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Neurochemical properties of neurospheres infusion in experimental-induced seizures

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ABSTRACT

Cell replacement through neural stem cells has been a promising alternative therapy for neurodegenerative diseases. It was evaluated the possible protect and/or prevent role of neurospheres in experimental models of epilepsy by the use of biomarkers of oxidative stress and histopathological analysis. After 1 h of the epileptic inductions by pilocarpine, pentylenotetrazole and picrotoxin, rats were infused with a suspension of 2×10^6 cells/0.25 mL, marked with Qtracker® 655, via caudal vein. In the control group epilepsy was not induced, but received the cell infusion under the same conditions of other groups. After 30 days, the rats were euthanized, and the removal of the brain was proceeded to later perform the assays oxidative stress and histopathology analysis. Thiobarbituric acid and nitrite levels were elevated in epileptic groups treated with neurospheres, and the levels of reduced glutathione, superoxide dismutase and catalase were reduced when compared to non-treated groups. The performance of oxidative enzymes from pilocarpine group treated with neurospheres showed slight increase. Histopathological evaluation observed distribution of neurospheres throughout the brain tissue, with viable cells and in process of differentiation in the pilocarpine group, but with differentiation and regeneration compromised in epilepsy by picrotoxin and pentylenetetrazole due to a microenvironment of oxidative stress. Neural stem cell therapy has a promising potential for protection in the pilocarpine epilepsy model, suggesting that the antioxidant system of neurospheres could reduce oxidative damage generated by seizure.

1. Introduction

The central nervous system has limited capacity to regenerate, especially when affected by neurodegenerative diseases (Pickard et al., 2011a), such as hippocampal sclerosis, which is the final pathological finding most commonly encountered in cases of partial epilepsy of different causes in adults, where there is a selective loss of neurons in areas of the dentate gyrus and the layer of pyramidal cells (Chang and Lowenstein, 2003).

Epilepsy is a set of neurological disorders that result from a large number of brain dysfunctions. Being characterized by recurring and spontaneous crisis, appearing suddenly and constituted of intermittent episodes of excessive electrical brain activity, following the corresponding clinical symptoms (Vezzani and Granata, 2005; Fabene et al., 2008). The brief characteristic involuntary movements of epilepsy can affect either only a part of the brain or the entire length of the two cerebral hemispheres (Trinka et al., 2015). The most common form of epilepsy syndrome is that, which affects the temporal lobe, initially affecting limbic system's structures (hippocampus and dentate gyrus). Their clinical importance is related to high prevalence of seizures, and to innumerable patients who do not respond to different therapeutic schemes (Scorza et al., 2005).

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Thus, the experimental models of pharmacologically induced epileptic seizures are quite relevant to properly mimic this syndrome in humans (Chang and Lowenstein, 2003; Scorza et al., 2005; Turski et al., 1983), being a useful tool for further physiopathological characterization of the process and to subsidize future pre-clinical trials related to new therapeutic approaches.

In this line, it is considered that the 21 st century is the century of technological convergence, once the great challenge of the researchers is concentrated in the development of cellular therapy as an alternative to repair damaged tissue areas (Blits and Bunge, 2006; Ryan and Federoff, 2007). Adult stem cells have been the subject of extensive research, due to the high plasticity displayed by these cells and their therapeutic potential for several pathological processes (De Kretser, 2007).

Therefore, the neural stem cells (NSCs) are shown as promising cell types in cell therapy, specifically through the neurospheres. Experiments are being carried out to analyze this potential (Kabos et al., 2002; Pickard et al., 2011b). These spheroidal bodies are made up of progenitor stem cells and pluripotent stem cells capable of self-renewal and generation of neurons (Anghileri et al., 2008; Radtke et al., 2009) glial cells and oligodendrocytes (Kang et al., 2006).

In this sense, numerous studies have been demonstrating therapeutic potential of bone marrow stem cells (BMSC) in the control of spontaneous recurrent epileptic seizures in the model of pilocarpine. During chronic epilepsy there is a decrease in concentration of several trophic factors that act in the proliferation of neural stem cells, what could contribute to the persistence of the seizures. Thus, approaches that increase the production of neurons in the epileptic hippocampus, such as BMSC infusion, might be promising to replace neuronal population's loss during the disease (Carrion et al., 2009).

Considering the consequences generated by the epileptic process in the cerebral hemispheres and the neurospheres self-renewal ability, the aim of this study was to evaluate the possible protection and/or prevention role of neural stem cells in experimental models of epilepsy, through oxidative stress biomarkers and qualitative histopathological analysis of *Wistar* rats' brain tissue.

2. Materials and methods

2.1. Animals and experimental delimitation

For all procedures performed in this research a total of 32 rats *Wistar* lineage (14 males and 18 females) were used. Weighing approximately 250 ± 50 g (2–3 months old), the animals were obtained from the animal facilities at Federal University of Piauí (CCA-UFPI). This study was carried out according to the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council of The National Academies, Washington, DC, 2008). All procedures were guided by the ethical principles established by the National Council for Animal Experimentation Control −CONCEA and in accordance with the national legislation in force (Act No. 11,794, August 08th, 2008 and law No. 9605 of February 12th, 1998). Furthermore, all experimental procedures in this study were approved by the Ethics Committee for Animal Use (CEUA/UFPI), under number 001/14 and efforts were made to minimize animal suffering and prioritize the welfare, according to the use of the 3Rs method adopted in the field of toxicology.

The animals were divided into three different models of epilepsy seizures induction: (A) Pilocarpine (PIL); (B) Pentylenotetrazole (PTZ); (C) Picrotoxin (PTX). Each experimental group was subdivided into epileptic rats treated (n = 4) and non-treated (n = 4) with neurospheres. In the control group (ABC4) epilepsy was not induced, but neurospheres were infused under the same conditions of the experimental groups. Therefore, the induction of seizures was held as follows: PIL (400 mg/Kg, IP) in groups A1 and A2 (Turski et al., 1983); PTZ (60 mg/Kg, IP) in groups B1 and B2 (Smith et al., 2007); PTX (3 mg/Kg, IP) in groups C1 and C2 (Bum et al., 2001). Time to onset of seizures, the number of seizures, peripheral and cholinergic signs of animals were measured for a period of one hour after the administration of the inducer drug. Infusion of neurospheres was made in groups A2, B2, C2, and in the other groups the infusion of saline solution (0.25 ml, IV). After 30 days, the animals were euthanized with sodium thiopental (CHEMIE®) in the lethal dose of 150 mg/kg/body weight (IM), preceded by the dose of 50 mg/kg/body weight ketamine (Rhobifarma Pharmaceutical Industry Ltda) associated with 2 mg/kg/body weight xylazine (Rhobifarma Pharmaceutical Industry Ltd), intramuscularly.

2.2. Isolation, culture and identification of NSCs

Four rats from *Wistar* lineage were euthanized for the collection, isolation, culture and expansion of neural stem cells in the lab of Stem Cell Culture (LABCelt - NUPCelt - UFPI).

The procedure for obtaining the cells followed the protocol described by Azari and coworkers (Azari et al., 2010), with modifications. Briefly, after euthanasia, the brain of each animal was transferred to a 10 cm Petri plate containing HEM to be sectioned and micro-dissected. Around 5.0 ml of trypsin were used for better digestion of tissues collected. After 7 min in a water bath, an equal volume of growth medium was added DMEM/F-12 for inactivation of trypsin. After centrifugation, the *pellet* was resuspended in 150 µl of neural medium (DMEM/F-Neural 12 + GlutaMax™ Gibco® Life Technologies ™, N-2 Supplement (100 x Gibco® Life Technologies ™), EGF [20 ng/mL] (Gibco® Life Technologies™), FGF-basic [10 ng/mL] (Gibco® Life Technologies ™), 0.2% sodium heparin and 10% penicillin-streptomycin (Gibco® Life Technologies™), passing by three successive centrifugations to 110 *g* for 5 min. The dissociated tissue was transferred to a culture bottle of 25 cm ^{2} containing 5 ml of neural medium. The cells were incubated at 37 °C, in wet atmosphere with 5% CO_2. The exchange of culture medium occurred every 3 days, being monitored daily for 7–10 days by which time neurospheres should have formed. NSCs generates spherical clusters of cells, so called neurospheres, which provide an excellent experimental system to study the proliferation/differentiation of CNS precursor cells. The generated neurospheres were quantified by computerized images analysis system (LEICA® QWIN D4000, UK-Cambridge).

NSCs identification was done by immunofluorescence, using the Nestin and GFAP antibodies. The neurospheres with 7 and 15 days were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 and then incubation in different tubes with primary antibody Nestin (1:100, Santa Cruz Biotechonoly) and GFAP (1:200 Santa Cruz Biotechonoly) for 1 h. Posteriorly, cell washing twice with PBS and incubated also in different tubes with the secondary antibody Goat anti-mouse IgG-FITC (Santa Cruz Biotechonoly) for 1 h and washed again three times with PBS. Having both markings prepared with blade cool for analysis in fluorescence microscopy.

2.3. Neurospheres tracking with the cell marker Qtracker® 655

The neurospheres cell culture with 15 days of development was trypsinized and centrifuged at 700 rpm for 5 min. The resulting *pellet* was resuspended in 1.0 ml of the growth medium (DMEM/F-12, GIBCO®). The homogenization of the components in the Qtracker® 655 Cell Labeling Kit (Invitrogen, Life Technologies, USA) was performed according to the manufacturer's specification. The neurospheres have been added to components of the cell marker Qtracker® 655, incubated in wet atmosphere with 5% CO_2, with 37 °C for 50 min and shaken in the vortex every 10 min. Then, 1.0 ml of DMEM/F-12 medium was added and centrifuged twice (110 *g* for 5 min), and the *pellet* resuspended in 1.0 ml of saline solution for subsequent animal infusion. Protocols were adopted to visualize the neurospheres-labeling in the brain tissues by fluorescence microscopy (BX41 fluorescent microscope - OLYMPUS).