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Article

Synergistic Reduction of *Staphylococcus aureus* Biofilms Using Povidone Iodine and Hydrogen Peroxide

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Abstract: *Staphylococcus aureus*, particularly its methicillin-resistant strains (MRSA), poses significant challenges in healthcare settings due to its propensity to form biofilms on various surfaces. These biofilms enhance bacterial survival and increase resistance to conventional treatments, complicating infection control efforts. This study investigates the efficacy of a novel approach combining povidone-iodine (PVP-I) and hydrogen peroxide (H₂O₂) to combat *Staphylococcus aureus* biofilm formation in hospital environments. A range of methodologies were employed, including biofilm formation assays, gene expression analysis, colony-forming unit (CFU) enumeration, and confocal microscopy, to assess the effects of PVP-I and H₂O₂, both individually and in combination. The results demonstrated a synergistic effect when these agents were combined, significantly reducing biofilm formation compared to the use of either compound alone. PVP-I, an iodine-based antiseptic, and H₂O₂, a potent oxidizing agent, each exhibit potential in disrupting bacterial biofilms when used independently. However, their combined application appears to enhance penetration into the biofilm matrix and increase overall antimicrobial activity. These findings have important implications for infection control strategies in healthcare settings. By harnessing the complementary mechanisms of PVP-I and H₂O₂, this approach offers a promising solution for preventing and eradicating biofilm-associated infections on medical devices and hospital surfaces. Additionally, the use of two agents with different modes of action may help mitigate the risk of developing antimicrobial resistance. This study lays the groundwork for future research aimed at optimizing concentrations, application methods, and assessing the long-term efficacy and safety of this combined approach. Ultimately, this research contributes to improved patient outcomes and a reduction in the economic burden associated with biofilm-related complications.

Keywords: *Staphylococcus aureus*; povidone-iodine (PVP-I); hydrogen peroxide (H₂O₂); biofilms; anti-biofilm activity

1. Introduction

Periprosthetic joint infection (PJI) represents one of the most severe complications following arthroplasty joint replacement (AJR) [1]. Bacterial pathogens are responsible for over 98% of PJIs, with *Staphylococcus aureus* and coagulase-negative staphylococci accounting for 50% to 60% of all cases [2,3]. *Staphylococcus aureus* (*S. aureus*) is the most prevalent cause of infections related to implanted biomaterials [4]. Particularly, methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a significant global health challenge in hospital settings. This Gram-positive bacterium is a leading cause of hospital-associated infections (HAIs), contributing substantially to patient morbidity, mortality, and escalating healthcare costs worldwide. The prevalence of MRSA varies considerably across different countries and regions. According to the World Health

Organization's (WHO) 2014 report on antimicrobial resistance, MRSA prevalence in hospitals exceeded 20% in all regions, with some areas reporting infection rates as high as 80%. In South Korea, the burden of hospital-acquired *Staphylococcus aureus* bloodstream infections (SA-BSI) is notably high. A national study conducted in 2011 estimated the incidence of hospital-acquired MRSA-BSI at 0.12 cases per 1,000 patient-days, compared to 0.04 cases per 1,000 patient-days for methicillin-sensitive *Staphylococcus aureus* bloodstream infections (MSSA-BSI). This translates to approximately 2,946 cases of MRSA-BSI and 986 cases of MSSA-BSI annually in hospitals with more than 500 beds. The mortality rate associated with these infections is alarmingly high, with MRSA-BSI at 31.9% and MSSA-BSI at 14.1%. The economic impact of these infections is also significant, with the additional cost per case estimated at \$20,494 for MRSA-BSI and \$6,914 for MSSA-BSI, leading to an annual total cost exceeding \$67.2 million. In recent years, MRSA prevalence in South Korean hospitals has increased, with MRSA accounting for 60-70% of all *Staphylococcus aureus* infections. This high prevalence underscores the urgent need for effective infection control measures within healthcare facilities to mitigate the spread and impact of MRSA [5]. Given the substantial clinical and economic burden of *Staphylococcus aureus* infections in South Korean hospitals, there is an urgent need to develop innovative strategies for the prevention and control of these infections. The use of povidone-iodine (PVP-I) and hydrogen peroxide (H_2O_2) has shown considerable potential in addressing these infections and offers several advantages over traditional methods. Iodine-based compounds, such as PVP-I, have long been recognized for their broad-spectrum antimicrobial activity. They are effective against a wide range of pathogens, including antibiotic-resistant strains like MRSA. These agents act by releasing free iodine, which penetrates microbial cell walls and disrupts critical cellular processes, leading to bacterial death. Their efficacy in reducing biofilm formation makes them particularly valuable in hospital settings [6,7]. Biofilms are complex systems comprising sessile bacteria encased within an extracellular polymeric substance (EPS), which serves as the structural scaffold of the biofilm. EPS also acts as a physical and chemical barrier, making it difficult for antibiotics to penetrate the biofilm, thus providing a protective shield against antimicrobial agents [8,9]. Hydrogen peroxide is a potent oxidizing agent that generates free radicals, causing oxidative stress and damage to bacterial cells. Research indicates that H_2O_2 is effective against various hospital pathogens, including *Staphylococcus aureus*. The ability of H_2O_2 to penetrate biofilms, along with its relatively low toxicity to human cells at appropriate concentrations, makes it an attractive option for infection control [10]. The combination of iodine-based compounds with H_2O_2 provides a synergistic approach to infection prevention and control [11–13]. This dual strategy has the potential to overcome the limitations of monotherapy, offering a more comprehensive solution to combat *Staphylococcus aureus* infections [14]. By targeting different aspects of bacterial survival and proliferation, this combination may enhance overall efficacy and reduce the risk of resistance development. Moreover, both agents are cost-effective and readily available, making them practical for implementation in hospital infection control programs. Their use could significantly reduce infection rates, thereby lowering patient morbidity and mortality, shortening hospital stays, and alleviating the economic burden on healthcare systems. This study investigates the effects of PVP-I and H_2O_2 , both individually and in combination, on the eradication of *Staphylococcus aureus* biofilm formation. By leveraging the complementary mechanisms of these two agents, we aim to develop a more effective strategy to combat biofilm-associated infections in hospital environments, ultimately improving patient outcomes and reducing the economic burden associated with these infections.

2. Results

2.1. Morphological Analysis of *Staphylococcus aureus*

Staphylococcus aureus was analyzed using Mannitol Salt Agar (MSA) and Gram staining. As a Gram-positive bacterium, *S. aureus* appeared purple under the microscope and exhibited a grape-like cluster arrangement, which is crucial for distinguishing it from other bacterial species. MSA is both a selective and differential medium used to isolate and identify *S. aureus*. It contains mannitol,

which *S. aureus* can ferment. The fermentation process produces acid, lowering the pH and changing the color of the phenol red indicator, resulting in yellow colonies. This color change differentiates *S. aureus* from other non-mannitol fermenting staphylococci, such as coagulase-negative staphylococci, which form pink colonies on MSA. These morphological and biochemical tests are critical for diagnosing and managing infections in clinical microbiology (Figure 1).

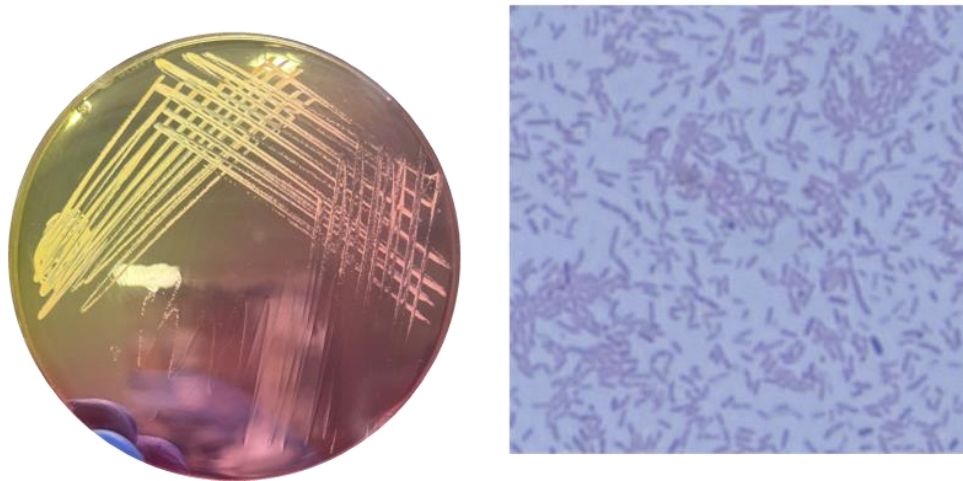


Figure 1. Morphology of *Staphylococcus aureus* in MSA medium.

2.2. Evaluation of PVP-I and H_2O_2 Effectiveness on Biofilm Removal

The effectiveness of PVP-I and H_2O_2 , both alone and in combination, in removing biofilms was assessed using a biofilm formation assay. After biofilm formation, the metal discs were treated with these agents. Quantitative analysis showed a slight reduction in biofilm biomass with individual treatments of PVP-I and H_2O_2 compared to the untreated control. However, the combination treatment with PVP-I and H_2O_2 led to a significant reduction in biofilm quality, indicating a synergistic effect. This combined treatment was notably more effective, highlighting its potential as a strategy for managing biofilm-related infections (Figure 2).

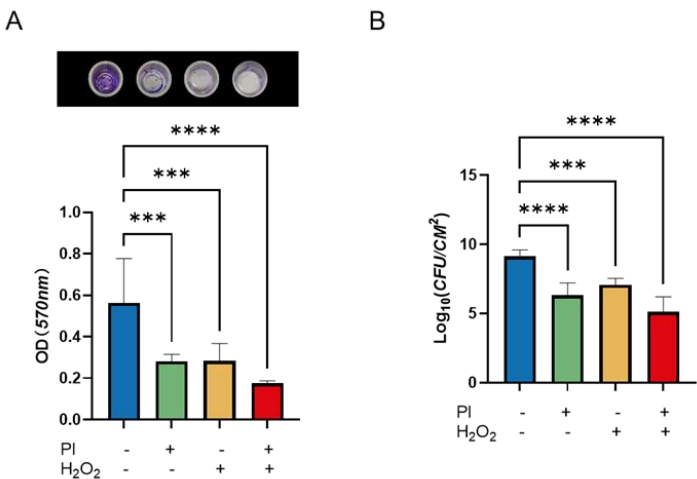


Figure 2. Biofilm formation Assay showing the reduction of biofilm in PI and H₂O₂ treated groups. Data are presented as the mean $\pm$ standard deviation ($n = 3$). The treatment group showed an approximate three-log reduction in CFU/cm² compared to the control group. Statistical significance is indicated as follows: ns = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to the control group.

2.3. Expression of Biofilm-Associated Genes Under PVP-I and H₂O₂ Treatment

The study also investigated the impact of PVP-I and H₂O₂ treatment on the expression of biofilm-associated genes. The levels of these genes were analyzed to understand their role in biofilm formation and maintenance under oxidative stress conditions. Results indicated that PVP-I and H₂O₂ treatment significantly modulated the expression of key biofilm-related genes, affecting biofilm structural integrity and resistance characteristics. These findings provide insights into the molecular mechanisms by which these agents influence biofilm dynamics and suggest potential therapeutic strategies for biofilm-associated infections (Figure 3).

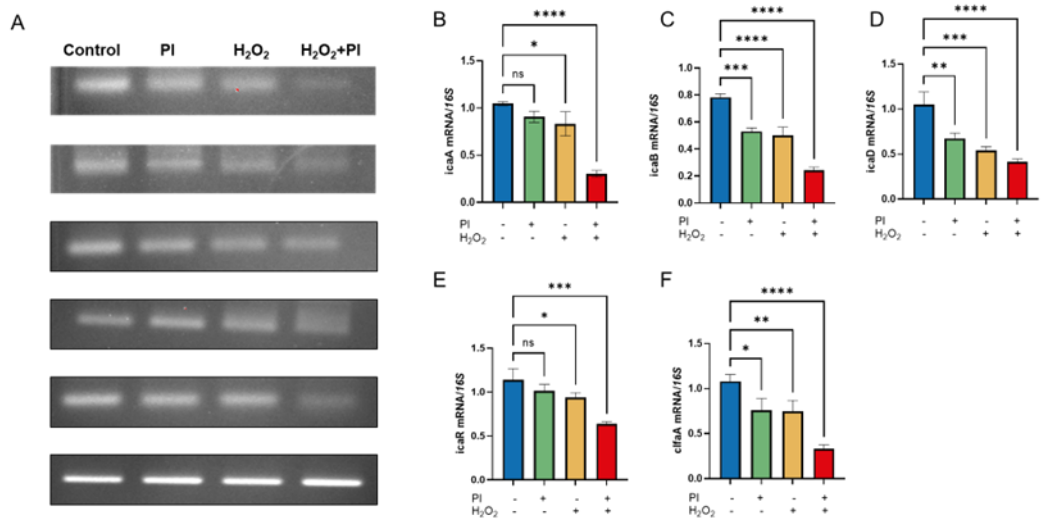


Figure 3. (A) Expression levels of biofilm-associated genes (icaABDR, ClfA) demonstrate a reduction in the PI and H₂O₂ treated groups (B) followed by quantitative analysis. Data are expressed as mean $\pm$ standard deviation (n = 3). Statistical significance is indicated as follows: ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 compared to the control group.

2.4. Live/Dead Assay of Biofilms Treated with PVP-I and H₂O₂

A Live/Dead assay was conducted to evaluate the effectiveness of PVP-I and H₂O₂, both individually and in combination, in removing biofilms. After treatment, the biofilms were assessed for viability. Quantitative analysis revealed a slight reduction in biofilm biomass with individual treatments of PVP-I and H₂O₂ compared to the untreated control. The Live/Dead assay demonstrated that both treatments increased the proportion of dead cells, as evidenced by an increase in red fluorescence. However, the combination treatment with PVP-I and H₂O₂ showed a more pronounced effect, significantly increasing the number of dead cells and thereby more effectively reducing biofilm viability. These findings underscore the potential of combining PVP-I and H₂O₂ in managing biofilm-related infections (Figure 4).

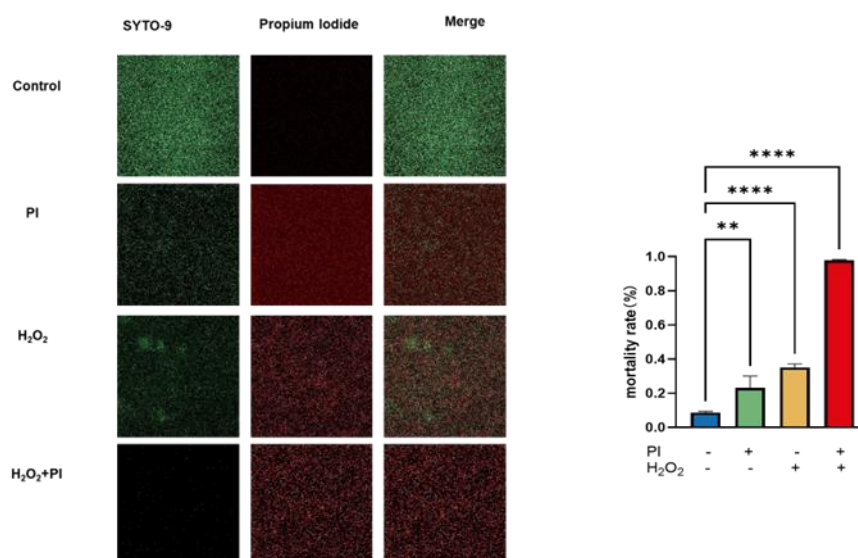


Figure 4. Confocal microscopy images from a Live/Dead assay demonstrate a reduction in biofilm viability in the PI and H₂O₂ treated groups, indicating increased cell death within the biofilms. Images were captured at 40x magnification with a scale bar of 100 μ m. Statistical significance is indicated as follows: ns = non-significant, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared to the control group.

3. Discussion

Biofilms pose a significant risk in hospital settings due to their ability to form on various surfaces and medical devices, leading to increased infection rates. Periprosthetic joint infections (PJI) are among the most severe complications following joint replacement surgeries[15–17]. The unique structure of biofilms makes it challenging for disinfectants like PVP-I to penetrate and eradicate the biofilm, often resulting in diminished antimicrobial effectiveness. Studies indicate that biofilm resistance to antimicrobial agents can be 100 to 1,000 times greater than that of planktonic bacteria[8,18]. Biofilms provide a protective environment for bacteria, rendering conventional antimicrobial treatments ineffective and leading to chronic infections. The prevalence of biofilm-forming microorganisms in hospital environments is high, with *Staphylococcus aureus*, coagulase-negative staphylococci, and *Pseudomonas aeruginosa* exhibiting biofilm formation rates of 100%[19]. This high prevalence underscores the clinical challenge posed by biofilms, which complicates patient management and infection control efforts. Continuous research and development of effective biofilm prevention and removal techniques are essential. In hospital settings, PVP-I and hydrogen peroxide have been explored as alternative strategies for removing infections caused by *Staphylococcus aureus*, including methicillin-resistant strains (MRSA). However, the efficacy of these agents in completely removing biofilms remains a challenge.

Recent studies provide compelling evidence of PVP-I and polyhexamethylene biguanide (PHMB) showing strong antibiofilm activity in vitro[20]. Both agents demonstrated significant activity against biofilms formed by various microorganisms, including MRSA and *Pseudomonas aeruginosa*. Notably, PVP-I exhibited a faster action against MRSA biofilms compared to PHMB, as evidenced by colony-forming unit (CFU) counts and microscopy analysis. Additionally, another study demonstrated that the combination of PVP-I and vancomycin effectively reduced early biofilm formation by MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) on titanium surfaces [21]. Research indicates that a 5-minute exposure to PVP-I can reduce CFU/cm² of *S. aureus* biofilm on prosthetic materials by approximately three orders of magnitude. This reduction was confirmed by confocal imaging and crystal violet staining. The study also noted reduced expression of biofilm-related genes such as *icaACDR* and *clfA*, suggesting that PVP-I effectively diminishes biofilm formation by affecting bacterial adhesion and virulence[6]. Some studies have shown that hydrogen peroxide can downregulate the expression of biofilm-related genes at certain stages of biofilm

development, such as the *sod* gene in *Pseudomonas aeruginosa*. However, the effectiveness of hydrogen peroxide in completely removing biofilms formed by *S. aureus* on infected implants is inconsistent. A recent study demonstrated that sustained application of hydrogen peroxide could effectively control biofilm growth and remove biofilms from dental water systems [22].

This study addresses the persistent challenge posed by biofilms in medical environments, particularly MRSA. These biofilms not only enhance bacterial survival but also confer resistance to conventional antimicrobial treatments, complicating infection control efforts. Our research explored the efficacy of combining PVP-I and H_2O_2 as a novel strategy for combating *S. aureus* biofilm formation. The results indicate a synergistic effect between PVP-I and H_2O_2 that surpasses the effectiveness of either agent alone. This synergy may stem from their complementary mechanisms: PVP-I, an iodine-based antiseptic, disrupts bacterial cell walls [23], while H_2O_2 , a potent oxidizer, generates reactive oxygen species that damage the biofilm's organic matrix, leading to its breakdown. The release of reactive oxygen species: H_2O_2 decomposes upon contact with biofilms to produce oxygen and water, while releasing highly reactive oxygen species. These species further damage microbial cells and organic matter within the biofilm. Disruption of microbial cells: The oxidative action of H_2O_2 and released reactive oxygen species can damage microbial cell membranes and internal structures, resulting in cell death. Physical detachment: As the organic matter and microorganisms within the biofilm are damaged, the biofilm structure becomes loosened, facilitating physical removal (e.g., washing)[24]. Therefore, theoretically, using PVP-I after H_2O_2 disrupts the biofilm structure can kill more bacteria[25,26]. The combined treatment likely enhances penetration into the biofilm matrix, increasing overall antimicrobial activity and effectively reducing biofilm biomass. These findings have significant implications for infection control practices in hospital environments. Utilizing the complementary mechanisms of PVP-I and H_2O_2 provides a promising approach to addressing biofilm-related infections on medical devices and hospital surfaces. Employing agents with different mechanisms of action may also help reduce the risk of antimicrobial resistance development, a key concern in managing biofilm-related infections. Although the results are promising, further research is needed to optimize concentrations, application methods, and evaluate the long-term efficacy and safety of this combination approach. Additionally, exploring the impact of this treatment on different *S. aureus* strains and other biofilm-forming pathogens could expand its applicability.

4. Materials and Methods

4.1. Bacterial Strains

The study utilized *Staphylococcus aureus* (*S. aureus*) strains. Representative colonies were obtained from a specified source and suspended in Tryptic Soy Broth (TSB; Fushenbio, Shanghai, China). The suspension was incubated overnight at 37°C with shaking at 200 rpm. After incubation, the bacterial suspension was collected and adjusted to a turbidity equivalent to the 1 McFarland standard. The suspension was then diluted 1:300 to achieve an approximate final cell concentration of 1×10^6 CFU/mL.

4.2. Biofilm Formation Assay

To prepare the overnight culture of *S. aureus*, the bacteria were cultured in TSB supplemented with 0.2% glucose. After adjusting the bacterial suspension, metal discs were placed in a 24-well tissue culture plate, with each well receiving 2 mL of the bacterial suspension to ensure complete coverage of the metal discs. The plates were incubated at 37°C with 5% CO_2 for 48 hours[27].

4.3. Treatment Procedures

After incubation, the metal discs were removed from the bacterial suspension and divided into four groups. Following multiple preliminary experiments, the final study employed 1% povidone-iodine (PVP-I) and 3.5% hydrogen peroxide (H_2O_2). The metal discs were exposed to either PBS, 1%

PVP-I, or 3.5% H₂O₂ for 5 minutes. For the combination treatment, the discs were first exposed to 3.5% H₂O₂ for 5 minutes, then transferred to 1% PVP-I for an additional 5 minutes. The experiment was repeated at least five times.

4.4. Colony-Forming Unit (CFU) Enumeration

Following treatment, the metal discs were placed in 1 mL of sterile PBS and subjected to ultrasonic treatment at 35 kHz for 15 minutes, with 10-second shaking intervals every 5 minutes. Serial tenfold dilutions were prepared, and 50 µL of the appropriately diluted samples were plated on Mannitol Salt Agar (MSA). The plates were incubated at 37°C for 24 hours, and CFU counts were determined by colony enumeration.

4.5. Biofilm Detection

After treatment with PVP-I, H₂O₂, and PBS, the wells were washed twice with PBS to remove any loosely attached bacteria. For biofilm staining, each metal disc was stained with 1 mL of 0.1% crystal violet solution. The plates were incubated at room temperature for 25 minutes. After staining, the dye was removed, and the discs were washed three times with PBS and dried at 37°C. The biofilm was then dissolved in 1 mL of 95% ethanol. Absorbance was measured at $\lambda = 590$ nm using a microplate reader, with the experiment repeated eight times[28].

4.6. Gene Expression Analysis (PCR)

Previous studies have linked PVP-I's biofilm inhibition to decreased transcription of the *icaADBC* operon and activation of *icaA* transcription repression in *S. aureus* [29–32]. Total RNA was extracted, and 0.5 µg of total RNA was used to synthesize first-strand cDNA in a 20 µL reaction mix containing 200 U M-MLV reverse transcriptase, 0.25 mM DTT, and 250 µM each of dATP, dCTP, dGTP, and dTTP. The reverse transcription conditions were as follows: initial incubation at room temperature for 10 minutes, followed by 12 cycles at 25°C for 30 seconds, 45°C for 4 minutes, and 55°C for 30 seconds, with a final 5-minute heat step at 95°C and storage at 4°C. The cDNA was then amplified using AccuPower® GreenStar PCR PreMix (Bioneer, Daejeon, South Korea) in a MyGenie™ 96/384 thermal cycler. Gene expression analysis targeted *clfA*, *icaA*, *icaB*, *icaD*, *icaR*, and 16S rRNA, with 16S rRNA used as the housekeeping gene for normalization and assessment[33].

4.7. Live/Dead Assay

Biofilms from control and treated groups were imaged using confocal microscopy to verify CFU data. The SYTO-9/PI Live/Dead BacLight Bacterial Viability Kit (Fushenbio, Shanghai, China) was used to visualize bacterial biofilms according to the manufacturer's instructions. SYTO-9 stains live bacteria green, while dead cells are stained red due to the uptake of propidium iodide (PI) through compromised cell membranes. After treatment, the biofilm samples were gently washed with sterile water to remove loosely attached bacteria and debris. The samples were then stained in the dark at room temperature for 15 minutes, washed with PBS to remove extracellular dye, and observed using the Zeiss confocal microscope.

5. Conclusions

In summary, this study provides significant contributions to improving patient outcomes and reducing the economic burden associated with biofilm-related complications. The synergistic use of povidone-iodine (PVP-I) and hydrogen peroxide (H₂O₂) represents a novel and effective strategy for managing biofilm-related infections, warranting further investigation and potential clinical integration.

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Hongyan: Assistance, Jong Keun Seon: supervision and assistance, Kyung Soon Park: conceptualization, supervision, project administration, final approval, and funding acquisition. All authors have read and agreed to this version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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