**TO THE EDITOR**

Chronic wounds remain a significant clinical challenge, with annual estimated expenditures in the United States alone being $28.1 billion (Sen, 2019), which is expected to increase with the projected 165% jump in diabetes by 2050 (Boyle et al., 2010). Recent studies have identified microbial infection as an important factor driving wound chronicity (Williams et al., 2018; Zhao et al., 2016). In these wounds, host antimicrobial and inflammatory defenses cannot eradicate pathogens, which then continue to recruit neutrophils and monocytes resulting in persistent proinflammatory signals that impair healing. Bacterial virulence factors are also linked to impaired healing. *Staphylococcus aureus* species are known to upregulate various proteases allowing for penetration into wound tissue (Kolar et al., 2013). Biofilm formation is a nearly universal ability shared by highly virulent pathogens and documented to be associated with wound chronicity (Kalan et al., 2019). Wound biofilms are difficult to eradicate owing to their resistance to penetration by neutrophils and their poor response to systemic antibiotics, which brings attendant risks, including bacterial resistance. With studies indicating that an estimated 65% and 80% of all microbial infections in humans are attributed to biofilms (Lewis, 2007), biofilm eradication remains a top challenge, and this underscores the need to develop nonantibiotic wound infection therapies that can also target the biofilm.

Although the selective serotonin reuptake inhibitor, fluoxetine (FLX), has been reported to have antimicrobial activity against some gram-positive bacteria in vitro (Munoz-Bellido et al., 2000) and diminish in vitro biofilm formation by *Proteus mirabilis* (Nzakizwanayo et al., 2017), these findings have not been translated to mammalian wounds or examined in clinically relevant models. The known effects of FLX on improving keratinocyte migration speeds and promoting a shift of the wound environment from proinflammatory to anti-inflammatory (Nguyen et al., 2019) combined with potential antimicrobial and antibiofilm activities present a potential for a multipronged therapeutic to improve healing in chronic wounds. To test this possibility, we used in vitro as well as a clinically translational human skin ex vivo wound infection model (Yoon et al., 2019) wherein human skin was collected following written informed consent under a University of California Davis Institutional Review Board–approved protocol. When *S. aureus*–infected human skin wound explants were treated with daily application of topical 0.2% FLX, scanning electron microscope images of the wound surface resembled those of the uninfected controls with the absence of detectable biofilm, which was clearly detectable in the untreated, infected wounds (Figure 1d). Treated wounds exhibited a greater than $10^5$-fold reduction in viable bacteria compared with the infected control (1.6 × $10^7$ vs. 6.6 × $10^{10}$ colony-forming units) (Figure 1e). Preparative innate responses were observed in the FLX–treated skin samples, including upregulation of proinflammatory cytokines IL-6 and TNF-α (Figure 1f).

The synergism of host response with topical FLX was examined using an in vivo murine wound infection model

---

**Abbreviation:** FLX, fluoxetine

**Accepted manuscript published online 15 December 2020; corrected proof published online 19 January 2021**

© 2021 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.

---

**TO THE EDITOR**

Chronic wounds remain a significant clinical challenge, with annual estimated expenditures in the United States alone being $28.1 billion (Sen, 2019), which is expected to increase with the projected 165% jump in diabetes by 2050 (Boyle et al., 2010). Recent studies have identified microbial infection as an important factor driving wound chronicity (Williams et al., 2018; Zhao et al., 2016). In these wounds, host antimicrobial and inflammatory defenses cannot eradicate pathogens, which then continue to recruit neutrophils and monocytes resulting in persistent proinflammatory signals that impair healing. Bacterial virulence factors are also linked to impaired healing. *Staphylococcus aureus* species are known to upregulate various proteases allowing for penetration into wound tissue (Kolar et al., 2013). Biofilm formation is a nearly universal ability shared by highly virulent pathogens and documented to be associated with wound chronicity (Kalan et al., 2019). Wound biofilms are difficult to eradicate owing to their resistance to penetration by neutrophils and their poor response to systemic antibiotics, which brings attendant risks, including bacterial resistance. With studies indicating that an estimated 65% and 80% of all microbial infections in humans are attributed to biofilms (Lewis, 2007), biofilm eradication remains a top challenge, and this underscores the need to develop nonantibiotic wound infection therapies that can also target the biofilm.

Although the selective serotonin reuptake inhibitor, fluoxetine (FLX), has been reported to have antimicrobial activity against some gram-positive bacteria in vitro (Munoz-Bellido et al., 2000) and diminish in vitro biofilm formation by *Proteus mirabilis* (Nzakizwanayo et al., 2017), these findings have not been translated to mammalian wounds or examined in clinically relevant models. The known effects of FLX on improving keratinocyte migration speeds and promoting a shift of the wound environment from proinflammatory to anti-inflammatory (Nguyen et al., 2019) combined with potential antimicrobial and antibiofilm activities present a potential for a multipronged therapeutic to improve healing in chronic wounds. To test this possibility, we used in vitro as well as a clinically translational human skin ex vivo wound infection model (Yoon et al., 2019) wherein human skin was collected following written informed consent under a University of California Davis Institutional Review Board–approved protocol. When *S. aureus*–infected human skin wound explants were treated with daily application of topical 0.2% FLX, scanning electron microscope images of the wound surface resembled those of the uninfected controls with the absence of detectable biofilm, which was clearly detectable in the untreated, infected wounds (Figure 1d). Treated wounds exhibited a greater than $10^5$-fold reduction in viable bacteria compared with the infected control (1.6 × $10^7$ vs. 6.6 × $10^{10}$ colony-forming units) (Figure 1e). Preparative innate responses were observed in the FLX–treated skin samples, including upregulation of proinflammatory cytokines IL-6 and TNF-α (Figure 1f).

The synergism of host response with topical FLX was examined using an in vivo murine wound infection model

---

**Abbreviation:** FLX, fluoxetine

**Accepted manuscript published online 15 December 2020; corrected proof published online 19 January 2021**

© 2021 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.
Figure 1. FLX inhibits planktonic growth and decreases biofilm mass of UAMS-1 in vitro as well as biofilm growth and inflammation in human skin ex vivo. (a) Growth of planktonic cultures and (b) biofilm cultures of UAMS-1 (starting concentrations of $10^6$ CFU/ml) grown in the presence or absence of 100 μM FLX and 0–100 μM FLX, respectively, and measured at OD$_{600}$ (planktonic) and OD$_{580}$ (biofilm biomass) over a 12-hour period. (c) Fold change in mRNA expression through real-time qPCR of UAMS-1 virulence genes *fib*, *sarA* *icaA* and *icaD*, and *hla*. (d) Scanning electron microscopy of human skin explants: noninfected, vehicle-treated wounds; wounds infected with $10^6$ CFU/ml UAMS-1; and wounds treated daily with UAMS-1 in the presence of 5% PEG vehicle or 0.2% FLX and harvested after 3 days. Bars = 5 μm. (e) CFU counts of wound homogenates. (f) Normalized mRNA expression of *IL6*, *TNFα*, *TGFβ*, *K16*, and *K17*. Expression is normalized to 16S (bacterial), *GAPDH*, and 18S (mammalian) housekeeping genes. In vitro data were derived from $n = 3$ biological replicates, each with technical triplicate. Human skin ex vivo data were derived from $n = 3$ different human skin donors, each with technical triplicates. Error bars represent mean ± SEM. Student’s t-tests were used for analyses, with statistical significance denoted: *$P < 0.05$. CFU, colony-forming unit; FLX, fluoxetine; K, keratin; OD, optical density; ND, not determined; PEG, polyethylene glycol.
Figure 2. FLX improves re-epithelialization of biofilm-coated, UAMS-1–infected wounds in vivo and modulates UAMS-1 and murine gene expression in the wound. (a) Wound re-epithelialization 7 days after surgery. (b) Representative wound sections harvested on day 7, stained with H&E. Black arrows indicate the original wound margin, and purple arrows indicate the advancing edge of the neoepidermis. Bars = 1 mm. (c) CFU counts of C57BL6 mouse spleen homogenates and homogenates of excised wound tissue. (d) Fold change in mRNA of UAMS-1 virulence genes fib, sarA, icaA and icaD, and hla in the murine wound tissues. (e) Normalized mRNA expression of fold change in mRNA expression of Arg1, Col3a1, iNos, Pdgfb, Ifng, Tnfa, and Il6 in the murine wound tissues. Expression is normalized to 16S (bacterial), Gapdh, and 18S (mammalian) housekeeping genes. Data were derived from n = 7 for control, n = 10 for UAMS-1, and n = 10 for UAMS-1 + FLX groups, and error bars represent mean ± SEM. Student’s t-tests with statistical significance denoted: *P ≤ 0.05. CFU, colony-forming unit; FLX, fluoxetine; ND, not determined.
Topical treatment of FLX began 2 days after bacterial inoculation to allow for the establishment of biofilm in the wound. Infected wounds treated daily for 5 days with 0.2% FLX demonstrated significantly improved re-epithelialization (15.8%, Figure 2a and b). Interestingly, although FLX treatment decreased the translocation of bacteria to the spleen, it did not alter wound viable bacterial counts (Figure 2c). The dampened inflammatory in vivo wound response with FLX treatment, as described previously (Nguyen et al., 2019) and evident by decreased purulence in FLX-treated wounds (Supplementary Figure S2), may explain the inability to replicate the in vitro and ex vivo antimicrobial effects in the in vivo model. Alternatively, FLX may have a more profound effect on the ability of planktonic organisms to form biofilms (Figure 1c) rather than altering the growth of organisms within the already formed biofilm within the wound. Molecular characterization of the wound tissue did, however, reveal upregulation of proreparative Pdgf-β that contributes to healing by its promigratory effects on keratinocytes, fibroblasts, macrophages, and neutrophils and angiogenic properties as well as Col3a1. The inflammatory cytokine IL-6 was also downregulated (Figure 2e).

Transcription of pathogen virulence factors was also interrogated in the wound tissue. Fib, implicated in biofilm formation by facilitating bacterial adherence to wound extracellular matrices (Bodén Wästfelt and Flock, 1995), was significantly downregulated in the FLX-treated wounds (Figure 2d). Also decreasing virulence is the FLX-mediated upregulation of sarA, a repressor of hla (Oscarsson et al., 2006) and a protease that is upregulated during deep tissue bacterial colonization (Anderson et al., 2018) (Figure 2d). Prolonged hla activity can rupture endothelial cells, allowing for hematogenous spread as seen in patients with pneumonia or wound infections (Inoshima et al., 2011). In this study, consonant with the observed decrease in hla expression, we found a four order of magnitude reduction in viable bacteria in the spleen of FLX-treated mice (2.4 × 10⁶ vs. 8.4 × 10² colony-forming units, Figure 2d). These results indicate that FLX inhibits the expression of several virulence factors that play a role in bacterial dispersal and subsequent systemic spread of infection.

In summary, these data suggest that topically applied FLX has the potential to alter infected cutaneous wounds by (i) directly inhibiting planktonic growth, (ii) limiting biofilm formation, (iii) improving wound re-epithelialization, and (iv) preventing hematogenous dissemination of bacteria from the wound site. These FLX-mediated responses suggest a role for topically administered FLX as a nonantibiotic adjunctive therapy in limiting some of the complications of bacterial dissemination from infected wounds such as cellulitis, sepsis, or osteomyelitis. Because FLX has a long history of safe use as a systemic drug, it may have a facilitated path to clinical translation as a topical wound therapy.

Data availability statement
Datasets related to this article can be found hosted at Mendeley with the following link: https://doi.org/10.17632/bf2bxzwxgf.1

ACKNOWLEDGMENTS
We acknowledge Michael Wong for assisting with patient consents for human skin tissue and the staff at the electron microscopy facility of the University of California, Davis, for sample preparation and imaging, We thank the University of California, Davis, Clinical and Translational Science Center staff for their assistance with biostatistical analysis. This study was supported in part by a Preclinical Development Award from the California Institute of Regenerative Medicine (PIC-08118) to RRI, a California Institute of Regenerative Medicine doctoral training grant (TG2-01163) to CN, and a University of California, Davis, Department of Dermatology Seed grant (RRI).


SUPPLEMENTARY MATERIALS AND METHODS

Bacterial strains and growth conditions

*Staphylococcus aureus* strain UAMS-1 clinical isolate (gift of Mark Smeltzer, University of Arkansas for Medical Sciences, Little Rock, AR) was cultured in tryptic soy agar and broth (BD Biosciences, San Jose, CA) and routinely grown with 0.1% casamino acids (Fisher Scientific, Hampton, NH) and in the growth rate studies, UAMS-1 was grown in DMEM (Thermo Fisher Scientific) supplemented with 0.1% casamino acids (Fisher Scientific) solved in DMEM supplemented with 10% fetal bovine serum, bacterial inoculum, or inoculum containing 0.2% FLX was added to each wound and cultured individually in a culture well at 37 °C and 5% carbon dioxide. The epithelial surface of explants was maintained at the air–liquid interface, wounds were treated, and media were changed daily. On day 3 of culture, explants were harvested.

Growth rate analysis assays

Colony-forming units (CFU) of liquid broth cultures of UAMS-1 were enumerated by spectroscopy, and suspensions were uniformly normalized to concentrations of 0.8–1.2 × 10⁸ CFU/ml. Growth rate analysis was performed in 96-well plates by measuring optical density values at 600 nm through the Spectramax Plus Microplate Reader (Molecular Devices, San Jose, CA) every 2 hours for a total of 12 hours. For the growth rate studies, UAMS-1 was grown in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 0.1% casamino acids (Fisher Scientific, Hampton, NH) and in the presence and absence of 100 μM fluoxetine (FLX) hydrochloride (Sigma Aldrich, St. Louis, MO). Relative quantification of biofilm formation by crystal violet staining

Broth cultures were spectroscopically enumerated and normalized to 0.8–1.2 × 10⁸ CFU/ml in a 96-well plate. After 24 hours of incubation at 37 °C, media and corresponding treatments were replaced. After 48 hours, the nonadherent planktonic cells were removed, and biofilm biomasses were washed once with ×1 PBS; fixed at 65 °C for 45 minutes; and then stained for 5 minutes at room temperature with a 0.1% crystal violet solution of gentian violet (Fisher Scientific), 1:1:18 mixture methanol, isopropanol, and ×1 PBS. Excess dye was removed and washed out three times with PBS. The stained biofilm was then sequestered and solubilized using 33% acetic acid and quantified by measuring the optical density at 580 nm. Biomass values were compared in the presence and absence of 100 μM FLX.

Human skin collection, inoculum preparation, wound induction, and culture

Ex vivo wound infection was established as we have reported (Yoon et al., 2019). Briefly, discarded human skin obtained at the time of elective cosmetic surgery was trimmed of any attached adipose tissue. Wounds were created using 3 mm punch instruments and excised into individual skin explants using 8 mm punch instruments. UAMS-1 was incubated for 15 hours in tryptic soy broth at 37 °C and then normalized to 0.8–1.2 × 10⁸ CFU/ml. Then, 10 μl of vehicle, 5% polyethylene glycol (Thermo Fisher Scientific) dissolved in DMEM supplemented with 10% fetal bovine serum, bacterial inoculum, or inoculum containing 0.2% FLX was added to each wound and cultured individually in a culture well at 37 °C and 5% carbon dioxide. The epithelial surface of explants was maintained at the air–liquid interface, wounds were treated, and media were changed daily. On day 3 of culture, explants were harvested.

Wounding and infection of C57BL/6J mice

The 11-week old female C57BL/6J wild-type mice (Catalog number 00064, Jackson Laboratory, Sacramento, CA) were used for this study. UAMS-1 inoculum was prepared as described earlier and normalized to 0.8–1.2 × 10⁸ CFU/ml. Two silicon splints, 10 mm in diameter, were glued and sutured onto the dorsum approximately 4 cm posterolateral to the nape; inside which two full-thickness circular excision wounds were generated by 8 mm biopsy punch and surgical scissors. Then, 20 μl PBS or bacterial inoculum corresponding to 1.6–2.4 × 10⁶ CFU/ml were added to each wound. Wounds were subsequently covered with Tegaderm (3M, Maplewood, MN) and sealed with New-Skin Liquid Bandage (New-Skin, Cedar Knolls, NJ). After 48 hours, 0.2% FLX in 20 μl of 5% polyethylene glycol dissolved in PBS vehicle or 20 μl of vehicle alone was applied to each wound daily for 5 days. Wound dressings were routinely examined for tears and repaired with Tegaderm as needed. Mice were euthanized 7 days after surgery, after which, skin, blood, and spleen were harvested.

Histological analysis and quantitation of wound re-epithelialization

Harvested skin was fixed for 12 hours in 4% paraformaldehyde, transferred to 70% ethanol, and then paraffin processed using the Sakura Tissue-Tek VIP processor 6 (Sakura Finetek, Torrance, CA). Sections of bisected wound tissue, 5-μm in size, containing the widest diameter of the wound were stained with Gill’s Hematoxylin III (American MasterTech, Lodii, CA) and Eosin (VWR, Radnor, PA) stains. Wounds were then imaged using the BioRevo BZ-9000 microscope and analyzed for re-epithelialization (Keyence Corporation of America, Osaka, Japan) as previously described (Yoon et al., 2019).

Scanning electron microscopy

Skin explants were fixed, dehydrated, gold sputter coated, and imaged as previously described (Yoon et al., 2019).

Bacterial enumeration

A quarter of each wound or half of each mouse spleen was placed in sterile PBS, homogenized using the BBX24 Bullet Blender (Next Advance, Troy, NY) and then were serially diluted from 10⁻¹ to 10⁻⁷ in triplicates, plated onto Luria-Bertani or tryptic soy agar plates, and incubated at 37 °C for 24 hours. The resulting colonies were counted and back calculated to enumerate CFUs.

Relative mRNA expression

Planktonic UAMS-1 cultures normalized to 0.8–1.2 × 10⁸ CFU/ml were cultured in six-well plates for 72 hours in the presence or absence of 100 μM FLX. The mRNA was isolated from these bacterial cell lysates and also from human and/or mouse wound tissue as indicated in the text, and the copy number of mRNA transcripts was quantitated and analyzed as previously described (Yoon et al., 2019). The gene targets were amplified using primer sequences listed in Supplementary Table S1.

Statistics

Three human skin donors and 30 mice were used for this study. Three mice were excluded from the study owing to complications during surgery, and in
total 27 mice were used in the final analyses. All in vitro assays were conducted in technical triplicate. Re-epithelialization of mouse wounds were analyzed by a scorer blinded to the treatment protocol. Genetic analyses were conducted by the CFX Maestro software (Bio-Rad, Hercules, CA); the source code is available on the product information page on the Bio-Rad website. Multiple t-tests were used to analyze planktonic growth curves, with Sidak–Bonferroni correction. The biofilm assays were analyzed using one-way ANOVA with Dunnett’s multiple comparison. Student’s t-tests were used for all other statistical analyses, with figure error bars represented as mean ± SEM and statistical significance set at *P < 0.05.

Study approval
Human skin tissues destined to be discarded were obtained from patients undergoing panniculectomy or lower body lift cosmetic procedures. Written consent was received for each patient under a University of California, Davis Institutional Review Board–approved protocol, and tissues were deidentified before use in the study. Mouse wounding and wound infection studies were approved under a University of California, Davis Institutional Animal Care and Use Committees approved protocol.

SUPPLEMENTARY REFERENCE

Supplementary Figure S1. Dose–response relationship of FLX in planktonic cultures of UAMS-1. Minimal inhibitory concentration of FLX against UAMS-1 was identified as 100 μM. CTRL, control; FLX, fluoxetine; OD, optical density.

Supplementary Figure 2. Murine-infected wound–representative images. Quantitative data are presented in Figure 2 legend. CTRL, control; FLX, fluoxetine.
Supplementary Table S1. Primer Sequences Used in the Study

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fib</td>
<td>GAATATGGTGCAGTCCACAATT</td>
<td>AAGATTTTGAGCTTGAATCAATTTTTGTCTTTTT</td>
</tr>
<tr>
<td>sarA</td>
<td>TTTTACCATGGCAATTACAAAAAT</td>
<td>TTTCTTTTGTTCGCTGATGTAT</td>
</tr>
<tr>
<td>icaA</td>
<td>CAATACTATTCCGGGTCTCTCCTCT</td>
<td>CAAGAAACTGCAATATCTTCCGGAATACAT</td>
</tr>
<tr>
<td>icaD</td>
<td>TCAAGCCCAAGACAGAGGGAAATA</td>
<td>ACACGATATAGCCATAAGTGCAGTTTT</td>
</tr>
<tr>
<td>hla</td>
<td>CAACTGATAAAAAAAATAGGCTGGAAAGTGAT</td>
<td>CTGGTGAABAACCTGAAGATAAATAGAG</td>
</tr>
<tr>
<td>agrB</td>
<td>TGCAAAAGTCTGGATATTTTGTTTACA</td>
<td>GGTATAGGTGCTTTTTGTCTTTGCT</td>
</tr>
<tr>
<td>16S</td>
<td>GGTCTGTAAGCTGACGCTGATGTG</td>
<td>GGTGACTACACGGGATCTGATCTCCT</td>
</tr>
<tr>
<td>il6 (human)</td>
<td>GGTACATCCTCGACGGGCATCT</td>
<td>GGTGCAGAGAGGAGGTGCAC</td>
</tr>
<tr>
<td>TNFα (human)</td>
<td>GAAGCATGATTECGGAGCTG</td>
<td>GATGCCAGAGAGGAGGTGCAC</td>
</tr>
<tr>
<td>TGFβ</td>
<td>CTAACTGGTGAACCCACACACG</td>
<td>TATCGCCAGGAATGTGGCTT</td>
</tr>
<tr>
<td>K16</td>
<td>GCCGCGCGAGATGGAAC</td>
<td>CTGCTGATCTGCTGAC</td>
</tr>
<tr>
<td>K17</td>
<td>AAGATCCGCTGACGCTACGAGAG</td>
<td>GATGTCGGCCTCACAACACAG</td>
</tr>
<tr>
<td>Tnfa (murine)</td>
<td>CCAGACACCATGACTCATGAC</td>
<td>CACTTGCGCTTGGCAGTAC</td>
</tr>
<tr>
<td>il6 (murine)</td>
<td>TAGTCCTCTACCCCAAATTCC</td>
<td>TTTGCTGCTTTGACCTTTCT</td>
</tr>
<tr>
<td>il10</td>
<td>ATGACGCTACACACTGATC</td>
<td>CCCATTTTTGCGAGTTCCT</td>
</tr>
<tr>
<td>Pdggβ</td>
<td>AAGTGGTGAGCAATATGAGCAACAC</td>
<td>CATGGGTGTGGCTAAAACCTTTGG</td>
</tr>
<tr>
<td>Col3a1</td>
<td>ACGTGAGGATGGGATGCAG</td>
<td>GGGTGGGGGACGCTGATG</td>
</tr>
<tr>
<td>nNos</td>
<td>GTTCGACCCCAACAATACCAGA</td>
<td>GGGACGCGCTGAGATGAC</td>
</tr>
<tr>
<td>Arg1</td>
<td>CCCAACACCAAGTTCCCTAGAG</td>
<td>AGGAGCTGCTATTAGGGAC</td>
</tr>
</tbody>
</table>

Abbreviation: K, keratin.