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Implants composed of digoxin and poly(ε-caprolactone): development, characterization, anti-proliferative and anti-angiogenic activities

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Drug delivery systems could be applied to locally treat cervical cancer, thus preventing the drawbacks of conventional therapy. In this study, anti-proliferative and anti-angiogenic effects of digoxin incorporated into poly(ε-caprolactone) implants were evaluated, aiming at the local treatment of cervical cancer. Implants were characterized, and the *in vitro* release profile of digoxin was demonstrated. Anti-proliferative and anti-angiogenic activities of digoxin were investigated by using chorioallantoic membrane and human cervix carcinoma (HeLa) cells, respectively. The chemical structure of digoxin and the semi-crystalline nature of poly(ε-caprolactone) were preserved after designing implants. The hydrophobicity of drug and polymer as well as the semi-crystalline structure provided a controlled diffusion of digoxin from implants. Digoxin released from implantable devices exhibited anti-proliferative activity against HeLa cells. The anti-angiogenic effect was also shown. Finally, implants composed of digoxin and poly(ε-caprolactone) could be applied as a therapeutic alternative to treat the early stage of cervical cancer, once they were able to locally control the release of this anti-angiogenic and anti-proliferative drug, minimizing its systemic side effects and toxicity.

1. Introduction

Digoxin is a digitalis glycoside with a narrow therapeutic range, and it is used as therapy for congestive cardiac insufficiency and arrhythmia. Digoxin is a plasma membrane inhibitor of Na^+/K^+-ATPase, which leads to a buildup of calcium ions in the intracellular environment, increasing myocardic contractions and cardiac frequency (Guan et al. 2014). The increased concentration of calcium ions inside the cell may lead to apoptotic mechanisms, resulting in cellular death (Guan et al. 2014; Alevizopoulos et al. 2014). Moreover, digoxin also inhibits the DNA topoisomerase activity, inducing apoptotic effects (Hashimoto et al. 1999; Prassas et al. 2008).

Providing a different perspective, Lopez-Lazaro et al. (2005) suggested that inhibition of Na^+/K^+-ATPase and concomitant inhibition of aerobic glycolysis (Warburg effect) may explain the anti-cancer effects of digoxin. The inhibition of glycolysis prevents the conversion of glucose to lactate in the cytosol, even in the presence of oxygen, leading to a reduced production of adenosine triphosphate (ATP) (Billiard et al. 2013), and consequently, activating cell death mechanisms. Additionally, digoxin exerts apoptotic effects in cancer cells by inhibiting the catalytic activity of topoisomerases I and II and the expression of transcription factors, such as protein 1 activator and NF-kB (Prassas et al. 2008). Finally, digoxin may also exert its antitumor activity by targeting the endothelium cells, leading to anti-angiogenic effects (Winter et al. 2015).

Anti-proliferative and apoptotic activities of digoxin have been reported in cancer lines and preclinical and clinical studies. Kometiani et al. (2005) confirmed the growth inhibitory effects of digoxin on breast cancer cells. Using the model of breast cancer Ehrlich ascites tumor in mice, Bogush et al. (2016) demonstrated that digoxin decreased the Na^+/K^+-ATPase activity, and as a result, inhibited the glycolysis in tumor cells. This drug also significantly increased the cisplatin efficiency against this ascitic form of cancer, suggesting that the combination of cytostatic drug and digoxin could be considered a promising alternative in cancer therapy. Clinical trials are currently being performed to measure the effects of digoxin, administered alone or in combination with other antitumor drugs, on the inhibition of the Na^+/K^+-ATPase in cancer (Durlacher et al. 2015). Altogether, these results show the anti-cancer potential of digoxin.

The treatment of early stage cervical cancer is based on surgery, which does not preserve fertility. However, recently, minimally invasive surgery and neoadjuvant chemotherapy have been indicated for the treatment of cervical cancer as an attempt to preserve fertility (Sato et al. 2016). In current clinical practice, different chemotherapeutic regimens and dosages are systemically administered; but severe adverse effects, toxic reactions and tumor resistance have been reported after the chemotherapy (Yin et al. 2012), leading to a significant impact on patient's life.

Considering that chemotherapeutic drugs are indicated to treat early stage cervical cancer, in this study, the activity of digoxin against the cervix cancer line was demonstrated after its incorporation into poly(ε-caprolactone) implants. The poly(e-caprolactone) is a biodegradable and biocompatible polymer that promotes long-term release of entrapped drugs due to its slow degradation (Tamaddon et al. 2015). It was hypothesized that the digoxin controlled released from these implantable devices could induce regression of cervical tumor cell growth due to the inhibition of Na^+/K^+-ATPase, the other concomitant mechanisms of cell death (Özdemir et al. 2016) and the growth of new vessels at the tumor site (Winter et al. 2015). It was also suggested that digoxin-loaded poly(ε-caprolactone) implants could locally inhibit the cervix tumor, minimizing not only the systemic adverse effects and toxicity but also the resistance of tumor cells.

2. Investigations and results

2.1. Preparation of implants composed of digoxin and poly(ε-caprolactone) (digoxin PCL implants)

Digoxin and poly(ε-caprolactone) (PCL) were dissolved in a mixture of acetonitrile and ethanol (1:1), followed by solvent evaporation. Then, the blended powder was molded into spherical implants at approximately 70 °C. This temperature provided the melting of polymer, and consequently, the entrapment of digoxin

particles into polymeric chains. The resultant monolithic implants were 9.98±0.04 mg in average weight, 0.77±0.02 mm in thickness and 4.85±0.05 mm in diameter (n = 10). The digoxin content into PCL implants was 99.87±1.40% (n = 10) and the variation limit of drug content was 6.76%. This value was lower than the pharmacopeic specification (15%) (United States Pharmacopeia 2009).

2.2. Characterization

2.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

Figure 1 shows the infrared spectra of pure digoxin (Fig. 1A), PCL implants (without drug) (Fig. 1B) and digoxin PCL implants (Fig. 2C).The infrared spectrum of pure digoxin revealed absorption bands at ~3400 cm^{-1} related to -OH stretching vibration; at ~3000–2900 cm^{-1} equivalent to C=CH_2 and CH_2 stretching vibration; at ~1750 cm^{-1}corresponding to -C=O stretching vibration; at ~1400 cm^{-1}attributed to conjugated C=C of aromatic groups; at ~1300 cm^{-1} equivalent to the bending mode of -CH_3 and at ~1200–1000 cm^{-1} corresponding to skeletal aromatic ring vibration. The FTIR results reported for pure digoxin were similar to those previously described (Sharma et al. 2010). The FTIR spectrum of PCL implants (without drug) showed characteristic absorption bands of this polymer such as at ~2950 cm^{-1} and at ~2850 cm^{-1} equivalent to asymmetric and symmetric -CH_2 stretching, respectively; at ~1750 cm^{-1} due to the -C=O stretching vibration; at ~1290 cm^{-1} attributed to the backbone C-C and C-O stretching modes in the semi-crystalline PCL and at ~1240 cm^{-1} and ~1160 cm^{-1} related to asymmetric and symmetric COC stretching vibrations, respectively. These absorption bands of PCL were previously described (Pereira et al. 2013). The FTIR spectrum of digoxin PCL implants evidenced the organic groups of polymer and the superposition of the majority of bands from the drug. The band at ~1750 cm^{-1}, equivalent to the carbonyl group from PCL and digoxin overlapped and its intensity was increased. Moreover, bands at ~3000–2800 cm^{-1} may be related to -CH_2 stretching from the polymer and drug. However, the absorption band at ~3400 cm^{-1}, related to -OH stretching vibration from digoxin, was shown in the spectrum of digoxin PCL implants. Finally, new bands for drug-loaded PCL implants were not detected.

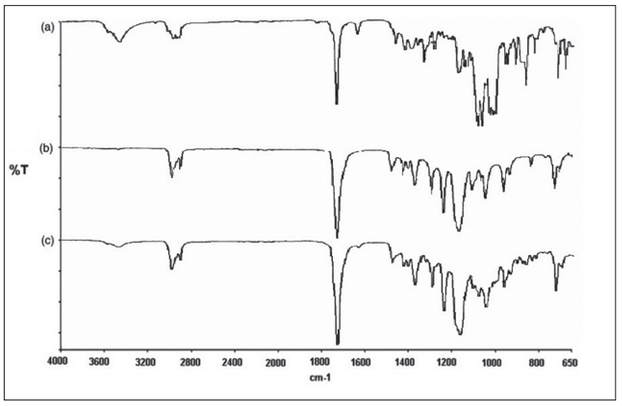

Fig. 1: FTIR spectrum of pure digoxin (A), PCL implants (without drug) (B) and digoxin PCL implants (C).

2.2.2. Differential Scanning Calorimetry (DSC)

Figure 2 exhibits DSC curves of PCL implants (without drug) (Fig. 2A), pure digoxin (Fig. 2B) and digoxin PCL implants (Fig. 2C). The DSC thermogram corresponding to PCL implants (without drug) exhibited a sharp peak at 63 °C, corresponding to the melting of a crystalline phase. The DSC results obtained were similar to those previously described (Fonseca et al. 2007). The DSC thermogram of pure digoxin showed an endothermic event

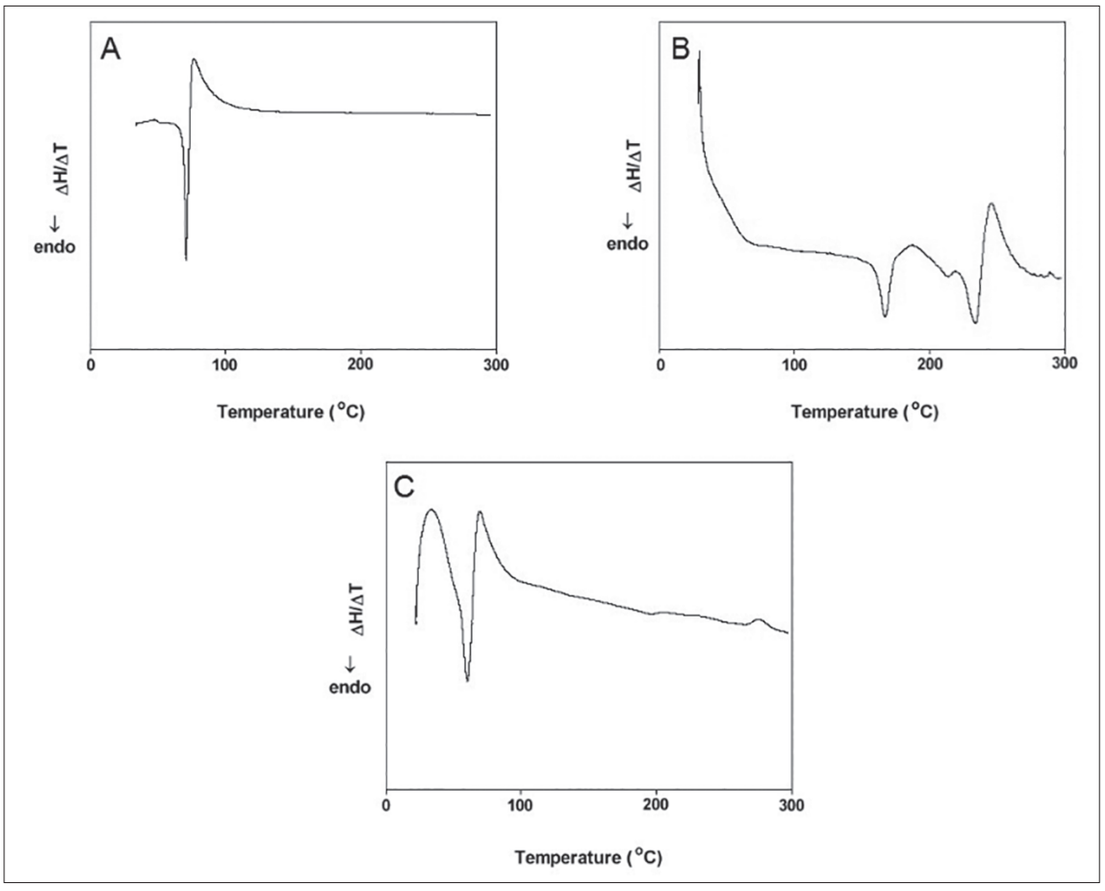

Fig. 2: DSC thermograms of PCL implants (without drug) (A), pure digoxin (B) and digoxin PCL implants (C).

at approximately 60 °C, corresponding to the adsorbed water in amorphous digoxin. The endothermic peak at 162 °C was due to the amorphous phase or glass state transition. This endotherm was followed by a large exothermic peak at approximately 175 °C related to the conversion of the amorphous form to the crystalline form, which subsequently melted with a sharp endothermic peak at 227 °C. The final exothermic event, around 240 °C, was also equivalent to another conversion of the amorphous state to the crystalline form, followed by degradation of the drug (Botha et al. 1992; Chiou et al. 1979). The DSC thermogram of digoxin PCL implants showed a large endothermic peak at approximately 63 °C equivalent to the melting of PCL and to the loss of absorbed water. The thermal events of digoxin could not be clearly visualized; however, small endothermic peaks could be identified at approximately 210 °C and 260 °C, which could be attributed to the digoxin melting and degradation, respectively.

2.3. Weight loss of PCL implants

Figure 3 indicates the degradation process of polymeric implants for 90 days in phosphate-buffered saline (PBS, pH 7.4). Accordingly, the weight loss of the PCL implants and digoxin PCL implants was approximately 96% and 98%, respectively, of their initial weight.

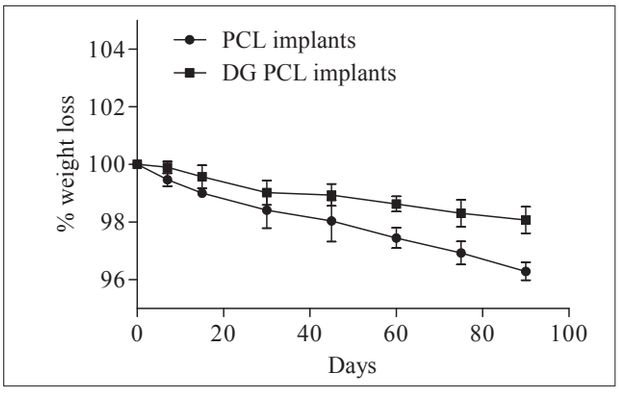

Fig. 3: In vitro weight loss (%) of PCL implants (without drug) and digoxin PCL implants. Results represent mean ± standard deviation (n = 5).

2.4. In vitro digoxin release from PCL implants

Figure 4 demonstrates the cumulative release of digoxin from PCL implants over a period of 90 days. The systems demonstrated a controlled and prolonged release of the drug without inducing an initial burst effect. The digoxin was released from polymeric implants in a constant rate at approximately 0.192 µg per day.

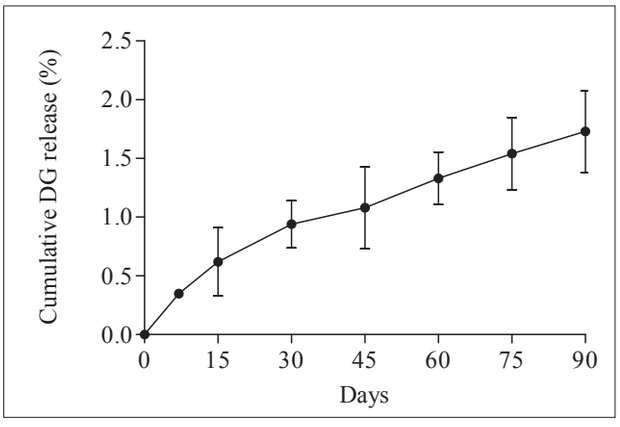

Fig. 4: In vitro release profile of digoxin fromPCL implants. Results represent the mean ± standard deviation (n = 5 for each time).

2.5. Hen's egg test-chorioallantoic membrane (HET-CAM) test - Anti-angiogenic effect of digoxin PCL implants and digoxin released from implants

Digoxin PCL implants and digoxin released from implantable devices after 15 days of incubation in PBS (pH 7.4) were applied on the CAM surface to verify the toxicity of these samples. After 5 min, the CAM was not damaged. However, the CAM showed signs of hyperemia, hemorrhage and intravasal coagulation after 24 h of being in direct contact with digoxin PCL implants and 2.88 µg of digoxin released from devices.

2.6. In vitro anti-proliferative activity of digoxin PCL implants and digoxin released from implants against HeLa cells

Figure 5 shows the anti-proliferative effect of digoxin released from PCL implantable devices and digoxin PCL implants in direct contact with HeLa cells, after 7 and 15 days in culture. The digoxin released from polymeric implants in the culture medium reduced the proliferation of HeLa cells at approximately 79% and 88%, after 7 and 15 days in culture, respectively. Digoxin PCL implants in direct contact with HeLa cells inhibited their proliferation around 81% and 92%, after 7 and 15 days of incubation, respectively. The statistical analysis (t-Student test) indicated that the inhibition of the tumor growth of digoxin from both groups was not different ($p < 0.05$).

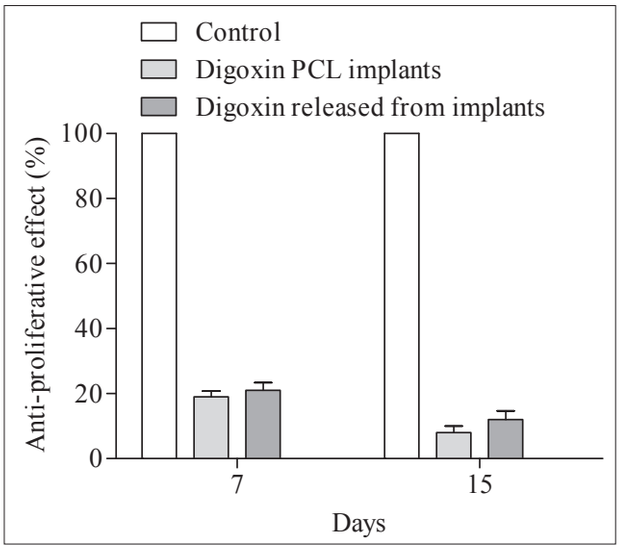

Fig. 5: Anti-proliferative effect of digoxin released from PCL implants and digoxin PCL implants after 7 and 15 days of incubation. Data were expressed as a percentage of the control group (HeLa cells in the culture medium), fixed as 100% ($p < 0.05$).

3. Discussion

Cervical cancer is extremely prevalent in women worldwide; and a high number of deaths can occur among those who are afflicted with this type of cancer. For local treatment of an early stage of cervical cancer, surgical procedures, radiation therapy and neoadjuvant chemotherapy are usually applied to increase survival rates of patients. However, systemic chemotherapy frequently induces adverse side effects and toxicity. Therefore, it makes sense to design an implantable device that is capable of controlling the delivery of the anticancer drug in effective therapeutic dosages within the cervical tumor, ensuring bioavailability. In addition, these implants should deliver the active chemotherapeutic agent through a vaginal route, preventing systemic side effects and toxicity, as well as increasing the therapeutic safety.

In this study, implants composed of digoxin and PCL were developed aiming at the local treatment of cervical cancer. Implants

were designed by the solvent evaporation method followed by the hot-molding process. The manufacturing technique enabled the formation of systems with a uniform distribution of digoxin into the polymeric matrix. The homogeneous distribution of the drug into implants enabled its delivery in a constant manner.

PCL was selected to compose the implantable devices, since it functions as a binder of the drug due to its viscosity when heated as high as 60 °C (Tamaddon et al. 2015). The entrapment of digoxin into the polymeric chains enabled the modulation of the drug released. In addition, the hydrophobic nature of PCL was also essential to induce the controlled delivery of digoxin from implants, which characterized the drug delivery systems.

Digoxin PCL implants were characterized with different analytical techniques to verify the chemical and morphological integrities of these substances. FTIR results demonstrated the presence of all bands corresponding to the polymer, which superimposed the digoxin bands, and also the absence of new bands. Therefore, the infrared indicated the dispersion of the drug into the polymeric matrix and the preservation of the chemical structure of these components. DSC results indicated that the semi-crystalline structure of PCL was preserved during the manufacturing technique of implants, since the melting point and melting enthalpy were similar in PCL implants (without drug) and digoxin PCL implants. Some thermic events of digoxin were evidenced in the thermogram of digoxin PCL implants, such as the melting point and degradation of the drug. However, these peaks exhibited lower intensities compared with the same peaks in the thermogram of pure digoxin, possibly due to the dispersion of the drug within the PCL network (Fernandes-Cunha et al. 2016). Moreover, peaks equivalent to the conversion of the amorphous form of digoxin to the crystalline form were not present in the thermogram of digoxin-loaded PCL implants, demonstrating that the entrapment of digoxin into the polymeric matrix prevented the structural modification of the drug (De Souza et al. 2016). Finally, the dispersion of digoxin within PCL chains partially changed its physical structure; however, this change did not induce chemical interactions between them.

The PCL implants (without drug) and digoxin PCL implants were submitted to the *in vitro* degradation study by immersing them into the PBS (pH 7.4) for 90 days. The PCL implants showed a low weight loss, since approximately 96% of the initial weight of polymeric devices remained after the period of incubation. The preservation of the semi-crystalline structure of PCL during the design of implants contributed to the slow rate of biodegradation of this polymer. The organization of the physical structure of PCL within the implantable devices restricted the hydrolysis of its ester bonds and, consequently, contributed to the low weight loss. Digoxin PCL implants showed a weight loss of approximately 98%. The dispersion of digoxin into polymeric chains probably restricted the water uptake due to the hydrophobicity of its molecule (Dressman and Reppas 2000). As a result, the hydrolysis of ester bonds of PCL and the rate of weight loss of polymer, presented in digoxin-loaded implants, were reduced when compared with those obtained for PCL implants.

The *in vitro* digoxin release study demonstrated that PCL implants modulated the lixiviation of the drug at a slow rate (0.192 µg per day) for a prolonged period. The degradation of PCL was not considered as the main factor that was responsible for the release of the drug, since this polyester is characterized by a very low hydrolysis rate (as evidenced by the weight loss study), which can vary from months to years (Merkli 1998). Consequently, the digoxin was released from PCL implants, probably by a diffusion mechanism controlled by the polymeric chains of implants. Furthermore, the low water solubility of digoxin (Dressman and Reppas 2000) may have induced a slow rate of drug diffusion. Finally, PCL implants did not provide an initial burst release of digoxin, which represented an important advantage of these systems, since the rapid release of digoxin could lead to toxicity. It was previously reported that digoxin induced severe ocular damage due to the high local dosage concentration of the drug (Winter et al. 2015). These toxic effects could be prevented by using sustained-release systems such as digoxin PCL implants.

The anti-angiogenic activity of digoxin was demonstrated in the HET-CAM test. The HET-CAM assay is based on the direct application of samples onto the chorio-allantoic membrane (CAM) and the observation of the reactions in vessels. These signs could be clearly observed, since the CAM is highly vascularized. In this study, the CAM was exposed to digoxin PCL implants and to digoxin released from PCL implants for 15 days over 5 min and 24 h. Accordingly, after 24 h, the digoxin demonstrated activity against the endothelial cells, since hyperemia, hemorrhage and intravasal coagulation were observed. This result corroborated with findings previously described by Winter et al. (2015), who have shown that the digoxin exerted a strong anti-angiogenic effect and this activity is dose dependent. Therefore, the controlled release of low concentrations of digoxin from PLC implants could induce the regression of the cervical tumor by inhibition of the growth of new vessels, which supply oxygen and nutrients to the developing tumor tissue.

Anti-proliferative and apoptotic activities of digoxin released from PCL implants were demonstrated against HeLa cells. Implantable devices provided the delivery of digoxin in concentrations that were cytotoxic to cancer cells. The digoxin leached from implants probably inhibited the enzyme Na^+/K^+-ATPase, which actively transports potassium ions into cells and sodium ions out of cells in a 2:3 stoichiometry. This inhibition increased intracellular calcium ions, leading to the death and apoptosis of these tumor cells (Lu et al. 2014). In addition, the digoxin possibly exerted its anti-proliferative effect by inhibition of glycolysis, suppression of catalytic activity of topoisomerases I and II and downregulation of expression of transcription factors, such as activator protein 1 and NF-kB (Prassas et al. 2008). Finally, and interestingly, the final responses of cancer cells and normal cells to digoxin differ due to the differential expression and activity of Na^+/K^+-ATPase subunits in tumor tissues compared with their normal counterparts. Indeed, it is established that malignant transformation is characterized by a significant increase in the activity of Na^+/K^+-ATPase (leakage theory) (Prassas et al. 2008; Kaplan 1978). Therefore, digoxin controlled delivered from PCL implants induced cytotoxicity against HeLa cells by multiple and synergic biochemical cascades; and its anti-proliferative and apoptotic effects could be concentrated, to a great extent, to the cervix tumor cells, not only due to the augmented expression of Na^+/K^+-ATPase in these cells but also due to the direct release of digoxin from PCL implants in the target site.

Cervical cancer can be carefully accessed by magnetic resonance imaging scans, which allow accurate measurements of the tumor with a precise definition (Shepherd 2012). After defining the tumor margins, digoxin PCL implants could be implanted in upper vaginal tissues and/or para-cervical vaginal tissues, near the tumor limits, and via the intra-vaginal route to locally provide controlled drug release. These systems enabled the delivery of digoxin in low concentrations, which could be enough to minimize/eliminate cervical tumor cells without inducing systemic side effects and/or toxicity. It was well documented that digoxin is a potent glycoside, which shows a narrow therapeutic range, and its systemic administration could lead to severe toxic effects (Souza et al. 2015). Furthermore, digoxin PCL implants showed an extended biodegradation profile not only due to the hydrophobic characteristic of polymer and drug but also due to the preservation of the polymeric semi-crystalline structure during the manufacture process of systems. These facts may lead to a prolonged exposition of drug to the tumor site. This exposition for a long period could be beneficial for minimizing the drug resistance of tumors. Drug resistance is the most commonly encountered phenomenon that limits successful cancer chemotherapy. The mechanism of drug resistance involves the drug efflux by transporters and anti-apoptotic defenses (Ling et al. 2016).

Finally, the anti-proliferative and anti-angiogenic activities of digoxin released from PCL implants could be applied to eliminate cervical cancer cells directly at the target site. Moreover, these implantable devices might avoid/minimize systemic side effects and toxicity as well as the drug resistance of the tumor. As a

consequence, the systems could reduce the necessity of an invasive surgery in the cervix that does not preserve fertility.

4. Experimental

4.1. Preparation of implants composed of digoxin and PCL

Digoxin (Sigma Chemical Co., USA) and poly(e-caprolactone) (PCL; MW ~ 14,000; density = 1.145 g/ml at 25 °C, Sigma Chemical Co., USA) were dissolved in a mixture of acetonitrile and ethanol (1:1) and the resultant solution was frozen under -80 °C. The mixture was lyophilized during 48 h (Pirani 78/1, Edwards of Brazil, São Paulo, Brazil). The resultant blend was collected and further molded into spherical implants (6 mm in diameter) by using a metallic mold at approximately 70 °C. Digoxin-loaded poly(e-caprolactone) implants (digoxin PCL implants) contained approximately 10% (w/w) of the drug corresponding to 1.0 mg of digoxin. Implants without drug were also prepared (PCL implants).

4.2. Determination of content of digoxin incorporated into PCL implants

The determination of digoxin into PCL implants was performed as follows: Ten implants were selected and weighed. Each implant was dissolved in 5 mL of acetonitrile and 70 mL of ethanol. The solution was sonicated for 10 min and the volume was adjusted to 100 mL with a hydro-alcoholic solution (1:1). Then, the solution was filtered. The standard solution of digoxin was also prepared as described earlier. The absorbance of resultant solutions was measured at 220 nm by using a PCL solution as blank. The spectrophotometric method of quantitation of DG was previously validated. The uniformity content of digoxin in implants was expressed as the percentage of pre-indicated value (approximately 1.0 mg). The relative standard deviation was also calculated.

4.3. Characterization

Infrared spectra were collected in a Fourier transform infrared spectrophotometer (FTIR; Perkin Elmer, model Spectrum 1000). Measurements were carried out by using the attenuated total reflectance (ATR) technique. Each spectrum was a result of 32 scans, with a resolution of 4 cm^{-1}.

DSC thermograms were obtained on a Mettler Toledo DSC (Switzerland). Samples were put into aluminum pans. The calorimeter was calibrated for temperature and heat flow accuracy by using pure indium melting (m.p. 156.6 °C and $\Delta H = 25.45$ J g^{-1}). The temperature ranged from 0 °C to 300°C, with a heating rate of 25 °C min^{-1} under nitrogen atmosphere.

4.4. Measurement of in vitro weight loss of PCL implants and digoxin PCL implants

The *in vitro* degradation study was evaluated by recording the weight loss of PCL implants (without digoxin) and digoxin PCL implants over 90 days in PBS (pH 7.4). Implants were placed in different tubes containing 3 mL of PBS (n = 5). Those tubes were placed inside a shaker incubator that was set at 37 °C and 30 rpm. At each time point (0, 7, 15, 30, 45, 60, 75 and 90 days), implants were retrieved from PBS, rinsed with deionized water, and vacuum-dried for 48 h before weight loss was analyzed. The percentage of weight loss was obtained by the ratio between the weight of implants both before and after incubation.

4.5. In vitro release of digoxin from PCL implants

Five digoxin PCL implants were immersed inside different tubes containing 24 mL of PBS (pH 7.4). The tubes were placed inside a shaker incubator that was set at 37 °C and 30 rpm. At predetermined intervals (7, 15, 30, 45, 60, 75 and 90 days), 3 mL of medium was sampled and 24 mL of fresh medium was immediately added to each tube. The release profile was evaluated as the cumulative percentage of drug released in the medium. The amount of digoxin leached was measured by using the spectrophotometric method previously validated (Silva et al. 2013).

4.6. HET-CAM

Fertilized hen's eggs were purchased from a poultry farm (Alimentos Rivelli, Brazil). Collected hen's eggs were incubated at 37±0.5 °C and 40±4% relative humidity for 10 days. The eggs were turned every day during incubation but were left in a horizontal position for several minutes to ensure that the embryo was properly positioned. On day 10, each egg was opened by cracking the underside of the egg against the edge of a plastic Petri dish. The chorio-allantoic membrane (CAM) was exposed, and samples 300 µL were placed directly onto the CAM's surface. After 20 s, samples were discarded and the CAM was carefully washed with HEPES buffer (pH 7.4) to ensure the total removal of the tested substance. The CAM was visually observed for five min and 24 h regarding the appearance of any of the following phenomena: hyperemia, hemorrhage and coagulation. Samples were PBS (pH 7.4) (negative control), 0.1 mol L^{-1} sodium hydroxide solution (positive control), digoxin PCL implants and digoxin leached from PCL implants in PBS (pH 7.4) for 15 days.

4.7. In vitro antitumor activity

HeLa cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, Scotland) that was supplemented with 10% fetal bovine serum (FBS; Hyclone), 60 mg mL^{-1} of streptomycin and 100 mg mL^{-1} of penicillin in a humidified atmosphere of 5% CO_2. Cells were seeded at 2 ´ 10^4 cells/cm^2. The culture medium was changed every 48 h to avoid nutrient depletion.

The effect on tumor cell proliferation was determined by using the MTT cell proliferation assay. The following samples were evaluated: digoxin PCL implants in direct contact with cells for 7 and 15 days and digoxin released from PCL implants after 7 and 15 days of incubation in the culture medium. Briefly, cells were plated in 96-well plates (1×10^5 cells/well) in RPMI 1640 medium that was supplemented with 10 %(v/v) FBS and incubated for 24 h at 37 °C in a humid atmosphere with 5% CO_2 to adhesion. After 24 h of settling down, the cells were washed with the culture medium and incubated in solutions containing digoxin released from PCL implants and digoxin PCL implants (n = 10 for each sample). After 48 h of incubation, the culture medium was removed and 100 µL of MTT (1 mg mL^{-1} in PBS) was added to each well. After 2 h of incubation at 37 °C, the cells were lysed with 100 µL of isopropanol, and absorbance values were measured at 550 nm by using a microplate reader Spectramax M5e (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate. The anti-proliferative effect was calculated by a percentage of reduction of tumor cells treated with digoxin when compared with tumor cells untreated with drug (control cells).

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Conflicts of interest: None declared.

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