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A surface modification of clozapine-loaded nanocapsules improves their efficacy: A study of formulation development and biological assessment

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ABSTRACT

This work aimed to develop nanocapsules (NC) coated with polysorbate 80 (P80), cationic chitosan (CS) or polyethylene glycol (PEG) using clozapine (CZP) as the drug model. The zeta potential, pH and encapsulation efficiency were directly affected by the CS coating. Using the bag dialysis method, the *in vitro* CZP release from CS-coated nanocapsules was similar to the PEG-coated at pH 7.4. Nanocapsules coated with PEG and CS exhibited an increased action duration compared to the P80-coated nanocapsules in pseudo-psychosis induced by D,L-amphetamine in rats. When comparing both groups, the group administered CS-coated nanocapsules showed better activity than the PEG-coated nanocapsules at 6, 10 and 12 h after D,L-amphetamine administration. The pharmacokinetic assessment in rats demonstrated that the observed half-lives were free CZP < P80-coated < PEG-coated˜CS-coated nanocapsules. Both the P80- and PEG-coated nanocapsules showed a statistically significant increase in their volume of distribution compared to free CZP. On the other hand, the cationic nanocapsules showed a decrease in total *clearance*. Together, these results indicate that the PEG and CS coatings are a promising delivery system for CZP in the treatment of schizophrenia.

1. Introduction

Schizophrenia is an important mental health issue that affects almost 1% of the global population. The phenothiazine and butyrophenone classes of drugs were first introduced in the 1950s [1]. Despite their efficacy against positive symptoms, extra-pyramidal effects led to the emergence of atypical antipsychotics, which are represented here by clozapine (CZP) [2]. CZP is effective against the positive and negative symptoms of schizophrenia. It blocks dopamine D_2 and $5HT_2$ receptors [2], and this serotoninergic antagonism results in fewer extra-pyramidal side effects [3]. In addition, CZP is a Biopharmaceutical Classification System class II drug with good absorption, although its bioavailability is less than 30% [1,4] due to poor solubility and extensive pre-systemic metabolism.

CZP is well known to decrease the risk of suicide in schizophrenic patients, including treatment-resistant patients [5], and to reduce the number and severity of physical assaults and overall aggression [6,7]. The main adverse effect of CZP is agranulocytosis [6]. In addition, hepatotoxicity and cardiotoxicity have been reported [1,5,6,8].

Nanocarriers are a promising strategy to target bioactive drugs to its biophase, reversing non-selective biodistribution. The surface of the nanoparticles can be functionalized using various agents to form the corona, which can modify their biological performance, such as for cerebral CZP delivery. The hydrophilic shell located on the nanoparticle surface prevents opsonization by steric

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repulsion of blood proteins [9,10] due to support by Polyethylene glycol (PEG) [10–12] and Polysorbate 80 (P80) [13–15]. Chitosan (CS) presents interesting biological characteristics, such as biocompatibility, biodegradability, mucoadhesion and cationic charges [16–18]. Our research group developed CZP-loaded P80-coated nanocapsules (NCs) and investigated their *in vitro* release and toxicity in the *Caenorhabditis elegans* model [19]. Nanoencapsulation extended drug release approximately three-fold and reversed the decrease in egg laying stimulated by a CZP solution. In addition, we showed that NCs coated with P80, PEG or CS are able to decrease CZP-induced brain damage to biomolecules, such as lipids, proteins and DNA, in chronically treated rats [15]. Considering these data, we aim to further assess the *in vitro* physicochemical characteristics of different CZP-loaded polymeric NC coatings, the impact of optimized formulations on the stereotypic behavior of a D,L-amphetamine-treated animal model, and the relation of the coatings with the *in vivo* pharmacokinetic profiles in rats.

2. Experimental section

2.1. Materials

Poly-(ε-caprolactone) (PCL, MM = 80.000), capric/caprylic triglycerides, Lipoid® S45, Polysorbate 80, Polyethylene glycol 6000 (PEG 6000), and chitosan (medium molecular weight, 85.7 deacetylation degree) were purchased from Sigma-Aldrich (Brazil). Acetone, acetic acid, formic acid, hydrochloric acid, sodium hydroxide, monosodium phosphate, ammonium acetate, and triethylamine were pharmaceutical grade. Clozapine (purity > 99%) was kindly donated by Cristália Indústria Farmacêutica (Brazil).

2.2. Methods

2.2.1. Nanocapsules suspensions formulations

CZP-loaded polysorbate-coated NCs (NCCZP) were prepared by interfacial deposition of the preformed polymer [19]. The organic phase contained the polymer PCL, medium chain triglycerides, the surfactant Lipoid S45® and CZP were dissolved in acetone and magnetically stirred at 40 °C. The organic phase was injected into an aqueous phase containing P80 and MilliQ® water under moderate magnetic stirring at room temperature. After 10 min, the acetone was removed, and the aqueous phase was concentrated by evaporation at 40 °C under reduced pressure using a rotavap (R-210/215 model, BÜCHI®). The final volume was adjusted to 10 mL to obtain a final concentration of 1.5 mg mL^{-1} CZP. All samples were prepared in triplicate batches. We subsequently obtained the CZP-loaded P80-coated NCs (NCCZP) or unloaded P80-coated NCs (NCBR).

The CZP-loaded CS-coated NCs (NCCZP-CS) were prepared using a CS solution that was previously solubilized in 1% acetic acid aqueous medium. To optimize the coating, three different concentrations of CS, 0.01, 0.05 and 0.1%, were initially assessed in the final suspensions. These solutions (1 mL) were slowly added to 9 mL of the NCCZP and NCBR suspensions under moderate magnetic stirring for 1 h at room temperature. The particle sizes and zeta potentials were measured for each formulation obtained. The corresponding blank formulations were also prepared (NCBR-CS).

To obtain the formulation coated with non-covalent PEG (NCCZP-PEG), the PEG 6000 polymer was added to the aqueous phase of the suspension to promote the adsorption of the polymer chains to the NC suspensions. The final concentration of PEG was 0.07%. NCBR-PEG was prepared without CZP.

2.2.2. Characterization of the nanocapsules suspensions

The pH, particle size, zeta potential, drug loading, encapsulation efficiency and morphology of the formulations were characterized. *Supplementary material* is available.

2.2.3. In vitro *release test*

In vitro release of CZP from the NCs was determined using the dialysis bag method (25 × 16 mm, 12,000 to 14,000 Da cut-off, Sigma-Aldrich). The release medium was 0.1 N HCl and phosphate-buffered saline (PBS)/ethanol (7:3, pH 7.4). The dialysis bags were immersed in the medium at 37 °C with horizontal shaking at 50 rpm. A CZP solution (1.5 mg/mL dissolved in PBS containing 30% ethanol) was also subjected to the release study to confirm that the diffusion of the CZP molecules across the membrane was not limited by the dialysis bag [19]. Two milliliters of the release medium were withdrawn at pre-determined times and analyzed by UV (UV 1800, Shimadzu®) at 274 nm. The experiments were performed in triplicate (n = 3).

The release profiles were plotted and fitted using different *in vitro* release models. The results of the *in vitro* drug release study were assessed using dissolution efficiency (DE) evaluation and independent/dependent models (see the *Supplementary material* for more details) [19,20].

2.2.4. Animal protocols

The experimental protocols for the pharmacodynamic and the pharmacokinetic studies were approved by the Animal Ethics Committee of UFSM (CIETEA-22/2010) and the Animal Ethics Committee of UNIPAMPA (023/2012), respectively. Both universities are affiliated with the Brazilian College of Animal Experimentation (COBEA) and adhere to the Principles of Laboratory Animal Care and international rules of ethics in research. All experiments were conducted in accordance with ethical and technical principles of animal experimentation established by the National Council for the Control of Animal Experimentation (CONCEA), Law n°. 11.794 of October 8th 2008, which establishes procedures for the scientific use of animals [21], and the Canadian Council on Animal Care [22]. Male Wistar rats weighing 250–280 g were used for all procedures. The animals were housed under a controlled 12 h light–dark cycle during the acclimation period, with access to water and food *ad libitum*. Wistar rats were purchased from the Central Animal Facility at UFSM.

2.2.5. Antipsychotic tests: pseudo-psychosis induced by D,L-*amphetamine (AMPH)*

Male Wistar rats (250–300 g) were used for the antipsychotic tests (n = 7), and the protocol was performed according to the method described by Benvegnú et al. [23] with some modifications. The animals were habituated in mirrored individual cages one hour prior to the beginning of the experiment. Two independent observers were positioned facing away from the animals. All groups received their first Amphetamine (AMPH) administration (8 mg/kg i.p.) at time 0 (hour 0). Thirty minutes after the AMPH administration, a single dose of 5 mg/kg body weight of each formulation was administered via the i.p. route: control (vehicle), CZP, NCBR, NCCZP, NCBR-PEG, NCCZP-PEG, NCBR-CS, and NCCZP-CS.

The groups that received blank NCs were used to verify the antipsychotic effects of the nanostructure used. To maintain AMPH activity, the rats received the second, third, fourth, fifth and sixth doses at 2, 4, 6, 8 and 10 h after the first AMPH administration. Trained observers quantified the stereotypic head behavior every 15 min according to the scaled scores developed by Ujike et al. [24]: 0, no head movement; 1, normal head movement and normal exploration; 2, increased rate of head movement with hyperactivity; 3, discontinuous, repetitive and stereotypic up-down head movement; 4, continuous, stereotypic head movement with an