

Reduction of *Staphylococcus epidermidis* adhesion to indwelling medical devices: a simple procedure

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Introduction

Staphylococcus epidermidis is a coagulase-negative *Staphylococcus* (CNS) that often colonises the skin and mucous membranes as an important part of its normal microflora. However, these staphylococci have emerged as the most frequently isolated pathogen in nosocomial sepsis. They are currently responsible for a larger number of indwelling medical device-associated infections than any other group of microorganisms, mainly due to their ability to adhere to biomaterial surfaces and to form biofilms.¹

Central venous catheter (CVC) infections, for example, are a noteworthy cause of morbidity and mortality and a main source of bacteraemia in hospitalised patients.² Infections caused by *S. epidermidis* are generally associated with immunocompromised, long-term hospitalised and seriously ill patients who represent very susceptible hosts.³ Initial adhesion of bacteria to the biomaterial surface is thought to be a key step in the colonisation of indwelling medical devices, and therefore ways to reduce it have been studied thoroughly over the past decades.

Owing to the increased resistance of microorganisms to antibiotics, and to host defence mechanisms, it is important to find alternatives to the use of antibiotics. Also, it is crucial to proceed with practices that minimise the risk of the emergence of multiresistant bacteria and reduce transmission of any microorganisms between patients.⁴

Significant attention has been given to the development of materials that inhibit bacterial adhesion and surface colonisation.⁵ Furthermore, the impregnation of biomedical devices with antiseptics has proved to be effective in reducing microbial surface adhesion, thus lowering the risk to patients of nosocomial infection.⁶ Moreover, the binding of specific natural substances (e.g., heparin) to the material surface has been one of the strategies attempted in order to reduce the extent of bacterial adhesion.⁷⁻⁹

Heparin is a proteoglycan that has strong anticoagulant activity,¹⁰ which is normally used to minimise thrombus formation¹¹ and extend the duration of catheter use.¹² Its strong hydrophilic nature prevents adhesion of bacterial cells, which may constitute a prime quality for an effective anti-adhesive coater.¹³ Nomura *et al.*¹⁴ compared the extent of

ABSTRACT

The present study aims to find a method to reduce *Staphylococcus epidermidis* adhesion to acrylic and silicone – two materials used commonly in medical devices – by heparin and gentian violet surface preconditioning. Different periods of heparin preconditioning are studied to evaluate the influence of preincubation time on the reduction of bacterial adhesion. A two-hour period was chosen and applied in the adhesion assays with either heparin or gentian violet. Squares of the materials with adherent cells were also analysed by scanning electron microscopy (SEM). Results of adhesion assays showed a significant reduction (53–90%, $P < 0.05$) in bacterial adhesion to silicone and acrylic after precontact with the conditioning substances. No statistical differences ($P > 0.05$) were found between the extent of adhesion on silicone coupons precontacted either with heparin or gentian violet for each of the strains tested. On acrylic, heparin was more efficient ($P < 0.001$) in reducing *S. epidermidis* IE186 adhesion than was gentian violet (85% and 53% reductions, respectively). Therefore, immersion of acrylic and silicone in heparin or gentian violet may constitute a simple and effective method by which to reduce *S. epidermidis* adhesion to medical devices.

KEY WORDS: Cross infection.
Gentian violet.
Heparin.
Staphylococcus epidermidis.

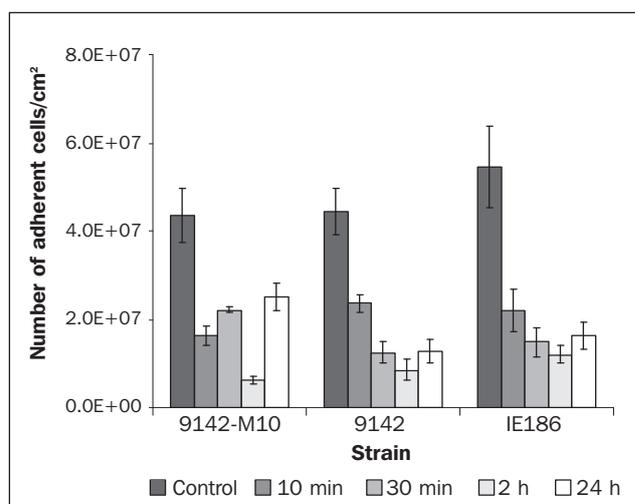


Fig. 1. Number of *S. epidermidis* cells adherent to control silicone coupons and to silicone coupons subjected to different heparin precontact times (10 min, 30 min, 2 h and 24 h) for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars) from three independent experiments.

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S. epidermidis adhesion between heparinised and non-heparinised PVC and silicone and the results showed reduced bacterial adhesion to heparinised surfaces.

Gentian violet (GV) is a triphenylmethane dye normally used to colour wood, silk or paper, and is also applied as a biological stain and antimicrobial agent.¹⁵ Gentian violet shows good bactericidal activity against some Gram-positive cocci (e.g., *Staphylococcus* species) and pathogenic yeasts such as *Candida* spp.¹⁶ It has been used topically to treat skin lesions¹⁷ and infections such as thrush in children and oropharyngeal candidiasis.¹⁸

The aim of the present study is to evaluate the ability of preconditioning of surfaces by heparin or GV to hamper *S. epidermidis* adherence, the ultimate goal being to find a simple method to minimise *S. epidermidis* adhesion and colonisation of indwelling medical devices, and thus reduce associated infections.

Materials and methods

Bacterial strains

Three strains of *S. epidermidis* were used. *S. epidermidis* 9142 is a clinical isolate and known surface polysaccharide intercellular adhesin (PIA)-producer identified as responsible for biofilm formation.¹⁹ Strain 9142-M10 is an isogenic mutant with a transposon inserted in the *ica* locus that encodes the proteins involved in PIA production and

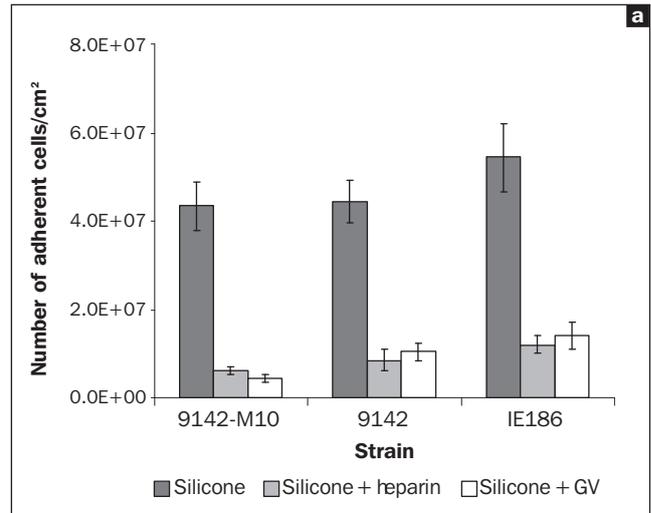
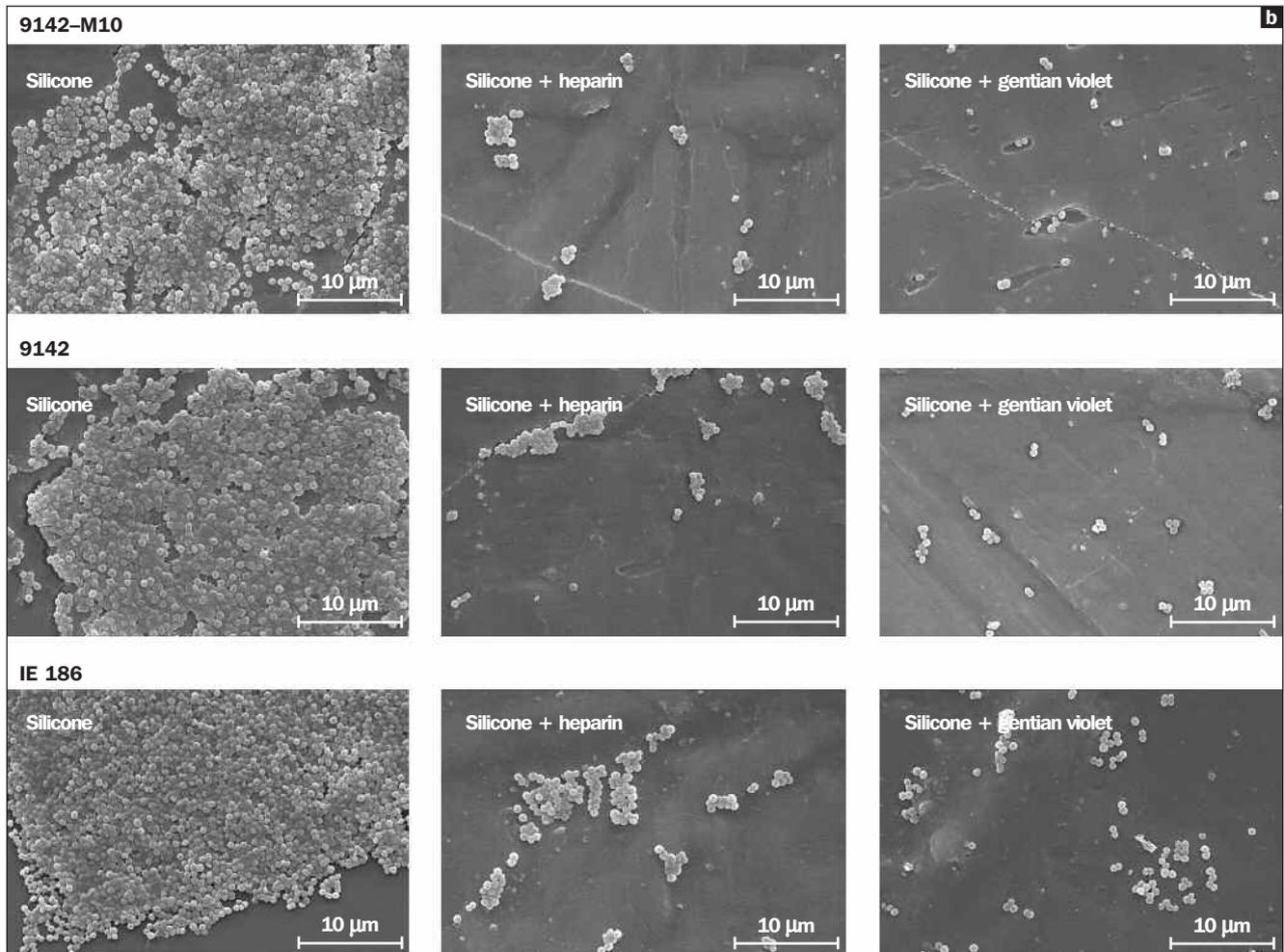


Fig. 2. Effect of heparin and gentian violet precontact on *S. epidermidis* adhesion to silicone. a) Number of *S. epidermidis* adherent cells to non-conditioned silicone coupons (control) and to silicone coupons precontacted with heparin or gentian violet (GV) for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars). b) Representative scanning electron micrographs of the extent of adhesion of the three *S. epidermidis* strains studied to silicone precontacted with heparin or gentian violet (original magnification x3000).



thus does not form biofilm. IE186 was a strain isolated from the blood of a patient with infective endocarditis. All strains were kindly provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA.

Media and growth conditions

For the adhesion assays, cells were first grown for about 36 h on plates of tryptic soy agar (TSA), followed by 24-h incubation in 15 mL of tryptic soy broth (TSB) at 37°C in an orbital shaker at 120 rpm (SI50; Stuart Scientific, Redhill, UK). The TSA and TSB media (Merck, Darmstadt, Germany) were prepared according to the manufacturer's instructions. A 50 µL sample of each suspension was transferred to 30 mL fresh TSB broth and incubated for 18 h at 37°C and at 120 rpm. Cells were then centrifuged for 5 min at 9000 xg at 4°C, and washed (x2) with saline solution (0.9% NaCl [w/v, Merck] in distilled water). The cell suspensions were adjusted to a final concentration of approximately 1×10^9 cells/mL, determined by absorbance at 640 nm.

Substrate preparation

Acrylic and silicone were used as substrate in the adhesion assays, having been cut into 2 x 2 cm squares. Prior to use, the coupons were washed several times with sterile distilled water and left to soak overnight. They were transferred to a new container with sterile distilled water and washed for 5 min with agitation, followed by 30-min immersion in a 70%

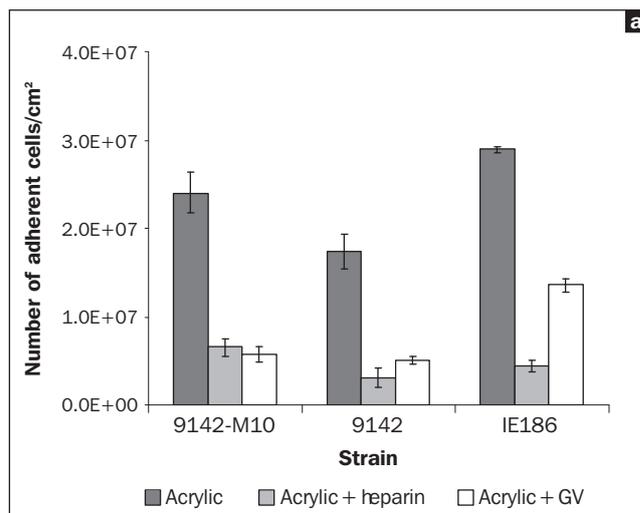


Fig. 3. Effect of heparin and gentian violet (GV) precontact on extent of *S. epidermidis* adhesion to acrylic. a) Number of *S. epidermidis* adherent cells to non-conditioned acrylic coupons (control) and to acrylic coupons precontacted with heparin or gentian violet (GV) for the three strains assayed (9142-M10, 9142 and IE186). Results represent means plus standard deviations (error bars). b) Representative scanning electron micrographs of the extent of adhesion of the three *S. epidermidis* strains studied to acrylic precontacted with heparin or gentian violet (original magnification x3000).

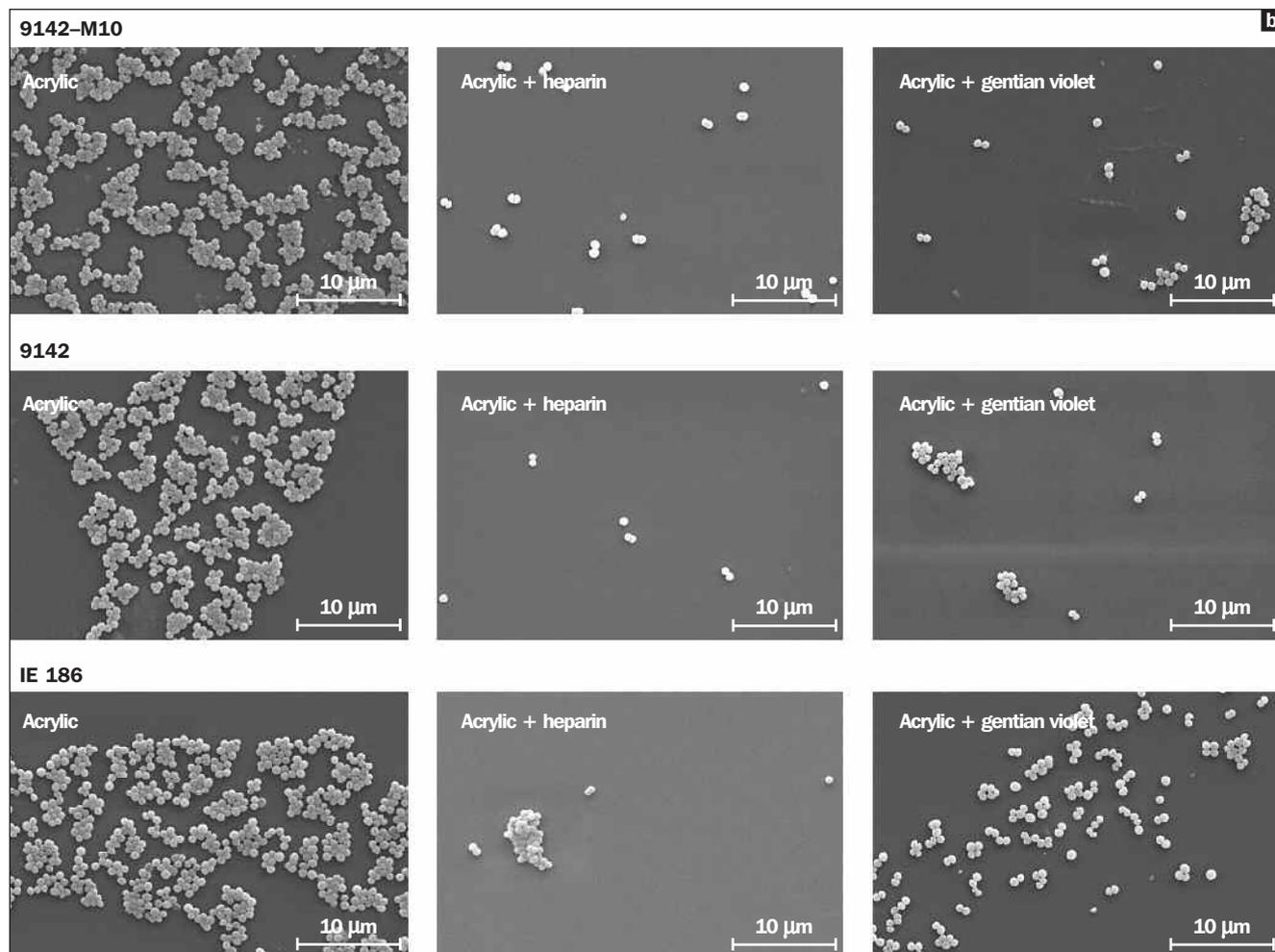


Table 1. Intra-assay coefficient of variation (CV [%]) of the effect of heparin precontact time on *S. epidermidis* adhesion to silicone.

Intra-assay CVs				
Precontact time	Assay	<i>S. epidermidis</i> 9142-M10	<i>S. epidermidis</i> 9142	<i>S. epidermidis</i> IE186
Control	1	17.08	7.73	16.32
	2	20.17	19.20	20.16
	3	6.46	10.66	20.12
10 min	1	8.50	3.26	15.46
	2	11.99	5.88	6.98
	3	20.04	1.59	22.11
30 min	1	0.84	1.51	17.56
	2	2.44	10.66	26.96
	3	1.18	6.25	25.31
2 h	1	14.17	34.94	4.69
	2	3.17	16.46	15.73
	3	21.21	16.33	4.38
24 h	1	17.68	11.99	21.42
	2	12.60	26.20	10.86
	3	10.96	12.79	19.18

Table 2. Inter-assay coefficient of variation (CV [%]) of the effect of heparin precontact time on *S. epidermidis* adhesion to silicone.

Inter-assay CVs			
Precontact time	<i>S. epidermidis</i> 9142-M10	<i>S. epidermidis</i> 9142	<i>S. epidermidis</i> IE186
Control	14.06	11.67	16.69
10 min	13.55	8.19	21.52
30 min	2.37	18.65	21.50
2 h	12.94	29.38	17.98
24 h	12.15	20.53	18.84

ethanol/sterile distilled water solution. Finally, the coupons were washed aseptically and individually with ultrapure sterile water and left to dry overnight at 60°C.

Heparin and GV precontact

The silicone and acrylic coupons were immersed in non-fractionated and low-molecular-weight heparin (5000 iu/mL; Leo Pharmaceutical Products, Ballerup, Denmark) diluted (1 in 4) in distilled water, or in 1% gentian violet (Merck), and left to soak for 2 h. To study the effect of heparin precontact time on bacterial adhesion, silicone coupons were also pre-incubated in heparin for 10 min, 30 min and 24 h, and then removed aseptically and left to dry overnight at room temperature.

Adhesion assays

Adhesion assays were performed as previously described.²⁰ Briefly, the preconditioned coupons were placed in six-well tissue culture plates (Sarstedt, Newton, NC, USA) containing 4 mL of a suspension of 1×10^9 cells/mL in 0.9% NaCl. Adhesion was allowed to occur for 2 h at 37°C in a shaker rotating at 120 rpm. Each coupon was then removed and washed by immersion to remove loosely attached cells. This procedure involved gentle transfer to a 100 mL glass beaker containing 50 mL distilled water for about 10 sec, followed

by transfer to a second 100 mL glass beaker containing 50 mL distilled water for 10 sec, and then to a third for 10 sec.²¹ The coupons were then left to dry at 37°C for about 1 h. All experiments were performed in triplicate and repeated in three independent assays.

Total cell counts of adherent bacteria

The dried coupons were stained with a 4'-6-diamidino-2-phenylindole (DAPI; Sigma, USA) solution (0.1 g/L) for 30 min. Each coupon was rinsed with distilled water and left to air-dry in the dark for 30 min. Adherent cells were visualised under an epifluorescence microscope (filter sensitive to DAPI fluorescence) coupled with a video camera. Enumeration of adherent cells was performed using automated enumeration software (SigmaScan Pro 5) and results were presented as number of adherent cells/cm².

Scanning electron microscopy

The coupons were dehydrated by 15-min immersion in increasing ethanol concentrations (10, 25, 40, 50, 60, 70, 80, 90 and 100% [v/v]), and then placed in a sealed desiccator. Samples were mounted on aluminium stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 scanning electron microscope (Leo, Cambridge, UK). Three fields were used for image analysis.

Statistical analysis

Results obtained (Tables 1–4) were analysed using SPSS software (Statistical Package for the Social Sciences Inc., Chicago) and all tests were performed with a confidence level of 95%. The Kolmogorov-Smirnov test was used to confirm a normal distribution of results. The effect of preconditioning time with heparin was analysed using a two-way ANOVA (repeated measures) with Wilks' Lambda test. Results of the adhesion assays were compared using one-way ANOVA by applying the Tukey multiple comparisons test. The independent samples *t*-test was also used to compare the effect of the same conditioning substance on acrylic and on silicone.

Results

Effect of heparin precontact time

The first approach aimed to assess the extent of adhesion of *S. epidermidis* strains to silicone coupons preconditioned with heparin for 10 min, 30 min, 2 h and 24 h. A statistically significant effect on the extent of adhesion of all *S. epidermidis* strains to heparin (Wilks' Lambda = 0.001, $P < 0.001$ for strain 9142-M10; Wilks' Lambda = 0.023, $P < 0.001$ for strain 9142 and Wilks' Lambda = 0.025, $P < 0.007$ for strain IE186) was observed. Figure 1 shows a significant difference ($P < 0.05$) between the number of cells adherent to the silicone control coupons and the extent of bacterial adhesion to all heparin precontacted coupons over several preconditioning times, and for all strains. Least adhesion was observed after 2 h of heparin precontact for all three strains assayed. Adhesion of strains 9142 and IE186 showed small variations with all the precontact periods tested, while strain 9142-M10 displayed greater variability of adhesion with the various heparin precontact times.

Effect of heparin and gentian violet precontact

Subsequent assays were performed with 2-h precontact for both conditioning substances.

In the case of silicone, a significant decrease ($P < 0.005$) in bacterial adhesion following the use of heparin or GV was observed (Fig. 2) for all strains studied ($P < 0.001$). No statistical differences ($P > 0.05$) were found between the extent of adhesion following precontact with either heparin or GV for each of the strains tested; however, on coupons precontacted with GV, adherence of strain 9142-M10 was significantly lower ($4.48 \times 10^6 \pm 9.44 \times 10^5$ cells/cm²) compared to strains 9142 ($1.04 \times 10^7 \pm 1.84 \times 10^6$ cells/cm²; $P < 0.001$) and IE186 ($1.39 \times 10^7 \pm 3.07 \times 10^6$ cells/cm²; $P < 0.001$). In the case of silicone precontacted with heparin, a statistical difference ($P < 0.001$) was observed between strain 9142-M10 ($6.16 \times 10^6 \pm 7.98 \times 10^5$ cells/cm²) and strain IE186 ($1.21 \times 10^7 \pm 2.17 \times 10^6$ cells/cm²), with strain 9142 presenting an intermediate level of reduction in the number of adherent cells ($8.51 \times 10^6 \pm 2.50 \times 10^6$ cells/cm², $P = 0.047$).

In the case of acrylic conditioning (Fig. 3), both precontacted surfaces showed significantly lower levels of adherent cells ($P = 0.001$) for all strains. With strain 9142-M10, the number of adherent cells to acrylic was almost independent of the conditioning substance used ($P = 0.984$). With strain 9142, the number of attached cells was less pronounced on acrylic precontacted with heparin ($3.07 \times 10^6 \pm 1.07 \times 10^6$ cells/cm²) than with GV ($5.04 \times 10^6 \pm 5.26 \times 10^5$ cells/cm²). In the case

of IE186, heparin showed significantly ($P < 0.001$) higher efficacy in acrylic conditioning, with only 4.36×10^6 cells adherent against 1.35×10^7 cells/cm² when conditioned with GV. Therefore, on acrylic substances, heparin preconditioning is slightly more efficient in reducing *S. epidermidis* adhesion than is GV.

In the acrylic surface control and the pre-incubated GV sample groups, the extent of adhesion of strain IE186 was significantly higher than the adhesion of strains 9142-M10 and 9142 ($P < 0.001$). On the other hand, following heparin pre-incubation, *S. epidermidis* 9142-M10 showed greater adherence ($P < 0.001$) than strains IE186 and 9142. Therefore, *S. epidermidis* 9142 was most affected by the preconditioning of acrylic with heparin and GV.

The number of adherent cells of all three *S. epidermidis* strains was markedly lower ($P < 0.001$) on acrylic than on silicone when the surfaces were not preconditioned. Moreover, when surfaces were precontacted with heparin, cells showed greater adherence to silicone than to acrylic, especially strains 9142 and IE186 ($P < 0.001$). A similar difference was observed for strain 9142-M10 ($P = 0.009$) and strain 9142 ($P < 0.001$) adherent to material coated with GV.

Discussion

One of the main sources of contamination of indwelling medical devices (e.g., catheters) is the skin insertion site. From this point, microorganisms migrate towards the intracutaneous tract on the exterior surface of the catheter, subsequently colonising it and causing sepsis.² Therefore, heparin or GV preconditioning of the inner and outer surfaces of such devices can act to prevent the development of infection.

The results of the present study agree with those of previous work on the use of such substances to prevent bacterial proliferation.^{7,8,14,16} Rose *et al.*⁵ also confirmed the ability of heparin preconditioning to reduce bacterial adhesion to cationic MPC co-polymers. Another study²² of *S. epidermidis* adhesion to intraocular lenses (IOLs) demonstrated that heparin treatment significantly reduced the extent of adhesion of these bacteria to regular polymethyl methacrylate (PMMA) IOLs, compared to antibiotic treatment. Similar results were obtained by Portolés *et al.*⁹ when performing adhesion studies of *S. epidermidis* to PMMA IOLs in the presence of heparin in solution.

These studies, together with the results of the present work, indicate that the preconditioning of indwelling medical devices with heparin may constitute a valid alternative to antibiotics, which are inefficient in reducing adhesion and biofilm formation on such surfaces. However, the mechanism by which heparin inhibits bacterial adhesion remains unclear, although it is not related to its action as an anticoagulant.²³ Heparin is composed of long hydrophilic chains and has a negative charge, and these factors are likely to play a role in inhibiting adherence.²⁴ *S. epidermidis* also has a negative charge, which may lead to the bacterium being repelled from the heparin preconditioned surface.

Saji *et al.*¹⁶ demonstrated a significant *in vitro* bactericidal effect for GV on methicillin-resistant *S. aureus* (MRSA) isolated from clinical specimens, as well as in the treatment of decubitus ulcers infected with MRSA. The present results also point to considerable efficacy against CNS such as

Table 3. Intra-assay coefficient of variation (CV [%]) of the effect of heparin and gentian violet (GV) precontact time on *S. epidermidis* adhesion to silicone and acrylic.

Intra-assay CVs				
Material	Assay	<i>S. epidermidis</i> 9142-M10	<i>S. epidermidis</i> 9142	<i>S. epidermidis</i> IE186
Silicone	1	17.08	7.73	16.32
	2	13.90	19.20	20.16
	3	11.75	6.81	9.55
Silicone + heparin	1	14.17	24.94	4.69
	2	3.17	16.46	25.73
	3	21.21	16.33	4.38
Silicone + GV	1	20.52	7.55	21.54
	2	23.71	22.91	26.53
	3	10.97	10.84	21.86
Acrylic	1	9.46	8.84	0.72
	2	10.09	12.49	0.88
	3	11.72	15.69	0.86
Acrylic + heparin	1	20.72	28.85	16.00
	2	16.07	23.72	13.36
	3	11.27	17.98	5.20
Acrylic + GV	1	5.13	15.76	7.29
	2	21.45	8.63	0.79
	3	10.60	3.88	3.55

Table 4. Inter-assay coefficient of variation (CV [%]) on the effect of heparin and gentian violet (GV) precontact time on *S. epidermidis* adhesion to silicone and acrylic.

Inter-assay CVs			
Material	<i>S. epidermidis</i> 9142-M10	<i>S. epidermidis</i> 9142	<i>S. epidermidis</i> IE186
Silicone	12.72	10.91	14.15
Silicone + heparin	12.94	19.38	17.98
Silicone + GV	21.06	17.66	21.98
Acrylic	9.24	11.21	1.17
Acrylic + heparin	14.91	14.95	15.02
Acrylic + GV	14.11	10.44	6.13

S. epidermidis. Therefore, GV seems to possess antimicrobial activity that can be used against an important group of pathogens normally associated with nosocomial infections.

In aqueous solutions, GV dissociates into positive and negative ions that pass through the cell wall of Gram-positive bacteria such as *S. epidermidis*. The positive ions then interact with negatively charged cell components such as peptidoglycan and DNA.²⁵ As GV is a mutagen and mitotic poison, cell growth is inhibited. This mode of action, as an antimicrobial agent, possibly explains the efficacy of GV against bacteria and yeasts, while showing only mild to moderate effects on mammalian cells. Chaiban *et al.*¹⁸ evaluated the cytotoxicity of an antiseptic combination of GV and chlorhexidine (Gendine) and reported no cytotoxicity against L929 mouse fibroblast cells.

In the present study, some differences were observed between strain 9142-M10, which is an isogenic mutant of the

biofilm-negative strain 9142, and strains 9142 and IE186, which have been defined as biofilm producers.^{20,26} Cerca *et al.*²⁰ showed that adhesion and biofilm formation are two features associated with the pathogenicity of infections associated with medical devices, and that high levels of initial adherence do not necessarily lead to strong biofilm formation. This can be due to the fact that biofilm formation depends mostly on cell-to-cell adhesion phenomena, which, in the case of CNS, are mediated by specific molecules such as PIA. On the other hand, initial adhesion also depends on several other bacterial cell surface factors. Thus, it is important to note that biofilm formation is not solely dependent on the extent of initial adherence of bacteria to the substrate, but that adhesion is a strain-dependent and not a species-dependent factor.

Nosocomial infections due to CNS such as *S. epidermidis* may be caused by factors including the use of indwelling

medical devices, previous antibiotic administration and infection control practices adopted by medical personnel.²⁷ The results presented in this work have potential clinical significance and show that both heparin and GV are effective in reducing bacterial adhesion. Pretreatment of indwelling medical devices with such substances therefore can act as an infection control measure and may constitute a successful means to reduce the incidence of nosocomial infection. □

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