The Effect of Pathophysiologic Glucose Concentration on Biofilm Growth In Vitro

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Introduction: Surgical site infections are the second most common cause of nosocomial infection in the United States and lead to longer hospital stays, higher mortality rates, and increased cost. Diabetes mellitus has been linked to postoperative and nosocomial infection by many authors. There have been several efforts to define target ranges for perioperative blood glucose in an effort to help curb post-operative infection rates. For most clinicians perioperative blood glucose control is a concern that revolves around host optimization. It is accepted that elevated blood glucose concentration leads to non-enzymatic glycosylation of cells in the body. This includes soft tissue that must be dissected and repaired during surgery as well as cells of the immune system. Previous authors have reported that elevated blood glucose levels effect both the cellular and humeral arms of the immune response thus compromising a host’s ability to combat a potential infection. It is also well known that prosthetic joint infections(PJI) arise from biofilm forming bacteria.

It has long been recognized that the growth rate of bacteria is dependent on the concentration of nutrients present in the media. It was not appreciated until recently that some of those nutrients may play a role as signaling molecules, affecting the behavior of the bacteria to which they are exposed. Recent research by multiple centers has suggested that the potential of bacteria to form biofilm is moderated by the concentration of glucose present in the media. Fluorescence studies suggest that the presence of increasing concentrations of glucose affects the agr pathway, leading to the formation of biofilm. It has also been suggested that the pH of the local cellular environment may play a role in this signaling process. Additional studies have found that in controlled conditions, there is a consistent increase in biofilm production across many different strains and bacterial types in media with high (1%) concentrations of glucose. The concentrations used to generate biofilms in many of these studies are in a greatly super-physiological range, however, and authors may not account for the presence of glucose in the media when they additionally supplement the media with glucose.

This study seeks to explore the relationship between biofilm formation and glucose concentration in a physiologic to pathophysiologica range. This study asks the question: What is the effect of pathological concentrations of glucose on biofilm growth?

Methods: Strain Selection:
Two standardized strains were selected for this work: Staphylococcus epidermidis (ATCC 35984) and Staphylococcus aureus (ATCC 49230, UAMS 1).

Biofilm Growth:
Biofilms were grown in accordance with the procedure documented by Kwasny and Opperman[15], excepting the use of a different media with varying glucose concentrations. Cells were grown overnight in lenox Broth (Alfa Aesar, Ward Hill, Massachusetts). Overnight cultures were then diluted 100fold with growth media supplemented with varying glucose concentrations. Final glucose concentrations ranged from 0-320 mg/dL. 200 mL of the bacterial dilutions were seeded in tissue culture treated 96 well plates (Sarstedt, Newton, North Carolina). Plates were then incubated at 37°C for 24-48hrs without shaking. Excess liquid culture was removed and biofilm were washed four times by gentle immersion in deionized water. Biofilm was then heat fixed at 60°C for 1hour. Biofilm was stained with 0.1% crystal violet dye (Best Science Supplies, Big Pine Key, Florida) for 15min.

Crystal violet dye was removed and the biofilm washed four times by gentle immersion in deionized water. Finally, Crystal violet dye was eluted with 30% acetic acid solution for 15 min. 200 uL of extracted dye was then transferred to unused wells in the 96 well plate and assayed by Fluostar Omega Multiplate Reader (BMG Labtech, Cary, North Carolina) at 600nm. Samples were intermittently photographed with a Nikon Macro Lens(Nikon, Melville, NY).

Statistical Analysis:
Amount of biofilm grown over time was analyzed with ANOVA with glucose concentration as a factor. Post-hoc analyses was performed with Tukey’s Test for multiple comparisons to identify similar groups. Standard Normal plots of residuals were constructed to determine if the ANOVA model was well behaved.

Results: Staphylococcus epidermidis was a much more robust biofilm former than staphylococcus aureus at all glucose concentrations(p<0.001, ANOVA). Both species tested demonstrated a statistically significant increase in the production of
biofilm with increased glucose concentration in the culture media (p<0.001, ANOVA). Staphylococcus epidermidis showed a consistent increase in biofilm formation with no clear break point. This is in distinction to Staphylococcus Aureus which did not demonstrate a substantial increase in biofilm production until a threshold was crossed between 200mg/dl and 220mg/dl (p<0.001).

**Discussion:** This data represents the first investigation to the author’s knowledge on the effect of glucose concentration within clinically relevant levels on biofilm formation. There are several limitations to this study. First, biofilm formation was only studied *in vitro*. Secondly, we did not track the pH of the growth media. While previous results have suggested that increased biofilm formation may in part be due to changes in pH when glucose is present, most experiments do not track this variable. It is also not apparent what the pH may be in any given wound environment, given differences in circulation, exposure, and wound conditions in various clinical situations. It was felt to be more appropriate to begin with a simple culture system to understand the behavior of the microbes, and we may add an arm including pH control in future work. Thirdly, we only studied two bacterial strains, there are several other types and strains of bacteria that are clinically relevant, and this effect may not happen in all bacteria. The results by Kwasny and Opperman, however, suggest that the tendency to form biofilm when exposed to glucose is conserved among the different gram positive organisms that they studied. Finally, our work involves a defined culture system that does not include many of the dissolved proteins and antibacterial peptides present in human blood and extracellular fluid. Our results suggest that the rate of biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis* is quite different, at least for the two strains studied. In the same time period, *Staphylococcus epidermidis* produced five times the amount of biofilm produced by *Staphylococcus aureus*. These results support that pathophysiological glucose concentrations would be expected to impact biofilm formation rates *in vivo*. These results suggest that the formation of biofilm *in vivo* is happening over hours to days after exposure, and that the presence of pathophysiological glucose concentrations, depending on other environmental variables and bacterial types, may reduce the time that the body has to respond to the pathogen before it is well established in biofilm. Thus, postoperative glucose management may be critical in controlling the subsequent PJI infection rate.

**Significance:** In vitro testing indicates a statistically significant increase in biofilm formation for strains of *Staphylococcus Aureus* and *Staphylococcus Epidermidis* as glucose concentration increases into the pathologic level.
Figure 1: Staphylococcus aureus growth as a function of glucose concentration.
Glucose concentration is reported in mg/dL. There is a significant trend of increased growth as the concentration increases.

Reference:
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