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PHD THESIS

NEW APPROACHES OF BIOFILM IN DISEASES FROM THE FIELD OF OTOLARYNGOLOGY

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RESUME

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Resume

Biofilms are heterogeneous structures formed by bacterial cells surrounded by a matrix and attached to solid surfaces. Because biofilms are particularly problematic in medicine, sharing knowledge about how different organisms form and disperse from biofilms and how biofilm microbes differ from planktonic ones is critical for the next generation of creative solutions.

Antibiotic resistance generally means an increase in the value of the minimum inhibitory concentration (MIC) of an antibiotic based on the permanent change of bacteria, for example, mutation or resistance acquired by horizontal gene transfer. In contrast, antibiotic tolerance is the ability of cells to survive the effect of an antibiotic due to a reversible phenotypic condition. The applicability of these terms and description are appropriate for planktonic cells, while for biofilms, the situation is different. In biofilms, antimicrobial tolerance is related to the growth mode of the biofilm. This is in contrast to bacteria that grow in planktonic culture, which will usually show susceptibility to antimicrobials. It must be recognized that biofilm formation is the natural state for the vast majority of bacteria *in vivo*. Consequently, antibiotic tolerance is a natural state of biofilms. Also, antibiotic resistance is a natural phenomenon, as bacteria have evolved to withstand the action of natural antibacterial products for billions of years in the absence of human activity.

Obtaining and characterization of biochemical agents of therapeutic interest in biofilm control

There are several strategies for overcoming antibiotic resistance, including reducing the widespread use of antimicrobials, collecting and analyzing data, developing new drugs and nanotechnology. Advances in nanotechnology have led to the synthesis of nano-sized organic and inorganic molecules with potential applications in industry, food, textile, medical and therapeutic fields. The development of new antimicrobial agents / nanometric nanocomposites can be used as an alternative strategy for overcoming antimicrobial resistance. The emergence of nanotechnology, the largest technological innovation in recent years, has modernized medicine. Demand for products derived from nanotechnology is constantly increasing. Nanotechnology, which is the innovative technology in the present scenario, can have a profound influence on improving human health. The increased durability, performance, strength, and other properties like flexibility and innovative physico-chemical properties of nanomaterials have been explored in the field of health.

Silver has been used since ancient times for its therapeutic effects, but the exact mechanism of action, despite the progress, is still in the debate stage. It is recognized that the antimicrobial potential is due to the positive charge of the silver ion, therefore the form used must be ionized. Even though in this form it is inert, when it comes in direct contact with the biological environment, it releases silver ions that interact with nucleic acids.

In the present paper, silver nanoparticles were biochemically synthesized by using total rosemary extracts, and were characterized by specific methods and were tested for the evaluation of antimicrobial activity and cytotoxic safety. Thus, the antimicrobial activity of biosynthesized silver nanoparticles was evaluated against gram-positive bacteria, gram-negative bacteria and fungi by the disk diffusion method. Following the testing of the two types of extracts, there was a negligible activity, at the concentrations tested (between 10-500 µg / mL), instead in the case of testing the silver nanoparticles, obtained biochemically, with the extracts from rosemary leaves, the antimicrobial activity was significant.

Exposure to silver nanoparticles as antimicrobial agents for biofilm control involves intact contact with tissues or administration; therefore, understanding their properties and assessing cytotoxicity is important for effective use in clinical applications. Cytotoxicity studies of nanoparticles are widely carried out on *in vitro* models due to their facile execution, control and interpretation, being the initial studies that mimic the conditions *in vivo*. Cytotoxicity can be studied *in vitro* through several qualitative and quantitative tests; however, quantitative tests are most appropriate because they quantify the number of living cells.

To evaluate the safety of the biosynthesized nanoparticles in the present study, additional preliminary tests were performed. Thus, the morphology and viability of two normal cell types were evaluated in the presence of extracts and silver nanoparticles. At low concentrations, human keratinocytes and human gingival fibroblasts were unaffected, whereas at the highest concentrations changes in cell shape and detachment in some cases of the cells from culture plate were observed.

Cell viability assays are an important step in toxicological assessments of new treatment methods and can provide significant data on cell death, metabolic activity and cell survival. The silver nanoparticles, together with the extracts used as biocatalysts in the syntheses, were tested to determine the effect in terms of viable cells on two healthy gingival cell lines, keratinocytes and fibroblasts. These lines are most often used in preliminary evaluations of compounds that have a direct connection with the buccal mucosa and the mucous membranes of the ENT sphere. The effect exerted depends on the dose tested: at low concentrations there is no noticeable effect on cell viability while with increasing the dose, an increase in the percentage of dead cells can be observed, especially at the highest concentration used (example - figure 1).

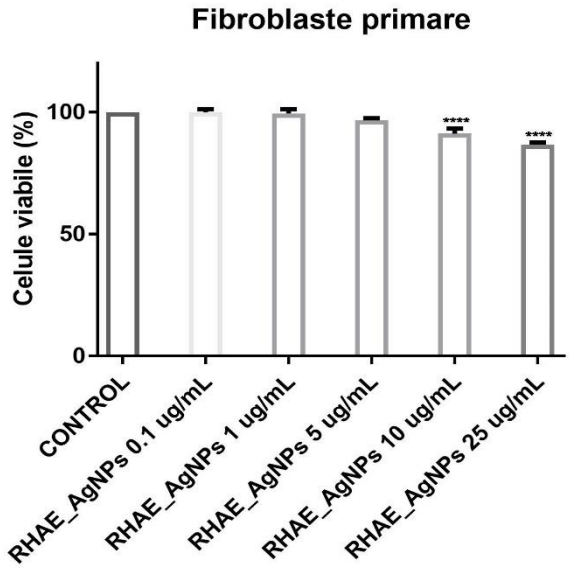

Figure 1. Percentages of viable fibroblasts resulting from stimulation with silver nanoparticles biosynthesized with hydroalcoholic extract of rosemary leaves

Antibiotics and extracts from plants. Synergism, antagonism and potentiation

This study aimed to test the antimicrobial activity of biosynthesized silver nanoparticles compared with antibiotics used for infections produced especially by *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Streptococcus pneumoniae* by different methods, namely: a) preclinical testing - on bacterial strains by the method of disk diffusion and anti-biofilm activity; and b) clinical tests - classical antibiotics on biological samples from current clinical activity. The anti-biofilm activity of silver nanoparticles obtained by biosynthesis was also studied in preclinical testing; the activity of silver nanoparticles in combination with antibiotics; antimicrobial activity and synergism. Biofilm formation was investigated *in vitro* by monitoring the binding of 0.1% crystal violet to bacterial cells adhered to a microtiter plate. The optical density value was used as an index to observe the ability to form biofilm in this organism. From the obtained data it was observed that the biofilm formed in the bacterial cells with a medium

concentration, which is associated with a high value of the optical density (example - figure 2).

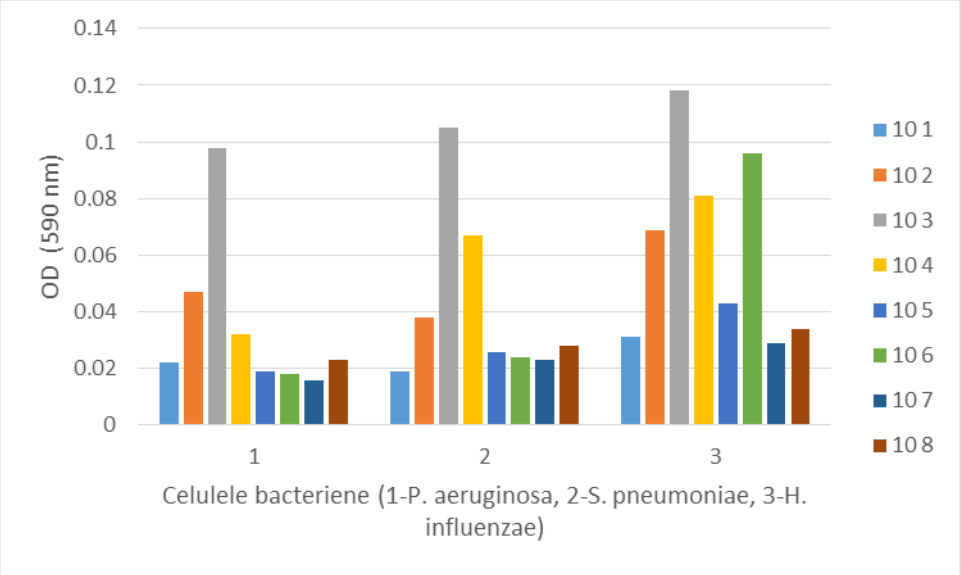

Figure 2. Biofilm formation in *P. aeruginosa*, *S. pneumoniae* and *H. influenzae* bacteria. It can be seen that the biofilm is formed in bacterial cells with a concentration of approximately 10^3 cfu / mL, as indicated by the high OD value.

The activity of silver nanoparticles biosynthesized with hydroalcoholic extract from rosemary leaves is the highest at the concentration of 10 µg / mL, with an increased inhibition rate for the three types of bacteria tested. Recently, various studies have indicated that silver nanoparticles can enhance the effect of antibiotics against sensitive and resistant bacteria, as well as reducing bacterial adhesion in the early stages of biofilm formation. In this study, the synergistic activity of the nanoparticles was evaluated with conventional antibiotics (ampicillin and amikacin) against gram-positive and gram-negative bacteria. The results presented here show that nanoparticles, in combination with antibiotics, increase the antimicrobial effect in an additive or synergistic way. Furthermore, cytotoxicity assays suggest that at low concentrations, silver nanoparticles and their combinations do not exhibit cytotoxic effects in healthy cells.

Statistical data and the relevance of the clinical approach in the area of biofilm related to diseases in the otolaryngology field

The management of ear, nose and throat infections requires a precise clinical and bacteriological diagnosis, followed by an initial empirical antimicrobial therapy, which can be adjusted once the identification of the causal organisms is available. The increasing antimicrobial resistance of many pathogens has made treatment of these infections more difficult.

A study on prescribing antibiotic pattern is an effective way to reflect the proper consumption of antibiotics. The present study focused mainly on the prescribing model of antibiotics in the ENT Section of the Timișoara Municipal Clinical Hospital. All patients who had ENT infections received medication prescriptions. The combination of amoxicillin and clavulanic acid was the most commonly prescribed antibiotic for conditions that did not require surgery. The predominantly isolated profile and bacteria highlight the need for continuous surveillance and reporting of the microbiology of ENT infections to guide clinicians to use appropriate antimicrobials for the etiologies under investigation. Almost all isolated bacteria, from patients requiring surgery, have shown considerable resistance especially to commonly used antibiotics, such as ampicillin, amoxicillin, and trimetoprim-sulfamethoxazole. Ciprofloxacin, amikacin, and gentamicin are effective against all bacterial isolates in the present study. The result of this study revealed that the antibiotic resistant bacteria recovered from the patients with infections become a major public health problem in the socio-economic case management. Due to the increased resistance to antibiotics, one can advocate waiting for results, especially if the patient has already treatment, which is often the case. National antimicrobial surveillance is strongly recommended to make the correct antibiotic recommendation, along with strict adherence to the antibiotic use policy to reduce the spread of drug-resistant microbes and associated complications in the country. Therefore, in parallel with the empirical treatment of infections, culture performance and antimicrobial sensitivity testing should be taken as routine and mandatory practice to properly manage infections, reduce

associated complications (individual and in the health system) and to reduce drug resistance.