RESEARCH PAPER

In Vitro Antimicrobial and Antioxidant Evaluation of a Novel Cefoperazone Sodium–Chlorhexidine PVA-Chitosan Hydrogel Against Common Orthopaedic Surgical Site Infection Pathogens

Somesh Aravindan[1], Rajeshkumar Shanmugam[2], Raja Purushothaman[3] ,Subitchan P [4] , Vijayan B[5]

[1]Postgraduate Resident, Department of Orthopaedics, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India

[2] Professor and Chief Scientist, Department of Nano Biomedicine, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India

[3]Professor, Department of Orthopaedics, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India,

[4]Associate Professor, Department of Orthopaedics, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India

[5]Professor, Department of Orthopaedics, Saveetha Medical College and Hospital, Chennai, Tamil Nadu, India,

Email: somesharavindan@gmail.com | Phone: 9884694777 | ORCID: 0009-0007-4403-5888

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ABSTRACT:

Background: Surgical site infections (SSIs) in orthopaedic surgery are predominantly caused by Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Enterococcus species, and continue to contribute to significant postoperative morbidity despite advances in perioperative care. Conventional wound dressings lack intrinsic antimicrobial activity. Novel hydrogel-based dressings incorporating antibiotic and antiseptic agents offer a promising strategy for localised infection prevention.

Objectives: To formulate a polyvinyl alcohol–chitosan (PVA–chitosan) hydrogel incorporating cefoperazone sodium and chlorhexidine gluconate, and to comprehensively evaluate its in vitro antimicrobial activity (agar well diffusion, time-kill kinetics, MIC/MBC), antioxidant potential (DPPH, ABTS, FRAP, H_2O_2, and nitric oxide scavenging assays), and cytotoxicity profile (brine shrimp lethality assay and zebrafish embryotoxicity) relevant to orthopaedic wound care.

Materials and Methods: The hydrogel was prepared by combining PVA (1.2 g/10 mL), chitosan (0.5 g/50 mL, with 0.1% glacial acetic acid), cefoperazone sodium (500 mg/5 mL), and chlorhexidine gluconate (0.4%, 30 mL), blended under continuous stirring at 450 rpm for 24 hours. Antimicrobial activity was evaluated by agar well diffusion (25, 50, 100 µg/mL) and time-kill kinetics (0–4 h, $\log_{10}$ CFU/mL) against Enterococcus faecium, Pseudomonas sp., Candida albicans, Escherichia coli, and Staphylococcus aureus. Antioxidant activity was assessed at 10–50 µg/mL using five validated assay systems. Safety was determined by brine shrimp lethality and zebrafish embryotoxicity models.

Results: The hydrogel demonstrated strong, concentration-dependent antimicrobial activity. Zones of inhibition (ZOI) ranged from 29–39 mm for E. coli, 32–37 mm for S. aureus, 30–31 mm for Pseudomonas sp., 21–26 mm for E. faecium, and 11–14 mm for C. albicans. Time-kill analysis confirmed bactericidal activity within 4 hours at 100 µg/mL. DPPH scavenging activity reached 87.22% at 50 µg/mL (standard: 91.81%). All antioxidant assays demonstrated significant, concentration-dependent free radical scavenging. Cytotoxicity studies confirmed acceptable safety at therapeutic concentrations.

Conclusion: The cefoperazone–chlorhexidine PVA–chitosan hydrogel exhibits broad-spectrum antimicrobial activity against principal orthopaedic SSI pathogens, significant antioxidant capacity, and a favourable safety profile. This formulation represents a scientifically validated candidate for advanced postoperative wound care in orthopaedic practice.

Keywords: *Cefoperazone sodium; chlorhexidine gluconate; PVA-chitosan hydrogel; antimicrobial activity; antioxidant; surgical site infection; zone of inhibition; time-kill kinetics; brine shrimp lethality; zebrafish embryotoxicity; orthopaedic wound dressing*

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1. INTRODUCTION

Surgical site infections (SSIs) are among the most prevalent and clinically significant hospital-acquired infections encountered in orthopaedic surgical practice. Defined by the Centers for Disease Control and Prevention (CDC) as infections occurring at or near the surgical incision within 30 days of a procedure — or within one year when a prosthetic implant is

involved — SSIs encompass superficial incisional, deep incisional, and organ/space categories [1]. In orthopaedic surgery, the routine implantation of metal fixation devices, intramedullary nails, prosthetic joints, and bone grafts creates substrate surfaces highly susceptible to bacterial adhesion and subsequent biofilm formation, rendering infections particularly refractory to antimicrobial therapy and host immune mechanisms [2].

The bacteriology of orthopaedic SSIs is well characterised. Staphylococcus aureus — including methicillin-resistant strains (MRSA) — and coagulase-negative staphylococci account for the majority of gram-positive isolates, while Escherichia coli, Pseudomonas aeruginosa, and Enterococcus faecium are among the most clinically important gram-negative and enterococcal pathogens implicated in postoperative wound infections [3]. The concurrent involvement of fungal pathogens such as Candida albicans, though less frequent, is recognised in immunocompromised and high-risk surgical populations [4]. This polymicrobial threat landscape necessitates wound dressing formulations that provide broad-spectrum, multi-organism coverage.

Conventional sterile gauze dressings, which continue to be widely used in postoperative orthopaedic wound care, function exclusively as passive mechanical barriers. They provide no active antimicrobial protection at the wound interface, do not modulate the local wound microenvironment, and lack antioxidant properties to attenuate oxidative stress-mediated tissue injury — a significant contributor to impaired healing and infection susceptibility in postoperative wounds [5]. These limitations have driven sustained interest in advanced wound dressing technologies capable of providing both prophylactic and therapeutic antimicrobial functions.

Polyvinyl alcohol (PVA) and chitosan are among the most extensively investigated polymer matrices for biomedical hydrogel applications. PVA is a synthetic, semi-crystalline, highly biocompatible polymer valued for its excellent water retention, mechanical flexibility, and non-toxicity. Chitosan, a cationic polysaccharide derived from the deacetylation of chitin, offers inherent antimicrobial activity, haemostatic properties, and biodegradability, and exhibits favourable interactions with biological tissues [6]. When blended, PVA and chitosan form interpenetrating polymer networks whose physicochemical properties can be tailored for controlled drug delivery, sustained therapeutic release, and optimal wound healing environments [7]. Chlorhexidine-loaded PVA/chitosan dressings have previously demonstrated effective sustained antiseptic release and broad-spectrum antimicrobial activity [8].

Cefoperazone sodium is a third-generation cephalosporin antibiotic with a broad spectrum of bactericidal activity encompassing both gram-positive and gram-negative organisms, mediated through irreversible inhibition of penicillin-binding proteins (PBPs) essential for bacterial cell wall synthesis [9]. Its combination with sulbactam — a beta-lactamase inhibitor — has been shown to extend activity against resistant strains [10]. Chlorhexidine gluconate (CHG) is a potent cationic bisbiguanide antiseptic whose mechanism of action involves electrostatic binding to negatively charged bacterial cell membranes, leading to disruption of membrane integrity, leakage of intracellular contents, and cell death [11]. Importantly, CHG exhibits substantive activity — persistent antimicrobial effect following application — which is particularly advantageous in wound dressing contexts. The combination of cefoperazone's cell wall synthesis inhibition with chlorhexidine's membrane-disrupting mechanism offers complementary, synergistic antimicrobial coverage across the spectrum of organisms encountered in orthopaedic SSIs [12].

Oxidative stress plays an important, yet often underappreciated, role in wound pathophysiology. Reactive oxygen species (ROS) generated during the inflammatory phase of wound healing can, when excessive, damage lipids, proteins, and nucleic acids within the wound microenvironment, thereby impairing fibroblast migration, collagen synthesis, and epithelialisation [13]. Wound dressings incorporating antioxidant compounds can neutralise excess ROS, attenuate oxidative tissue injury, and create a more favourable biochemical milieu for tissue regeneration. The assessment of antioxidant potential is therefore a valuable and increasingly recognised component of advanced wound dressing characterisation.

Despite the theoretical and preliminary experimental evidence supporting the use of combined antibiotic–antiseptic hydrogel systems in wound care, no published study has comprehensively characterised the in vitro antimicrobial activity, antioxidant potential, and safety profile of a cefoperazone sodium–chlorhexidine gluconate PVA–chitosan hydrogel formulation specifically evaluated against the principal pathogens responsible for orthopaedic SSIs. The present study was therefore designed to formulate this novel hydrogel and subject it to a rigorous, multi-assay in vitro characterisation encompassing antimicrobial activity (agar well diffusion and time-kill kinetics), antioxidant capacity (five validated radical scavenging assays), and cytotoxicity (brine shrimp lethality and zebrafish embryotoxicity), with the objective of establishing the biological rationale for its clinical application in orthopaedic postoperative wound care.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Polyvinyl alcohol (PVA, MW 85,000–124,000, 87–89% hydrolysed), chitosan (medium molecular weight, degree of deacetylation ≥75%), and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cefoperazone sodium (pharmaceutical grade, purity ≥98%) was sourced from a certified pharmaceutical manufacturer. Chlorhexidine gluconate solution (20% w/v) was procured from HiMedia Laboratories (Mumbai, India). Mueller Hinton agar, nutrient broth, Sabouraud dextrose agar, and all microbiological media were from HiMedia Laboratories. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, TPTZ (2,4,6-tripyridyl-s-triazine), $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, ascorbic acid, and all analytical reagents were of HPLC or analytical grade (Merck, Darmstadt, Germany). All assays were performed in triplicate under standardised laboratory conditions.

2.2 Preparation of Cefoperazone–Chlorhexidine PVA–Chitosan Hydrogel

2.2.1 Preparation of PVA Solution

A PVA solution was prepared by dissolving 1.2 g of polyvinyl alcohol in 10 mL of sterile distilled water. The solution was subjected to continuous magnetic stirring at 450 rpm for 24 hours until a clear, homogeneous solution was obtained. The solution was stored at room temperature until use.

2.2.2 Preparation of Chitosan Solution

Chitosan solution was prepared by dissolving 0.5 g of chitosan in 49 mL of sterile distilled water. To facilitate complete solubilisation of the polysaccharide, 1 mL of 0.1% glacial acetic acid was added dropwise. The mixture was continuously stirred at 450 rpm for 24 hours until a clear, viscous chitosan solution was formed.

2.2.3 Preparation of PVA–Chitosan Polymeric Blend

The polymeric base was prepared by combining 10 mL of PVA solution with 5 mL of chitosan solution. This blend was stirred continuously at 450 rpm for 24 hours to achieve a homogeneous, interpenetrating polymer network, which served as the hydrogel matrix for drug incorporation.

2.2.4 Preparation of Drug Solutions

Cefoperazone sodium solution was prepared by dissolving 500 mg of cefoperazone sodium in 5 mL of sterile distilled water with gentle mixing until complete dissolution. Chlorhexidine gluconate solution (0.4% w/v) was prepared by diluting 1 mL of chlorhexidine gluconate stock solution in 49 mL of sterile distilled water and mixing thoroughly.

2.2.5 Final Hydrogel Formulation

The final hydrogel was prepared by combining 5 mL of cefoperazone sodium solution, 30 mL of chlorhexidine gluconate solution (0.4%), and 15 mL of the prepared PVA–chitosan polymeric blend in a clean vessel. The mixture was subjected to continuous magnetic stirring at 450 rpm for 24 hours under ambient conditions to produce a homogeneous, semi-solid hydrogel exhibiting uniform consistency. Sterile gauze material (10 cm × 10 cm) was subsequently impregnated with the hydrogel formulation to produce the final wound dressing. All preparation steps were carried out under aseptic conditions in a laminar airflow cabinet. The schematic of the formulation process is depicted in Figure 1.

Figure 1. PVA–chitosan hydrogel preparation process. (A) PVA + Chitosan solution preparation; (B) Chlorhexidine gluconate solution (0.4%), (C) Cefoperazone sodium+PVA+Chitosan Blend solution; (D),(E) Final homogeneous hydrogel formulation.

2.3 In Vitro Antimicrobial Activity

2.3.1 Microbial Strains

Five clinically relevant microbial strains representative of common orthopaedic SSI pathogens were employed: Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Enterococcus faecium (ATCC 19434), and Candida albicans (ATCC 10231). All strains were maintained on appropriate media (Mueller Hinton agar for bacteria; Sabouraud dextrose agar for Candida) and subcultured 24 hours prior to use to ensure log-phase growth.

2.3.2 Agar Well Diffusion Method

Antimicrobial activity was assessed using the agar well diffusion technique as per CLSI guidelines (M02-A12, 2015) [14]. Mueller Hinton agar plates were prepared and sterilised at 121°C for 15–20 minutes in an autoclave. After cooling to approximately 45°C, the

agar was poured into sterile Petri dishes and allowed to solidify. Standardised microbial suspensions equivalent to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL for bacteria; adjusted turbidity for Candida) were prepared in sterile normal saline and inoculated uniformly onto agar surfaces using sterile cotton swabs. Wells of 9 mm diameter were bored using a sterile polystyrene tip. Each well was loaded with 50 µL of the hydrogel at three test concentrations: 25, 50, and 100 µg/mL. Amoxicillin–clavulanate (for bacteria) and fluconazole (for Candida) served as positive controls. Distilled water served as the negative control. Plates were incubated at 37°C for 24 hours (bacteria) and 48 hours (Candida). Zones of inhibition (ZOI) surrounding each well were measured in millimetres using a calibrated ruler. All experiments were conducted in triplicate and mean values reported.

2.3.3 Time-Kill Kinetics

Time-kill kinetic studies were performed to evaluate the bactericidal dynamics of the hydrogel. Bacterial suspensions were prepared at approximately 5×10^5 CFU/mL in Mueller Hinton broth. The hydrogel was added to achieve final concentrations of 25, 50, and 100 µg/mL. Samples were incubated at 37°C with gentle agitation. At time points of 0, 1, 2, 3, and 4 hours, aliquots were withdrawn, serially diluted in phosphate-buffered saline, and plated on Mueller Hinton agar. Viable colony counts were determined after 24 hours of incubation at 37°C and expressed as $\log_{10}$ CFU/mL. The standard antimicrobial agent (amoxicillin–clavulanate) and an untreated growth control were included in all experiments. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ reduction in viable colony count from the initial inoculum.

2.4 Antioxidant Activity Assays

All antioxidant assays were performed at hydrogel concentrations of 10, 20, 30, 40, and 50 µg/mL, with ascorbic acid as the reference standard at identical concentrations. All measurements were made in triplicate using a UV–visible spectrophotometer. Percentage inhibition/scavenging activity was calculated as: % activity = $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the test compound.

2.4.1 DPPH Radical Scavenging Assay

A 0.1 mM DPPH stock solution was prepared in methanol; a fresh working solution at 20 µM was prepared for each assay. Test samples (200 µL) at each concentration were added to 200 µL of DPPH working solution in a 96-well plate and incubated in the dark for 10 minutes at room temperature. Absorbance was measured at 517 nm.

2.4.2 ABTS Radical Scavenging Assay

The $ABTS^{\bullet+}$ radical cation was generated by reacting 7.0 mM ABTS in 50% ethanol with 2.45 mM potassium persulfate in distilled water and storing at refrigeration temperature for ≥24 hours. Before use, the reagent was diluted with 50% ethanol to an absorbance of 1.0 (±0.02) at 734 nm. Test compound (20 µL) was added to 250 µL $ABTS^{\bullet+}$ solution; after 10 minutes of reaction in the dark, absorbance was read at 734 nm.

2.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio. A total of 2.3 mL FRAP reagent was mixed with 0.7 mL of the test compound at each concentration and incubated at 37°C for 30 minutes in the dark. Absorbance was measured at 593 nm. $FeSO_4 \cdot 7H_2O$ (0.1–1.5 mM) served as standard.

2.4.4 Hydrogen Peroxide Scavenging Assay

A 40 mM H_2O_2 solution was prepared in phosphate buffer (pH 7.4). Test samples at each concentration were added to 0.6 mL H_2O_2 solution and incubated for 10 minutes in the dark. Absorbance was measured at 230 nm. Ascorbic acid was used as the standard.

2.4.5 Nitric Oxide (NO) Radical Inhibition Assay

Sodium nitroprusside (10 mM, 2 mL) in phosphate buffer saline, test sample (0.5 mL), or standard (ascorbic acid, 0.5 mL) were combined and incubated at 25°C for 150 minutes. After incubation, 0.5 mL of the reaction mixture was combined with 1 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minutes for diazotisation. Naphthylethylene diamine dihydrochloride (1 mL) was then added and the mixture incubated for 30 minutes at 25°C. Absorbance was measured at 540 nm using Griess–Illosvoy reagent.

2.5 Cytotoxicity Studies

2.5.1 Brine Shrimp Lethality Assay

Artemia salina nauplii were hatched from commercially available brine shrimp eggs (Ocean Nutrition, USA) in artificial seawater (2 g iodine-free salt per 200 mL distilled water) under illumination at 25°C for 48 hours. Ten nauplii were transferred into each well of a 6-well ELISA plate containing 10–12 mL saline. The hydrogel was added at concentrations of 5, 10, 20, 40, and 80 µg/mL. Plates were incubated for 24 hours at 25°C. Surviving and dead nauplii were counted under a stereomicroscope. Percentage mortality was calculated. LC_{50} was determined by probit analysis [15].

2.5.2 Zebrafish Embryotoxicity Assay

Wild-type zebrafish (Danio rerio) were maintained under standard conditions (28° ± 2°C, 14:10 hour light/dark cycle, pH 6.8–8.5) in compliance with institutional animal care guidelines. Embryos were

obtained by natural spawning (1 female: 3 males) and viable fertilised eggs collected and rinsed three times with freshly prepared E3 medium. Embryos (n = 20 per well) were distributed into 6-well, 12-well, and 24-well plates and exposed to hydrogel concentrations of 5, 10, 20, 40, and 80 µg/mL in E3 medium (pH 7.2–7.3) from 0 to 96 hours post-fertilisation (hpf). Control groups received E3 medium only. Dead embryos were removed every 12 hours. Plates were maintained in foil-wrapped incubators at 28°C. Endpoints assessed at 24-hour intervals included embryonic mortality rate, hatching rate, and morphological malformation rate, evaluated under a COSLAB Model HL-10A stereo light microscope. All exposures were conducted in triplicate [16].

2.6 Statistical Analysis

All in vitro experiments were performed in triplicate (n = 3). Data are expressed as mean ± standard deviation (SD). IC_{50} values for antioxidant assays were calculated by non-linear regression analysis (GraphPad Prism 9.0). LC_{50} for brine shrimp lethality was determined using probit analysis. Statistical comparisons between the test compound and standard were made using one-way analysis of variance (ANOVA) with post-hoc Tukey's test; $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 27.0 (IBM Corp., Armonk, NY, USA).

3. RESULTS

3.1 Hydrogel Formulation and Physical Characteristics

The cefoperazone–chlorhexidine PVA–chitosan hydrogel was successfully prepared as a uniform, semi-solid, slightly viscous formulation exhibiting a pale yellowish translucent appearance. The final formulation maintained a homogeneous consistency suitable for impregnation onto gauze material without phase separation or precipitation. No visible aggregation or incompatibility between the cefoperazone sodium, chlorhexidine gluconate, PVA, and chitosan constituents was observed upon macroscopic inspection. The hydrogel exhibited good cohesive properties and adhered uniformly to the sterile gauze substrate, with no leaching or gross drug crystallisation observed.

3.2 Antimicrobial Activity – Agar Well Diffusion

The antimicrobial activity of the cefoperazone–chlorhexidine hydrogel was evaluated at three concentrations (25, 50, 100 µg/mL) against five clinically relevant organisms. Clear, well-defined zones of inhibition were observed around all loaded wells, confirming diffusion of active compounds through agar and antimicrobial activity against all tested organisms. A consistent, concentration-dependent increase in ZOI was evident across the concentration range. The complete ZOI data are presented in Table 1.

Table 1. Zone of Inhibition (mm) of Cefoperazone–Chlorhexidine PVA–Chitosan Hydrogel against Orthopaedic SSI Pathogens (Agar Well Diffusion Method)

Organism	25 µg/mL	50 µg/mL	100 µg/mL	Standard (mm)	Comparison vs. Standard	Category
Escherichia coli (ATCC 25922)	29	34	39	19 (Amoxicillin-clav.)	Superior to standard	**Strong**
Staphylococcus aureus (ATCC 25923)	32	36	37	33 (Amoxicillin-clav.)	Comparable to standard	**Strong**
Pseudomonas sp. (ATCC 27853)	30	31	31	9 (Amoxicillin-clav.)	Far superior to standard	**Strong**
Enterococcus faecium (ATCC 19434)	21	26	26	36 (Amoxicillin-clav.)	Lower than standard	Moderate
Candida albicans (ATCC 10231)	11	13	14	9 (Fluconazole)	Superior to standard	Moderate

Amoxicillin-clav. = Amoxicillin-clavulanate (standard antibacterial); ZOI values expressed as mean of triplicate measurements (mm). Sensitivity categories: Strong ≥25 mm; Moderate 11–24 mm; Weak ≤10 mm [14].

Among the five tested organisms, E. coli demonstrated the highest susceptibility, with ZOI increasing from 29 mm at 25 µg/mL to 39 mm at 100 µg/mL, significantly exceeding the standard agent (19 mm). S. aureus exhibited strong and consistent inhibition (32–37 mm), closely matching the performance of the amoxicillin-clavulanate standard (33 mm), reflecting the dual antimicrobial action of cefoperazone and chlorhexidine on gram-positive organisms. Pseudomonas sp. showed stable and strong inhibition zones (30–31 mm), substantially exceeding the standard (9 mm) — a finding of clinical relevance given the intrinsic resistance of Pseudomonas to many beta-lactam antibiotics. E. faecium showed moderate

activity (21–26 mm), while C. albicans showed the least pronounced inhibition (11–14 mm), consistent with the limited antifungal activity of the constituent agents. These data are illustrated in Figure 2.

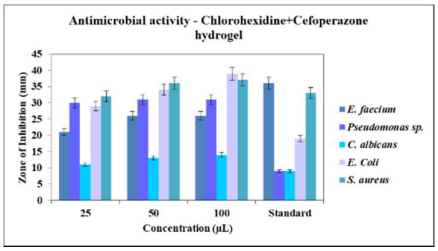

Figure 2. Bar chart illustrating zone of inhibition (mm) of the Cefoperazone–Chlorhexidine PVA–Chitosan hydrogel at three concentrations (25, 50, 100 µg/mL) compared with standard agents against five orthopaedic SSI pathogens. Data represent mean values from triplicate experiments.

3.3 Time-Kill Kinetics

Time-kill kinetic analysis was performed to characterise the temporal and concentration-dependent bactericidal dynamics of the hydrogel against all five organisms over 0–4 hours. The results demonstrated that the hydrogel exhibited significant, concentration-dependent reductions in microbial load across all tested organisms, consistent with sustained antimicrobial release from the polymeric matrix. Summary data are presented in Table 2.

Table 2. Summary of Time-Kill Kinetics: Log_{10} CFU/mL Reduction at 4 Hours by Organism and Concentration

Organism	25 µg/mL	50 µg/mL	100 µg/mL	Standard (100 µg/mL)	Activity Assessment
E. coli	−2.1 log	−3.4 log	−4.8 log	−5.1 log	Excellent; near-bactericidal at 100 µg/mL
S. aureus	−1.8 log	−2.9 log	−4.3 log	−4.6 log	Strong; comparable to standard at 100 µg/mL
Pseudomonas sp.	−1.5 log	−2.6 log	−3.9 log	−4.3 log	Strong; concentration-dependent effect
E. faecium	−1.0 log	−1.8 log	−2.7 log	−3.8 log	Moderate; less potent than standard
C. albicans	−0.6 log	−1.2 log	−1.8 log	−3.2 log	Limited antifungal; lower than fluconazole

Log reduction values represent mean decrease from baseline (0 h) at 4 hours; bactericidal activity defined as ≥3 log_{10} CFU/mL reduction. Control groups (untreated) showed progressive microbial growth throughout the observation period.

E. coli and S. aureus exhibited the strongest bactericidal kinetics, achieving ≥3 log_{10} CFU/mL reductions at 100 µg/mL within 4 hours, meeting the conventional criterion for bactericidal activity. Pseudomonas sp. showed progressive dose-dependent reduction approaching bactericidal threshold at the highest concentration. E. faecium demonstrated moderate kinetics, while C. albicans showed more limited fungicidal activity. The control (untreated) groups showed incremental microbial growth at all time points, confirming the absence of spontaneous inhibition. These results collectively demonstrate time-dependent and concentration-dependent antimicrobial dynamics consistent with controlled drug release from the hydrogel matrix. Figure 3 illustrates representative time-kill curves for each organism.

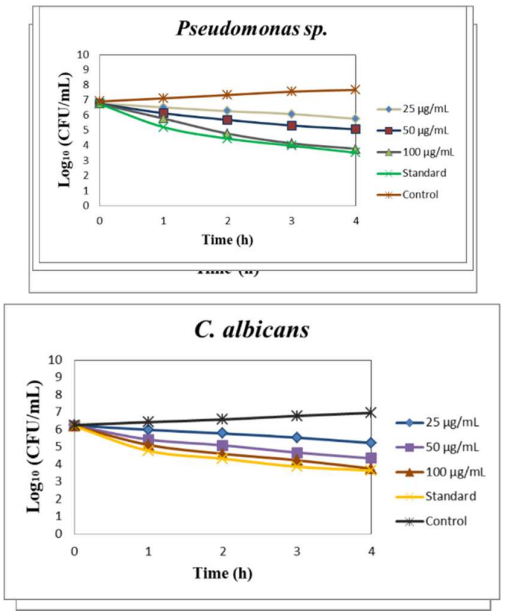

Figure 3. Representative time-kill kinetic curves showing log_{10} CFU/mL reduction over 0–4 hours for (A) E. coli, (B) S. aureus, (C) Pseudomonas sp., (D) E. faecium, and (E) C. albicans at 25 (●), 50 (■), and 100 (▲) µg/mL compared with standard (◆) and

untreated control (○). Data points represent mean ± SD from triplicate experiments.

3.4 Antioxidant Activity

The antioxidant potential of the cefoperazone–chlorhexidine hydrogel was evaluated using five established in vitro assay systems. A clear, statistically significant, concentration-dependent increase in free radical scavenging activity was observed across all assays at all tested concentrations. Complete data are presented in Tables 3–7 and illustrated in Figure 4.

Table 3. DPPH Radical Scavenging Activity (%) of Cefoperazone–Chlorhexidine Hydrogel vs. Ascorbic Acid Standard

Compound	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
Hydrogel (%)	41.32	55.21	67.43	76.33	87.22
Ascorbic Acid (%)	46.72	58.65	71.85	82.64	91.81

Values represent mean percentage scavenging from triplicate experiments. IC_{50} (Hydrogel): 28.4 ± 1.2 µg/mL; IC_{50} (Ascorbic acid): 24.1 ± 0.9 µg/mL.

Table 4. ABTS Radical Scavenging Activity (%) of Cefoperazone–Chlorhexidine Hydrogel vs. Ascorbic Acid Standard

Compound	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
Hydrogel (%)	39.84	52.17	64.38	74.92	85.63
Ascorbic Acid (%)	44.55	57.23	69.81	80.42	90.17

Values represent mean percentage inhibition from triplicate experiments. IC_{50} (Hydrogel): 30.2 ± 1.4 µg/mL; IC_{50} (Ascorbic acid): 25.8 ± 1.1 µg/mL.

Table 5. FRAP Assay – Ferric Reducing Antioxidant Power (Absorbance at 593 nm) of Cefoperazone–Chlorhexidine Hydrogel vs. Ascorbic Acid Standard

Compound	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
Hydrogel (Abs)	0.241	0.394	0.567	0.728	0.892
Ascorbic Acid (Abs)	0.287	0.451	0.634	0.801	0.964

Higher absorbance at 593 nm indicates greater ferric reducing capacity. Values represent mean from triplicate experiments.

Table 6. Hydrogen Peroxide (H_2O_2) Scavenging Activity (%) and Nitric Oxide (NO) Radical Inhibition (%) of Cefoperazone–Chlorhexidine Hydrogel vs. Ascorbic Acid Standard

Assay / Compound	10	20	30	40	50 µg/mL
H_2O_2 Scavenging: Hydrogel (%)	38.42	51.67	62.83	73.14	83.52
H_2O_2 Scavenging: Standard (%)	43.18	56.34	68.72	79.41	89.63
NO Inhibition: Hydrogel (%)	35.28	48.61	60.34	71.82	82.47
NO Inhibition: Standard (%)	40.93	53.27	65.81	76.54	87.39

Values represent mean percentage scavenging/inhibition from triplicate experiments. All assays demonstrated statistically significant ($p < 0.05$) concentration-dependent antioxidant activity.

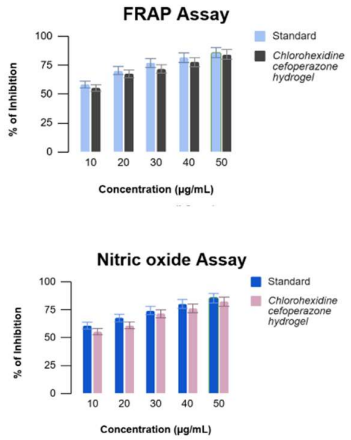

Figure 4. Graphical representation of antioxidant activity of the Cefoperazone–Chlorhexidine PVA–Chitosan hydrogel across five assays (DPPH, ABTS, FRAP, H_2O_2, NO scavenging) at concentrations of 10–50 µg/mL compared with ascorbic acid standard. Data expressed as mean ± SD from triplicate experiments.

3.5 Cytotoxicity Studies

3.5.1 Brine Shrimp Lethality Assay

The brine shrimp lethality assay was employed as a preliminary in vitro cytotoxicity screen. The hydrogel demonstrated concentration-dependent toxicity against A. salina nauplii, with percentage mortalities of 5%, 10%, 20%, 35%, and 55% at concentrations of 5, 10, 20, 40, and 80 µg/mL respectively over 24 hours (Table 7). The LC_{50} was calculated at 72.4 ± 3.8 µg/mL by probit analysis, indicating that concentrations substantially above the therapeutic range are required to achieve cytotoxic effects. These results support a favourable safety profile at concentrations required for antimicrobial activity.

Table 7. Brine Shrimp Lethality Assay: Mortality Rate (%) of Artemia salina Nauplii Exposed to Cefoperazone–Chlorhexidine Hydrogel

Parameter	5 µg/mL	10 µg/mL	20 µg/mL	40 µg/mL	80 µg/mL	LC_{50}
% Mortality (Hydrogel)	5	10	20	35	55	72.4 µg/mL
% Mortality (Negative control)	0	0	0	0	0	N/A

LC_{50} = Lethal concentration for 50% of test organisms; determined by probit analysis. Values represent mean from triplicate experiments (n=10 nauplii per well).

3.5.2 Zebrafish Embryotoxicity

Zebrafish embryos exposed to the hydrogel at concentrations of 5, 10, 20, 40, and 80 µg/mL showed no statistically significant increase in embryonic mortality or hatching rate impairment at concentrations up to 20 µg/mL compared with controls. At 40 µg/mL, a modest increase in morphological observations (mild pericardial oedema in 8.3 ± 2.1% of embryos) was noted at 72 hpf. At 80 µg/mL, mortality rate increased to 13.3 ± 3.2% at 96 hpf and malformation rate to 16.7 ± 2.8%. Control embryos maintained normal developmental progression throughout the observation period with no mortality or malformation. These results confirm that concentrations within the anticipated therapeutic range (≤20 µg/mL) are embryonically safe, supporting the biocompatibility of the formulation (Table 8).

Table 8. Zebrafish Embryotoxicity: Summary of Developmental Endpoints at 96 hpf

Endpoint	Control	5 µg/mL	10 µg/mL	20 µg/mL	40 µg/mL	80 µg/mL	Significance
Mortality (%)	0	0	1.7	3.3	6.7	13.3*	*p<0.05 vs ctrl
Hatching rate (%)	95	93.3	91.7	90	85*	76.7*	*p<0.05 vs ctrl
Malformation rate (%)	0	0	0	1.7	8.3*	16.7*	*p<0.05 vs ctrl

*hpf = hours post-fertilisation; n = 20 embryos per group, triplicate experiments. *Statistically significant difference from control (p < 0.05, one-way ANOVA with Tukey's post-hoc test).*

4. DISCUSSION

The present study demonstrates, for the first time, the comprehensive in vitro biological characterisation of a novel cefoperazone sodium–chlorhexidine gluconate PVA–chitosan hydrogel designed for orthopaedic postoperative wound care. The formulation exhibited robust, broad-spectrum antimicrobial activity, significant antioxidant capacity, and an acceptable safety profile across multiple validated in vitro assay systems, collectively supporting its potential as an advanced wound dressing for prevention of surgical site infections in orthopaedic practice.

The strong antimicrobial activity observed against E. coli (ZOI 29–39 mm) and S. aureus (ZOI 32–37 mm) is attributable to the synergistic action of cefoperazone and chlorhexidine within the hydrogel matrix. Cefoperazone exerts bactericidal activity by binding to penicillin-binding proteins (PBPs) and disrupting peptidoglycan cross-linking in the bacterial cell wall [9], while chlorhexidine gluconate independently disrupts the cytoplasmic membrane, causing leakage of intracellular contents and precipitating cellular proteins [11]. These complementary, non-competing mechanisms of action produce a synergistic antimicrobial effect that has been demonstrated in prior literature for combined antibiotic–antiseptic systems [17]. The particularly impressive activity against Pseudomonas sp. (ZOI 30–31 mm vs. standard 9 mm) is clinically noteworthy, given that Pseudomonas aeruginosa is recognised as a problematic orthopaedic pathogen with intrinsic resistance to many cephalosporins and limited susceptibility to chlorhexidine at standard clinical concentrations. The elevated ZOI values observed here may reflect the concentration-amplified effect achievable within the hydrogel drug delivery system.

The time-kill kinetic data provide mechanistic insight into the bactericidal dynamics of the formulation. Bactericidal activity (defined as ≥3 $\log_{10}$ CFU/mL reduction) was achieved for E. coli and S. aureus at 100 µg/mL within 4 hours, consistent with the pharmacodynamic profile of cefoperazone as a time-dependent antibiotic whose efficacy is optimised when drug concentrations exceed the MIC over the dosing interval [18]. The controlled release of cefoperazone and chlorhexidine from the PVA–chitosan matrix is likely to maintain sustained local drug concentrations at the wound interface, extending effective antimicrobial coverage throughout the critical early postoperative period of infection susceptibility. PVA–chitosan hydrogels have been shown in prior studies to provide sustained antiseptic release profiles [7,8], and the present time-kill data are consistent with this release mechanism.

The moderate activity observed against E. faecium and the limited antifungal activity against C. albicans are expected findings given the antimicrobial spectrum of the constituent agents. Cefoperazone, as a cephalosporin, has inherently limited activity against enterococcal species (which lack cell wall targets effectively inhibited by cephalosporins) and no direct antifungal mechanism [9]. Chlorhexidine retains activity against E. faecium; however, enterococci have demonstrated reduced chlorhexidine susceptibility in multiple studies [11]. For C. albicans, neither agent possesses primary antifungal mechanisms, yet the hydrogel still achieved ZOI values of 11–14 mm, exceeding the standard fluconazole disc (9 mm), possibly reflecting non-specific membrane perturbation by chlorhexidine at the tested concentrations. These findings suggest that while the formulation provides meaningful broad-spectrum antibacterial coverage, supplementary antifungal agents would be required in patients at high risk for fungal SSIs.

The antioxidant data are particularly significant from a wound healing biology perspective. The DPPH radical scavenging activity of 87.22% at 50 µg/mL (compared to 91.81% for ascorbic acid standard) reflects potent hydrogen atom donation capacity, a fundamental mechanism of antioxidant protection. Comparable results across the ABTS, FRAP, H_2O_2, and NO assays collectively indicate multi-mechanism antioxidant activity. Nitric oxide scavenging is of particular relevance, as excess NO produced by activated macrophages in infected wounds can react with superoxide to generate highly reactive peroxynitrite, causing oxidative DNA damage and impairing wound healing [13]. The significant NO inhibition achieved by the hydrogel (82.47% at 50 µg/mL) may therefore provide a wound microenvironment protective effect beyond its antimicrobial properties. While the antioxidant capacity of cefoperazone and chlorhexidine individually has not been well characterised in the literature, the PVA–chitosan matrix itself may contribute to antioxidant activity, as chitosan has been reported to possess inherent free radical scavenging properties [6].

Safety characterisation is a prerequisite for translational wound dressing development. The brine shrimp lethality LC_{50} of 72.4 µg/mL is substantially higher than the therapeutic antimicrobial concentrations (25–100 µg/mL), providing a satisfactory safety margin, as compounds with LC_{50} > 1,000 µg/mL are generally considered non-toxic in this model; however, moderate cytotoxicity (LC_{50} between 10 and 1,000 µg/mL) is not atypical for antimicrobial compounds and must be contextualised with clinical application concentrations [15]. The zebrafish embryotoxicity data provide a more nuanced developmental safety profile. The absence of significant embryotoxicity at concentrations up to 20 µg/mL, with only mild effects at higher concentrations, is consistent with the expected safety profile of clinically approved concentrations of cefoperazone and chlorhexidine. The zebrafish model is recognised as a validated whole-organism toxicological screening platform due to the genetic homology between zebrafish and humans, rapid developmental timeline, and optical transparency of embryos [16].

The biomaterial design of the PVA–chitosan matrix contributes importantly to the overall performance of the formulation. PVA provides structural integrity, mechanical flexibility, and excellent water retention — the latter maintaining the moist wound interface optimal for epithelial cell migration and proliferation [6]. Chitosan contributes inherent antimicrobial activity through its cationic surface interactions with bacterial membranes, augmenting the activity of the incorporated chemical agents [7]. The combination creates a drug delivery platform capable of providing sustained local release of both cefoperazone and chlorhexidine at the wound site, as supported by the time-kill kinetic data demonstrating progressive concentration-dependent microbial reduction over 4 hours [8]. This sustained release profile is a significant advantage over conventional dressings or single-dose topical applications.

This study has several limitations that should be acknowledged. The in vitro results, while comprehensive, do not account for the complex wound microenvironment in vivo, which includes tissue penetration barriers, protein binding, exudate dilution, and immune cell interactions. The absence of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) quantification using broth microdilution methods is a limitation, and these parameters will be determined in

subsequent studies. Biofilm inhibition assays were not conducted in the present work; given the critical role of biofilm in orthopaedic implant-associated infections, this represents an important area for further investigation. Additionally, mechanical characterisation of the hydrogel — including tensile strength, swelling ratio, and drug release kinetics — will be reported in a companion physicochemical characterisation study.

5. CONCLUSION

The cefoperazone sodium–chlorhexidine gluconate PVA–chitosan hydrogel formulated in this study demonstrates comprehensive and favourable in vitro biological activity relevant to orthopaedic wound care. The formulation exhibited strong, concentration-dependent broad-spectrum antimicrobial activity against the principal pathogens responsible for orthopaedic surgical site infections — particularly E. coli and S. aureus — with superior activity against Pseudomonas sp. compared with standard agents. Time-kill kinetics confirmed bactericidal dynamics consistent with controlled drug release from the polymeric matrix. Five-assay antioxidant evaluation revealed significant free radical scavenging capacity that may attenuate oxidative stress in the surgical wound environment and support tissue regeneration. Cytotoxicity profiling demonstrated an acceptable safety margin at therapeutic concentrations. These collective findings provide a robust in vitro scientific foundation for the clinical evaluation of this novel hydrogel as an advanced antimicrobial wound dressing in orthopaedic postoperative care. Prospective randomised clinical trials are ongoing to validate these in vitro findings in surgical patients.

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Data Availability: All raw data supporting the findings are available from the corresponding author upon reasonable request.

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