiostat as a vascular access site closure device. Significance was assessed by one-way ANOVA, demonstrating that the data collected during the experiments are statistically significant with $p \leq 0.001$

Results	Stage					
	1	2	3	4	5	6
Primary success (%)	0	100	99	98	98.5	0
Secondary technical success (%)	0	100	99	98	99	0
Clinical success (%)	0	100	99	98	97	0
Surgical success (%)	0	100	100	98.8	98	0
Ambulatory after 24 h (%)	0	100	100	100	100	0
Recovery of function (%)	0	100	98.8	98	96	0

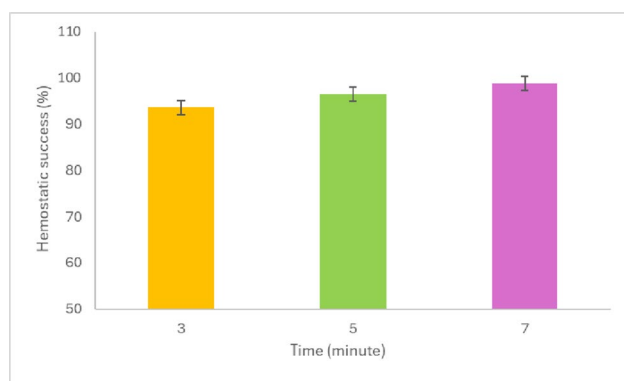


Fig. 8 Graphical representation to evaluate the hemostasis time achievement at different time intervals

equal to 100% in all AOCF patients at various stages of the disease. No patient experienced adverse effects during the procedure.

Evaluation of Hemostatic Activity

At the end of the 7-min observation period, hemostatic success of Axiostat-treated lesions was 99% in patients with AOCF. In most cases of failure, bleeding occurred in patients using an antiplatelet and anticoagulant therapy (not discontinued before surgery). Controlling for differences in pretreatment hemorrhage level, the median time to hemostasis was approximately 3 min after use of the dressing combined with manual compression by the surgeon, especially in patient with Rutherford's stage 3. At the end of the 7-min observation period, hemostatic success of Axiostat-treated lesions was 98.9% (Fig. 8).

Discussion

The aim of this study was to evaluate the physicochemical properties and biological performance of the commercially available chitosan-based haemostatic sponge Axiostat. The originality and novelty of this study are evident on three main levels (methodological, microbiological and clinical), addressing specific gaps not covered by the manufacturer's clinical trials. In vitro results show that the material has antibacterial and antioxidant activity, effectively absorbs fluids and has a porous structure. Cell culture experiments indicate that it is biocompatible, supports cell proliferation and migration, and helps reduce oxidative stress. Although these results are consistent with the known properties of chitosan-based wound dressings and in vivo results confirm its safety and performance in surgical practice, it should be emphasized that independent and rigorous validation (not conducted by the manufacturer) is essential for the scientific community to confirm the claimed performance and

discover new potential uses. The added value of this work lies in exploring the multifunctional properties of chitosan in the 5 × 5 sponge format. The specific investigation of antioxidant capacity and swelling behaviour under controlled conditions provides data that is lacking in commercial clinical trials. In fact, despite the well-known antioxidant properties of chitosan as a polymer, there are no actual studies in the literature carried out on Axiostat 5 × 5 to evaluate this capacity. Furthermore, the identification of marked efficacy against *Klebsiella pneumoniae* represents a point of scientific merit, since the data reported in the literature show that the antibacterial capacity of Axiostat gauze has been tested on Gram-positive bacterial strains such as *Staphylococcus aureus* (ATCC 29737), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341), and gram-negative strains such as *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 14169), and *Salmonella abony* (ATCC 6) [14]. Tests on *Klebsiella pneumoniae* are not mentioned in any study. Therefore, this study identifies a specific spectrum of action against a multi-resistant pathogen that is critical in hospital environments, providing clinicians with a rational basis for choosing this device. The use of human keratinocytes (HaCaT) for cytotoxicity testing, combined with actual application in endovascular procedures, closes the circle between molecular safety and clinical efficacy. In the literature, the cytotoxicity of Axiostat gauze has only been tested on Balb/c 3T3 mammary cell lines [14]. The haemostatic success rate for lesions treated with Axiostat was 98.9% within 7 min. In our study, the rate of haematoma at 24 h was 15%. Although this figure may appear significant in absolute terms, it must be strictly contextualised in light of the characteristics of the enrolled population, which was heavily influenced by severe cases of Chronic Peripheral Arterial Occlusive Disease (CPAOD) and particularly aggressive antithrombotic regimens. Patients with CPAOD have stiff, calcified and poorly elastic arterial walls that hinder the natural closure of the arterial puncture site. When this condition is combined with the effects of anticoagulant and antiplatelet drugs, the vascular access site remains vulnerable to micro-haemorrhages in the hours following primary haemostasis. This explains why, despite the device's immediate effectiveness in stopping bleeding, a haematoma may form in the medium term. Furthermore, in such endovascular procedures, the length of time the introducer remains in the femoral artery is also one of the crucial factors influencing the risk and severity of a haematoma at the access site. The longer the introducer remains in place, the more time the blood (made extremely fluid by antiplatelet agents and/or anticoagulants) has to exploit arterial pressure and seep millimetre by millimetre into the surrounding tissues, giving rise to extensive and deep subcutaneous haematomas. To properly assess the

clinical safety of Axiostat, this result must be compared with the established literature on other haemostatic methods: manual compression (MC) and mechanical vascular closure devices (VCDs). In the literature, the use of manual compression alone in patients undergoing dual antiplatelet therapy (DAPT) or active anticoagulation shows haematoma rates comparable to ours, ranging from 8% to almost 18%. Although large multicentre studies, such as that conducted by Jakobsen et al., indicate that the use of any VCD significantly reduces the overall incidence of haematomas compared with MC (1.7% vs 3.6%), whilst also reducing the proportion of minor haematomas (1.3% vs 2.7%) and those requiring transfusions (0.2% vs 0.3%) or surgical revision (0.2% vs 0.5%), it should be emphasized that these case series include a wide range of patients and do not focus exclusively on groups at high risk of arterial complications [17, 18]. In the study conducted by Mayer et al., although it was demonstrated that VCDs drastically reduce the mechanical time required to achieve initial haemostasis compared with manual compression (approximately 1 min vs 12 min), this did not eliminate the incidence of haematomas and bleeding in the following days, particularly in patients on anticoagulant therapy. The researchers highlighted that the primary composite endpoint of vascular complications related to the access site occurred more frequently in patients on oral anticoagulant therapy (OAT) than in those not on OAT. Haematomas were observed more frequently in patients on oral anticoagulant therapy (8.7%) than in those not on OAT (5.2%) [19]. However, the true clinical superiority of non-invasive topical patches over rigid VCDs becomes apparent when analyzing major complications. Invasive intramural devices have a technical failure rate of between 1.1 and 8.7% and carry a significant risk of severe vascular complications, such as pseudoaneurysms, arterial wall infections and acute vessel occlusions. The use of a topical, external technology such as Axiostat completely eliminates the risk of such catastrophic events. The clinical utility of Axiostat as an advanced haemostatic biomaterial is strongly supported by its unique biochemical mechanism and comparative clinical data. In a clinical study conducted by Kabeer et al., aimed at evaluating pre-hospital haemorrhage control in acute trauma, the haemostasis time achieved with Axiostat was significantly shorter than that of the group using conventional cotton gauze (4.68 ± 1.04 min versus 18.56 ± 5.04 min, respectively) [20]. Furthermore, there was a reduction of approximately 50% in blood loss from the wound site in cases treated with Axiostat compared to the control group (4.80 g versus 13.70 g). This superior efficacy stems from the electrostatic interaction between the chitosan matrix (positively charged) and the polyanionic domains (negatively charged) present on the membranes of erythrocytes and platelets. Unlike traditional vascular closure

devices (VCDs) or standard manual compression (MC)—which show reduced efficacy and an increased failure rate in patients undergoing dual antiplatelet therapy (DAPT) or systemic anticoagulation (heparins, warfarin, DOACs)—Axiostat's independent mechanism retains its haemostatic capacity even in the absence of functional fibrin or thrombin. When compared with other widely used surgical biomaterials, Axiostat's biocompatibility profile offers distinct advantages. For example, oxidized regenerated cellulose (ORC), whilst effective in internal surgery, acts by lowering the local pH to induce protein precipitation. This acidic microenvironment can trigger local inflammatory responses and delay the healing of perivascular tissue. In contrast, Axiostat operates at physiological pH, minimizing inflammation and preserving the integrity of the surrounding soft tissues. Gels and alginates are materials that act as passive haemostatics, merely absorbing fluids and providing a mechanical scaffold for endogenous platelet activation; their use is therefore strictly dependent on the efficiency of the patient's native coagulation cascade. Furthermore, gelatins undergo significant volumetric expansion following hydration, introducing risks of neurological or vascular compression if confined to narrow anatomical spaces. Chitosan does not exhibit macroscopic volumetric changes, thus avoiding compromise of adjacent structures. Despite the clinical advantages described, it is important to recognize several inherent technical and operational limitations of the gauze. For example, unlike mechanical VCDs (e.g. Angio-Seal, Perclose ProGlide) which seal the arteriotomy autonomously, Axiostat functions as an adjunct device requiring operator-dependent manual compression. Therefore, if the initial pressure is insufficient, unstable or applied at the wrong point, the electrostatic seal will not form correctly, leading to haemostatic failure. The standard Axiostat device is validated for arterial and venous access up to a maximum of 7–8 French (typical in diagnostic radiology and interventional cardiology). For larger gauges—such as those used in complex procedures such as transcatheter aortic valve implantation (TAVI), endovascular aneurysm repair (EVAR) or the insertion of ventricular assist devices (e.g. Impella)—Axiostat alone is not sufficient to ensure vascular wall sealing and must be used in conjunction with mechanical suture systems. Furthermore, due to the highly hygroscopic nature of lyophilized chitosan, the packaging must remain completely intact until the exact moment of use. Early exposure to ambient moisture or liquid droplets triggers the gelation reaction prematurely, compromising the dressing's adhesive properties and drastically reducing its clinical efficacy. For all these reasons, future research should focus on optimizing the integration of chitosan-based dressings with non-occlusive compression techniques (patent compression), in order to minimize the risk of

vascular thrombosis, particularly in radial artery access. Furthermore, clinical studies aimed at investigating the synergy between Axiostat and mechanical occlusion systems for large-bore catheters could extend its applicability to complex structural cardiac procedures. One could also consider functionalizing this device with bioactive substances to make it even more promising in the biomedical field.

Conclusions

This study has demonstrated that the Axiostat hemostatic patch possesses a broad range of beneficial properties, both in laboratory settings and in clinical applications. In vitro plate-based ZOI assays, confirmed its antibacterial potential, particularly against *Klebsiella pneumoniae*, a pathogen commonly associated with hospital-acquired infections and revealed a significant antioxidant capacity through DPPH and ROS assays. The patch's porous microstructure, coupled with its high-water absorption capacity, supports rapid fluid uptake, an essential feature for effective hemostasis. Moreover, its biocompatibility was evident in promoting keratinocyte proliferation and migration, further suggesting a role in enhancing tissue regeneration and wound healing. From a clinical perspective, the patch proved highly effective in managing arterial access closure in patients undergoing endovascular procedures, including those with advanced peripheral artery disease. The high rate of primary technical success, rapid achievement of hemostasis, and early return to ambulation reflect its reliability and ease of use. Importantly, the device maintained a favorable safety profile, even among patients with comorbidities and ongoing antithrombotic therapy. Although minor complications such as hematomas and pseudoaneurysms were observed, these were manageable and not directly attributable to the device itself. In summary, Axiostat emerges as a promising multifunctional hemostatic material with applications that extend beyond bleeding control to include infection prevention and tissue healing support. While these results are encouraging, further research, particularly through large-scale, randomized clinical trials—will be essential to fully establish its clinical efficacy and broaden its adoption in various surgical and emergency care settings.

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ing—review & editing, F.C. and R.C.; Supervision, R.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Institutional Review Board Statement Ethics review and approval were waived for this study due to its retrospective nature and because it was based exclusively on anonymized clinical data collected during routine clinical practice, without any additional diagnostic or therapeutic interventions. Pursuant to Italian law (Legislative Decree No. 196/2003, as amended by Legislative Decree No. 101/2018, Article 110 of the Privacy Code) and in line with AIFA Decision No. 425/2024, Ethics Committee approval is not required for retrospective analyses of anonymized data derived from routine clinical practice that do not involve experimental protocols or deviations from standard care. All patients provided written informed consent for treatment and for the use of their anonymized clinical data for scientific purposes. The in vitro experiments were conducted exclusively in an academic research laboratory and did not involve human or animal subjects.

Informed Consent The patients provided informed consent for publication according to D.Lgs. 196/2003.

Competing Interests The authors declare no competing interest.

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