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Research Article
Modulation of Virulence Factors of *Staphylococcus aureus* by Nasal Decongestants

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Abstract

Background and Objective: When applied, nasal decongestants may reach cells of *Staphylococcus aureus*, a bacterium that commonly colonizes the mucosa. The aim of this study was to evaluate experimentally the effect of corticoids (budesonide, dexamethasone and triamcinolone), alpha-agonists (naphazoline, oxymetazoline, fenoxazoline and xylometazoline) on biofilm formation and secretion of staphylococcal haemolysin and proteases. **Materials and Methods:** Commercial formulations had their preservatives neutralized and were added to the culture broth in which the strain ATCC®25923™ was grown. The biofilm production was measured after 72 h of incubation. This study investigated the proteolytic and haemolytic activities of culture supernatant for the interval between the 48-72 h of incubation. **Results:** It was obtained that there are interactions amongst decongestants and *S. aureus* as biomass reductions (budesonide, triamcinolone and fenoxazoline), protease activity (triamcinolone), specific protease activity (triamcinolone), haemolytic index (budesonide, triamcinolone and dexamethasone) and specific haemolytic index (budesonide, triamcinolone and dexamethasone). On the other hand, xylometazoline increased the rate of haemolysin secretion. **Conclusion:** These interactions may albeit not in a unanimous manner and be beneficial to decongestants users.

Key words: *Staphylococcus aureus*, nasal decongestants, biofilm, protease, haemolysin

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nasal decongestants are among the most commonly dispensed prescriptions in retail pharmacy and its global market moves about US$ 7 billion year^{-1} and alpha-adrenergic agonists and corticosteroids for local release correspond to approximately 70% of this market[1].

Nostrils, local application of pharmaceutical forms of decongestants are areas normally colonized by *Staphylococcus aureus* even in healthy individuals[2-5]. This nasal colonization has been identified as a risk factor for later development of endogenous infections[6,7]. Therefore, the influence of these drugs on nasal *S. aureus* populations deserves a better evaluation.

The aim of this study was to evaluate whether local release of corticosteroids (budesonide, dexamethasone and triamcinolone) and α-adrenergic agonists (naphazoline, oxymetazoline, fenoxazoline and xylometazoline) in the formulation of numerous decongestants interfere with (i) Biofilm formation, (ii) Production of staphylococcal haemolysin and (iii) Proteases.

MATERIAL AND METHODS

The conduct of this study was approved by the institutional committee of ethics in study (protocol 6128/11) and blood donors were informed about the meaning of the study and of possible risks.

The concentrations of steroids in commercial formulations are 640 μg mL^{-1} budesonide, 546 μg mL^{-1} dexamethasone disodium phosphate and 400 μg mL^{-1} triamcinolone acetonide according to information provided by the manufacturers. In the case of dexamethasone as the nebulizer formulation also contains phenylephrine and neomycin, this study use the injectable formulation with concentration adjusted to mimic the nasal spray. The concentrations of decongestants containing are 1 mg mL^{-1} naphazoline, 500 μg mL^{-1} oxymetazoline, 1 mg mL^{-1} fenoxazoline and 1 mg mL^{-1} xylometazoline according to information provided by the manufacturers.

It was employed the *S. aureus* ATCC®25923™ which is a reference strain for antimicrobial testing.

Neutralization of preservatives: Preservatives in nasal decongestants were neutralized to avoid any bias in the results. The preservatives used in the formulations are benzalkonium chloride and parabens which neutralization was carried out with the letheen broth used to grow the bacterium.

Preparation of strain: *Staphylococcus aureus* was grown in BHI broth at 37°C, 150 rpm, capnophilic conditions (80% N_2, 10% O_2 and 10% CO_2) for 24 h. Grown cells were resuspended in sterile water until turbidity near to tube No. 0.5 of McFarland standards which corresponds to approximately[8] to 1×10^8 CFU mL^{-1}.

Determination of the inhibitory activity: Amounts of 25.7 mg of Letheen broth powder were diluted in 1 mL of each decongestant and in 1 mL of deionized water for control. All solutions were filtered in membrane (0.22 μm porous size) and collected in sterile tubes. Aliquots of 100 μL of bacterial suspension were added to each tube that were incubated at 37°C in 10% CO_2 for 24 h. Microbial growth were assessed visually and compared with control tube[9].

Biofilm formation: As the bacterium grew in all concentrated decongestant solutions concluded that Minimum Inhibitory Concentrations (MICs) if any should be above those found in commercial presentations. Then, decongestants were used at a final concentration of 1:10 commercial formulation diluted in Letheen Broth (LB). As a control, used LB without decongestants. Preparations were filtered through a membrane (0.22 μm porous size) and collected in sterile bottles. Twenty-four wells polystyrene plates received 1 mL aliquots of bacterial suspension (~1×10^8 CFU mL^{-1}) and were incubated in normoxia at 37°C and 100 rpm. After 2 h, wells were washed with sterile water and 1 mL aliquots of LB with decongestants were added. Plates were statically incubated at 37°C in capnophilic conditions in order to mimic nostril's surface conditions. After 24 h, supernatants were aspirated and each well received new 1 mL aliquots of LB with decongestants. Plates returned to incubation for more 24 h at 37°C in capnophilic conditions. This step was repeated once more. After 72 h of incubation, supernatants were carefully aspirated and transferred to sterile 2 mL microtubes which were centrifuged at 10,000×g. Biofilm supernatants were analyzed for haemolytic and proteolytic activities.

Biofilms were stained with 50 μL of 0.4% crystal violet (in 12% EtOH) for 5 min. Each plaque was gently washed until the supernatant stayed clear and with no dye. Biofilm biomasses were estimated colorimetrically at 595 nm after elution with 1% SDS.

Haemolytic activity: Aliquots 30 mL of blood were collected from healthy male donors. Cellular content was washed with Hanks balanced salt solution (HBSS: 4.2 mM $NaHCO_3$, 5 mM KCl, 0.4 mM KH_2PO_4, 138 mM NaCl, 0.34 mM Na_2HPO_4, 5 mM glucose and pH 7.0). Red cell preparations were performed

within the 1 h after collection and experiments were performed in the period upto 4 h post-purification[10].

Erythrocyte preparations with 40% haematocrit were mixed (1:1) with biofilm supernatants and shaken gently at 37°C for 16 h in capnophilic conditions. Haemolytic activities of supernatants of biofilms were determined by colorimetrically at 545 nm. Indexes of haemolysis (IH) were determined as ratios of haemoglobin released in experimental groups compared with those released by erythrocytes (40% haematocrit) combined (1:1) with letheen broth (Prepared in 2 M HCl).

Specific haemolytic indices were determined by dividing the haemolytic activity by estimated biomasses ($1SHI = HI\ biomass^{-1}$).

Proteolytic activity: Azocasein was dissolved at 5 mg mL^{-1} in a buffer containing 50 mM tris-HCl (pH 5.0), 200 mM NaCl, 5 mM $CaCl_2$, 0.05% triton X-100 and 0.01% sodium azide. Aliquots of 400 µL of such solution were mixed with 100 µL of biofilm supernatants and left to incubate for 2 h at 37°C. Protein digestions were stopped by adding 150 µL of 20% trichloroacetic acid and rapid mixing. After 30 min at room temperature, tubes were centrifuged at 16,000×g (3 min) and the pellets were discarded. Supernatants were mixed with equal volumes of 1 M NaOH and $OD_{440\ nm}$ were determined.

One unit of enzyme activity was arbitrarily defined as the amount of enzyme required to increase the absorbance in 0.001 min^{-1} of digestion. The specific proteolytic activity was calculated to provide the amount of enzyme units by the absorbance of crystal violet retained in biofilms.

Statistical analysis: All tests described above were carried out in triplicate in at least three different situations. Numeric data were tabulated in MSExcel® spreadsheets (Microsoft Co.). Data were tested for normality of distribution by the Levene index and submitted to simultaneous multiple comparisons by the Tukey test. A threshold of 0.05 was considered to establish statistical differences between groups.

RESULTS AND DISCUSSION

Locally active corticosteroids and alpha-agonists are commonly indicated as accessories in the therapy of chronic sinusitis[11,12]. However, there are indications that their abuse may lead to an increase in the population of *S. aureus* with direct involvement in events of rhinosinusitis[13]. A relationship between these drugs and bacterial virulence has not yet been fully established which makes this a pioneering study in the field and even being a screening study.

General consideration about drug concentrations: Since, decongestants did not decrease planktonic growth, it has been inferred that MICs were not obtained. The subsequent tests were performed at concentrations 10-fold lower than those of the commercial formulations. This was taken arbitrarily since, it does not have any information about the mucosal retained concentrations after instillation and clearance of such formulations.

Locally active corticosteroids: Dexamethasone has increased the average biomass of biofilms ($p = 0.0062$). However, exposure to triamcinolone ($p<0.0001$) and budesonide ($p<0.0001$) decreased the final biomasses in Fig. 1 and 2. In principle, any inhibitory activity of these steroids on *S. aureus* has not been expected since, these drugs are used for purposes such as clearing the airways[14], reducing symptoms of allergies[15] and decreasing postoperative edema[16] without any reference to a supposed antimicrobial activity. However, De Vries *et al.*[17] have already obtained that a budesonide-based formulation reduced bacterial growth. From a therapeutic perspective such anti-biofilm property is appreciable because in addition to its primary function as a decongestant and it could also control the pathogen growth. Nevertheless, as commercial formulations containing benzalkonium chloride and parabens were employed, it became plausible to hypothesize that such preservatives have compromised the growth. It had even been previously proposed as an explanation for the antibacterial activity in "de Vrie's" manuscript. However, there is well-founded evidence that components of the letheen broth eradicate antimicrobial action of preservatives[18-20]. In addition, the manufacturer states do not add any preservative in the budesonide-containing formulation.

Triamcinolone induced a remarkable reduction of proteolytic activity compared to control ($p<0.0001$) (Fig. 1). Also, in relation to this parameter, budesonide and dexamethasone did not diverge from each other ($p = 0.8050$) or in relation to control ($p\geq0.1525$). As triamcinolone promoted concomitant reduction in biomass and proteolytic activity, it was incurred in decreased specific proteolytic activity. It might be interpreted as a reduction in the rate of proteases secretion by bacterial load. This side effect should be further explored because it seems to be beneficial. On the other hand, the specific proteolytic activity of budesonide was far superior to control ($p = 0.9125$). Despite the reduction of biomass and the glucocorticoid maintained unchanged the protease secretion rate. This finding deserves some attention since, budesonide is considered as a safe drug[21-24].

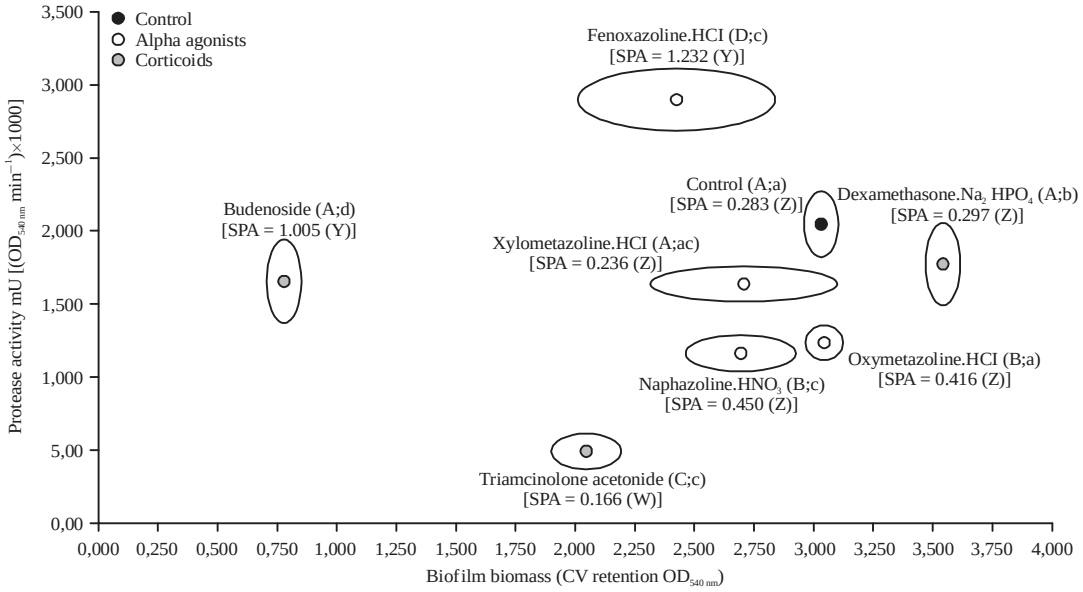

Fig. 1: Cartesian distribution of proteolytic activities of supernatants by biomasses of 48-72 h old *Staphylococcus aureus* ATCC®25923™ biofilms grown in presence of nasal decongestants. Dotted areas surrounding arithmetical averages indicate 95% confidence intervals for proteolytic activities (y axis) vs biomasses (x axis). SPA: Specific proteolytic activities (proteolytic activity × biomass^{-1}). Different majuscule letters A, B, C and D after drug names indicate differences (Tukey test, $p<0.05$) for proteolytic activities. Different minuscule letters a, b, c and d after drug names indicate differences (Tukey test, $p<0.05$) for biomasses. Different majuscule letters Y, W, X and Z after SPA indicate differences (Tukey test, $p<0.05$) for such parameter

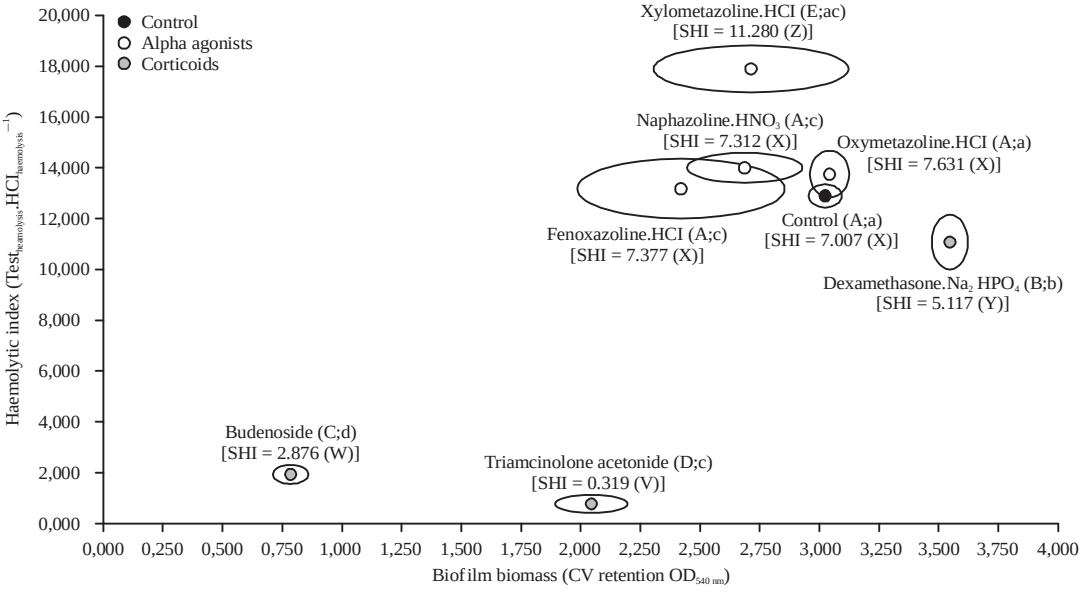

Fig. 2: Cartesian distribution of haemolytic indexes of supernatants by biomasses of 48-72 h old *Staphylococcus aureus* ATCC®25923™ biofilms grown in presence of nasal decongestants. Dotted areas surrounding arithmetical averages indicate 95% confidence intervals for haemolytic activities (y axis) vs biomasses (x axis). SHI: Specific haemolytic indexes (haemolytic index × biomass^{-1}). Different majuscule letters A, B, C and D after drug names indicate differences (Tukey test, $p<0.05$) for haemolytic indexes. Different minuscule letters a, b, c and d after drug names indicate differences (Tukey test, $p<0.05$) for biomasses. Different majuscule letters V, Y, W, X and Z after SHI indicate differences (Tukey test, $p<0.05$) for such parameter

Interestingly, the three steroids promoted significant reductions in haemolytic behavior when compared to control ($p \leq 0.0239$) with 18.23% for dexamethasone, 88.24% for budesonide and 97.18% for triamcinolone (Fig. 2). These reductions reflect in specific haemolytic activities that also were decreased ($p \leq 0.01034$).

Reductions in haemolytic indexes and in specific proteolytic activities are very interesting and corroborate with previous assumption that some steroid molecules can promote "Detoxification" of staphylococcal alpha-toxin (alpha-haemolysin)[25,26], a pore-forming toxin that acts on membranes of target cells[27] depending on a specific activation by purinergic signaling[28].

The mechanisms by which the steroid-dependent inhibition of α-toxin occurs are not completely understood. It is believed that the toxin affects punctually the membrane fluidity inserting itself into phosphatidylcholine portion[29]. Steroids can compete for the binding sites of the α-toxin. In addition, this binding competition can reduce the amount of caveolin molecules necessary for the activity of the α-toxin and resulting in fewer erythrocytes lysed because of pore formation by α-toxin[30].

Alpha-agonists: There has occurred a slight reduction in turbidity of cultures under the influence of fenoxazoline (data not quantified). However, this reduction has not been considered as a significant fact because turbidity remained considerable.

Oxymetazoline, naphazoline and xylometazoline induced average biofilm masses that did not vary among themselves ($p>0.050$) as well as compared to control ($p>0.050$). However, exposure to fenoxazoline led to a reduced biomass when compared with control and oxymetazoline ($p<0.050$) but not with naphazoline and xylometazoline ($p>0.050$) (Fig. 1). Any inhibitory activity of fenoxazoline was not expected since, this drug is indicated only for airways clearing[31] and as vasoconstrictor eye drops[32] without any mention of an alleged antimicrobial activity. From a therapeutic perspective, this antimicrobial property could be appreciable, since in addition to its primary function as a decongestant it could also reduce the growth of a recognized pathogen. Moreover, as preservative neutralization was performed and reductions in biofilm/planktonic populations were not observed for other decongestants which also contains benzalkonium chloride at close concentrations and any influence of preservatives was not taken in account.

Fenoxazoline was the agonist that showed increments in specific proteolytic activity ($p<0.050$) (Fig. 1). This implies that with less cells, inversely and greater amounts of proteases were secreted. The mechanisms by which this phenomenon occurs are to be established in future studies, once this study only screened for virulence shifts and not to infer mechanisms of action.

It was observed that xylometazoline promoted significant increases in specific haemolytic index (Fig. 2). This is due to the induction of increased secretion of haemolysins and not to reductions in biomass. Naphazoline and fenoxazoline occupied an intermediate in this index as result of reduction in biomass with maintenance of haemolytic indexes.

CONCLUSION

This study concluded that an interaction amongst decongestants assessed and *S. aureus* can be beneficial to the patient because they promote, albeit not unanimous, biomass reductions (budesonide, triamcinolone and fenoxazoline), protease activity (triamcinolone), specific protease activity (triamcinolone), haemolytic index (budesonide, triamcinolone and dexamethasone) and specific haemolytic index (budesonide, triamcinolone and dexamethasone). On the other hand, xylometazoline increased the rate of haemolysin secretion.

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