UNIVERSITY OF AGRICULTURAL SCIENCES AND VETERINARY MEDICINE CLUJ-NAPOCA
DOCTORAL SCHOOL OF VETERINARY MEDICINE

Ph.D. THESIS

Nanoparticle coatings for controlled release from a balloon catheter

(SUMMARY OF Ph.D. THESIS)

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Resume

Peripheral artery disease (PAD) is a systemic atherosclerotic disease that affects over 202 million people worldwide and over 8 million Americans, being one of the leading causes of mortality in the United States (Beckman et al., 2011, Patel et al., 2015, Jaff et al., 2010, Song et al., 2019).

Currently, there is a significant global epidemiological transition, and atherosclerotic cardiovascular diseases represent a major concern. According to a research conducted by Fowkes and his colleagues (2017)(Fowkes et al., 2017), this condition is characterized by obstruction of the arteries in the lower extremities by atherosclerotic deposits, which can lead to occlusion and reduced blood flow in that area, thus limiting mobility and functionality. This condition is considered to be one of the major cardiovascular diseases (Marrocco and Bush, 2010, Song et al., 2019). Intermittent claudication is the most common symptom in patients with PAD, but this symptom is often ignored and not properly treated, leading to a decrease in the quality of life and functional capacity of patients, even when asymptomatic (Konishi et al., 2019, Patel et al., 2015). Ischemia can cause limb amputation in the most severe cases (Beckman et al., 2011, Jaff et al., 2010, Patel et al., 2015). People with peripheral arterial disease are at high risk of developing a heart attack, stroke, or dying from cardiovascular problems.

In the treatment of peripheral artery disease in the lower limbs, the recommended procedure is revascularization which can be performed through an endovascular method called angioplasty. This procedure involves dilating the obstructions in the arteries using a balloon and, in some cases, placing a stent (Barrett et al., 2017, Norgren et al., 2007, Tendera et al., 2011, Urasawa et al., 2011). Angioplasty is a medical procedure that involves mechanically widening a narrow or obstructed blood vessel caused by atherosclerotic deposits(Analysis, 2010). As peripheral arteries have a small diameter, balloon angioplasty is preferred over implanting metal stents(Thukkani and Kinlay, 2015).

Balloon angioplasty allows for slow stretching of the vessel to increase the size of the lumen(Analysis, 2010). Unfortunately, balloon angioplasty can cause stretching and tension on the blood vessel wall, and the resulting injury can trigger a series of cellular events that lead to the formation of a new injury(Dugas et al., 2019). The phenomenon of restenosis (re-narrowing of the blood vessel) after stent implantation continues to be an important aspect in clinical practice (Ali et al., 2019). It is believed that early restenosis and the slow development of intimal hyperplasia in stented blood vessels may be associated with deep vessel wall injuries, which involve a fracture of the internal elastic lamina(Van Den Berg, 2017). Hyperplasia involves inflammatory processes, migration and division of smooth muscle cells, as well as accumulation of extracellular matrix (Dugas et al., 2019, Goyal et al., 2012). These processes contribute to the thickening of the blood vessel wall, which can lead to the obstruction of blood flow (Van Den Berg, 2017).

Current protocols for prevention and therapy of restenosis after vascular angioplasty or stenting are based on the sustained release of an antiproliferative drug into the vessel wall(Werk et al., 2008). Drug-coated balloons (DCBs) have gradually emerged in the treatment of peripheral arterial disease (Cortese et al., 2016, Tepe et al., 2015, Tepe et al., 2008, Werk et al., 2008) and coronary in-stent restenosis (Doshi et al., 2019). The concept of DCBs is based on the rapid healing of the vessel wall due to the quick release of the drug. DCBs require three fundamental elements: a semi-compliant angioplasty balloon, an anti-proliferative drug, and a new active substance delivery system (Doshi et al., 2019). A paclitaxel chemotherapy agent-eluting DCB has been approved by the FDA.

Paclitaxel is a lipophilic treatment that quickly and tightly binds to tissue, leading to rapid absorption and retention at the cellular level at the site of administration. However, the use of paclitaxel has major disadvantages, including systemic toxicity (Dugas et al., 2019; Marzullo et al., 2009), premature release of paclitaxel before reaching the site of the lesion due to direct application of the drug on the surface of the balloon (Dugas et al., 2019; Marzullo et al., 2009) and delayed re-endothelialization, as demonstrated in animal studies using paclitaxel-coated stents (McFadden et al., 2004; Farb et al., 2001). Re-endothelialization is a fundamental process in vascular lesions after interventional therapy, which is essential for preventing excessive neointimal proliferation(Liu et al., 2019). Recently, the FDA has issued a series of warnings regarding the use of paclitaxel-coated balloons in the peripheral treatment of arterial disease, after a late signal of increased mortality was identified in patients treated with such devices(Fda, 2019). According to studies, the relative risk of 5-year mortality was 1.57, representing a 57% relative increase in mortality for patients treated with paclitaxel-coated balloons. This signal raised concerns about the safety and efficacy of using paclitaxel in the peripheral treatment of arterial disease (FDA, 2019). As a result, research has focused on developing methods for the controlled delivery of other anti-proliferative agents in place of paclitaxel.

Based on the above mentioned, this thesis is structured into two chapters and presents a summary of available literature on the use of nanotechnology in human and veterinary medicine, as well as the synthesis of PLGA nanoparticles. It provides an overview of the current state of knowledge in the field of nanotechnology. The first chapter provides general aspects about nanotechnology, including the synthesis and encapsulation of active substances in polymeric nanoparticles. Additionally, the advantages and disadvantages of using nanoparticles, particularly biodegradable PLGA nanoparticles, are examined. Finally, the applications of nanomedicine are discussed.

The first chapter presents general aspects of nanotechnology, including the synthesis and encapsulation of active substances in polymeric nanoparticles. Additionally, the advantages and disadvantages of using nanoparticles, particularly the biodegradable PLGA nanoparticles, are examined. Finally, the applications of nanomedicine are discussed. Chapter two focuses on the synthesis of PLGA nanoparticles and presents three methods: Emulsion diffusion, Nanoprecipitation, and Emulsion Evaporation, each describing the polymer concentration, molecular weight of the polymer, molar ratio of the copolymer, solvents, surfactant, homogenization speed and time, as well as cryoprotectants. All of these are presented along with the methodology, advantages, and disadvantages. These two chapters provide a complete picture of the use of nanotechnology in medicine and the importance of this field currently. The second part of the doctoral thesis focuses on its purpose and motivation, as well as three original studies aimed at implementing a new delivery system for anti-proliferative substances, represented by quercetin and resveratrol, using PLGA nanoparticles. The conclusions, recommendations, and innovative aspects are also included. The thesis concludes with a bibliography citing 137 titles.

The aim of the first study was to develop an efficient delivery system for active substances with anti-proliferative properties, to be used in the minimally invasive treatment of peripheral artery diseases, without causing adverse effects. This delivery system must allow for efficient uptake of the active substances and demonstrate increased anti-proliferative activity and minimal toxicity to vascular cells. To achieve this goal, PLGA nanoparticles were synthesized, both empty and loaded with quercetin or triacetyl resveratrol, using a single synthesis technique, the emulsion evaporation method. The nanoparticles were characterized in terms of morphology using Transmission Electron Microscopy (TEM), and the size distribution and Zeta potential were determined using Dynamic Light Scattering (DLS). In the same study, cell proliferation was evaluated using a colorimetric immunoassay for quantifying

cell proliferation, based on the measurement of Invitrogen Hoechst nuclear substance incorporation with cell permeability emitting blue fluorescence when bound to the smooth muscle cells' DNA of rat aortic cells.

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Study 2 aimed to improve the new delivery system developed in Study 1. Nanoparticles made of PLGA were synthesized, and the antiproliferative active substances were represented by polyphenols: quercetin, acetylated quercetin, rhamnazin, and triacetyl resveratrol. By adjusting the positive charge of the delivery system, the nanoparticles were designed to be biocompatible and biodegradable. Other parameters were modified to provide sustained release of the encapsulated polyphenols (e.g. quercetin was acetylated in the lab to achieve adequate encapsulation and sustained release of the active substance). The PLGA nanoparticles loaded with polyphenols were characterized using DLS and TEM. This study aimed to evaluate the controlled release of the active substances from the synthesized nanoparticles. The release protocol involved dialyzing the samples and analyzing them via high-performance liquid chromatography (HPLC). The effect of the polyphenol-loaded nanoparticles was evaluated using a colorimetric immunoassay for quantifying cell proliferation, based on measuring BrdU incorporation during DNA synthesis. The incorporation of 5-bromo-2'-deoxy-uridine (BrdU) into aortic smooth muscle cells from rats was measured using a commercial cell proliferation assay kit. The cytotoxicity of the delivery system is a major barrier to delivering active substances, so the viability of smooth muscle cells was evaluated, and the relative levels of ATP

were measured to test the cytotoxicity of the nanoparticles. The synthesized polymeric nanoparticles containing quercetin and resveratrol exhibited a spherical shape.

After resuspension in nanopure water, the nanoparticles had a mean size of 83.6 ± 3.2 and a polydispersity index (PDI) of 0.177 ± 0.027. The positive charge of the nanoparticles was designed to enhance their interaction with the negatively charged phospholipid membrane of vascular cells. The PLGA nanoparticles with pNP(Q) and pNP(TAR) were synthesized at a size of 102.7 nm and 90.6 nm, respectively. Their size met the requirements for cellular uptake. All polyphenols were separately encapsulated in PLGA nanoparticles to allow for comparison, but a combination of them was also performed. Treatment with rhamnazin-loaded PLGA nanoparticles significantly reduced cell proliferation by up to 40% at 24 hours at all concentrations. At 24 hours, inhibition of cell proliferation was also observed with the combination of nanoparticles loaded with rhamnazin and triacetyl resveratrol (TAR). At 48 hours, PLGA nanoparticles loaded with TAR at a concentration of 0.3mg/km showed an inhibition of proliferation of about 20%. Only particles loaded with active substances significantly reduced proliferation, and at 72 hours, only pNP loaded with acetylated quercetin maintained its inhibitory effect. It is noteworthy that at 72 hours, the nanoparticles showed a significant increase in cell proliferation. Cell viability was evaluated on smooth muscle cells as in the first study and was assessed as relative levels of ATP compared to untreated cells. This was maintained or even increased at most doses. However, at a concentration of 0.1 mg/ml in the case of quercetin-loaded nanoparticles, ATP values decreased by 5-10%. The release profile for all three active substances encapsulated in nanoparticles was measured over 6 days. A significant effect of treatment, time, and significant interactions between treatment and time were demonstrated at different concentrations of nanoparticles loaded with active substances. Given that a primary cell culture was used, excessive cell death or proliferation may be due to their instability. Formulations with incorporated active substances showed an accentuated release on the first day, followed by a more gradual release of the active substances over the entire 6-day period. While rhamnazin was rapidly released, the release was slightly delayed when acetylated quercetin was used, with 85% being released by day 3.

Study 2 aimed to improve the new delivery system for the active substances used in the first experiment. The active substances loaded into nanoparticles were acetylated quercetin, rhamnazin (a quercetin derivative), and resveratrol. Nanoparticles were synthesized at a size that allowed for their incorporation via cellular endocytosis and showed an extended release period. Cell viability was maintained or even increased at certain concentrations, and the antiproliferative effect was demonstrated by incorporating the BrDU stain. The release of the active substances from the nanoparticles demonstrated that rhamnazin was rapidly released, while the release was slightly delayed when acetylated quercetin was used.

The aim of the latest study was to incorporate quercetin into PLGA nanoparticles, using different formulations compared to the previous two studies, in order to improve their characteristics. The ultimate goal of this study was to design a new drug-coated balloon using a polymeric nanodelivery system for sustained release of polyphenols that reduce restenosis, but with reduced toxicity compared to chemotherapy agents. The development of PLGA nanoparticles with entrapped quercetin, dimethoxy quercetin (ramnazin), and quercetin covalently attached to PLGA was desired. In this study, PLGA-Eudragit RL-100 nanoparticles were synthesized and PLGA nanoparticles were conjugated with quercetin. The polyphenol-loaded PLGA nanoparticles were characterized using DLS and TEM. The protocol for releasing the entrapped active substances from the PLGA nanoparticles was performed using the same method as in studies 1 and 2. To evaluate cell proliferation in rat aortic smooth muscle cells, a brief balloon inflation and nanoparticle delivery simulation was performed using DMEM culture medium with a 2-hour incubation at 37°C, as determined from the results of studies 1 and 2.

Calcification can be a frequent complication after angioplasty, which can lead to less favorable outcomes. This is because calcification can lead to a reduction in arterial compliance, which can limit the vessel's ability to expand to allow adequate blood flow. Additionally, calcification can lead to artery dissections, which can result in reduced blood flow or obstruction. Moreover, calcification can lead to acute vessel recoil, which can be problematic for surgery and may require additional treatment (Fitzgerald et al., 1992).

A secondary aim of the present study was to allow the adherence of pNP to calcified lesions. Vascular calcification is a common condition in PAD and may be more extensive than in coronary artery disease (Narula et al., 2020). The accumulation of calcium and phosphate in the intimal and medial layers of the vessel is typical of patients with PAD, especially those with chronic kidney disease and diabetes(Karwowski et al., 2012). Calcification can be a frequent complication after angioplasty, which can lead to less favorable outcomes. This is because calcification can lead to a reduction in arterial compliance, which can limit the vessel's ability to expand to allow adequate blood flow. Additionally, calcification can lead to artery dissections, which can result in reduced blood flow or obstruction. Moreover, calcification can lead to acute vessel recoil, which can be problematic for surgery and may require additional treatment (Fitzgerald et al., 1992). Furthermore, late loss of lumen after paclitaxel-coated balloon angioplasty has been shown to correlate with circumferential calcification (Fanelli et al., 2014), and hypotheses suggest that such results are due to the inability of the calcified lesion to absorb paclitaxel.

Thus, in the experiments targeted in study 3, it was tested whether coating with pNP was capable of strong adherence to cells in which calcium accumulation was experimentally induced. Balloon catheters were coated with polymeric nanoparticles using an ultrasonic method, with the characteristics of the nanoparticles, loading of active substance, uniformity of coverage, and release of active substances determined. Quantification of pNP and loading of active substances used gravimetric analysis and HPLC. Regarding statistics, all data from figures are expressed as means ± standard deviation. For most biological studies, experiments were designed to compare the effects of both types of dose and pNP. Thus, data were analyzed using two-way ANOVA with Bonferroni posthoc tests using GraphPad Prism software. The exception is that, for experiments aimed at testing cellular adhesion under calcifying growth conditions, three conditions were compared: calcification, washing, and pNP treatment. Thus, in this case, a three-way ANOVA was performed (with Bonferroni's posthoc tests). The cationic characteristics of pNP were provided by adding a cationic polymer, Eudragit RL100, during pNP synthesis. By adjusting the positive charge quantity of the system, pNP was designed to be biocompatible and biodegradable and was found to meet the ideal specifications for cellular absorption and maintaining a continuous release period. PLGA nanoparticles with pNP(eQ), pNP(eR), as well as conjugated quercetin (pNP(cQ), were developed to a size of 101 nm. All polyphenols were separately trapped in PLGA pNP to allow for comparison. Similar to previous experiments, incorporated quercetin was rapidly released within the first 24 hours, except this time the active substance was trapped separately in pNPs. It was no longer embedded together with resveratrol in its methylated form (Dugas et al., 2019). However, covalent attachment of quercetin delayed its release, as indicated by the sustained release and a longer release profile. The methylated derivative of quercetin (rhamnazin) with increased hydrophobicity provided somewhat sustained release of quercetin, although not as prolonged as pNP containing Q attached covalently. In the latter case, the release was sustained for a total of 6 days, which is beneficial because vascular healing as well as cellular events contributing to restenosis begin within the first 7 days after angioplasty (Kenagy, 2011).

In this experiment, an ultrasound coating method was used that allowed the new delivery system to generate a uniform layer. This coating technique is intended to minimize the non-specific release of the active substance into the bloodstream and improve long-term retention of the active substance in the vascular tissue, but such a limitation needs to be studied in future animal experiments. To create an effective angioplasty balloon with active substances, several key factors need to be considered, such as optimal delivery of the active substances, the use of biocompatible nanoparticles with sustained release, and the use of particles chemically attached with quercetin. Fluorescence microscopy can also be used to verify the uniformity of the surface and the loading of active substances.

The studies have presented certain limitations, such as a focus on short-term observations, the use of in vitro methods, uncontrolled variables, and testing on a limited number of balloons. Additionally, the production of cracks in nanoparticle layers during drying could affect the efficacy of the treatment. Further studies are needed to investigate the cracking of nanoparticle coatings for angioplasty balloons, as this phenomenon only occurs in thick nanoparticle layers. Surface uniformity may be affected by the number of layers, but current methods for evaluating uniformity may be inconclusive. Manipulation of balloons and other nanoparticle-coated materials can lead to defects in the coating layer, and future investigations should compare results from fluorescence microscopy with other imaging methods. In vivo studies with balloon angioplasty models are needed to test the pharmacokinetics of drug release at the vascular wall. If cracking is a problem, the use of plasticizing compounds in additional formulations to reduce coating fragility during drying could be considered.

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