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Effect of Human Insulin on the Formation of Catheter-Associated *E. coli* Biofilms

Balbina J. Plotkin[1*], Zijian Wu[1], Kathleen Ward[1], Shaven Nadella[1], Jacalyn M. Green[2], Benny Rumnani[1]

[1]Department of Microbiology and Immunology, Midwestern University, Downers Grove, USA
[2]Department of Biochemistry, Midwestern University, Downers Grove, USA
Email: [*]bplotk@midwestern.edu

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Abstract

Biofilm formation is essential for the survival and growth of *Escherichia coli* in catheter-associated infections. Individuals with type 2 diabetes mellitus can excrete insulin and/or glucose in their urine. This population also has an increased incidence of urinary tract infections. The focus of this study was to determine if the composition of Foley catheter material affects biofilm formation by *E. coli* in a model system for type 2 diabetes mellitus. Rubber (lubricious-coated), silicon-coated, silver-coated and nitrofurazone-coated catheter segments (5 mm; n = 6) were tested. Catheter segments were added to *E. coli* ATCC25922 (10^4 CFU/ml, final concentration) in artificial urine alone, or with insulin (40 μU/ml) and/or glucose (0.1%). After incubation (18 h, 37˚C, in air and anaerobically) the level of catheter-associated biofilm was determined by crystal violet staining (Abs_{550nm}). Statistical analysis was done by ANOVA with post-hoc analysis (Tukey). Neither nitrofurazone-coated nor silver-coated catheters supported the formation of *E. coli* biofilm, regardless of growth condition tested. In contrast, under aerobic biofilm formation on silicon catheters was significantly higher ($p < 0.05$) than that on sterile catheter alone. In addition, glucose with insulin induced significantly more biofilm ($p < 0.05$) than *E. coli* controls. Biofilm formation was also significantly increased ($p < 0.05$) under anaerobic conditions on lubricious-coated rubber catheters as compared to sterile catheters. These results may aid in the development of a catheter material that can prevent biofilm formation, or alternatively guide choice of catheter material for individuals shedding insulin in their urine.

Keywords

***Escherichia coli*, Insulin, Catheters, Diabetes Mellitus, Biofilm, Electronegativity**

[*]Corresponding author.

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1. Introduction

Catheter-associated urinary tract infections (UTIs) account for eighty percent of all healthcare associated UTIs [1]-[6]. Catheterized patients who develop a UTI are overall three times more likely to die than similarly infected patients without a catheter. Of the catheterized population, individuals with diabetes mellitus have increased mortality from catheter-associated infections as compared to non-diabetic individuals [7]. However, the underlying reason for increased incidence of morbidity and mortality has not been fully defined. A possible contributing factor is the presence of insulin, in addition to glucose, in urine [8]-[14]. Depending on an individual's disease status, excess insulin is excreted in the urine in both the presence and absence of glucose. Insulin excretion can occur when there is dysregulation of insulin production in response to increased blood glucose levels, peripheral insulin resistance, or, as in the case of insulin-dependent diabetes mellitus, excess insulin levels at the pharmacokinetic peak immediately post-injection [8]-[14]. Although glucose has been shown to be associated with increased bacterial growth and biofilm formation, the role urinary insulin plays in catheter-associated infections has not been explored. [7] [15]

As the most common etiologic agent of UTIs across all patient populations, *E. coli* is particularly recognized as a cause of nosocomial UTIs [2] [3] [9] [16]-[19]. *E. coli* is also one of the most common causes of bacterial sepsis, typically occurring secondary to a UTI. A virulence factor commonly associated with *E. coli* infection is the ability to form a biofilm [6]. These biofilms can complicate patient health and medical procedures because they are difficult to prevent and eliminate. Biofilms are aggregated colonies of bacteria living together and protected by an extracellular matrix (e.g. capsular polysaccharide) that is excreted by the bacterial population [20]. Characteristics of bacterial biofilms include nutrient and oxygen gradients [21]. This variation in environmental conditions results in phenotypic alterations typically triggered by quorum signaling.

Quorum signaling is intercellular bacterial communication that relays information to individual organisms regarding the density of the bacterial population and the suitability of the environment for survival. The molecules that are utilized as quorum signals can be intra-species, inter-species, or inter-kingdom in origin [22] [23]. Human recombinant insulin (insulin-r) functions as an inter-kingdom quorum-signal compound for *E. coli* [24] [25]. In *E. coli*, insulin-rquorum signaling has been shown in previous studies to play an important role in the regulation of *E. coli* chemotactic responses, growth rates, and biofilm formation [24] [25]. In the presence of glucose, insulin-r enhances *E. coli* biofilm formation [25]. However, whether insulin and glucose affect biofilm formation under oxygen limitation (anoxic conditions) is not known. This gap in our understanding of biofilm formation represents a significant deficit since the oxygen levels at mucosal surfaces, and certainly in catheter interiors, would be extremely hypoxic to anoxic, respectively. The focus of this study is to determine if catheter composition and oxygen levels affect biofilm formation by *E. coli* in a model for type 2 diabetes mellitus. Results from this study may indicate which catheter material is optimal for utilization in individuals with uncontrolled type 2 diabetes mellitus.

2. Materials and Methods

2.1. Bacterial Isolate and Growth Conditions

E. coli ATCC 25922, a highly stable quality control strain shown to recognize and respond to insulin-r as a quorum-signaling compound, was used for all studies [24] [25]. Bacterial stock was maintained in skim milk medium at −80˚C until use. For catheter biofilm formation assays, filter sterilized artificial urine (AU), freshly prepared each day, was used. The composition of the AU was (g/l): $CaCl_2$, 0.65 g; $MgCl_2$, 0.65 g; NaCl, 4.6 g; Na_2SO_4, 2.3 g; $Na_3C_3H_5O(CO_2)_3$, 0.65 g; $Na_2C_2O_4$, 0.02 g; KH_2PO_4, 2.8 g; KCl, 1.6 g; NH_4Cl, 2.0 g; urea, 12.0 g; creatinine 1.1 g; TSB 3.0 g; $NaSO_4$, 2.3 g; pH 6.2 (Sigma-Aldrich) [26]-[28]. For surface electronegativity determinations (adherence to glass) *E. coli* was grown, as previously described for other assessments of cell surface characteristics, in peptone (0.01 gm/ml) yeast nitrogen base broth (PYNB; pH 7 and pH 5.5) [24] [25].

2.2. Catheters Tested

Four types of 16 fr/ch commercially available catheters were used for the study (C.R. Bard, Inc, Covington, GA). The types tested were rubber (lubricious-coated), silicon-coated, hydrogel®, Bactigard® silver alloy-coated and nitrofurazone-coated catheters (**Figure 1**). Catheter shafts were aseptically cut into 5 mm segments (n = 8/growth condition). Segments were placed in wells (1/well) of 24 well plates containing 1.5 ml of bacterial

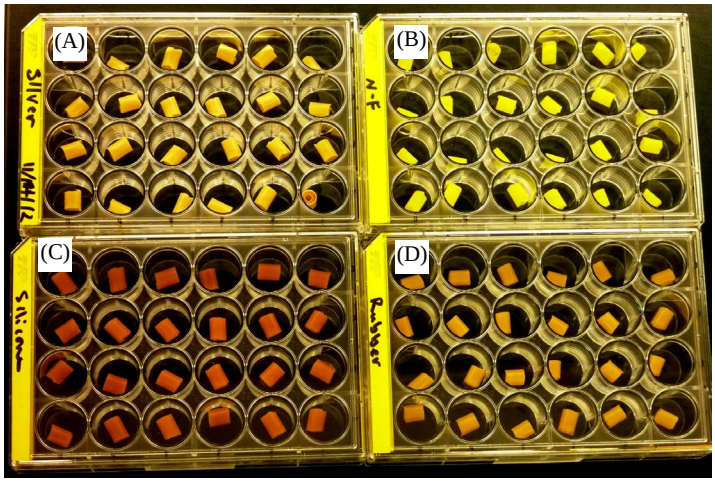

Figure 1. Catheter segments (5 mm) used for determination of effect of catheter composition and insulin, glucose and anaerobiosis on the formation of *E. coli* catheter associated biofilm. (A) Silver catheter; (B) Nitrofurazone catheter; (C) Silicon catheter; (D) Rubber catheter.

suspension (10^5 CFU/ml) in AU alone, AU with and without insulin-r (40 μU/ml; Humulin® R, Eli Lilly and Co., Indianapolis, IN) and/or glucose (0.001 g/ml). After incubation (air and anaerobic conditions; 37˚C; static; 24 hr) the segments were washed with gentle agitation (4x, PBS, pH 6) then stained (crystal violet; Troy Biologics). Unbound stain was removed by extensive washing (PBS, pH 6). Catheter segments were then destained (absolute ethanol, 3 ml/catheter in 15 ml sealed conical centrifuge tube; intermittent agitation, three weeks). The level of catheter-associated biofilm, as measured by crystal violet absorbance, was determined (Abs_{550nm}). Negative controls (n = 8 for each catheter type) consisted of catheter segments incubated in the absence of bacteria in AU alone, or with glucose (0.001 g/ml) and/or insulin-r (40 μU/ml) then processed as described for test catheter segments.

2.3. Biofilm Release

To determine if insulin can reverse *E. coli*'s sessile (biofilm) state and enhance its return to planktonic state (biofilm release), rubber and silicon catheter segments produced and incubated in the bacterial suspension, as described above, were washed with gentle agitation (4x, PBS, pH 6) then placed in PBS alone (control) or inPBS with insulin-r (40 μU/ml). After incubation (30 min; 37˚C), the catheter segments were processed as described above. The level of catheter-associated biofilm as measured by crystal violet absorbance was determined spectrophotometrically (Abs_{550nm}).

2.4. Surface Electronegativity

To determine the effect of insulin on *E. coli*'s relative surface electronegativity, its adherence to glass (an electronegative substrate) was determined [29] [30]. Overnight cultures in PYNB alone, or with insulin-r (200 μU/ml) and/or glucose (0.001 g/ml) were inoculated (10^5 CFU/ml) into homologous medium with and without insulin-r and/or glucose. These bacterial suspensions (0.5 ml) were placed in flat bottom 24 well plates containing sterile, acetone washed glass coverslips (round, 12 mm). After incubation (18 hr; 37˚C), the coverslips were removed, washed extensively, stained with crystal violet and dried. The dye was removed from the coverslips (ethanol, 0.5 ml) and Abs_{590nm} determined. All assays in were done in quadruplicate and repeated at least twice.

2.5. Statistical Analysis

Each experiment was performed at least in triplicate and repeated twice. Whenever possible, experiments were coded and performed in a blinded fashion. Statistical analysis was done by ANOVA (InStat, GraphPad Software Inc.) with post-hoc analysis (Tukey-Kramer). Mean values were considered significantly different at $p < 0.05$.

3. Results

3.1. Effect of Insulin-r and/or Glucose on Catheter-Associated Biofilm

Of the catheters tested, neither nitrofurazone-coated nor silver-coated catheters supported the formation of *E. coli* biofilm, regardless of growth condition tested (**Figure 2(C)** and **Figure 2(D)**). In contrast, under aerobic conditions, biofilm formation on silicon catheters (**Figure 2(A)**) was significantly higher ($p < 0.05$) than sterile catheter alone. In addition, the presence of glucose and insulin together resulted in significantly more biofilm ($p < 0.05$) than *E. coli* alone or in the presence of either insulin or glucose alone. However, the presence of insulin-r and/or glucose did not significantly affect the level of biofilm formation on silicon catheter material. Biofilm formation was also significantly increased ($p < 0.05$) on lubricious-coated rubber catheters (**Figure 2(B)**). However, the biofilm permissive conditions differed from silicon catheters in that biofilm formation was significantly ($p < 0.05$) increased under anaerobic conditions as compared to sterile catheters. Also, the levels of biofilm in the absence and presence of insulin and/or glucose were similar.

3.2. Effect of Insulin-r and/or Glucose on Relative Surface Electronegativity of *E. coli*

Previous studies show that insulin together with glucose enhances *E. coli* surface hydrophobicity [25]. To determine if *E. coli* association with silicon catheter segments is related to insulin-mediated changes in *E. coli* surface electronegativity, the adherence to glass (silicon), a negatively charged surface, was measured under various environmental conditions (**Figure 3**) [29] [30]. The more adherent the cells, the more positively charged

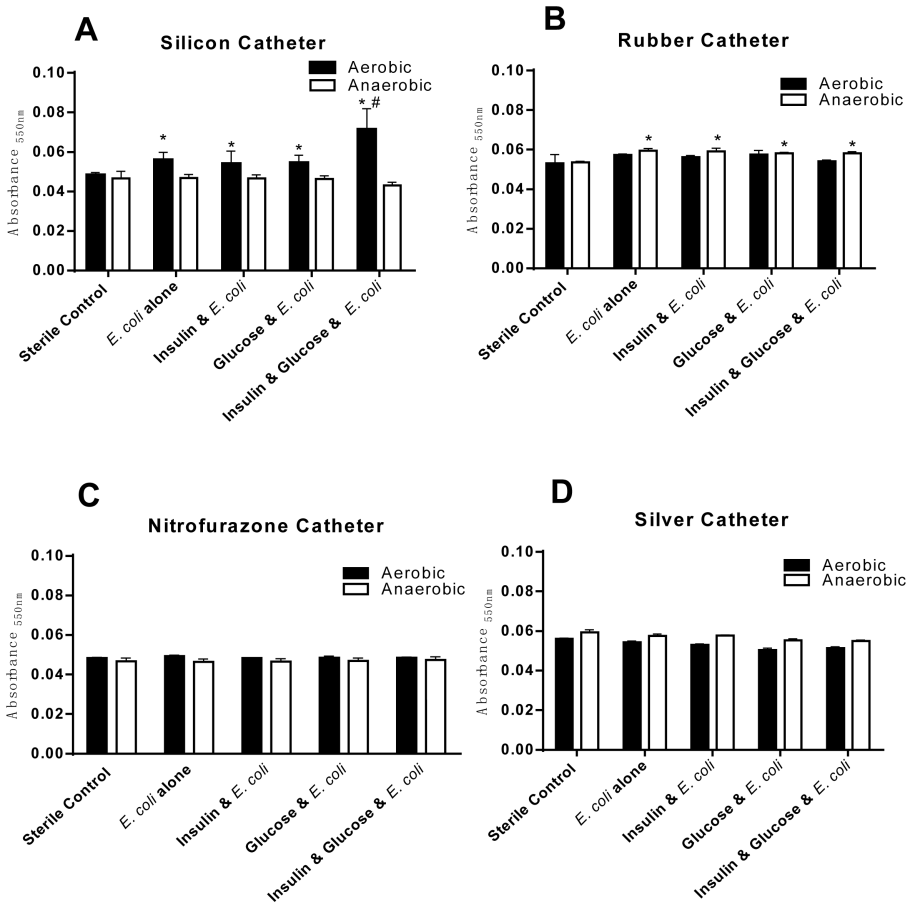

Figure 2. Effect of catheter composition and insulin, glucose and anaerobiosis on the formation of *E. coli* catheter associated biofilm. (A) Silicon catheter, (B) Rubber catheter, (C) Nitrofurazone catheter, (D) Silver catheter. *: indicates significantly different ($p < 0.05$) from sterile control catheter segment.

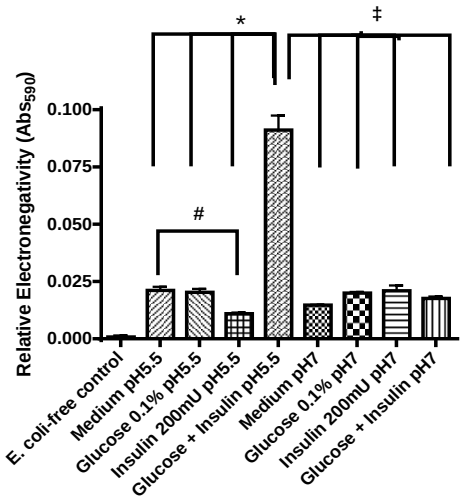

Figure 3. The effect of insulin-r and pH on *E. coli*'*s* adherence to glass in the presence and absence of glucose. Overnight cultures of *E. coli* K12 ATCC 25923 in yeast nitrogen base with 1% peptone (pH 7.0 and pH 5.5) were inoculated (10^4 CFU/ml) into homologous medium with and without insulin-r and/or glucose. Bacteria (0.5 ml) were placed in flat bottom 24 well plates containing acetone washed glass coverslips and grown for 18 hr; 37˚C. After incubation, the coverslips were removed, washed extensively, stained with crystal violet and dried. The unbound dye was removed from the coverslips (ethanol, 0.5 ml) and Abs_{590} measured. All assays in were done in quadruplicate and repeated at least twice. *, #, and ǂ: indicates significant difference ($p < 0.05$). *: indicates glucose + insulin, pH 5.5 is significantly different from all other conditions at pH 5.5. #: indicates insulin, pH 5.5 is significantly different from medium, pH 5.5. ǂ: indicates glucose + insulin, pH 5.5 is significantly different from all other conditions at pH 7.0.

their cell surface. Glucose alone, regardless of the pH, and acidic vs. physiological pH had no effect on bacterial adherence to glass. In contrast, the combination of glucose and insulin at pH 5.5, but not pH 7.0 resulted in a significant ($p < 0.05$) increase in bacterial adherence to glass as compared to medium alone, medium with added insulin or medium with added glucose. There were no changes in adherence at physiological pH. Interestingly, at pH 5.5 insulin alone significantly ($p < 0.05$) inhibited biofilm formation.

3.3. Effect of Insulin-r on Preformed Catheter-Associated Biofilm

Previous work showed that insulinacts as a chemorepellent for *E. coli* [25]. The ability of insulin to promote the planktonic or swimming state of biofilm-associated *E. coli*was determined for silicon (**Figure 4**) and rubber catheters (data not shown). Regardless of incubation condition, insulin did not affect the level of catheter-associated biofilm on either rubber or silicon catheter material.

4. Discussion

Type 2 diabetes mellitus is a common medical condition. Patients with type 2 diabetes mellitus develop peripheral insulin resistance. Their bodies respond by secreting more insulin, leading to hyperinsulinemia. This excess insulin is excreted in the urine in both the presence and absence of glucose, depending on disease state and glycemic control. These individuals also exhibit a higher incidence of UTIs, as compared to individuals with a normal metabolism or those with type 1 diabetes mellitus. Previous studies have shown that insulin and glucose modulate *E. coli* behavior including formation of biofilms. Biofilm is a substance composed of a matrix that protects bacteria from antibiotics and other environmental onslaughts. It is also the virulence factor associated

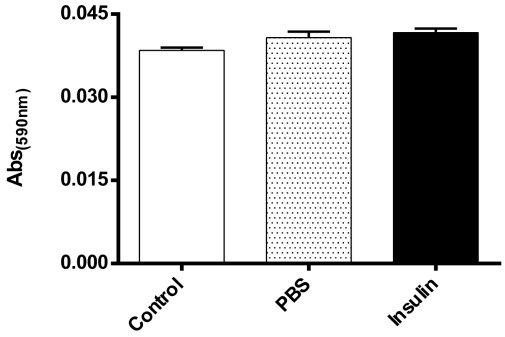

Figure 4. Effect of insulin on preformed biofilms on silicon catheter segments. Silicon catheter segments with preformed biofilm (18 hr, 37˚C) were either processed for determination of biofilm levels (control) or washed and incubated an additional 30 min, 37˚C in PBS (pH 6.0) or PBS with insulin (40 μU/ml) before levels of catheter-associated biofilm was measured.

with 80% of all infections, including catheter-associated UTIs and sepsis [31] [32]. The ability to form biofilms is essential for establishment of *E. coli* catheter-associated UTIs. We have reported that recombinant human insulin affects *E. coli* biofilm formation in a manner that is dependent on substrate and microenvironment, *i.e.*, nutritionally rich vs. minimal medium. Essentially, biofilm formation on plastic was more robust in the presence of minimal nutrient availability. Biofilm formation on a hydrophobic substrate (plastic) and bacterial growth were also affected by variations in concentrations of normally occurring constituents of urine. This is analogous to *in situ* biofilm formation on urinary tract catheters where the sole nutrient is urine, a nutritionally minimal environment. Determining whether or not there is an effect of insulin and glucose on bacteria colonizing catheter material is important in understanding the optimal catheter material for use in this patient population. Results from this study indicate that insulin in combination with glucose enhances biofilm formation on rubber and silicon catheter segments. These increased biofilm levels positively correlate with the impact of insulin and glucose on *E. coli* surface hydrophobicity. Similarly, insulin and glucose increase *E. coli*'s adherence to rubber and its positive surface charge, thus enabling increased bacterial association with negatively charged silicon-coated catheters. In addition, the degree of aerobiosis has an effect on biofilm formation on these two types of material, indicating that examination of catheter material for its permissibility of biofilm should be tested under both aerobic and reduced oxygen (anaerobic) environments. This is particularly important since the lumen of the bladder and catheters have reduced oxygen concentrations. In addition, manipulation of urine pH through diet or through use of pharmaceuticals offers yet another possibility for regulating formation of *E. coli* catheter-associated biofilms. The study does possess some limitations. First, a single strain of *E. coli* was used for all experiments. While this is a widely used quality control strain that enables comparison to other studies, it is possible that results might vary among *E. coli*. Similarly, the use of artificial urine enabled better control and comparison of conditions, but again, may not completely and accurately reflect clinical conditions. In conclusion, these results may aid in the development of a catheter material that can prevent biofilm formation, or alternatively guide choice of catheter material for individuals shedding insulin in their urine.

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