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Biofilm Dispersal and Wound Infection Clearance With Preclinical Debridement Agents

Rebecca Elizabeth Schneider[1,2] | Jason Valentino Hamdan[3] | Kendra Pauline Rumbaugh[1,2]

[1]Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, Texas, USA | [2]Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, Texas, USA | [3]Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas, USA

Correspondence: Kendra Pauline Rumbaugh (kendra.rumbaugh@ttuhsc.edu)

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ABSTRACT

Biofilms complicate wound care by causing recurrent infections that are often resistant to debridement and are highly antibiotic-tolerant. We investigated whether the addition of a biofilm dispersal agent could improve the efficacy of debridement. The previous studies have indicated that a glycoside hydrolase cocktail of alpha-amylase and cellulase can act as a potent biofilm dispersal agent. With in vitro and ex vivo *Pseudomonas aeruginosa* biofilm models, we compared glycoside hydrolases against other, clinically relevant, enzymatic debridement agents (papain, bromelain, and collagenase). Glycoside hydrolase biofilm dispersal was dose-dependent. However, at doses of 1% or above, glycoside hydrolases outperformed, or were comparable, to other enzymatic debridement agents. With our in vivo surgical wound infection model, we evaluated biofilm dispersal using infection dissemination as a proxy. We found that sharp debridement followed by multiple glycoside hydrolase treatments enhanced biofilm dispersal. Furthermore, a single dose of glycoside hydrolase in combination with debridement decreased infection load in acute wounds. Similarly, when we treated established 5-day-old infections, we saw a decrease in infection load and no infection dissemination. Overall, our data suggest that debridement enhances the efficacy of a topical antibiotic ointment, allowing for greater infection clearance.

1 | Introduction

Chronic wounds impact the lives of millions worldwide. In the United States alone, of the over 30 million patients diagnosed with diabetes mellitus, 15%–25% suffer from chronic foot ulcers [1, 2]. More than 1-in-10 of diabetic foot ulcers will require amputation [1]. Treatment of chronic wounds is impeded by stalled healing, rampant inflammation, and persistent infection [2–4]. Currently, the most effective treatment available is debridement: the removal of dead, necrotic, or infected tissue, including slough, fibrin, and foreign material, from the wound [2, 5, 6]. Although debridement is widely considered essential for the healing of chronic wounds, this treatment strategy can still be improved upon [7]. One debridement session is often insufficient to clear a lasting infection, and patients repeatedly return for additional follow-up procedures, with some cases requiring years of treatment [4, 8]. Furthermore, wound care is expensive, both in the billions of dollars spent by the healthcare system and in the reduced quality of life for people enduring this chronic condition [5, 9].

Texas Tech University Health Sciences Center, Lubbock, Texas.

Summary

- Wound clearance and antibiotic efficacy are enhanced by combining sharp debridement with glycoside hydrolases, an enzymatic cocktail of alpha-amylase and cellulase that acts as a dose-dependent dispersal agent.
- This study aimed to determine whether the addition of a dispersal agent could improve current debridement methods.
- We utilised an in vitro 24-well-plate biofilm assay and an ex vivo and in vivo murine surgical wound infection model.
- Our results suggest that glycoside hydrolases are dose-dependent and cause equivalent biofilm dispersal as clinically relevant enzymatic debridement agents.
- Furthermore, combining sharp debridement with glycoside hydrolase treatment causes greater biofilm dispersal, improves wound clearance, and enhances topical antibiotic efficacy.

Debridement encompasses a large category of treatment strategies that range from highly specialised to broadly nonspecific. Surgical or sharp debridement is regarded as the current gold-standard wound care [2, 10–12]. This technique allows skilled medical professionals to methodically remove nonviable wound tissue; however, this requires the use of medical facilities, specialised surgical equipment, and local anaesthetics [2, 6, 10]. Additionally, infections in chronic wounds can be difficult to diagnose or visualise; therefore, certain infections may spread beyond visibly necrotic tissue [8, 12–14]. Alternative debridement strategies include biological with medical maggots (*Lucilia sericata*), mechanical with precision water jets or wet-to-dry bandages, autolytic with endogenous enzymes, or enzymatic with various proteolytic combinations [7, 12, 15]. Although less specific, the relative ease of enzymatic debridement agents offers patients a treatment option that can be administered outside the clinic [5, 12, 15]. However, most enzymatic agents (bromelain, papain, papain-urea, collagenase, etc.) still have considerable side effects including pain, bleeding, and damage to healthy surrounding tissue [7]. Moreover, additional studies are needed to explore how effectively debridement combats a major hindrance to the healing of chronic wound: biofilms.

Biofilms are strong impediments to the treatment of chronic wounds [9, 13, 16]. These recalitrant infections have challenged the biomedical community to improve current debridement approaches to include novel treatments, such as biofilm dispersal agents [9, 13, 16, 17]. Biofilms include matrices of carbohydrates, proteins, lipids, and extra-cellular nucleic acids (eDNA and eRNA) that can be synthesised by microbes or scavenged from the environment (e.g. the human host) [9, 13]. Biofilm dispersal has been explored as a strategy to clear infections by degrading the protective matrix and exposing more vulnerable dispersed cells to clearance by immune cells or antibiotic treatment [9]. A glycoside hydrolase (GH) cocktail of alpha-amylase and cellulase enzymes that target carbohydrate linkages has demonstrated effective biofilm dispersal capability [18–20]. We hypothesized that the addition of GH as a biofilm dispersal agent could enhance the efficacy of current debridement techniques by improving the infection clearance. This study tested GH as a potential enzymatic debridement agent with in vitro and preclinical in vivo murine surgical wound models.

2 | Materials and Methods

2.1 | Bacterial Strains

The bacterial strains utilised in this study included wild-type MPAO1 and a bioluminescent PAO1 strain carrying the luminescence reporter plasmid pQF50-lux. These strains have been previously described [21, 22]. Overnight cultures and subcultures of the PA strains were grown in brain–heart infusion (BHI) broth in bevelled flasks with shaking at 200 rpm at 37°C.

2.2 | Debridement Agents

The following enzymes were purchased from Fisher Scientific: alpha-amylase from *Bacillus subtilis* (MP Biomedicals, Cat No 0210044725, ~165 U/mg), cellulase from *Aspergillus niger* (MP Biomedicals, Cat No 0215058325, ~150 U/mg), and collagenase (COL) from *Clostridium histolyticum* (MP Biomedicals, Cat No ICN15070501). Enzymes purchased from Sigma-Aldrich included bromelain (BRO) from pineapple stem (Cat No B4882-25G, ~3 U/mg) and papain (PAP) (Cat No, ~3 U/mg). Enzymes were suspended in phosphate-buffered saline (PBS) to produce a solution of the desired percent (*w*/*v*) concentration (calculated as g solute/mL solvent) or a standardised enzymatic activity (U/mL) concentration. GH solutions consisted of a 1:1 ratio of alpha-amylase and cellulase. The concentrations of the debridement agents were based on the previous data from our laboratory that indicated that treatment with a 10% (*w*/*v*) solution of GH caused a large biofilm dispersal event in our murine model [18, 21]. All debridement agents were tested at 10%, 1%, 0.1%, and at concentrations allowing for a standardised activity level of 3 U/mL. For debridement agents with a solubility limit below 10% (collagenase and bromelain), the agents were tested at concentrations equal to or below the maximum solubility concentration. The enzyme solutions were utilised in the in vitro, ex vivo, and in vivo models.

Surgical tools utilised for sharp debridement were purchased from Fisher Scientific. Forceps curved medium point (Cat No 16-100-110) and Bruns bone curette 00 (Cat No 10192-268) were utilised for the in vivo model.

2.3 | In Vitro Well Plate Biofilm Model and Crystal Violet Assay

Twenty-four-well plates were utilised for the in vitro biofilm formation assay. Individual wells were filled with 1 mL of BHI and inoculated with 10^4 colony-forming units (CFU) of PAO1 from overnight cultures in 100 μL of PBS. CFU quantification was determined through serial dilution and colony enumeration on Pseudomonas Isolation Agar (PIA). The well plates were incubated statically at 37°C for 48 h to allow for formation of a biofilm. After 48 h of growth, any remaining BHI and planktonic

cells were removed, and the wells containing the biofilms were washed with PBS. The biofilms were then treated with 1 mL of enzymatic solution or PBS vehicle control for 3 h at 37°C under static conditions. A total of eight replicates were performed for each treatment. The PBS vehicle was utilised as a negative control for the enzymatic solutions to account for any potential effects from the solvent alone.

A crystal violet (CV) staining assay was utilised to assess biofilm degradation: specifically, biofilm mass was quantified through staining. For the CV assay, biofilm remaining after enzymatic treatment was washed with PBS and stained with 1% CV solution for 30 min. Following staining, excess CV was washed off with PBS and the stained biofilms were eluted with 95% ethanol for 1 h. The ethanol-eluted stains were diluted 1:10 in PBS in a new 96-well plate and quantified by reading absorbance at OD 595 nm.

2.4 | Murine Surgical Wound Infection Model

Wild-type, adult, female Swiss Webster mice (aged 6–8 weeks) were utilised in the murine surgical wound infection model as previously described [18, 19, 21, 23–25]. Mice were anaesthetised with isoflurane, an inhaled anaesthetic. The backs of the mice were shaved and depilated and a full-thickness, 1.5 × 1.5 cm section of dermis was excised. The wounds were covered with a semipermeable polyurethane dressing (OPSITE dressing) and inoculated with 100 μL of 10^4 CFU PAO1 suspended in PBS. Mice were allowed to recover on heating pads and monitored for signs of distress.

2.5 | Ex Vivo Biofilm Dispersal

Forty-eight hours (acute infection) or 120 h (established infection) after inoculation, mice were euthanized, and wound tissue was collected for ex vivo biofilm dispersal experiments. Wound tissue were suspended in 1 mL of enzymatic solution and incubated statically at 37°C for 3 h. Following treatment, the remaining wound tissue was removed from the solution, transferred to a homogenising tube with 1 mL of PBS and homogenised at 5 m per second for 3 min. Solutions containing dispersed bacteria and remaining wound tissue were serially diluted for CFU/g tissue quantification on Pseudomonas Isolation Agar (PIA).

2.6 | In Vivo Debridement, Biofilm Dispersal/Dissemination, and Antibiotic Treatment

Forty-eight hours (acute infection) or 120 h (established infection) after inoculation, the mice were divided into the following treatment groups: sharp debridement alone; combination sharp debridement and PBS; combination sharp debridement and GH; GH alone; and PBS alone. The mice were treated with one or a combination of the following: sharp debridement with a curette and forceps for 5 min; enzymatic debridement with 1-h topical treatments (0.2 cc doses) of 10% (w/v) GH; and/or 1-h topical treatments (0.2 cc doses) of PBS vehicle controls. For combination treatment groups, sharp debridement was administered first, directly followed by enzymatic or vehicle control treatments. For groups treated with antibiotic therapy, a topical combination of neomycin (3.5 mg per gram ointment)/bacitracin zinc (400 units per gram ointment)/polymyxin B (5,000 units per gram ointment) was applied over the full surface of the wound immediately following debridement, and a new OPSITE dressing was applied. Following treatment, mice were monitored overnight and assessed the following day for sepsis indicators with the Murine Sepsis Score (MSS) as previously described [26–28]. Mice were euthanized: wound tissue was collected for wound bacterial load and internal organs (lungs, liver, and spleen) were extracted for evaluation of disseminated bacterial infection. The extracted wounds and internal organs were homogenised at 5 m per second for 3 min and serially diluted for CFU/g tissue quantification on PIA.

2.7 | Statistics

Statistical analyses were conducted with GraphPad Prism 10 (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance (ANOVA), post hoc Mann–Whitney tests, or Fisher's Exact tests were utilised to compare group means. p-values < 0.05 were considered statistically significant.

3 | Results

3.1 | Biofilm Dispersal Efficacy of Debridement Agents

To determine the potential of GH as an enzymatic debridement agent, the biofilm dispersal efficacy of GH was compared to that of clinically relevant enzymatic debridement agents. The debridement agents were first tested against biofilms formed in a 24-well-plate in vitro assay. This in vitro assay allowed us to assess efficacy of the debridement agents against biofilm formed exclusively from bacterial-derived components. When the remaining biofilm was analysed with a CV stain, GH treatment revealed a dose-dependent trend in which 10% GH treatments removed more biofilm than 1% GH treatments, and both GH treatments eliminated greater amounts of biofilm compared to the PBS vehicle control (Figure 1A). By comparison, degradation of biofilm by PAP at the same concentrations was less effective than GH (Supplementary Figure 1) and less dose-dependent (Figure 1A,B). At a standardised concentration of 3 U/mL, PAP and BRO were effective biofilm dispersal agents compared to PBS control (Figure 1C).

To assess dispersal efficacy against biofilms composed of both bacterial and host components, we utilised our murine surgical wound infection model. Forty-eight hours after wounding and infection, the mice were euthanized. The wound tissue was extracted and submerged in 1 mL of GH, PAP, COL, or BRO in a PBS solution at a range of concentrations for 1 h, after which percent dispersal was determined. GH demonstrated biofilm dispersal efficacy (Figure 2A), and both GH and COL demonstrated a gradual decrease in efficacy with decreasing concentrations (Figure 2A–C). In contrast, PAP maintained high levels of biofilm dispersal despite decreasing concentrations (Figure 2B). At a standardised activity level of 3 U/mL, the debridement agents dispersed biofilm with varying efficacy, but all four debridement

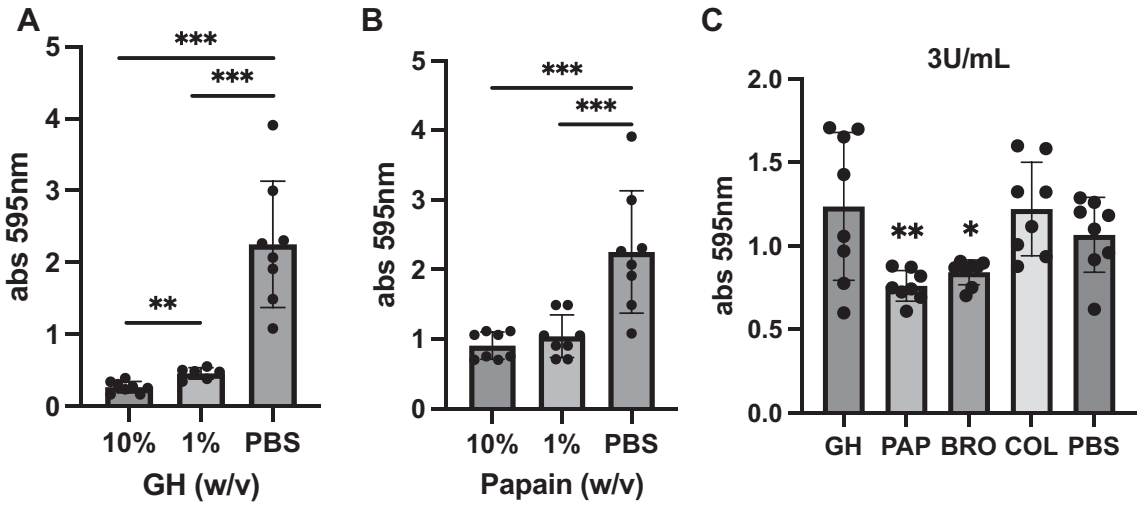

FIGURE 1 | Enzymatic debridement agents degraded in vitro-grown biofilms. PAO1 biofilms were grown statically in BHI in a 24-well plate for 48 h and were treated with GH or PBS vehicle control (A) a crystal violet stain of remaining biofilm revealed that GH biofilm degradation was dose-dependent. (B) Biofilm reduction from PAP treatment was dose-independent. (C) At a standardised concentration of 3 U/mL, PAP and BRO treatments caused a reduction in biofilm compared to PBS control. Data were analysed with a one-way ANOVA and Mann–Whitney tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the PBS vehicle control.

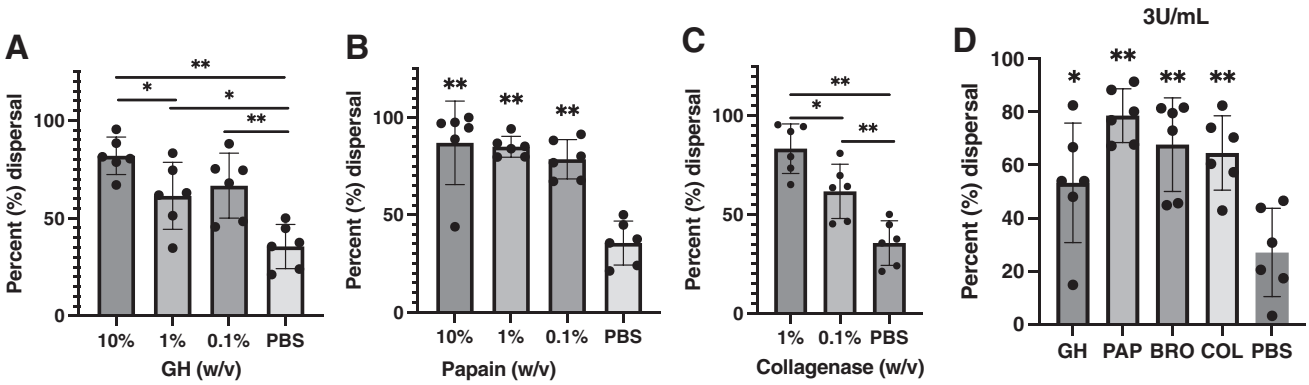

FIGURE 2 | Enzymatic debridement agents dispersed biofilms in wound tissue, ex vivo. The wound beds from mice infected for 48 h with PAO1 were surgically extracted and were suspended in enzymatic treatments for 3 h. Percent dispersal was calculated as follows: [(CFU/mL dispersed fraction)/((CFU/mL dispersed fraction) + (CFU/mL remaining biofilm))] * 100%. (A) GH dispersal was dose-dependent but remained effective at lower doses. (B) Papain dispersal was dose-independent. (C) Collagenase dispersal was dose-dependent (D) at a standardised enzymatic activity level (3 U/mL), GHs, papain (PAP), bromelain (BRO), and collagenase (COL) dispersed biofilm with varying efficacy. Data were analysed with a one-way ANOVA and Mann–Whitney tests. *$p < 0.05$, **$p < 0.01$ compared to the PBS vehicle control.

agents remained effective biofilm dispersal agents (Figure 2D). These data suggest that is GH a dose-dependent biofilm dispersal agent. Moreover, GH is comparable to clinically relevant enzymatic debridement agents, particularly at higher concentrations.

3.2 | Infection Dissemination After Debridement

Having established GH as an effective biofilm dispersal agent, we next assessed the efficacy of GH in vivo. Based on our ex vivo results, we selected the 10% GH concentration because it demonstrated the highest biofilm degradation and dispersal efficacy of the various GH concentrations (Figures 1A and 2A). Previous studies in our laboratory have established that high levels of biofilm dispersal in vivo lead to infection dissemination [21, 23]. Thus, infection load in the internal organs and sepsis symptoms provide reliable measures of biofilm dispersal in our murine model.

To determine whether biofilm dispersal was equivalent to or could be enhanced by other debridement methods, GH treatment was compared to sharp debridement. Mice with 48-h-old wound infections were treated with GH, sharp debridement, PBS vehicle control, or a combination of both debridement strategies. Wounds treated with a combination of sharp debridement followed by multiple consecutive GH treatments (GH + SD) had an average of a 1-log increase in infection load compared to sharp debridement (SD) and a 1.5-log increase compared to the PBS vehicle control (Figure 3A). Both GH and GH + SD groups had large internal organ infection loads (Figure 3B). This is indicative of high levels of infection dissemination in both the GH and GH + SD treatment groups

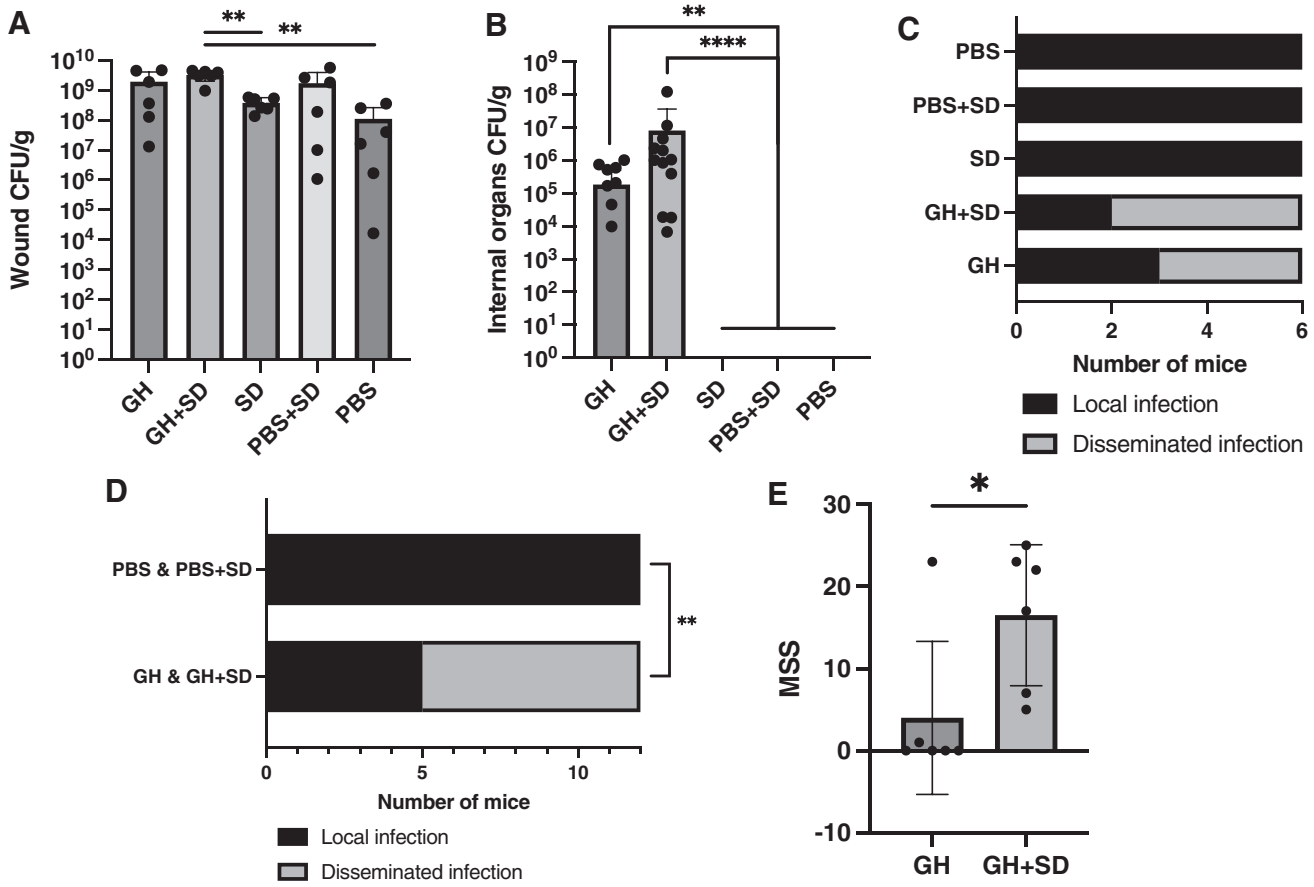

FIGURE 3 | Multiple doses of GH caused large dispersal events that could be increased with other debridement strategies. Mice with 48-h-old PAO1 wound infections were treated with various debridement strategies and assessed for systemic infection up to 24 h later. Treatments included one or a combination of the following: sharp debridement (SD) with a curette and forceps; enzymatic debridement with 10% (*w*/*v*) GH (three 0.2 cc/h doses); and/or PBS vehicle controls. (A) Wound infection load was elevated in the GH + SD group compared to SD alone or PBS vehicle control. (B) Groups treated with GH and GH + SD had high internal organ PA loads compared to SD, PBS + SD, or PBS. (C, D) Disseminated infection occurred in only GH and GH + SD groups. (E) Sepsis symptoms were more severe in the GH + SD compared to GH alone. Data were analysed with a one-way ANOVA and Mann–Whitney tests or Fisher's exact test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

compared to the PBS and PBS + SD groups, which experienced no dissemination (Figure 3B–D). Mice with severe disseminated infections (Figure 3B–D) begin displaying sepsis symptoms which can be evaluated with the Murine Sepsis Score (MSS). The GH + SD group experienced elevated average MSS of 16.5 compared to GH treatment with an MSS of 4 (Figure 3E). Together, these data suggest that multiple GH treatments cause a large biofilm dispersal event that leads to systemic infection dissemination. Furthermore, the biofilm dispersal efficacy of GH is enhanced through combination with sharp debridement.

3.3 | Infection Reduction With Debridement

Based on a dose-dependent biofilm dispersal efficacy for GH (Figure 2A–D), we explored whether a single dose of 10% GH caused less infection dissemination than multiple doses (Figure 4C,D). Forty-eight-hour-old wound infections were treated with sharp debridement, a single dose of GH or PBS, or a combination of the debridement strategies. Wounds in the GH + SD treatment group showed a decrease in infection load compared to GH, SD, and a no treatment (NT) control (Figure 4A). The GH + SD group had up to a 3.5-log reduction in bacterial load compared to an untreated wound infection, a significant difference compared to GH, SD, or PBS treatment (Figure 4B). Mice treated with a single dose (1X txt) of GH and GH + SD also displayed only moderate levels of infection dissemination, indicated by decreased internal organ infection loads compared to multiple (3X Txt) GH treatments alone or in combination with SD (Figure 4C,D).

The previous studies with our murine model indicate that uninfected wounds transition through different stages of healing from 2 to 5 days post-injury [29]. Additional evidence from our murine model supports that bacteria in chronic wounds infections (defined as at least 4-day infections) have vastly different virulence gene expression compared to bacteria in acute infections (2-day wound infections or burn infections) [30]. Therefore, we endeavoured to determine whether combination GH + SD treatment would result in infection clearance for established 5-day (120 h) infections. Mice treated with sharp debridement followed by multiple doses of GH (GH + SD) had reduced infection loads compared to NT controls (Figure 5A). All debridement treatments had at least a 1-log reduction from NT controls and GH + SD treatment resulted in up to a 3.5-log

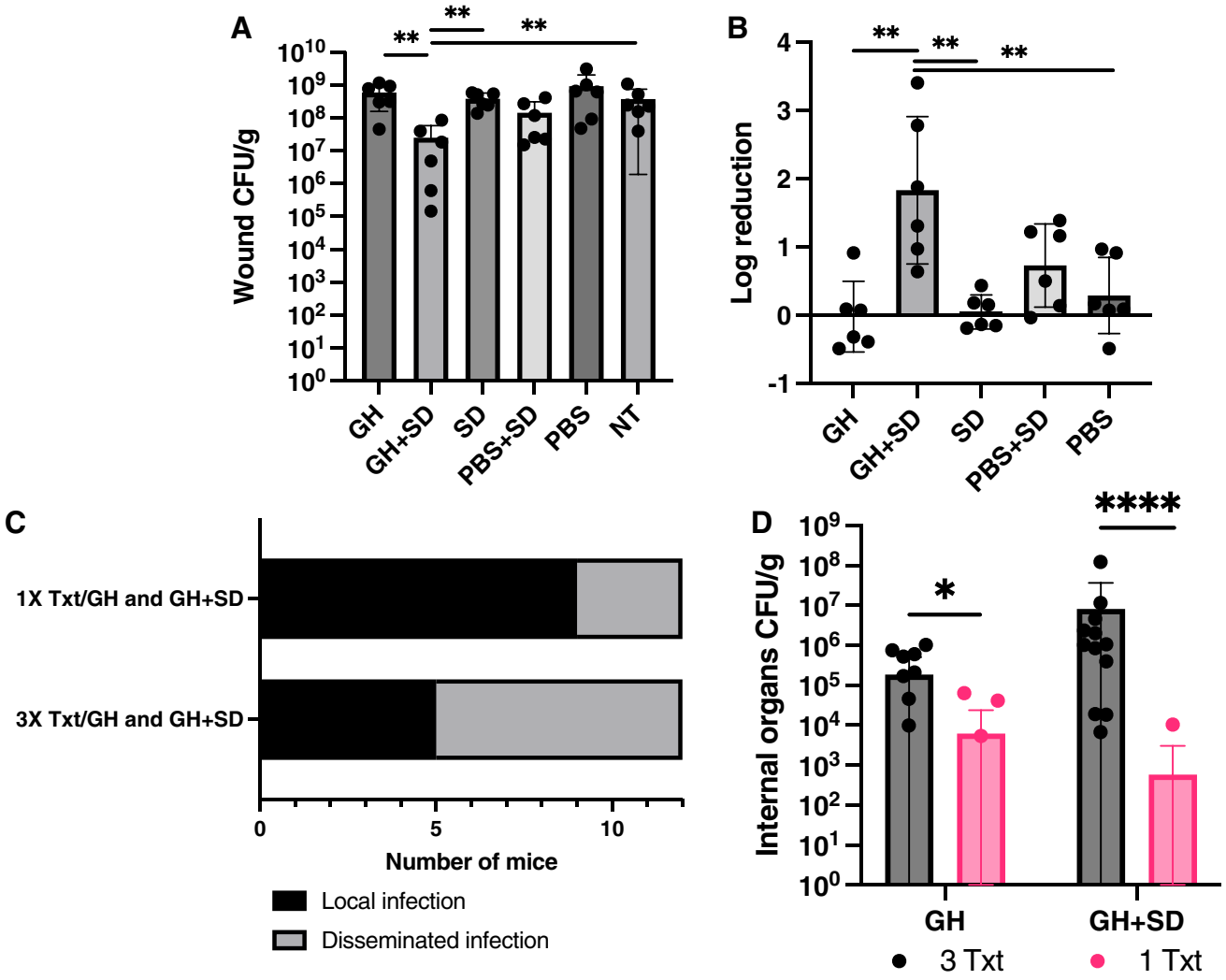

FIGURE 4 | Single dose of GH combined with sharp debridement improved wound clearance in acute 48-h-old infections. Mice with 48-h-old PAO1 surgical wound infections were treated with various debridement strategies and assessed for systemic infection up to 24 h later. Treatments included one or a combination of the following: Sharp debridement (SD) with a curette and forceps; enzymatic debridement with 10% (*w*/*v*) GH (one 0.2 cc/h dose); and/or PBS vehicle controls. (A) wound infection load was decreased in the GH + SD group compared to SD, GH, or no treatment (NT) groups. (B) Wound infection log reduction was assessed compared to the no treatment control (NT) wound load for all groups. Groups treated with GH + SD displayed the greatest infection load reduction of the treatment groups. (C, D) Groups treated with one dose (1Txt) GH and GH + SD had decreased dissemination levels and internal organ PA loads compared to multiple doses (3 Txt). Data were analysed with a one-way ANOVA and Mann–Whitney tests or Fisher's exact test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

reduction in bacterial load (Figure 5B). Most dramatically, mice with 120-h-old wound infections treated with GH and GH + SD displayed no dissemination of infections compared to mice with acute 48-h-old infections that received the same treatment (Figure 5C). Together, these data suggest that a single GH treatment has a lower risk of infection dissemination than multiple consequecutive treatments in acute infections. Furthermore, more established infections also are unlikely to disseminate, allowing GH biofilm dispersal to effectively aid in wound infection reduction.

3.4 | Enhanced Antibiotic Efficacy With Debridement in Established Infections

Antibiotic tolerance is one of the greatest challenges of treating biofilm infections [31–34]. Biofilm wound infections are often nonresponsive to topical antibiotic treatments [31–34]. We investigated whether debridement could increase the efficacy of a neomycin sulfate/bacitracin zinc/polymixin B sulfate (NEO) topical antibiotic, which may be used for the treatment of wound infections [2, 35, 36]. For acute 48-h-old infections, we found no significant difference in wound infection load between NEO alone and NEO combined with debridement strategies (Figure 6A). NEO was able to effectively reduce infection load in the wounds of the NT control group by up to 6 logs in all groups (Figure 6B). For 120-h-old infections, NEO alone was less effective at clearing the wound infection (Figure 6C); however, when NEO was combined with debridement strategies, there was a reduction in wound infection load (Figure 6C,D). All debridement strategies combined with NEO had up to a 6-log reduction of an NT wound infection, a significant difference compared to the 4-log reduction with NEO alone (Figure 6D). These data suggest that

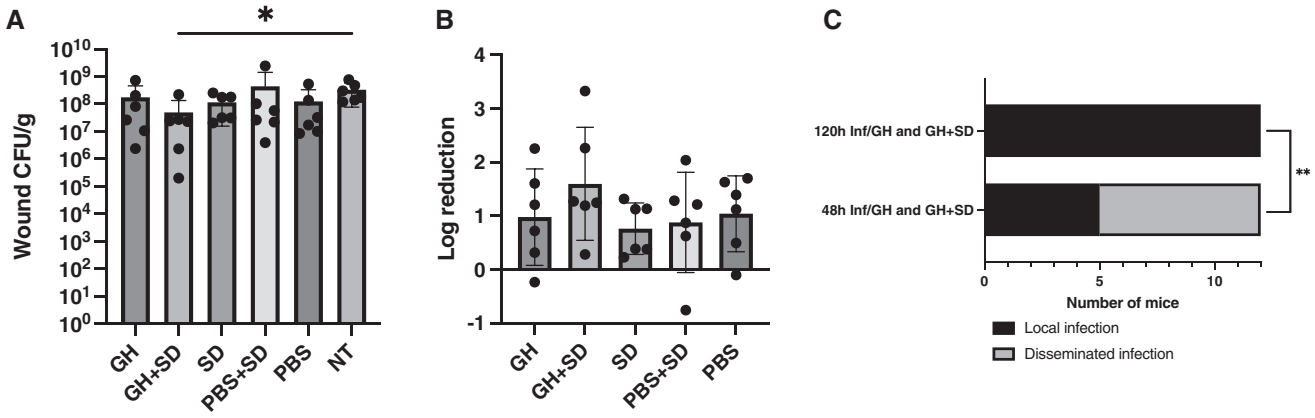

FIGURE 5 | Established 120-h-old infections showed improved wound infection clearance and reduction in disseminated infections with combined GH-sharp debridement treatment. Mice with 120-h-old PAO1 wound infections were treated with various debridement strategies and assessed for systemic infection up to 24 h later. Treatments included one or a combination of the following: sharp debridement (SD) with a curette and forceps; enzymatic debridement with 10% (w/v) GH (three 0.2 cc/h dose); and/or PBS vehicle controls. (A) Wound infection load was decreased in the GH + SD group compared to the no treatment (NT) group. (B) Wound load log reduction was assessed compared to the NT control wound load for all groups (C) mice with 120-h-old infections treated with GH or GH + SD experienced no infection dissemination, a significant reduction compared to 48-h-old infections. Data were analysed with a one-way ANOVA and Mann–Whitney tests or Fisher's exact test. *$p < 0.05$, **$p < 0.01$.

debridement can enhance topical antibiotic efficacy in established wound infections.

4 | Discussion

Debridement remains one of the most effective strategies for promoting healing in chronic wounds [37, 38]. In this study, we wanted to determine the efficacy of GH as a debridement agent. Compared to clinically utilised enzymatic debridement agents, GH degraded biofilms in both an in vitro model where all EPS components are synthesised by bacteria and an ex vivo wound model where both bacteria-derived and host-derived EPS is present. In the bacteria-derived in vitro model, GH outperformed Papain in biofilm degradation at concentrations of 1% and above. By comparsion, collagenase, which should selectively target the host component of collagen, was ineffective against in vitro biofilms. This demonstrates that at sufficient concentrations, the biofilm dispersal capacity of GH is equivalent to or exceeds that of clinically relavent enzymatic dispersal agents, particularly when targeting bacteria-derived biofilm components. This is reinforced by the results from Watters et al. which found alpha-amylase to be as effective at reducing *S. aureus* biofilm biomass as bromelain or papain in an in vitro human plasma model [17].

Currently, sharp debridement is considered the most effective treatment for chronic wounds [38, 39]. A skilled surgeon can be highly effective at removing visibly necrotic and infected tissue. However, we wanted to investigate whether the addition of a biofilm dispersal agent could target remaining biofilm missed during sharp debridement, thus improving treatment outcome. Our laboratory has established that a large dispersal event in our murine surgical wound model can lead to infection dissemination in acute infections [21, 23]. Thus, we used bacterial dissemination as a proxy for dispersal. When sharp debridement was combined with multiple GH treatments, wound infection load was elevated and infection dissemination and sepsis increased. This suggests that sharp debridement can be complemented with GH enzymatic debridement to increase biofilm dispersal in a wound infection.

We have shown that GH biofilm dispersal (with concominant dissemination) is dose-dependent. Therefore, with a decrease from multiple doses to a single dose of GH, we saw a more moderate level of dissemination with no sepsis symptoms in an acute wound infection. Furthermore, mice treated with sharp debridement and GH showed a decrease in wound infection load. This suggests that when sharp debridement removes slough and dead tissue, it allows GH to more effectively disperse the biofilm imbedded in live tissue. Furthermore, a lower level of biofilm dispersal does not overwhelm the immune response and instead allows for enhanced infection clearance.

Older and chronic infections are stalled in the healing process, often with high levels of inflammation [40]. We theorised that an enhanced immune response of older infections could reduce risk of infection dissemination with biofilm dispersal. When we treated established five-day infections with sharp debridement and GH, we found both a total elimination of infection dissemination and a reduction in wound infection load. These data reinforce that more established infections have a low risk of infection dissemination when treated with GH biofilm dispersal agents. Future experiements will explore the potential of inflammatory responses in acute and established infections to emlinate dispersed bacterial cells, thus preventing infection dissemination and promoting infection clearance.

Biofilms have been shown to display high antibiotic tolerance [41–43]. However, dispersal agents have been explored as a potential means of reducing antibiotic tolerance through the degradation of the protective biofilm matrix and the release of antibioitic-susceptible dispersed bacterial cells [41–44]. Other research groups have demonstrated that biofilm dispersal agents, such as nitric oxide (NO) which trigger active dispersal by modulating levels of the intracellular messenger cyclic-di-guanosine

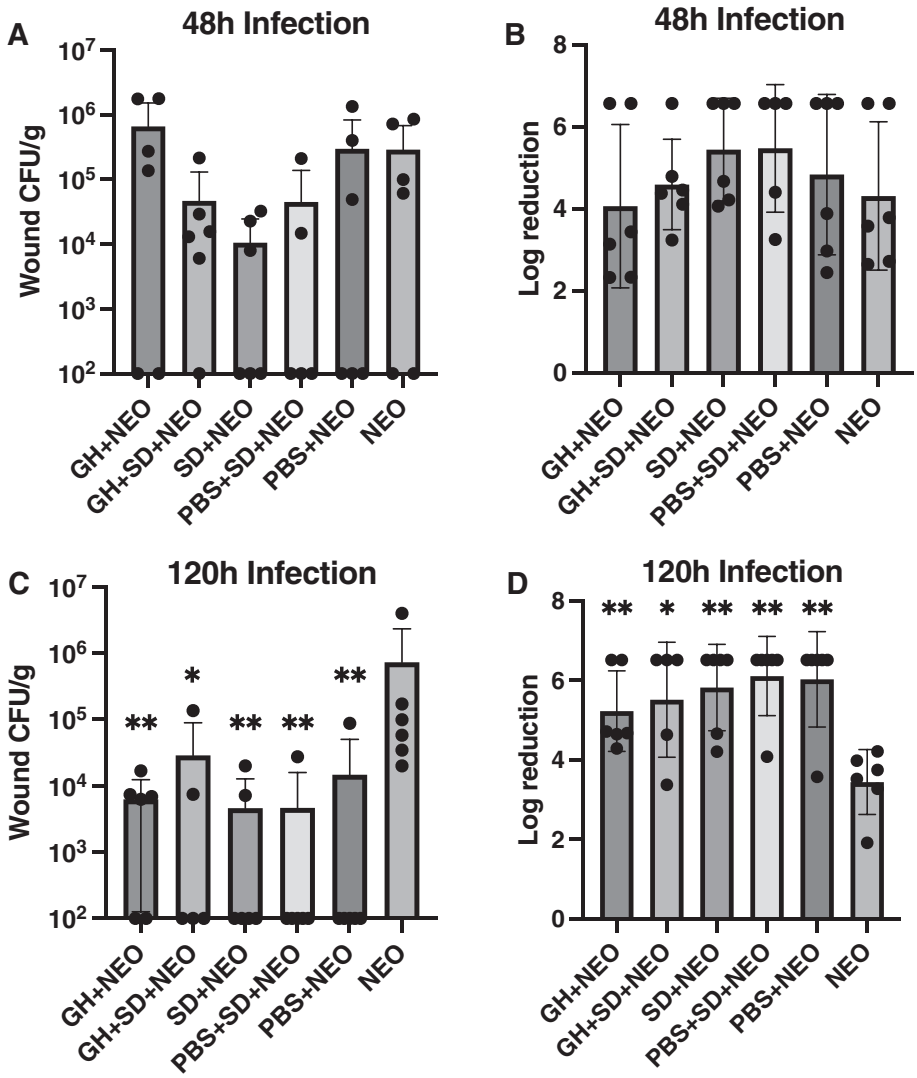

FIGURE 6 | Clinically relevant debridement strategies enhanced antibiotic efficacy in established 120-h-old infections. Mice with 48 h and 120 h-old PAO1 wound infections were treated with various debridement strategies combined with topical neomycin sulfate/bacitracin zinc/polymixin B sulfate (NEO) combination antibiotic and assessed for systemic infection and wound infection load up to 24 h later. Debridement treatments included one or a combination of the following: sharp debridement (SD) with a curette and forceps; enzymatic debridement with 10% (w/v) GH (one 0.2 cc/h dose); and/or PBS vehicle controls. Topical NEO was applied across the surface of the wound immediately following debridement treatments. (A, B) NEO alone and in combination with debridement strategies effectively reduced infection load in acute 48-h-old infections (C) debridement with all debridement strategies increased wound clearance compared to NEO alone in established 120-h-old infections. (D) Wound load log reduction was assessed compared to no treatment (NT) control wound load for all groups. All debridement strategies showed a greater infection load log reduction compared to NEO alone in 120-h-old infections. Data were analysed with a one-way ANOVA and Mann–Whitney tests. *$p < 0.05$, **$p < 0.01$ compared to NEO alone.

(C-di-GMP), have been effective at reducing both biofilm mass and antibiotic tolerance [41, 45, 46]. For acute infections, we found that debridement agents did not increase the efficacy of a topical antibiotic: this suggests the topical antibiotic effectively eliminated the weaker biofilms in the acute infections. However, in more established 120-h infections, all debridement strategies increased the efficacy of the topical antibiotic. This suggests that older infections have more robust biofilms and thus greater antibiotic tolearance. Furthermore, debridement has been utilised clinically for wound preparation, encouraging chronic wound infections to revert to acute infections by disrupting biofilm formation and thus opening a therapeutic window [8, 12, 47]. This aligns with our results which could suggest that debridement and biofilm dispersal agents allow topical antibiotics to be as effective against chronic wound infections as they are against acute infections.

There are limitations that must be considered for our in vivo murine surgical wound infection model. Humans and mice share the healing mechanism of granulation and reepthelialization, but tissue contraction is a feature that distinguishes murine healing from that of humans [48, 49]. While this limitation would not have a strong impact on acute infections, it is an important consideration for future longitudinal chronic wound healing studies. Furthermore, our monomicrobial *P. aeruginosa* model could be improved through the addition of other bacterial

and fungal pathogens (e.g. *Staph aeurus* or *Candida albicans*). Polymicrobial interactions are more representative of chronic wounds and could impact the efficacy of treatments. GH treatment is still in the preclinical stages, and further benchscience experimentations need to be conducted prior to any clinical use.

In conclusion, the addition of a glycoside hydrolase biofilm dispersal agent to sharp debridement causes greater biofilm dispersal, improves wound clearance, and enhances topical antibiotic efficacy.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.