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PhD THESIS

ASPECTS RELATED TO THE SAFETY PROFILE OF CARDIOVASCULAR DRUGS INDICATED IN CERTAIN PATHOLOGICAL CONDITIONS AND PREGNANCY ASSOCIATED

A B S T R A C T

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ABSTRACT

Pregnancy induces a series of more or less significant physiological and anatomical changes in the cardiovascular system, from the beginning (first trimester) to the postpartum period. From changes in cardiac output to structural changes, the heart undergoes progressive adaptive remodeling in most cases. Mechanisms involving increased blood volume, signaling from progesterone, and vascular endothelial growth factors lead to cardiac myocyte hypertrophy and increased angiogenesis. At the same time, during pregnancy, certain conditions can develop that can endanger the life of patients. Cardiovascular disease and most of its related disorders are the leading causes of maternal morbidity and mortality. Currently, the driving factors contributing to this trend are related to increasing age and rising rates of obesity. The use of cardiovascular drugs during pregnancy has increased significantly in recent years taking into account: (a) the fact that more and more women are delaying conception, which leads to higher rates of hypertensive disorders during pregnancy, and (b) the increase in the number of women with congenital cardiovascular disease who become pregnant. Most drugs are not teratogenic when used in therapeutic doses. Due to the constantly changing physiology during pregnancy, plasma drug levels may increase leading to adverse effects. Therefore, the use of drugs during pregnancy is not without risks, and the analysis of possible effects should be part of the counseling of women with pathologies (especially cardiovascular diseases) who want to become pregnant or who are already pregnant.

Beta-blockers are considered fourth-line therapy outside of pregnancy, but due to their fetal and neonatal safety profile they are frequently prescribed, and labetalol is the recommended first-line agent for the treatment of both chronic hypertension and acute hypertensive emergencies throughout gestation. Some cardiovascular drugs, such as digoxin, require extra caution because they have little effect on the mother during pregnancy due to the increased clearance of the drug and the increased unbound fraction of digoxin. Most antiarrhythmic drugs, including beta blockers, calcium channel blockers, digoxin, etc. they are compatible with breastfeeding because they have little transfer into breast milk.

The importance of maternal diet for the health of the fetus is well known. The nutritional status of the fetus depends to a large extent on the maternal intake, and nutrient deficiencies can lead to congenital malformations and damage to the mother's health. The use of dietary supplements during pregnancy is very common although knowledge about the safety and/or effectiveness of the supplements is not fully understood. At the same time,

during pregnancy most of the time, expectant mothers often focus on diet, frequently using food supplements. Information related to their use during pregnancy is often contradictory in terms of safety and/or effectiveness. Recommendations are generally made taking into account safety data and tolerable doses established for pregnant women. During pregnancy, the skin is also affected, and the most feared disease is melanoma diagnosed during the fertile period, known as pregnancy-associated melanoma. Different biological mechanisms are involved, and these are closely related to hormonal and immune status and increased lymphangiogenesis.

Therefore, the detailed study of the effects exerted on certain reference cells of two established cardiovascular drugs (labetalol and digoxin) as such and in combination with molecules of natural origin was chosen.

The present thesis is structured according to methodological norms into two main parts: the general part and the special part. In the general part, the latest data from the specialized literature are presented with reference to: (a) hypertension in pregnancy, specific aspects and conventional treatments (classes of drugs used in the treatment of gestational hypertension, beta-blockers in the treatment of gestational hypertension), (b) the use biocompounds in therapeutics, natural resources and gestational hypertension (major classes of compounds used in hypertension, plants and phytocompounds used in pregnancy-associated hypertension) and (c) pregnancy-associated melanoma and the antitumor potential of consecreated drugs.

Taking into account the complexity related to cardiovascular drugs, dietary supplements and diseases that intervene in pregnancy (eg hypertension or skin malignancies), the present work had three specific scientific objectives, namely: (a) contributions to the completion of the safety profile of of labetalol - evaluation through preclinical studies, in vitro, of the effects exerted by labetalol (as such and in combination with folic acid and folate) on myoblasts and hepatocytes (b) contributions related to the antitumor effects of digoxin - evaluation through preclinical studies, in vitro and in ovo of the effects exerted by digoxin (as such and in combination with betulinic acid) on melanoma cells, (c) analysis of the data related to the antitumor effects exerted by labetalol and digoxin. The special part is structured in five chapters, namely: contributions related to the specific action of synthetic and natural cardioprotective drugs on cell viability and morphology; evaluation of the actions exerted by labetalol and its combinations with folates on cell migration; the influence of digoxin and its combinations with betulinic acid on apoptotic processes; the influence of digoxin and its combinations with betulinic acid on blood vessels and a detailed study of the antitumor effects of established drugs - digoxin and labetalol. The paper also contains 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 hypothesis was based on the fact that the concomitant administration of labetalol-folic acid or folate during pregnancy could lead to harmful side effects, which, to our knowledge, have not been intensively investigated to date. Therefore, a current experimental study was to present an in vitro toxicological profile of labetalol associated with folic acid and folate using healthy myoblasts and hepatocytes as models for compound-induced cardio- and hepatotoxicity. The effects of labetalol, folic acid, folate and their combinations on the viability of healthy myoblasts were evaluated following prolonged treatment at 72 hours. Labetalol induced a dose-dependent decrease in myoblast viability, with the most prominent effect at the highest concentration, 150 nM, folic acid decreased myoblast viability in a concentration-dependent manner up to ~82% (50 nM), folate caused a significant increase in the percentage of viable cells at the lowest concentration of 0.2 nM, while at the highest concentration (50 nM) viability was reduced to ~83%. A similar trend was observed in liver cells. The impact of the labetalol-folate combination on myoblast viability was concentration-dependent: labetalol (50 nM) together with folate (25 nM and 50 nM) exerted a stimulatory effect over time at a concentration of 150 nM labetalol reduced viability was observed cell phones. The impact of labetalol combined with folic acid on myoblast viability was insignificant. In hepatocytes, a reduction in cell viability was observed following the combination of labetalol (50 nM and 150 nM) with folic acid (0.2 nM) and folate (50 nM), while the other treatment regimens led to cell viabilities similar to untreated cells. Morphological assessment did not indicate significant confluence changes in myoblasts and hepatocytes after 72-hour treatment with labetalol-folic acid and labetalol-folate combinations.

In vitro labetalol-folic acid and labetalol-folate combinations did not exert toxicity (cardiac or hepatic) at known plasma concentrations. Moreover, the combination of labetalol-folate improved myoblast viability, while the combination of labetalol-folic acid demonstrated cardiac healing properties, suggesting a possible benefit to cardiac function resulting from their co-administration, which should be explored in future studies.

To assess whether labetalol, folic acid, folate and their combinations interfere with myoblast migration, a wound healing assay was performed. The wound healing rate of untreated cells reached ~66% (out of 100%, indicating complete scratch closure) after 24 h. Compared to control, single treatment of cells with labetalol and folic acid inhibited wound healing at both concentrations tested after 24 h of treatment. Folate, on the other hand, exerted an inhibitory effect at 25 nM, while it slightly stimulated wound regeneration at 50 nM. The results also suggest that all combinations except LB 150 nM–FA 50 nM (wound healing rate ~53%) stimulate cell migration and wound healing after 24 h of treatment.

Compared to untreated cells, the strongest stimulatory effects were recorded when labetalol (at concentrations of 50 and 150 nM) was combined with 0.2 nM folic acid with wound healing rates of ~85% and ~88%, respectively . The main conclusion of the study is that the labetalol-folic acid combination demonstrated cardiac healing properties, suggesting a possible benefit for cardiac function resulting from their co-administration, which should be explored in future studies.

Another major objective was to evaluate the effects of digoxin, betulinic acid and the combination of the two substances on human melanoma cells (SK-Mel-28 and RPMI-7951). To determine the cytotoxic potential of the two compounds and their combination, viability was determined 24 hours after stimulation with digoxin (5, 10, 25 and 50 nM), betulinic acid (1, 5, 10 and 25 μM) and the combination digoxin (5, 10, 25 and 50 nM) + betulinic acid 10 μM. Digoxin was observed to cause a slight reduction in cell viability in SK-Mel-28 melanoma cells: at a concentration of 5 nM, cell viability was found to be around 99%, while at concentrations of 10, 25, and 50 nM, viability remained relatively constant at around 81%. Also on this type of melanoma cells, betulinic acid showed a concentration-dependent cytotoxic effect: at a concentration of 1 μM, the percentage of viable cells decreased to about 63%. As a result of combining digoxin with betulinic acid, it was observed that the highest concentrations of digoxin tested (25 and 50 nM) together with a concentration of 10 μM betulinic acid produced a more pronounced cytotoxic effect than the two compounds tested individually. A similar effect was also observed in RPMI-7951 melanoma cells. Digoxin reduced the percentage of cell viability in a concentration-dependent manner: at concentrations of 5 and 10 nM, cell viability was similar to control cells, but at concentrations of 25 and 50 nM, viability decreased to approximately 88% and 82%, respectively. Betulinic acid decreased the number of viable cells also in a dose-dependent manner with the note that the effect of betulinic acid on cell viability was much less pronounced in this type of melanoma cells. The combination of digoxin and betulinic acid had a more pronounced cytotoxic effect than each compound used individually. Thus, even at the lowest concentration tested, the percentage of viable cells decreased to a value of approximately 74%, while at the highest concentration tested, the percentage of cell viability decreased to a value of approximately 23%. Morphological changes observed included: a round appearance of cell shape, detachment of cells from the plate, and a decrease in cell confluence, data which are consistent with the results obtained in the case of the viability assay. At low digoxin concentrations (5 and 10 nM), the combination index values obtained indicated an antagonistic effect, whereas at higher concentrations (25 and 50 nM), the combination index values indicated a synergistic effect. For the human melanoma cell line the combination index values were all below 1, indicating a strong synergistic effect.

Data from this study revealed that a digoxin+betulinic acid combination (50 nM+10 μM) showed a more pronounced cytotoxic effect than the compounds tested individually on both types of tumor cells, resulting in a decrease in cell viability and morphological changes . These results provide a basis for future pharmaco-toxicological studies to deepen the synergistic effect exerted on melanoma cells and to establish the biological mechanisms involved.

Investigation of the changes occurring in nuclei and actin fibers after stimulation with betulinic acid (10 μM), digoxin (50 nM) and their combination was carried out to gain a deeper understanding of the mechanisms by which these compounds act on melanoma cells. In SK-Mel-28 cells, betulinic acid and digoxin cause only a slight condensation of chromatin and actin fibers, while when they are combined, the number of nuclei decreases and massive condensations of nuclei and actin fibers occur, indicating the onset of a similar process apoptosis. Regarding the effect of betulinic acid on nuclei and actin fibers of RPMI-7951 cells, at a concentration of 10 μM it causes a slight condensation of nuclei and actin fibers. In the case of digoxin, a relatively low impact on nuclei and actin fibers was observed, but the combination of the two compounds was shown to have a strong effect on the condensation of nuclei and actin fibers, also causing the formation of apoptotic bodies. Data from this study revealed that a digoxin+betulinic acid combination (50 nM+10 μM) showed a more pronounced cytotoxic effect than that of the compounds tested individually and changes in nuclei and actin fibers indicating an apoptotic-like effect. These data provide a basis for future pharmaco-toxicological studies to deepen the synergistic effect exerted on melanoma cells and to establish the biological mechanisms involved.

The hen's egg chorioallantoic membrane was used as a biological model to evaluate the vascular toxic potential of betulinic acid (10 μM), digoxin (50 nM) and their combination. Regarding the test samples, betulinic acid had an irritation score of 0.75, digoxin had a score of 1.09 and the combination had a score of 0.52. At the level of the vascular plexus, a slight intravascular coagulation was observed, followed by a slight vascular lysis following the administration of betulinic acid, digoxin showed a stronger irritating effect on the vascular system compared to betulinic acid. Vascular coagulation and vascular lysis were identified to be more pronounced in this case. However, the combination of the two compounds did not lead to significant changes, the only notable effect being a slight intravascular coagulation. Despite this fact, none of the compounds nor their combination showed significant irritant effects at the vascular level, and the viability of chicken embryos was not affected even after 24 hours of application. Data from this study revealed that no toxic effects on blood vessels were observed. These data provide a basis for future pharmaco-toxicological studies to deepen the synergistic effect exerted and to establish the biological mechanisms involved.

In recent years, research studies have focused on investigating the anticancer potential of several molecules used therapeutically to treat various pathologies. Thus, cardiotonic glycosides and especially digoxin came to the fore as promising molecules in the treatment of cancers, their targeted effect being investigated at the molecular level. Moreover, there is evidence that beta-blockers, both non-selective (labetalol, propranolol, carvedilol) and selective (nebivolol, atenolol), show activity in the treatment of cancer. Therefore, the last study included the analysis of data related to the antitumor effects exerted by labetalol and digoxin, and the main conclusions that can be drawn are presented below. Cardiotonic glycosides and beta-blockers are drug classes widely known for their benefits in cardiovascular disease, with therapeutic utility in certain conditions and in pregnant women. Due to their established actions, in recent years attention has been directed towards the antitumor effect of cardiotonic glycosides and non-selective beta-blockers. Thus, the aim of the present study was to highlight the anticancer activity of digoxin and labetalol, both in vitro and in vivo, in order to further evaluate their effects and study in more detail their mechanisms of antitumor action. Analyzing the data from the specialized literature and the original results obtained, it can be said that digoxin has a series of antitumor effects on a wider spectrum of malignancies, while labetalol has been much less studied for these effects.