Cytotoxicity evaluation of indomethacin-loaded polymeric nanoparticles in a human breast adenocarcinoma cell model

Avaliação da citotoxicicidade de nanopartículas poliméricas contendo indometacina em modelo celular de adenocarcinoma de mama humano

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RESUMO

Novas pesquisas indicam que anti-inflamatórios podem ser aplicados como agentes anti-cancerígenos como indometacina para hepatocarcinoma humano, canceres de colon e estômago. Como sabe-se, indometacina possui efeitos adversos gastrointestinais, cardiovasculares e renais. Uma vez que cancer de mama tem alta incidência e não há estudo da indometacina carreada em nanopartículas para esta aplicação, este estudo envolve o desenvolvimento de nanocapsulas de poli-epsilon-caprolactona carregadas com indometacina para a redução de citotoxicidade como agente quimioprotetor para cancer de mama. O nanocarreador foi preparado por método de deposição interfacial e sua caracterização foi realizada por determinação de pH, diâmetro médio e índice de polidispersão por espalhamento dinâmico de luz, potencial zeta por mobilidade eletroforética, eficiência de encapsulação por método de cromatografia líquida de alta eficiência e seu ensaio de citotoxicidade com linhagem de células queratinócitos (HaCaT) e células de cancer de mama (MCF-7). As formulações branca (C-NC) e contendo indometacina (Ind-OH-NC) mostraram leve pH ácido, diâmetros em torno de 200 nm e PDI<0,2 com potencial zeta em torno de -20 mV e eficiência de encapsulação de 99% (1 $mg.mL^{-1}$), cujo coeficiente de distribuição indicou efeito de permeação e retenção (efeito EPR). Ambas formulações não foram citotóxicas às células HaCaT, provando serem seguras às células normais e Ind-OH-NC teve uma permeação concentração e tempo-dependente e teve eficácia em reduzir a viabilidade celular da linhagem MCF-7.

Palavras-chave: Nanocarreador, Câncer de mama e Viabilidade celular.

ABSTRACT

New researches indicates that anti-inflammatories can be applied as anti-cancer agents as indomethacin for human hepatocarcinoma, colon and stomach cancers. As known, indomethacin has gastrointestinal, cardiovascular and renal adverse effects. Once breast cancer has a high incidence and there is no study of indomethacin carried into nanoparticles for this propose, this study involves the development of indomethacin-loaded polyepsilon-caprolactone (PCL) nanocapsules for reduction of cytotoxicity as chemoprotective for breast cancer. The nanocarrier was prepared by interfacial deposition method and its characterization was performed by the determination of pH, z-average diameter and polydispersity index by dynamic light scattering; zeta potential by electrophoretic mobility; encapsulation efficacy by high performance liquid chromatography method and its cytotoxicity assay with a keratinocyte cell line (HaCaT) and breast cancer cells (MCF-7). The blank (C-NC) and indomethacin-loaded formulations (Ind-OH-NC) showed a slightly acid pH, sizes around 200 nm and PDI<0.2 with zeta potential around -20 mV and encapsulation efficacy of 99% (1 mg mL^{-1}), which distribution coefficient indicated permeation and retention effect (EPR-effect). Both formulations were not cytotoxic to HaCaT cells, proving to be safe for normal cells and Ind-OH-NC has concentration and time-dependent permeation and had efficacy in reducing cell viability of MCF-7 lineage.

Keywords: Nanocarrier, Breast cancer, Cellular viability.

1 INTRODUCTION

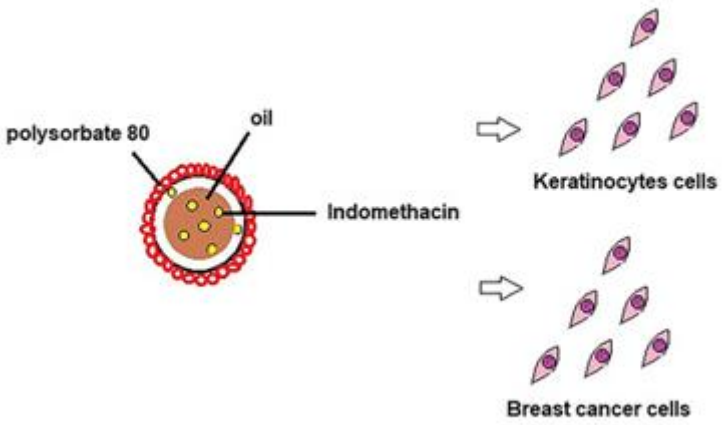
In Brazil, breast cancer has one of the highest incidences, which usual therapies promotes cytotoxicity (BRASIL, 2020; ROCHA *et al.*, 2020). The use of non-steroidal anti-inflammatory drugs (NSAIDs) as promising anti-cancer agents is growing, once the tumoral microenvironment shows inflammatory responses (RAHME *et al.*, 2005; ACKERSTAFF *et al.*, 2007). The NSAIDs act by the non-selective inhibition of cyclooxygenases enzymes (COX-1, related to homeostasis and COX-2, related to the synthesis or prostaglandins, growth factors, cytokines and hormones) and that impact in the cancer dissemination (ACKERSTAFF *et al.*, 2007; BERNARDI *et al.*, 2009 [a]; DUPERYRÓN *et al.*, 2013).

Indomethacin is known by its low solubility and high permeability, besides its gastrointestinal, cardiovascular and renal complications which could be avoid if it is included in a nanoparticle device (BERNARDI *et al.*, 2009 [a]; DUPERYRÓN *et al.*, 2013).

Free indomethacin has been studied upon human hepatocarcinoma cells (Hep-G2), colon and stomach models and, associated with doxorubicin upon breast cancer cell line (MDA-MB-435) showing that the indomethacin can reduce invasion of MDA-MB-435 and modify choline and triglycerides metabolism (ACKERSTAFF *et al.*, 2007; ARISAWA *et al.*, 2011). In nanocarriers, literature mentioned polymeric micelles of dextran-indomethacin containing paclitaxel with sustained release to MCF-7/ADR cells (JI *et al.*, 2017), and polyepsilon-caprolactone (PCL) nanoparticles used with glioma cell lines lead to antiproliferative effect due to arrest cell cycle progression and, applied to glioblastoma in rats (POHLMANN *et al.*, 2008; BERNARDI *et al.*, 2009 [a]; BERNARDI *et al.*, 2009 [b]).

As observed, there is no study so far present, with polymeric nanoparticles loaded with indomethacin for human breast cancer treatment and, considering these, this study was designed to investigate the antineoplasic potential of polymeric nanoparticles loaded with indomethacin for human breast cancer therapy, using as model the HaCaT and MCF-7 cell lines (Figure 1).

FIGURE 1. Illustrative model of indomethacin-loaded nanocapsules evaluated upon HaCaT and MCF-7 cells.



2 MATERIAL AND METHODS

Material

PCL (Mw 14,000 g mol^{-1}, Mn 10,000 g mol^{-1}, Sigma Aldrich), polysorbate 80 (Tween 80®, Alpha Química), capric/caprylic triglyceride (CCT, Embacaps), indomethacin (99% purity, Sigma Aldrich), sorbitan monostearate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylltetrazolium bromide (MTT, Sigma Aldrich), trypan blue (Sigma Aldrich), Dulbecco's modified eagle medium (DMEM, Sigma Aldrich) and Tamoxifen (citrate salt, Sigma Aldrich), penicilin and streptomicin (Merck) and fetal bovine serum from Gibco (Thermo Fischer Scientific, Massachusetts, USA).

High performance liquid chromatography method

A liquid chromatography (HPLC) method was adapted (POHLMANN *et al.*, 2020) and employed a chromatograph Shimadzu LC equipment (Shimadzu Corporation, Japan) with guard column Cartridges C18 (4.0 x 3.0 mm, Phenomenex), column C18 (250 mm x 4.6 mm x 5 µm, Phenomenex) and UV-VIS detector at room temperature. The mobile phase consisted of acetonitrile:metanol:water at pH 5.45 1:8:1 (v/v), flow rate was 1.0 mL min^{-1} and the injection volume was 20 µL. The mother solution was prepared in methanol:water 1:1 (v/v) and the curve solutions were diluted in the mobile phase in the ranged from 1 to 30 µg mL^{-1} with detection at 267 nm.

Preparation of nanocapsules

Two formulations of nanocapsules suspensions were prepared by interfacial deposition, according (BERNARDI *et al.*, 2009 *b*): drug-unloaded nanocapsules (C-NC)

and indomethacin-loaded nanocapsules (Ind-OH-NC), as demonstrated in Table I, evaporated at 40ºC (Yamato Rotatory Evaporator with BM510 Water bath and RE801 modulus, USA) to a final volume of 10 mL.

TABLE I. Formulation composition.

Materials and quantities	C-NC	Ind-OH-NC
PCL (g)	0.1059	0.102
Sorbitan monoestearate (g)	0.078	0.078
CCT (oil) (mL)	0.330	0.33
Indomethacin (g)	0	0.010
Acetone (mL)	28	28
Polysorbate 80 (g)	0.08	0.08
Water (mL)	53	53

Footnote: PCL: polyepsilon-caprolactone, CCT: capric/caprylic triglyceride.

Physical-chemical characterization of nanocapsules

The pH was determined using a potentiometer (DM-22, Digimed Analytical Instrumentation, Brazil), previously calibrated with standard solutions, and the results were expressed as mean ± SD of the triplicate.

For drug extraction, the formulations (500 µL) were dispersed in tetrahydrofuran (THF, 1 mL) and acetonitrile (3.5 mL) and stirred in vortex (AP56, Phoenix, Brazil) by 5 minutes, followed by filtration at 0.45 µm and injected (obtaining the total drug concentration). For the concentration of the drug in the continuous phase, a sample of each formulation was submitted to ultrafiltration-centrifugation (ultrafiltration units, Millipore®, 10 kDa, Irland) for 10 minutes at 5000 rpm (Microcentrifuge NT805, Brazil, n=3, obtaining the free drug concentration). Encapsulation efficiency (EE%) was calculated by dividing the difference of drug content and drug concentration in the continuous phase by the drug content, and multiplying by 100.

Measurements of z-average diameter (mean hydrodynamic diameter), polydispersity index (relative variance, PDI) and diffusion coefficient (Log D) were determined by the dynamic light scattering (DLS) in a Malvern Zetasizer instrument (NanoZS, ZEN 3600 model, Malvern Instruments, UK, 25ºC, backscatter detection at 173º) and each sample (20 µL), without previous treatment, was diluted in 10 mL of ultrapure water and potassium phosphate buffer at pH 7.4 and pH 5.5 (0.45 µm, Millipore®, dilution of 500 times, n=3).

The zeta potential was determined by laser Doppler electrophoresis (DLS, NanoZS, ZEN 3600 model, Malvern Instruments, UK, 25ºC), where each sample (20 µL)

was diluted in 10 mmol L^{-1} sodium chloride aqueous solution (10 mL), and placed in the folded capillary cell for analysis (n=3).

Cytotoxicity in cell cultures

The solutions and dispersions of each sample (culture medium, indomethacin solution at 26.7 µM (IC_{50} value, data not shown), C-NC and Ind-OH-NC (1 to 100 µM), DMSO at 0.25 mM and Taxomifen at 76.9 µM were previously prepared in an Eppendorf diluted in the culture medium and transferred directly to the respective wells in a final volume of 200 µL.

The cytotoxicity of the samples was evaluated using keratinocytes HaCaT (ATCC® PCS-200-001 TN ™) and MCF-7 (ATCC® HTB-22) cell lines cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose level, supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin and maintained in an atmosphere of 5% CO_2/95% air at 37ºC, seeded in 96-well plates (2.10^4 cells per well) 24h before the application of the materials.

After 24, 48 and 72h of the treatment's application, the contents of the wells were removed and a MTT solution at 5 mg mL^{-1} was added and incubated for 3h. Then, the formazan crystals formed by tetrazolium cleavage were dissolved with dimethyl sulfoxide (DMSO) and the absorbance was recorded at 570 nm (Anthos 2010 Instrulab, Software Adapt). The cell viability was calculated using Equation 1:

Cell viability (%) = $(Abs_{sample}/Abs_{control})100$,

were Abs_{sample} is the absorbance of cells treated with different formulations and $Abs_{control}$ is the absorbance of control cells (incubated with cell culture medium). The results are expressed as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test in the Excel program (values of $p<0.05$ were considered significant).

3 RESULTS AND DISCUSSION

HPLC method

The HPLC method was validated according to the International Conference on Harmonization (ICH, 2005), showing a linear calibration curve of indomethacin in the range of 1 to 30 µg mL^{-1}, with correlation coefficient of 0.999 (y = 48190x-17287; retention time = 4.5 minutes, LD = 0.299 µg mL^{-1} and LQ = 0.997 µg mL^{-1}). The method was linear, specific, reproductive and exact (p = 0.011, Fcalc. = 4.55 and Fcrit. = 3.00).

Characterization of nanocapsules

The pH measurements of the nanocapsules formulations, showed a slightly acid pH: 4.96 ± 0.11 for C-NC and 4.78 ± 0.10 for Ind-OH-NC. Drug content was of 1 mg mL^{-1} (DC = 1.09 ± 0.12 mg mL^{-1}), encapsulation efficiency of 99.04% and drug loading (%DL) of 2.11 ± 0.24. The formulations were evaluated by dynamic light scattering and by electrophoretic mobility, as shown in Table II.

TABLE II. Z-average hydrodynamic diameter and polydispersity index (PDI) and Log D of formulations diluted in potassium phosphate buffer conditions by dynamic light scatering and, zeta potencial (ζ) by eletrophoretic light scattering of nanocapsules formulations, obtained in triplicate (n=3) and expressed as mean ± stardard deviation.

Formulation	Z-average diameter (nm)	PDI	Log D	ζ (mV)
C-NC	218.33 ± 1.32	0.173 ± 0.01	2.25 ± 0.01	-21.0 ± 0.72
Ind-OH-NC	197.46 ± 2.05	0.134 ± 0.02	2.49 ± 0.02	-18.7 ± 0.85
C-NC at pH 7.4	221.96 ± 2.13	0.189 ± 0.02	2.21 ± 0.02	*
C-NC at pH 5.5	183.30 ± 7.77	0.159 ± 0.01	2.69 ± 0.11	*
Ind-OH-NC at pH 7.4	186.56 ± 1.33	0.104 ± 0.02	2.63 ± 0.01	*
Ind-OH-NC at pH 5.5	197.16 ± 2.45	0.119 ± 0.02	2.5 ± 0.03	*

Footnote: PDI: polydispersity index, Log D: distribution logarithm, ζ: zeta potencial. (Refraction index: IR_{PCL}= 1.46 and absorption = 0.010). *Not evaluated.

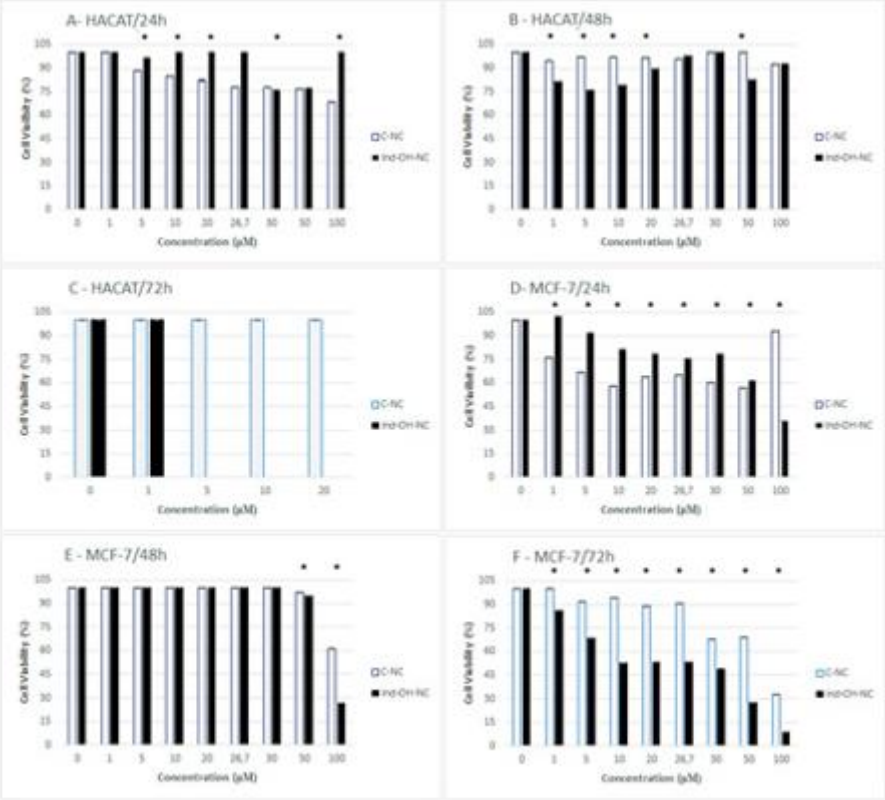
Ind-OH-NC formulation was also quantified after 49 days of storage at room temperature, showing 229.53 ± 0.58 nm, PDI = 0.167 ± 0.01, ζ = -19.3 ± 1.67 mV and %EE was 98.92%.

The physical-chemical characteristics of nanoparticles formulations are in accordance with the literature, showing homogeneity of the system (200 nm, PDI<0.2), good physical stability (-20 mV) due to electrostatic repulsion of particles and steric effect and good encapsulation efficiency (99.04%) (BERNARDI *et al.*, 2008; BERNARDI *et al.*, 2009[b]; POHLMANN *et al.*, 2020).

Cytotoxicity assay

Firstly, the nanoparticles cytotoxicity was evaluated upon HaCaT and MCF-7 cells in 24, 48 and 72h after treatments application, according to Figure 2.

FIGURE 2. C-NC (blank nanocapsules) and Ind-OH-NC (nanocapsules loaded with indomethacin) cell viability study in HaCaT cells (A, B and C) and in MCF-7 cells (D, E and F) for 24, 48 and 72h, respectively, assessed by MTT assay (n=3, triplicate) using the culture medium was as control. Symbol (*) represents the statistical differences ($p<0.05$) between the samples C-NC and Ind-OH-NC (ANOVA and Tukey test, HaCaT/24h (A): F = 2.928, Fcrit = 1.882, p = 0.002 and HSD = 0.686; HaCaT/48h (B): F = 5.125, Fcrit = 1.882, p = $1.1x10^{-5}$ and HSD = 0.651; HaCaT/72h (C): F = 16.343, Fcrit = 2.510, p = $8.67x10^{-7}$ and HSD = 0.460; MCF-7/24h (D): F = 13.133, Fcrit = 1.882, p = $3.5x10^{-11}$ and HSD = 0.271; MCF-7/48h (E): F = 6.397, Fcrit = 1.882, p = $8x10^{-7}$ and HSD = 0.175 and MCF-7/72h (F): F = 22.93, Fcrit = 1.882, p = $4x10^{-15}$ and HSD = 0.266). The results are expressed by concentration applied in the cell culture by well.



It was not possible to obtain results of 72h in HaCaT cells from samples C-$NC_{26,7-100}$ and for Ind-OH-NC_{5-100}.

Indomethacin has a high diffusion coefficient (Log D), around 3 at pH 5.5, so it has higher distribution to lipophilic membranes as well as high ulcerogenicity to stomach (FILARETOVA, TAKEUCH, 2012). Although, when carried into nanoparticles, its toxic effects are proved to be reduced (OUSHITOMI *et al*., 2014; RIASAT *et al*., 2016). The C-NC formulation increased its Log D when exposed to acidic pH, showing that can be probably more easily absorbed in a cancer region due to lipophilic affinity. Ind-OH-NC had no great modification in its Log D in different pHs (around 2.5), represents a facilitator for the drug diffusion to the tissues. Also, particles with diameters around 200 nm are related to have longer circulation time and low clearance reflecting the EPR-effect (permeation and retention effect) (LETCHFORD, BURT, 2007).

The cytotoxicity assay demonstrated that the treatments evaluation applied to HaCaT cells were not cytotoxic, except by C-NC_{100} (68.7% of cell viability) in 24h of exposure. This may have occurred due to the presence of some constituent in the formulation, but according the literature, PCL and CCT are non-toxic substances and Tween 80® can present some cytotoxicity although its concentration was very low (ARECHABALA *et al.*, 1999; FRANCO *et al.*, 2017). No sample showed citotoxicity to HaCaT cells in 48h or 72h, even the negative controls, proving that the nanocarrier is safe for epithelial normal cells.

To MCF-7 cells, after 24h, treatments showed reduction in the cell viability when exposed to free indomethacin solution (68.1%) and C-NC_{5-50} (varying from 66-57% of cell viability), although the C-NC_{100} was not cytotoxic. Ind-OH-NC_{50-100} were cytotoxic (61.4 and 35.8%, respectively), DMSO at 0.5%, Tamoxifen at 76.9 µM and H_2O_2 at 0.25 mM showed low cytotoxicity (66.5, 69.6% and 72.83%, respectively). At 48h, it was demonstrated cytotoxicity from C-NC_{100} (61.32%), Ind-OH-NC_{100} (26.28%) and H_2O_2 at 0.25 mM (37.99%). It is interesting to observe that C-NC_{5-50} that were cytotoxic at 24h, but were not at 48h and C-NC_{100} that was not cytotoxic at 24h, reduced the cell viability in 48h of exposure. These behaviors can be explained by protein aggregation from the environment, that would reduce the cell viability and with more time of exposure, cells found an equilibrium where they could have a recovery in their survival (MOORE *et al.*, 1999). After 72h, the treatments C-NC_{30-100} (68-33%), Ind-OH-NC_{5-100} (68-9%), DMSO at 0,5% (60.24%), tamoxifen at 76.9 µM (31.30%) and H_2O_2 at 0.25 mM (12.94%) showed cell viability reduction. These results suggest concentration and time-dependent permeation and efficacy of the indomethacin-loaded nanoparticles. The fact that C-NC_{30-100} showed cytotoxicity to MCF-7 in 72h may have occurred by time-dependent permeation. So, it was possible to produce homogeneous polymeric nanocapsules containing 1 mg mL^{-1} of indomethacin which demonstrated to be secure for normal keratinocytes cells, while the formulation containing indomethacin controlled MCF-7 cells proliferation and survival probably by EPR-effect. The death mechanism by which indomethacin act in MCF-7 cells will be studied next.

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