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In vitro oxidant effects of D-glucosamine reduce adhesion and biofilm formation of *Staphylococcus epidermidis*

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ABSTRACT

Staphylococcus epidermidis is a common pathogen in medical device-associated infections. Its major pathogenic factor is the ability to form adherent biofilms. In this work, three *S. epidermidis* strains isolated from infected catheters were chosen with the objective of investigating the effect of D-glucosamine (D-Glu) on reactive oxygen species (ROS) production, adhesion and biofilm formation. The chemiluminescence and nitroblue tetrazolium reduction assays were used to determine ROS production by planktonic *S. epidermidis* and the microtiter plate assay to quantify *in vitro* biofilm formation. D-Glu generated a dose-dependent increase in ROS in planktonic cells with maximum stimuli at a concentration of 0.05 mM, and reduced adhesion and biofilm formation. On the other hand, glucose showed an anti-oxidative stress action and promoted biofilm adhesion and growth. This study suggests a potential application of D-Glu against infections associated with indwelling medical devices, since the oxidative stress caused by this hexosamine in planktonic *S. epidermidis* contributed to reducing biofilm formation.

Key words: *Staphylococcus epidermidis*, D-glucosamine, glucose, reactive oxygen species, biofilm

RESUMEN

Los efectos oxidantes de la D-glucosamina *in vitro* reducen la adhesión y formación de biofilms de *Staphylococcus epidermidis*. *Staphylococcus epidermidis* es un patógeno común en infecciones asociadas a dispositivos médicos. Su factor de patogenicidad más importante es la capacidad para formar biofilms. Se trabajó con tres cepas de *S. epidermidis* aisladas de catéteres, con las que se efectuaron ensayos de quimioluminiscencia y de reducción de azul de nitrotetrazolio, para determinar la producción de especies reactivas del oxígeno (ERO) en *S. epidermidis* planctónico, y ensayos dirigidos a cuantificar la formación de biofilm *in vitro*, empleando placas multipocillos. La D-glucosamina generó un aumento dependiente de la dosis en la producción de ERO en las células planctónicas, con un estímulo máximo a una concentración de 0,05 mM. Este aumento condujo a la reducción de la adhesión y de la formación de biofilm. La adición de glucosa, en cambio, mostró un efecto anti estrés oxidativo y promovió la adhesión y el crecimiento de biofilm. Este estudio sugiere una posible aplicación de la D-glucosamina contra las infecciones asociadas a dispositivos médicos, ya que el estrés oxidativo provocado por esta hexosamina contribuyó a una menor formación de biofilm.

Palabras clave: *Staphylococcus epidermidis*, D-glucosamina, glucosa, especies reactivas del oxígeno, biofilm

Staphylococcus epidermidis is an important cause of infections associated with diverse biomaterials with biofilm-producing strains emerging as important pathogens, especially in patients with implanted devices. An increasing number of research studies have focused on the role of biofilm formation. Biofilm-producing strains have displayed greater adhesive abilities in comparison to non-producing ones. Clinical isolates of these bacteria are frequently found to produce catheter-associated infections and also post-surgical infections, with a markedly high proportion being biofilm-producing strains. Biofilm is the most important factor by which these species adhere and colonize catheters. The biofilm-forming capability of this bacterium seems to be favored by the presence of the *ica* operon, since the biofilms of ica-negative strains are less extensive (9, 11).

The fact that *Staphylococcus* has become the most common cause of nosocomial bacterial infections can be associated with the high antimicrobial resistance that it presents in biofilm (13). The optimal antimicrobial treatment of foreign-body infections is difficult to define because biofilm is resistant, even to drugs specially chosen to combat staphylococci. It is now well documented that efforts to eradicate bacterial biofilms are often unsuccessful, with removal of the infected device being required (10).

Since the production of reactive oxygen species (ROS) by *S. epidermidis* has not been studied in relation to biofilm formation, it could be useful to investi-

gate the different factors that participate in the biofilm development with the aim of impeding the colonization of medical devices. Even though, formed biofilms were associated to lower sensitivity to the stress caused by diverse agents like antibiotics (1, 4), it is actually necessary to know the susceptibility of *S. epidermidis* to stress prior to biofilm formation.

The present study was designed to address the issues of *S. epidermidis* adhesion and inhibition of biofilm formation with respect to the generation of oxidative stress. For this purpose, *in vitro* methods of ROS production were developed to correlate biofilm formation with the alteration of ROS production in order to assess the effect of glucose or D-glucosamine (D-Glu) on *S. epidermidis* capacity to develop biofilm, since it had been previously described that the adherence of *Candida albicans* to HET1-A cells was partially inhibited in the presence of D-Glu, but not of D-glucose, L-fucose or D-galactose (7).

Three *S. epidermidis* clinical strains isolated from infected catheters were investigated in this study. Strains were collected at Tránsito Cáceres de Allende Hospital in Córdoba, Argentina. All strains were identified following the Clinical and Laboratory Standards Institute (CLSI) indications. *S. epidermidis* adhesion was studied by incubating sterile glass or polystyrene (PE) slides with 3 ml of each strain (OD_{600} 1) diluted 1/100 in phosphate-buffered solution (PBS) plus 15 mM glucose or D-Glu cultured for 12 h in static condition. Then, the slides were rinsed four times with PBS, fixed and stained with Crystal Violet (CV) to test the initial adhesion. The number of attached bacteria was calculated by microscopy of 20 fields of 1000x and the mean number of bacteria and the standard deviation were calculated. The biofilm-forming ability of the strains was measured by determination of adhesion to 96-well polystyrene plates; the *S. epidermidis* strains were grown for 18 h in trypticase soy broth (TSB) before being diluted (1:100) with TSB plus glucose or D-Glu. Then, 200 µl of these bacterial suspensions with the same OD_{600} were seeded in each well of flat-bottomed microtiter plates (96-well, Greiner Bio-One, Germany), and then incubated without agitation for 24 h at 37 °C. After incubation, the supernatant was eliminated and the plate was rinsed four times with PBS. After drying by air flow at 37 ° C, staining for adherent biofilms was performed using CV (2 %). Then, the excess of CV was removed and cells were rinsed three times with 200 µl PBS (pH 7.2) before drying for 24 h at room temperature. A quantitative assessment of the biofilm formation was obtained by extracting the CV with 200 µl of ethyl alcohol and acetone (8/2, v/v) per well. The intensity of the coloration was determined at 595 nm using a microplate reader (Model 680 BioRad, Hercules, CA). All strains were tested in three independent experiments on different days. The average OD_{595} nm value was determined by three replicates and was interpreted by the following scale: positive (>0.24), weak (>0.12 and <0.24) or negative (<0.12) (12). The biofilm biomass unit (BBU) was arbitrarily defined with 0.1 OD_{595} nm = 1 BBU.

The bacterial oxidative stress was measured by chemiluminescence in *S. epidermidis* cultured in TSB for 24 h at 37 °C was adjusted to OD_{600}~1 in PBS pH 7.2. Then, 0.3 ml of bacterial suspension was incubated with 0.3 ml of 0.145 mM bis-*N*-methylacridinium nitrate (lucigenin), 0.3 ml of (50, and 0.05 mM) D-glucosamine or of (50 and 0.05 mM) glucose, plus 0.1 ml of dimethyl sulfoxide (DMSO). Controls were performed with bacteria in the absence of antibiotics. The light emitted by ROS was expressed as relative light units (RLU) at different times in seconds, after subtraction of the background. The area under the curve (AUC) was calculated by the Origin computer program for the representations of RLU versus seconds.

Intracellular ROS by Nitroblue Tetrazolium (NBT) reduction was determined in a bacterial suspension (400 µl OD_{600} ~1) in TSB was incubated with 100 µl of 0.05 mM D-Glu and 0.5 ml of 1 mg/ml NBT for 30 min at 37 °C. Then, 100 µl of 0.1 M HCl was added and the tubes were centrifuged at 1 500 g for 10 min. The pellets were treated with 400 µl of DMSO to extract the reduced NBT, and finally, 800 µl of PBS was added. Reduced NBT was measured as blue formazan at 575 nm (intracellular ROS). Each experiment was performed simultaneously in triplicate for each time assayed. To carry out the statistical analysis, we calculated the difference between the average of the three measurements for each strain and the average control value and then tested whether these differences were statistically significant by ANOVA, following the Student-Newman-Keuls test.

The results of the microscopic analysis of initial adhesion showed that glucose enhanced the number of attached bacteria in 12 h; however, D-Glu reduced the adhesion to glass and PE (Table 1). These observations are according to Enache *et al*. (1996), where D-Glu inhibited the adherence of *C. albicans* to HET1-A cells by 40%.

A quantitative analysis showed that the *S. epidermidis* cells attached to 96-well polystyrene plates exhibited weak or negative biofilm formation in the control (according to the scale described above). The

Table 1. Glucose and D-glucosamine (D-Glu) effect on initial adhesion of *Staphylococcus epidermidis* to glass and polystyrene (PE) slides. The number of attached bacteria was calculated by using 20 fields of 1000x microscopy and the mean number of bacteria and the standard deviation were calculated

Strain	Initial adhesion (bacteria/field) to PE			Initial adhesion (bacteria/field) to glass		
	Control	glucose	D-Glu	Control	glucose	D-Glu
848	430±113	1500±200	19±2	8±4	866±88	0
1569	14±4	600±80	0	0	77±31	0
2786	18±2	87±11	0	488±55	1200±100	0

attachment of samples with glucose after 24 h of incubation was significantly increased ($p < 0.05$), but 24 h of incubation with D-Glu significantly reduced their attachment (Figure 1).

The assays performed by chemiluminescence showed that *S. epidermidis* produced a detectable amount of ROS in planktonic conditions. The normal production of ROS expressed as AUC calculated by the Origin computer program for the representations of RLU versus seconds by planktonic bacteria was reduced by the incorporation of glucose mainly in 1569 and 2786 strains (Figure 2.a). However, D-Glu produced an increase in ROS in three strains even at 0.05 mM, the lowest concentration assayed. The AUC obtained for each concentration of D-Glu is indicated in Figure 2.b. It is evident that the three strains exhibited the maximum ROS production for 0.05 mM, which declined at high concentrations.

The intracellular ROS showed a rise with D-Glu, which produced an appreciable increase in ROS in the bacterial cytoplasm during the NBT. In order to summarize the effects of D-Glu, the average percentage of increases in intracellular ROS for each strain with respect to the control without this hexosamine was calculated in 15 % for strain 848, 11 % for strain 1569 and 16 % for strain 2786 (data not shown).

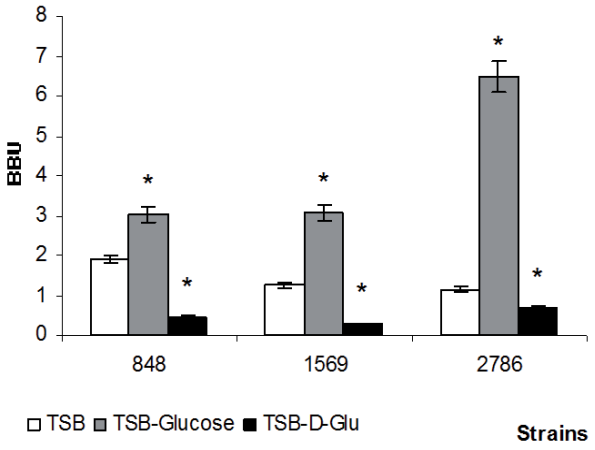

Figure 1. Biofilm formation of *Staphylococcus epidermidis* strains from 24 h at 37 °C, determined by Cristal Violet staining, tested in three independent experiments and expressed as biofilm biomass unit (BBU). *$p < 0.05$

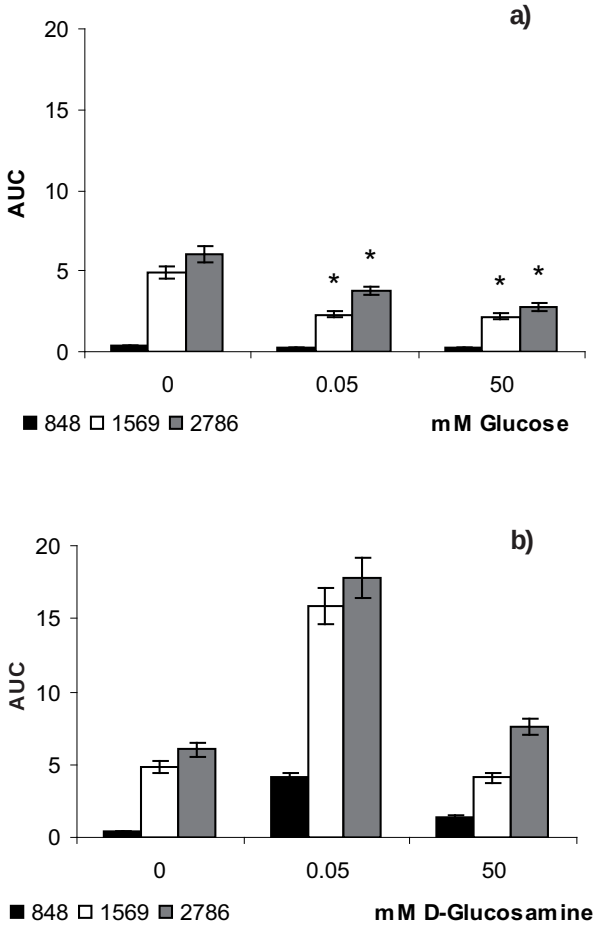

Figure 2. Oxidative stress calculated by the variation of area under the curve (AUC) in an Origin computer program for the representations of RLU versus seconds in chemiluminescence assay provoked by a) incubation with glucose 0.05 and 50 mM b) incubation with D-glucosamine 0.05 and 50 mM. Each experiment was performed in triplicate. *$p < 0.05$ significance respect to control

The analysis of this work must be done taking into consideration that bacterial adherence is a problem for implanted devices that motivated the investigation of attenuating factors, like the inhibition by mixture of immunoglobulins observed on stainless steel surfaces (8). Actually, factors that interfere with the adherence must be investigated since it is the first step of biofilm formation (5). Further aspects need to be identified for the purpose of clarifying the features that affect biofilm formation, and in this regard, oxidative stress was little investigated.

In this paper, the clinical isolates exhibited different abilities to adhere to surfaces, and glucose increased adhesion to glass and PE, while D-Glu reduced adhesion to both surfaces. A parameter that was controlled is the dynamic condition, since shaking greatly reduced biofilm formation. This aspect was considered when a static culture was selected as the method to obtain an important biofilm, bearing in mind that the aim of this study was to find a substance that reduces adhesion and subsequent biofilm formation, as a contribution to a future drug application and to understand the mechanisms involved in the colonization of biomaterials. The results obtained indicate that since the strains were exposed to the same culture conditions for all experiments, the quantity of biofilm produced by each strain was dependent on the presence of glucose or D-Glu. Evidently, glucose favored biofilm formation, while D-Glu decreased this capacity in the three strains studied, and this benefic effect was associated with the increase of ROS production in planktonic cells.

Actually, it is accepted that cellular response to environmental, physiological, or chemical stress is dose-dependent, and it is the key to survival following injuries. In that sense, the results obtained with D-Glu 50 mM were coherent with previous publications, in which an excessive stress caused ROS reduction as a consequence of high bacterial damage (2, 3).

In addition, D-Glu presents a pronounced antioxidant effect in eukaryotic cells, with a multiple scavenging capacity, because it acts on superoxide and hydroxyl-radical. However, the capacity of humans to synthesize D-Glu declines with age and this compound can be employed as a drug to alleviate oxidative stress. D-Glu is considered a therapeutic agent for inflammatory diseases useful for the prevention and treatment of osteoarthritis, which was recently reported to be non-toxic and without side effects (14). Furthermore, D-Glu favorable effects could be amplified with the results of this work as an antibiofilm agent.

In addition, the low production of ROS expressed as AUC observed with glucose can be related to the decrease in oxygen consumption, as a consequence of the anaerobic metabolism induced by glucose in biofilms in agreement with previous reports (6).

The potential application of D-Glu to reinforce different antibiotics could be useful to develop substances against biofilms. The present results provide better understanding of the processes that interfere adherent biofilms on inert surfaces and indwelling medical devices. Furthermore, this study could contribute to the treatment of infections associated to *S. epidermidis* biofilms by the possible application of D-Glu.

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