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PHD THESIS

CONTRIBUTIONS RELATED TO THE ROLE OF ANTIBIOTICS IN TUMOR PROCESSES - A PERSPECTIVE OF PRECLINICAL ANALYSIS WITH RELEVANCE IN THE CLINICAL APPROACH

– R E S U M E –

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RESUME

Antibiotic class drugs are considered a cornerstone of modern medicine, and their discovery has led to a spectacular approach to infectious diseases. However, their excessive use in recent years, worldwide, has generated a critical public health problem, and microbial resistance correlated with the loss of antibiotic action is a challenge for specialists in the field. Finding new innovative therapeutic approaches to counter bacterial resistance is urgently needed, and natural compounds with antibacterial effects could be considered a promising option. The role played by antibiotics in tumor processes and their interrelationship with the microbiota are a debated topic that is still far from being elucidated. Therefore, the present thesis provides a global perspective on antibiotics in terms of evolution from a historical perspective with emphasis on the main classes of antibiotics and their adverse effects; deepens the connection between antibiotics and microbiota, emphasizing the dual role played by antibiotics in tumorigenesis. In addition, the use of natural compounds with antibacterial/antitumoral properties as potential alternatives to classical antibiotic therapy is discussed.

Natural compounds have an extraordinary chemical diversity, which is responsible for a multitude of biological effects, and therefore can be considered the most promising resources for the discovery and development of new antibacterial/antitumoral drugs. Recent research in the field of antitumor therapy and antibiotics has highlighted the fact that some antibiotics can cause apoptosis of cancer cells, thus preventing their growth and metastasis. On the other hand, some studies have drawn attention to the fact that the consumption of antibiotics can cause a disruption of the saprophytic microbial flora. The gut microbiome plays a significant role in the curative approach to cancer. Therefore, the consumption of antibiotics can cause, in addition to the destruction of the intestinal flora, a decrease in the immune system and a promotion of inflammatory processes, all of which have pro-tumor effects and lead to a decrease in the effectiveness of antitumor treatment. Thus, antibiotics can be seen as a double-edged sword, exerting a beneficial effect in cancer therapy as well as a pro-cancer effect, posing a risk to humans.

Considering the above and taking into account the complexity of the beneficial and less beneficial effects associated with antibiotics (e.g., antibacterial action/excess consumption leading to microbial resistance, antitumor action/pro-tumor effects), the present work had three specific scientific objectives, as presented in the following:

(1) Evaluation of the effects exerted by ampicillin on pharyngeal carcinoma cells (Detroit-562 cells) in terms of viability, morphology, cell migration, and nuclear and actin fiber structure - considering the hypothesis that broad-spectrum antibiotics exert harmful effects on human microbiota, and pharyngeal carcinoma may be affected by such changes.

(2) Evaluation of the effects exerted by tetracycline on pharyngeal carcinoma cells. Therefore, cell viability and morphology, as well as the structure of actin fibers and nuclei and the ability of cells to migrate, were examined.
(3) Analysis of the combination between thyme essential oil and tetracycline regarding: (a) antimicrobial activity against Gram-positive (*Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram- negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and (b) cytotoxic activity on human colorectal adenocarcinoma cells.

The current thesis is structured in accordance with methodological norms into two main parts: the general part and the special part. In the general part, the newest data from the specialized literature are described with reference to: (a) general aspects regarding conventional antibiotics (brief history, classification of antibiotics, aspects related to the toxicity of antibiotics), (b) natural compounds with antibacterial effect (with the accent on the classes of natural compounds with antimicrobial action) and (c) antibiotics and cancer (antibiotics as cancer therapy, antibiotics as cancer promoters, potential pro-tumoral mechanisms of action related to antibiotics). The special part is structured in three chapters, as follows: preclinical evaluation of the effect of ampicillin on pharyngeal carcinoma cells; preclinical evaluation of the effect of tetracycline on pharyngeal carcinoma cells; assessment of the biological effects of the association between tetracycline and thyme essential oil. The paper also presents a part of general conclusions and particular contributions and ends with bibliographic references that support the information presented and the original results obtained.

The first chapter from the experimental part is related to the preclinical evaluation of the effect of ampicillin on pharyngeal carcinoma cells. In terms of incidence, head and neck cancer ranks sixth, and almost half of cases are oral cancers. Although significant progress has been made in the management and treatment of these cancers, the five-year survival rate for oral cancer still falls below 50% in most countries. Given the heterogeneity of head and neck cancers, treating these conditions can be a challenge for specialists in the medical domain. Different factors contribute to the development of oral cancer, including smoking, exposure to ultraviolet rays, chronic inflammation, and certain bacterial or viral infection. Therapeutic protocol options for oropharyngeal cancers include chemotherapy, radiation therapy, and surgery. An immunotherapeutic approach to treat these types of cancer is the latest treatment option but it is still accompanied by a number of unknowns adverse reactions. The major disadvantage associated with antitumor therapy is the occurrence of toxic reactions both locally and systemically. In some cases, both chemotherapy and radiotherapy can lead to the need for antibiotic cure. Most of the antibiotics used in this case are broad-spectrum antibiotics, which increase the risk of changing the physiological

microbiome. In recent years, studies have focused on the role of the microbiota in the pathogenesis and response to treatment in a wide variety of diseases, including cancer. Regarding throat cancer, such as pharyngeal cancer, there is little evidence regarding the role of the microbiota. A substantial difference was found between the microbiota of patients with throat cancer and that of healthy patients, thus emphasizing the important role played by the microbiota in this type of cancer. Antibiotics are considered to be the cornerstones of modern medicine, but currently, they are associated with problems related to bacterial resistance and, as a result, are becoming more and more ineffective day by day. At the same time, antimicrobial resistance can be associated with a significant virulence and transmission, playing a crucial role in the global spread of resistant bacteria. There is considerable controversy regarding the use of ampicillin in cancer patients. Despite the fact that ampicillin can be used prophylactically in cancer patients, it is not known exactly what effect it can have on the development and proliferation of tumor cells. Therefore, there is conflicting evidence regarding the relationship between ampicillin and tumorigenesis. On the one hand, some studies provide evidence that ampicillin has antitumor properties. However, there is evidence in the literature that ampicillin has a pro-tumour effect, leading to stimulation of tumor cell proliferation, increasing tumor size. Considering the hypothesis that broad-spectrum antibiotics exert harmful effects on human microbiota, and pharyngeal carcinoma may be affected by such changes, the main objective of this study was to evaluate the effects of ampicillin on pharyngeal carcinoma cells. Effects on viability, morphology, cell migration, and nuclear and actin fiber structure were assessed. Ampicillin did not show a significant cytotoxic effect; in fact, at the lowest concentrations tested (10, 25, and 50 μM), cell viability increased compared to untreated control cells. Meanwhile, concentrations of 75 and 100 μM resulted in a slight decrease in viability, but the decrease was not significant, around 94%. Cell morphology, as well as nuclear structure and actin fiber organization, did not show important differences compared to those observed in non-antibiotic-stimulated cells.

The second chapter is based on the preclinical evaluation of the effect of tetracycline on pharyngeal carcinoma cells. Today, pharyngeal cancer is a major problem facing mankind, largely due to alcohol consumption and smoking. There has been considerable interest in examining the causal relationship between microbiota composition and cancer development. As a result, different changes in the microbiota have been shown to be associated with the development of different types of cancer. It has been documented that the intestinal microbiota plays a significant role in the development of gastric cancer. In contrast, the relationship between oral dysbiosis and oral cancer is not fully understood. It has been proposed in the literature that the microbial influence on the cancer process can be mediated through different mechanisms of action, of which the most recognized mechanism

is the induction of chronic inflammation. In addition to this mechanism of action, bacteria can also influence the process of cell proliferation and inhibit cell apoptosis, thus contributing to the development of several types of cancer. Recent studies have shown that some antibiotics may be useful in the treatment of cancer by: (a) promoting apoptosis in cells; (b) inhibiting the proliferation of cell lines; and (c) prevention of metastasis. There is controversy surrounding the use of antibiotics in the treatment of cancer patients. In addition to altering the microbiota, antibiotics also reduce immunity and promote inflammation, which contributes to tumor development and decreased treatment effectiveness. Several antibiotics with antitumor activity, which are known to have a strong anti-proliferative effect on tumor cells, have been discussed in the literature. There are several common mechanisms linked to tetracyclines affecting tumors, including inhibition of mitochondrial protein synthesis, inhibition of matrix metalloproteinases, inhibition of nuclear factor kappa signaling, etc. Tetracyclines also inhibit the formation of new blood vessels. Based on the above, the current study investigated the effects of tetracycline on pharyngeal carcinoma cells. Therefore, cell viability and morphology, as well as the structure of actin fibers and nuclei and the ability of cells to migrate, were evaluated. The concentrations selected for the *in vitro* study were between 10 μM and 100 μM. Based on the results, tetracycline causes a decrease in cell viability directly proportional to its concentration. Cell viability decreased to about 71% at a concentration of 10 μM, while at a concentration of 100 μM the viability decreased to about 46%. To gain a deeper understanding of the effects of the antibiotic on pharyngeal carcinoma cells, cell morphology was analyzed. Tetracycline causes morphological changes and a decrease in cell confluence in a concentration-dependent manner. Morphological changes were observed at all concentrations studied, including (i) cell rounding, (ii) cell detachment from plates, and (iii) decreased confluence and cell number. The effects of the highest concentration on cell morphology were most obvious. Considering that cell migration is a characteristic of tumor cells, the present study evaluated the impact of tetracycline tested in three concentrations (10, 50 and 100 μM) on cell migration. The effects of tetracycline were evident in the inhibition of cell migration as well as morphological changes. There was a correlation between the tested concentration and the decrease in the ability of the cells to migrate. As a result, at a concentration of 10 μM, the closure rate decreased to about 27% compared to about 64% for control cells. At concentrations of 50 and 100 μM, greater decreases in cell migration were observed. As a more comprehensive picture of the mode of action of tetracycline at the cellular level, fluorescent immunocytochemistry was used to observe changes at the level of actin fibers and the nucleus after stimulation with tetracycline in three concentrations (10, 50 and 100 μM) for 72 h. Actin fibers were visualized using Rhodamine-Phalloidin staining. A change in the distribution of actin fibers was observed in tetracycline-treated cells compared to control

cells. Throughout the cells, actin fibers were highly concentrated at the edges, indicating a condensed state. While unstimulated cells had uniform distributions of actin fibers throughout the cell. Regarding the effect on nuclei, tetracycline caused a strong condensation of chromatin, the appearance of apoptotic bodies and a decrease in the number of nuclei. Concentrations of 100 µM resulted in the most significant changes. The cytotoxic effects of tetracycline may result from a variety of possible biological mechanisms, including: (a) inhibition of mitochondrial protein synthesis; (b) inhibition of matrix metalloproteinases; (c) impairment of angiogenesis; (d) eradication of cancer stem cells; and (e) increasing the sensitivity of tumor cells to radiotherapy by down-regulating DNA-dependent protein kinase.

The third chapter was focused on the assessment of the biological effects of the association between tetracycline and thyme essential oil. To meet the challenges of antibiotic resistance, a key approach is to stimulate the discovery of bioactive substances at an early stage. Completing the pharmacological actions of classical antibiotics with substances of natural origin is intensively studied to achieve efficiency in the clinic, correlated with a real objective of developing a new generation of chemotherapeutic drugs derived from natural sources, considering their unparalleled chemical diversity. The resistance developed by Gram-positive and Gram-negative bacteria to several drugs has made them difficult to treat and/or even untreatable with currently available antibiotics. Moreover, it is of major importance to identify new targets and new classes of antibiotics that can deal with drug-resistant bacterial pathogens. This requires basic research to discover new gaps and develop new antibiotic approaches. The aim of the present research was to evaluate a combination of thyme essential oil and tetracycline regarding: (a) antimicrobial activity against Gram-positive (*Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and (b) cytotoxic activity on human colorectal adenocarcinoma cells. The sensitivity of bacteria to thyme essential oil and its combination with tetracycline, determined by the disk diffusion method, revealed that the essential oil exhibits increased dose-dependent activity in all tested strains, except *P. aeruginosa*. The maximum zone of inhibition revealed by thyme essential oil was at the highest concentration tested (50µL/mL) against *S. aureus* and *K. pneumoniae*, while the combination with tetracycline resulted in a slight increase in diameter. To determine the cytotoxic effect of thyme essential oil and tetracycline, different concentrations were tested on the human colorectal adenocarcinoma cell line. Cell viability was assessed using the MTT assay at 48 h intervals. In the case of tetracycline, a slight decrease in cell viability was observed only at the highest concentration tested (10µg/mL). In contrast, thyme essential oil exerted a greater than 30% decrease in cell viability at the highest concentration tested (50µL/mL). Regarding the evaluation of the

highest concentration of tetracycline and three different concentrations of thyme essential oil on the behavior of the cells, the values recorded were between 98-84%.

The main conclusions are presented in the following.

- All experiments were conducted according to well-established and recognized protocols: cell viability with the MTT test, migration capacity through an automated procedure, changes at the nucleus level through nuclear labeling to reflect apoptotic processes.
- Certain particular effects exerted by the antibiotic were identified, namely: an increase in cell viability was recorded at the lowest concentrations of antibiotic tested and a slight reduction of it at the highest concentrations tested; cell migration was not statistically significantly affected in the case of stimulation of cells with antibiotics compared to unstimulated cells and no pro-apoptotic processes were observed in the nuclei.
- tetracycline has a concentration-dependent cytotoxic effect, characterized by a decrease in cell viability, as well as morphological changes characteristic of apoptosis (condensation of the nucleus and actin fibers, the appearance of apoptotic bodies).
- tetracycline has been shown to be a potential antitumor agent, but further studies are needed to clarify its biological mechanism of action and determine its safety profile.
- Further studies are needed to fully understand how tetracycline affect the throat microbiota and how this microbiota contributes to pharyngeal cancer.
- The chemical composition of thyme essential oil was evaluated by the gas chromatography-mass spectrometry method, the antioxidant capacity by the DPPH assay, the antibacterial and synergistic properties were determined by the disk diffusion method, and the cytotoxic activity by quantifying viable cells by using the MTT test.
- Thyme essential oil has increased antioxidant activity, antibacterial potential against both Gram-positive and Gram-negative bacteria, especially S. aureus and K. pneumoniae at the highest concentration tested, also having a synergistic effect when when combined with tetracycline.
- Cells treated with the essential oil showed a dose-dependent reduction in the viability of colorectal adenocarcinoma cells, while the combination with tetracycline resulted in a significantly attenuated decrease in viability.
- The association of the biological product with the classical antibiotic tetracycline demonstrated the preservation of its cytotoxic properties and the increase of antimicrobial activity against *S. aureus*, *E. coli* and *K. pneumoniae*.