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Staphylococcus epidermidis biofilm growth in response to glucose: Implications for diabetic implantable device infections

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SUMMARY The association between high bacterial infection rates and diabetes mellitus (DM) has been well established, where DM specifically predisposes individuals to frequent and severe biofilm-associated implantable device infections (IDI). Diabetic hyperglycemia, or elevated blood sugar, is accepted to contribute to this phenomenon and thus has prompted questions regarding how biofilm development is enhanced in a glucose-rich environment. *Staphylococcus epidermidis* is the species responsible for most IDI, however, few studies have evaluated the effect of excess glucose on the biofilm formation of this bacterium and have not considered how low oxygen and pH, the physiologically relevant characteristics of IDI, may affect results. Here, *S. epidermidis* was grown in media containing 0, 20, 200, and 2000 mg/dL of glucose under aerobic and anaerobic conditions. Biofilm mass was quantified using the microtiter crystal violet assay and media pH was measured prior to and following biofilm growth. Glucose significantly increased biofilm mass only in anaerobic conditions at 2000 mg/dL, where increasing glucose concentrations corresponded with progressive media acidification. These data identify a relationship between oxygen condition, reduced pH, and enhanced *S. epidermidis* biofilm mass under excess glucose conditions, and thus provide insight into different variables that may contribute to the link between IDI and DM.

INTRODUCTION

Hyperglycemia, or the presence of abnormally elevated blood glucose levels, is a common symptom of the metabolic disorder, diabetes mellitus (DM). Hyperglycemia is implicated in impaired immune system processes (1-5), and thus the frequent and debilitating infections seen in DM are considered a consequence of complex interactions between high blood glucose levels, host immunity, and the characteristics of the involved microbes (6,7). Bacterial species are most commonly the causative pathogens for DM infections (6, 8-10), which evidently pose a major challenge to diabetic health, especially in the case of biofilm formation. Biofilm is a surface-adhered aggregate of bacterial cells that is coated in a protective layer of extracellular polymeric substances (11) which among other factors, gives biofilms considerable resistance to antimicrobials and the immune response (12, 13). High glucose environments reportedly enhance overall biofilm growth (14, 15) and extracellular polymeric substances production (16). Correspondingly, hyperglycemic diabetics experience more frequent and severe biofilm-associated infections in contrast to those with sufficient glycemic control (17-19). In comparison to non-diabetics, those with DM are at a greater risk for specific biofilm-associated infections including periodontitis (20), osteomyelitis (21, 22), and implantable device infections (IDI) (23-26). IDI occur when biofilm forms on indwelling prosthetics and their surrounding tissue, and are most often caused by the gram-positive skin commensal, *Staphylococcus epidermidis* (27-30). Evaluating the influence of variable blood glucose levels on *S. epidermidis* biofilm growth is

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thus an important area of research if the relationship between IDI and DM is to be fully established.

In addition to glucose level, how other environmental conditions contribute to optimized *S. epidermidis* biofilm growth, such as oxygen availability and pH, must be understood to address the burden of IDI on DM. The stimulatory effect of glucose on biofilm has been evaluated previously for many bacterial species (31), where hyperglycemic glucose concentrations (>200 mg/dL) (32) have been shown to enhance *S. epidermidis* biofilm mass (33-35) and restore the species' capacity to form biofilm (36). In addition, *S. epidermidis* biofilm reportedly grows best in low pH (37, 38) and microaerobic to anaerobic conditions (39, 40). Despite indwelling medical devices being surrounded by near-hypoxic tissues (41-43) and infected implant sites being generally acidic (44), all previous studies evaluating the effect of glucose on *S. epidermidis* biofilm growth have failed to measure environmental pH or anaerobic biofilm growth (15, 33-35). Additionally, an evident issue regarding these studies is the inconsistent results which likely arise from differences in methodology, such as the chosen *S. epidermidis* strain, incubation time, or growth substrate. Here, the effect of increasing glucose availability on *S. epidermidis* biofilm in the presence and absence of oxygen was quantified, along with changes in pH following biofilm formation, to aid in elucidating the heightened severity and risk of IDI in those with DM and complement the limited literature. As the glucose level was raised, pH was predicted to progressively lower while anaerobic and aerobic *S. epidermidis* biofilm mass increased, where anaerobic biofilm growth would consistently surpass that of aerobic at each glucose concentration.

METHODS AND MATERIALS

Biofilm mass assay. *S. epidermidis* (Ward's Science, 470176-542) biofilm growth was quantified using a microtiter crystal violet assay as described previously (34) with slight modification. Briefly, *S. epidermidis* was cultured overnight on tryptic soy agar and used to inoculate 3 mL of glucose-free lennox broth. Bacteria were cultured overnight at 37°C with shaking at 150 rpm. Lennox broth containing 0, 20, 200, or 2000 mg/dL glucose was inoculated with *S. epidermidis* to 0.08 OD_{600} where 200 µL of each broth was plated (six wells per glucose condition) in two separate tissue-treated polystyrene 96-well plates (Corning, 353072). The 200 mg/dL glucose condition is representative of the hyperglycemic state (32). A seventh well per glucose condition contained only the respective broth as a contamination control. All broth was adjusted to pH 7 via a pH probe, however, the broth was detected as pH 8 when pH detection strips were used for the tracking of pH changes before and after biofilm growth. One plate was placed in an anaerobic chamber containing two oxygen gas packs (BD Biosciences, 260268) (anaerobic growth condition), and the other in a shallow dish lined with moistened paper towel and loosely covered with aluminum foil to reduce media evaporation (aerobic growth condition). The pH of one representative well per glucose condition (1 well out of 6 replicates) was measured before and after static incubation (67 hours at 37°C) via pH detection strips (Fisher Scientific, 1095350007) for two separate plates (n=2). Following incubation, plates were washed with dH_2O (3x) and left inverted for ~30 minutes. Biofilms were heat fixed via 1 hour of 60°C static incubation and treated with 200 µL of 0.1% crystal violet solution (Fisher Scientific, 548-62-9) for 15 minutes. Washing was repeated and 200 µL of 30% acetic acid solution was left on biofilms for 15 minutes to elute the crystal violet stain. Biofilms were not agitated during the crystal violet staining and elution process. Mass was quantified by measuring the resulting acetic acid solution with a NanoDrop 2000c spectrophotometer (ThermoScientific, ND2000CLAPTOP) at 600 nm (n=3), where the contamination control OD value was subtracted from that of each individual well within the same glucose treatment to ensure the OD readings reflected absorbance from biofilm growth only.

Statistical analyses. Significant differences in biofilm mass were determined by a two-way ANOVA and post hoc Tukey's test. The normal distribution and homoscedasticity of data were confirmed using data visualization, Shapiro-Wilk's test, and Levene's test. All analyses were performed via R programming with R studio software (45).

RESULTS

Biofilm mass. *S. epidermidis* biofilm formed in all four glucose media concentrations under both aerobic and anaerobic growth conditions (Figure 1). Respective mean biofilm mass (OD_{600} ± SEM) at 0, 20, 200, and 2000 mg/dL glucose were as follows: 0.8 ± 0.28, 0.6 ± 0.19, 1.3 ± 0.22, 1.8 ± 0.36 (aerobic), and 0.1 ± 0.04, 0.2 ± 0.01, 1.0 ± 0.08, 1.8 ± 0.18 (anaerobic) (Figure 1). Significant differences ($p<0.01$) in biofilm mass were found only between 2000 versus 0 and 20 mg/dL glucose in anaerobic conditions. Anaerobic and aerobic biofilm mass were not significantly different at any glucose concentration (Figure 1).

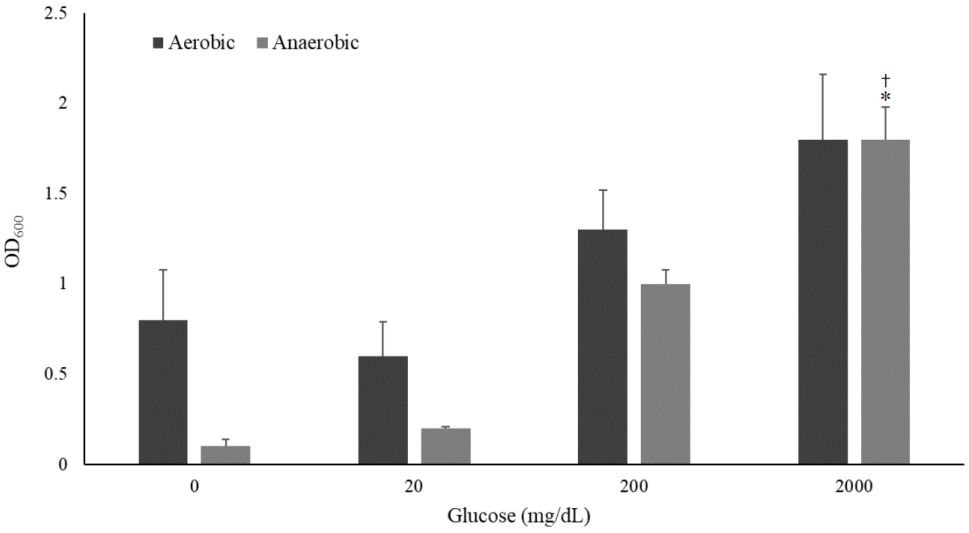

FIG. 1 Glucose Increases Anaerobically Grown *S. epidermidis* Biofilm. Biofilm growth in aerobic (dark grey) and anaerobic (light grey) conditions as quantified by a microtiter crystal violet assay. Biofilm mass significantly increased only in the presence of 2000 mg/dL within the anaerobic condition. Error bars represent mean + SEM (n=3), where *$p<0.01$ compared to anaerobic, 0 mg/dL glucose condition, and †$p<0.01$ compared to anaerobic, 20 mg/dL glucose condition according to a two-way ANOVA and post hoc Tukey's test.

Biofilm media acidification. In the aerobic and anaerobic growth conditions, pH was reduced from the initial broth reading of 8 (before biofilm growth) only in the 200 and 2000 mg/dL glucose concentrations (Figure 2). Respective mean pH values at 0, 20, 200, and 2000 mg/dL glucose were as follows: 8.0, 8.0, 5.5, 4.3 (aerobic), and 8.0, 8.0, 4.8, 4.3 (anaerobic) (Figure 2).

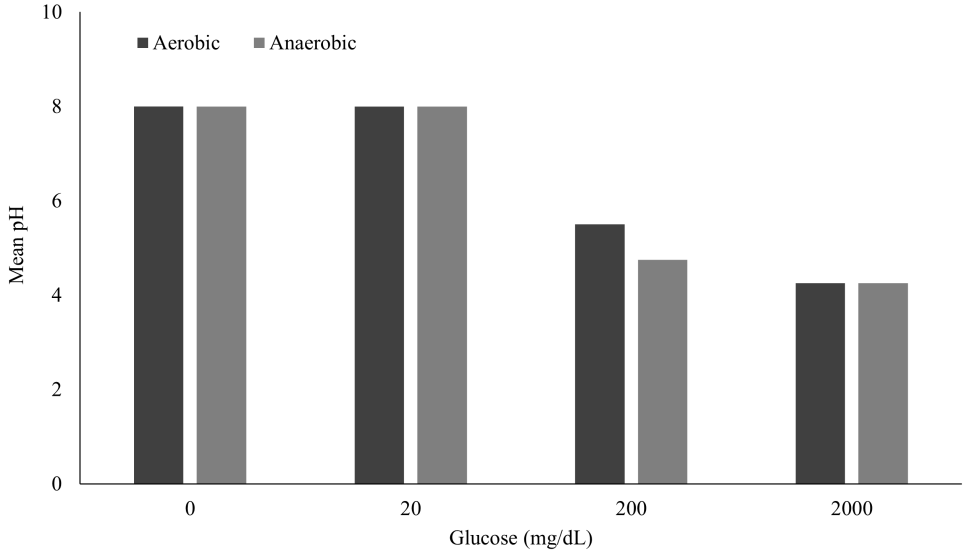

FIG. 2 *S. epidermidis* Biofilm Growth in Aerobic and Anaerobic Conditions is Characterized by Comparable Media Acidification. Acidity measurements were performed via pH detection strips following aerobic (dark grey) and anaerobic (light grey) biofilm growth in a microtiter crystal violet assay in which each broth (0, 20, 200, or 2000 mg/dL glucose) prior to growth was detected as pH 8 (n=2).

DISCUSSION

Previous literature evaluating the influence of glucose on *S. epidermidis* biofilm growth is heavily limited, characterized by contradictory results, and fails to explore how oxygen conditions and changes in pH may contribute to the enhanced risk of IDI in those with hyperglycemia. To contribute to the current body of evidence while also introducing a novel oxygen variable and tracking of pH, the effect of 0, 20, 200, and 2000 mg/dL glucose on *S. epidermidis* anaerobic and aerobic biofilm mass was investigated. *S. epidermidis* biofilm in

both oxygen conditions was hypothesized to significantly increase with each glucose concentration, where anaerobically grown biofilm would be larger than that of aerobic in all growth media conditions, yet a significant increase was detected only from 0 and 20 mg/dL to 2000 mg/dL in the anaerobic growth condition (Figure 1). Significantly more biofilm at 2000 mg/dL supports the hypothesis that higher glucose availability will enhance biofilm mass and aligns with 2000 mg/dL reportedly increasing *S. epidermidis* biofilm cell density previously (33). However, no significant effect of glucose at 200 mg/dL in either oxygen growth condition was unexpected given this concentration represents the hyperglycemic state (32) and the increased rate of bacterial infections in DM is established as a consequence of hyperglycemia (6, 7). Although this finding matches that of a recent study (15), others have reported a significant effect at both 200 (34) and 250 mg/dL glucose (35). In addition, *S. epidermidis* biofilm has been shown to grow better anaerobically than aerobically, which is contradictory to the findings of this study where no significant difference was found between the biofilm grown with or without oxygen for each glucose condition. Importantly, *S. epidermidis* was still viable after incubation in the 0 mg/dL glucose condition, indicating that less biofilm mass in the lower glucose conditions (0 and 20 mg/dL) is not a consequence of *S. epidermidis* being unable to grow in reduced glucose environments (Supplementary Figure S1 and S2). Unexpected results may have arisen from the chosen methodology; the microtiter crystal violet assay is one of the most widely used techniques for biofilm quantification (46, 47), to such an extent that a minimum information guideline has been recently developed (48), however, it has been argued that the required washing of biofilms and nonspecific binding of crystal violet to both planktonic and biofilm cells may cause overestimation or underestimation of true biofilm mass (46, 47). This assay was also employed in each previous study (15, 33-35) and thus may contribute to the contradictory findings. In addition to this, evaluating distinct *S. epidermidis* strains may cause different biofilm mass readings, given *S. epidermidis* metabolism (49) and biofilm-forming capacity (49, 50) have been shown to change according to strain. Importantly, those reporting the contrasting significant (34) and insignificant (15) effect of glucose on *S. epidermidis* biofilm at 200 mg/dL utilized the same crystal violet technique and *S. epidermidis* strain, and therefore emphasize the limitations of the microtiter crystal violet assay. Given this, the unexpected findings reported here are likely a consequence of this approach and the use of a different *S. epidermidis* strain, which demonstrates the need for more reliable and comparable methodological approaches in future studies measuring *S. epidermidis* biofilm growth.

An important factor to consider when comparing *S. epidermidis* biofilm growth is oxygen availability, yet previous studies have not indicated their oxygen condition, where it can only be assumed that aerobically grown biofilm was quantified (15, 33-35). *S. epidermidis* is a facultative anaerobe (40), but forms biofilm best in anaerobic conditions (39, 40) and therefore *S. epidermidis* biofilm mass can only be reliably compared if oxygen conditions are the same. Further, the relationship between IDI and DM is arguably best addressed by investigating anaerobically grown *S. epidermidis* biofilm. This is because device implantation results in near-hypoxic regions within surrounding tissues (41-43) and this wound site hypoxia is exacerbated in diabetics due to reduced circulation caused by the common DM symptoms, angiopathy and neuropathy (51). Given *S. epidermidis* attachment and biofilm growth is improved in little to no oxygen (39, 40), IDI may occur more often in diabetics because of the enhanced hypoxia, where the infection becomes more severe due to a consistent supply of excess glucose via hyperglycemia. In addition, another IDI-relevant species, *Pseudomonas aeruginosa* (52), has been shown to gain more antibiotic resistance when grown anaerobically (53, 54) and in an environment with excess glucose (55, 56). Although further research is needed to confirm if *S. epidermidis* responds similarly to glucose and anaerobic conditions in terms of antimicrobial resistance, based on the data presented here, implanted devices in those with DM may be an optimal growth environment for *S. epidermidis* through an interaction between tissue hypoxia and hyperglycemia.

In addition to oxygen availability, environmental pH can also impact *S. epidermidis* biofilm growth (37, 38). Here, pH decreased with each glucose increase beginning at 20 mg/dL in both oxygen growth conditions (Figure 2). The pH changes are assumed a result of acidic byproduct accumulation via glucose catabolism (57), where lactic acid and carbon dioxide are responsible for acidity in the anaerobic and aerobic conditions, respectively (58).

Biofilm growth in both anaerobic and aerobic conditions resulted in comparable media acidification at each glucose concentration, with the only difference occurring at 200 mg/dL (Figure 2). No significant difference in biofilm mass between oxygen conditions was found for this glucose level (Figure 1), however, the greater acidity in anaerobic growth may suggest there was more biofilm formed in the absence of oxygen that was masked by the limitations of the microtiter crystal violet assay.

The purpose of tracking pH was to determine whether increased biofilm mass corresponded to decreased pH, due to all previous studies evaluating the effect of glucose on *S. epidermidis* biofilm failing to report pH changes (15, 33-35), and acidity being known to affect biofilm growth (59). It has been hypothesized that in addition to energy production, bacteria that prefer low pH utilize excess glucose to create a more favourable growth environment through metabolism-induced acidification of their surroundings (60). Given low pH has been shown to enhance *S. epidermidis* biofilm growth (37, 38), it is possible catabolism of excess glucose by *S. epidermidis* biofilm is driven based on the desire to lower environmental pH and therefore optimize growth conditions. IDI are already more acidic than normal physiological levels (44), and in consideration with this hypothesis, diabetics may frequently contract IDI because the surplus of glucose available to *S. epidermidis* via hyperglycemia can be metabolized to both increase biofilm mass and further acidify the biofilm microenvironment to maintain the infection. The advantage of glucose metabolism is well documented across many bacterial species, especially with respect to virulence (61). Glucose is not only shown to be the optimal carbon source for performing important infection-related processes such as toxin expression (62), antibiotic resistance development (63), and bacteriocin production (64), but bacteria can also alter their glucose metabolism rate to increase their chances of survival. For instance, gut microbiome *Escherichia coli* reportedly raise their glucose catabolism to lower intestinal pH in a manner that impairs the growth of competing bacteria (65, 66). In a similar fashion, *S. epidermidis* may modify environmental pH through excess blood glucose utilization in a hyperglycemic individual to enhance and sustain IDI. Acidity may therefore play an important role in the severity of IDI in DM, however, this topic requires further research that more specifically and reliably evaluates how low pH is affecting *S. epidermidis* biofilm mass.

Limitations In addition to the microtiter crystal violet assay, this study is limited by the pH detection methods, monospecies *in vitro* approach, media evaporation that occurred in the aerobic growth condition, and sample size. The pH detection strips used could not distinguish small differences in acidity or detect a pH beyond 4. Given this, more comprehensive and specific pH detection methods may have revealed a lower pH at each glucose concentration of the anaerobic condition, which would support previous literature (39, 40) and the original hypothesis that anaerobic biofilm mass would consistently surpass that of aerobic. Further, *in vitro* monospecies biofilm does not represent typical biofilm infections which are usually polymicrobial (67) and would be affected by additional factors such as temperature, osmolarity, ion concentrations, and the presence of other nutrients (68). Biofilm mass could vary depending on the growth rates of different species present within the biofilm and also the chosen growth substrate. In addition, the use of a humidified dish only reduced the level of media evaporation occurring in the aerobic condition rather than completely removed it. Given this, the nutrients available to *S. epidermidis* uncontrollably fluctuated in aerobic growth, causing high variability in biofilm mass which likely compromised the capacity to reach statistical significance in this condition. Since *S. epidermidis* biofilm can grow both anaerobically and aerobically in glucose (39), increasing glucose availability was expected to correspond to significantly more biofilm in each growth condition. If media evaporation was controlled for, a significant effect of glucose at 2000 mg/dL in the aerobic condition may have been reached, potentially along with significantly more growth in the anaerobic condition in comparison to aerobic throughout all glucose treatments. These results would support *S. epidermidis* being a known facultative anaerobe that grows biofilm best in anaerobic conditions (39, 40). Finally, a small sample size was used in this study for both assays. The pH data were based on an n=2, which does not allow for statistical analyses and therefore additional tests are needed to confirm whether the differences in pH across glucose conditions were significant. The biofilm mass assay was limited to n=3, which upon further

replication with controlled media evaporation, may have resulted in a significant effect of glucose at the clinically relevant 200 mg/dL glucose treatment in either oxygen condition. This result would be consistent with the hypothesis that an interaction between oxygen, pH, and excess glucose contributes to the increased frequency and severity of IDI in those with DM.

Conclusions Here, the effect of 0, 20, 200, and 2000 mg/dL glucose on *S. epidermidis* biofilm mass in anaerobic and aerobic conditions was evaluated to complement the currently contradictory and heavily limited body of literature on this topic and further explore variables that may contribute to the burden of IDI on diabetics. Elevated glucose significantly increased anaerobically grown *S. epidermidis* biofilm mass and increasing glucose concentration resulted in progressive media acidification for both oxygen conditions. This data provides a link between hypoxia, low pH, and excess glucose in enhanced *S. epidermidis* biofilm growth, and therefore reveals a potentially important interaction that may explain how those with DM experience more frequent and severe IDI.

Future Directions In addition to replication of this study with an increased sample size and multiple reliable quantification methods, including a maintained pH condition for both oxygen levels would more directly address whether reduced pH, hypoxia, and elevated glucose create an optimal environment for *S. epidermidis* biofilm growth.

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