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Development and evaluation of liposomal formulation containing nimodipine on anxiolytic activity in mice

Lina Clara Gayoso e Almendra Ibiapina Moreno [a,b], Giselle Zayra da Silva Oliveira [a], Isabella Macário Ferro Cavalcanti [b], Nereide Stela Santos-Magalhães [b], Hercília Maria Lins Rolim [a], Rivelilson Mendes de Freitas [a,*]

[a] Laboratory of Experimental Neurochemistry Research, Federal University of Piaui, Teresina, PI, Brazil
[b] Immunopathology Keizo-Asami Laboratory, Federal University of Pernambuco, Recife, PE, Brazil

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ABSTRACT

Nimodipine has been investigated in the treatment of anxiety. Its administration, however, presents a number of limitations, particularly by low bioavailability, low aqueous solubility and photosensitivity. These difficulties can be resolved by the use of nanometer-scale pharmaceutical carriers. The goal of the present study was thus to develop a liposomal formulation containing nimodipine (NMD-Lipo) and evaluate anxiolytic activity using models of anxiety (open-field, light and dark and elevated plus-maze test). The results suggest that administration of NMD-Lipo has no sedative or muscle relaxant effect in animals, since there was no reduction in the number of crossings, grooming and rearings. The increased residence time of the animals treated with NMD-Lipo in the bright field is a reflection of the anxiolytic-like activity of the formulation. Furthermore, the reduction in residence time of rodents treated with the combination of flumazenil and NMD-Lipo in the illuminated box suggests that NMD-Lipo acts on benzodiazepine receptors. The increase in the number of entries and length of stay in the open arms of mice treated with NMD-Lipo suggests that anxiolytic activity of formulation and reduction in number of entries and length of stay in open arms of rodents treated with a combination of flumazenil and NMD-Lipo suggest that NMD-Lipo act on benzodiazepine receptors.

1. Introduction

Anxiety is an emotional state that is part of human existence, since normal circumstances in people's lives, such as the development of some kind of physical and mental suffering, as well as changes in everyday life, may be associated with its onset. It is a type of emotion that has been shaped by natural selection, since it makes people alert to impending dangers (Richey et al., 2010). However, anxiety may cease to be a natural occurrence and progress to a pathological condition when it occurs disproportionately to the triggering event that causes it or when there is no apparent reason for its onset (Salomons et al., 2010).

Pathological anxiety is characterized by excessive and uncontrollable worry about a considerable number of factors, in which the individuals involved have experienced at least three of the following symptoms: feeling keyed up or on edge, sleep disturbance, muscle tension, being easily fatigued, difficulty concentrating or having one's mind go blank, and irritability (Maack et al., 2012). The pathological form is debilitating, reduces the quality of life of patients and is associated with an increased risk of death and suicide (Zou et al., 2012).

Treatments currently applied for anxiety disorders include pharmacotherapy and cognitive behavioral therapy (Bartley et al., 2013). The pharmacological treatment of pathological anxiety consists of the use of benzodiazepines, buspirone and antidepressants. Although this drugs shows great efficacy in the therapy of pathology, its administration has many drawbacks. For example, benzodiazepines can cause some side effects such as amnesia, induction of dependence and sedation which cause inconveniences for the patients (Raupp et al., 2008). The search for new therapeutic agents with anxiolytic properties is therefore of paramount importance.

Research has shown that the excessive flow of calcium through the membrane, which results in increased levels of intracellular ion, may play a role in the pathophysiology of affective disorders (Maigaard et al., 2012), epileptiform activity (N'Gouemo, 2013) and in the induction of anxiety (Kumar et al., 2012). From this perspective, the application of nimodipine, a selective antagonist of L-type calcium channels, has been investigated in the treatment of numerous neurological disorders (Yanpallewar et al., 2004).

Nimodipine has high lipophilicity and hence easily crosses the blood brain barrier. Studies have concluded that this drug has the ability to increase cerebral blood flow and its use in the treatment of ischemia present in numerous pathologies affecting the brain (Aslan et al.,

* Corresponding author at: Departamento de Bioquímica e Farmacologia, Universidade Federal do Piauí - UFPI, Campus Universitário Ministro Petrônio Portella, Programa de Pós-Graduação em Ciências Farmacêuticas, Bairro Ininga, Teresina, Piauí, CEP: 64.048-901. Brazil. Tel.: +55 86 3215 5870.
E-mail address: rivelilson@pq.cnpq.br (R.M. de Freitas).

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2009), besides being useful in the therapy of mood disorders (Frye et al., 2003; Pazzaglia et al., 1995) and in the treatment of senile dementia (Chalikwar et al., 2012), and in displaying anticonvulsant properties (Marinho et al., 1997; Mikati et al., 2004; Nascimento et al., 2005).

However, the administration of nimodipine has a number of limitations, owing chiefly to its high first-pass effect in the liver, which results in decreased bioavailability, low aqueous solubility and photosensitivity (Sun et al., 2013). These difficulties can be overcome through the use of nanometer-scale pharmaceutical carriers. These nanosystems are useful tools to improve the pharmacokinetic profile of drugs that have limited pharmaceutical applicability (Santos-Magalhães and Mosqueira, 2010). Furthermore, nanotechnology is great in improving the therapy of diseases that affect the central nervous system because the drugs applied in those treatments normally cannot cross the blood–brain barrier and could substantially benefit from the use of nanocarriers (Wong et al., 2012).

Based on these findings, the goal of the present study was twofold: the design of a liposomal formulation containing nimodipine and the evaluation of the drug's anxiolytic effects tested in three animal models of anxiety (open field, the light and dark and the elevated plus-maze test).

2. Material and methods

2.1. Material

Cholesterol (Chol), trehalose, nimodipine, diazepam and flumazenil were purchased from Sigma-Aldrich (St. Louis, USA). Soybean phosphatidylcholine (PC) (Lipoid S 100®) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Solvents and other chemicals were supplied by Merck (Darmstadt, Germany).

2.2. Production of liposomal formulation derived from nimodipine (NMD-Lipo)

Liposomes containing nimodipine (NMD-Lipo) were prepared using the method of hydrating the lipid film (Lira et al., 2009) at the Immunopathology Keizo-Asami Laboratory, Federal University of Pernambuco. NMD-Lipo was produced using the lipids soybean phosphatidylcholine and cholesterol (117.6 mM) at 8:2 ratio and drug concentration of 1.0 mg/mL. These constituents were dissolved in a mixture of chloroform: methanol (3:1 v/v) under magnetic stirring. The solvents were removed by vacuum evaporation at 80 rpm for 60 min at 37 ± 1 °C, resulting in a thin lipid film. This film was then hydrated with 10 mL of pH 7.4 phosphate buffer solution resulting in the production of large multilamellar vesicles (MLV). This liposomal suspension was then subjected to sonication (Vibra Cell, Branson, USA) at 200 W and 300 Hz for 40 s to obtain small unilamellar liposomes (SUV).

2.3. Characterization of NMD-Lipo

After 24 h of production, NMD-Lipo was characterized by evaluating its features: macroscopic aspects, pH, particle size, polydispersity index, zeta potential, drug content and encapsulation efficiency. The pH of the liposomes was measured using a digital pH meter (Bioblock Scientific 99, Prolabo, Paris, France) at room temperature. The particle size and polydispersity of the liposomes were determined using photon correlation spectroscopy (Particle Analyzer™ Delsa Nano S, Beckman-Coulter, USA). For this analysis 300 μL of the liposomal suspension was diluted in 1 mL of deionized water (Milli Q Plus, Millipore, USA). The zeta potential of the liposomes, corresponding to the surface charge of the vesicles, was measured using a Zetatrac NC-148 apparatus (Microtrac, USA). A sample of the liposomes (50 μL) was diluted in 5 mL of deionized water before analysis.

The content of nimodipine in liposomes was determined using UV spectroscopy at 237 nm. A standard curve of nimodipine was prepared at concentrations of 0.5, 1, 2, 3, 4, 5 and 6 μg/mL of nimodipine using methanol as solvent. Subsequently, an aliquot of liposomes (30 μL) was diluted in methanol to a final concentration of theoretical 3 μg/mL of nimodipine.

The encapsulation efficiency of nimodipine into liposomes was determined by the technique of ultrafiltration/ultracentrifugation using Ultrafree® units (Millipore, USA). A liposomal sample aliquot (400 μL) was transferred to filtering units and subjected to ultracentrifugation at 8776 *g* for 1 h. The amount of encapsulated nimodipine was obtained from the difference between the total quantity measured in the formulation and that of the filtrate obtained after centrifugation. The readings were performed at 237 nm.

2.4. Studies of anxiolytic activity of NMD-Lipo

2.4.1. The experimental units

Animal models of anxiety are applied for the evaluation of anxiolytic or anxiogenic compounds, as well as the identification of their mechanisms of action and study of the neurobiology of disease. We used male Swiss mice 2 months of age and weighing 25–30 g, from the Central Animal Facility of the Center for Agricultural Sciences, Federal University of Piauí. The animals used in the experiment remained on the premises of the Experimental Neurochemistry Laboratory Research, for 7 days, for proper acclimatization. The experimental units received water and diet (Labina ®) *ad libitum* and were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) and temperature (25 ± 1 °C). The experimental protocols and procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA/UFPI No. 014/11).

2.4.2. Treatments

The mice were divided into thirteen groups of eight animals each and treated intraperitoneally as follows: 0.9% saline (negative control), diazepam at a dose of 2 mg/kg (positive control), nimodipine at doses of 0.1, 1 and 10 mg/kg (groups Free NMD 0.1, 1 and 10 respectively), NMD-Lipo at doses of 0.1, 1 and 10 mg/kg (groups NMD-Lipo 0.1, 1 and 10 respectively), flumazenil at a dose 2.5 mg/kg (group Flu), flumazenil in combination with diazepam (group Flu + DZP) and flumazenil in combination with NMD-Lipo (groups Flu + NMD-Lipo 0.1, Flu + NMD-Lipo 1, and Flu + NMD-Lipo 10). The behavioral assessments were carried out 30 min after drug administration.

2.4.3. Open field test

The motor activity of the animals was observed by means of an acrylic open field (transparent walls and black floor, 30 × 30 × 15 cm) and divided into nine equal quadrants based on the model described by Archer (1973). After 30 min of treatment, the animals were placed, one at a time, in the center of the field for quantification of the number of crossings with four legs (spontaneous locomotor activity), number of self-cleaning behavior (grooming) and the number of lifting (rearing) without abutting the wall during the period of 5 min.

2.4.4. The light and dark test

The apparatus used is made of acrylic divided into two compartments (bright field and dark field) that communicate through a small door 5 by 5 cm (Crawley, 1985). The dark field (black acrylic, 27 × 18 × 29 cm) is poorly lit. The bright field (acrylic, 27 × 18 × 29 cm) is illuminated by ambient light. Each animal was observed for 5 min. The parameter used is the dwell time in the bright field in seconds.

2.4.5. Elevated plus-maze test

The elevated plus-maze for mice (Lister, 1987) consists of two opposing open arms (30 × 5 cm) and two closed (30 × 25 × 5 cm), likewise opposing cross-shaped arms. The open and closed arms are connected by a central platform (5 × 5 cm) high and 45 cm from the floor. The animals were placed in the center of the apparatus with the