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Trophic Performance of Neurons of the Prefrontal Cortex of Newborn Wistar Rats in Culture After Addition of Phosphatidylserine

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Abstract

Introduction: The surface of the cortex is formed by bodies of neural cells that process all the information that the brain receives and sends, enabling man to think, plan, coordinate thoughts and actions, perceive visual and sound patterns and use languages. The prefrontal cortex (CPF) is a complex and highly developed region of the neocortical regions of the human brain, being considered the frontal cortex association cortex. **Objective**: To analyze the effects of supplementation with different dosages of Phosphatidylserine on the neuroprotection and plasticity of nerve cells in the prefrontal cortex of cultured newborn Wister rats. **Methods**: the experimental procedures were conducted under license from the Ethics Committee on Animal Experimentation (CEEA) of the State University of Rio Grande do Norte (UERN), with opinion number 015/2016. The cellular material of the cerebral cortex was collected from 2 newborn Wistar rats, 2 days old. Then cultivated in different groups, one control (D10 = G1) and the others,

containing different concentrations of phosphatidylserine, PS (G2 = 1 µg, G3 = 2 µg and G4 = 3 µg). Scanning electron microscopy (SEM) was used to evaluate growth and cell morphology after 72 hours. To verify normality, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used and to confirm the results, two post-tests were performed - Tukey and Bonferroni - with the difference results with $p < 0.05$ being considered statistically significant. **Results**: We evidenced that the group G4 treated with 3 µg of the phosphatidylserine conditioned medium, presented significant morphological growth when compared to the groups G1 = D10, G2 = 1 µg and G3 = 2 µg, suggesting that this phosphate may act promoting neuroprotection and plasticity to neurons of the cerebral cortex, in culture. Conclusion: The neuroprotective effect and the preservation of neuroplasticity of cells in culture are notorious. By the way, phosphatidylserine promoted the maintenance of cell expansion in the experimental groups, mainly in the group treated with conditioned medium of 3µg, allowing the more expressive identification of the cell morphology, indicating its action in the neuroprotection of the cerebral cortex cells.

Introduction

Phosphatidylserine is a phosphoglycered composed of two fatty acids and a polar group bound to a glycerol molecule. This polar group is composed of a phosphate group, which binds to the glycerol molecule, and a serine molecule bound to phosphate through the hydroxyl group of its group. This compound has an asymmetric carbon atom and can be designated either as D-glycerol-1-phosphate or as L-glycerol3-phosphate. This polar group is composed of a phosphate group, which binds to the glycerol molecule, and a serine molecule bound to phosphate through the hydroxyl group of its group (VANCE *et al*., 2005).

PS consists of a high fat content and essential biological quality as a nutrient for the body. It is distributed throughout the body, as all cells need it equally and plays a key role in stability and impulse conduction through the cell membrane. However, although it is distributed throughout the body, in the brain accumulates a greater amount of FS, and within it, in the cerebral cortex or gray matter. This indicates, in percentage terms, the importance of this nutrient in nerve function, as a neurotransmitter and essential nutrient for the maintenance of sinatic activity (SALEM, 1995).

The main aspect of PS that underlies its known physiological functions is not its creation or destruction, but its location: PS exposure on the surface of mammalian cells is physiologically significant because it is usually completely absent in extracellular space (BEVERS & WILLIAMSON, 2016). There are two basic enzymatic activities that regulate the distribution of SF between the two leaflets. One is responsible for removing PS from the external leaflet by ACTIVE TRANSPORT DEPENDENT ONP; the relevant proteins are members of the type IV subfamily of type P ATPases.

It is known that its importance is linked to the function of the cell membrane, because phospholipids (such as FS) are critical elements for its ideal function. The electrical potential and its transmission to along the cell wall and this to other membranes, are some of the basic functions of the membrane. Membrane proteins are essential for performing these processes, and PS is important to regulate actions of these proteins. To get an idea, myelin is rich in PS and the content of this phospholipid increases twice as much gray matter of the brain, from birth to old age. Under normal conditions, practically all PS of a cell is located inside the cell plasma membrane (HAREL-ADAR *et al*., 2011).

Thus, the aim of this study was to analyze the effects of supplementation with differentiated dosages of Phosphatidylserine on the neuroprotection and plasticity of neurons of the prefrontal cortex of newborn Wistar rats in culture.

Methods

To perform the experiment, two newborn rats (Wistar - Rattus novergicus) were used, with a age of 02 days to obtain the hippocampuses. The project met the standards for carrying out animal research with all procedures, through the Ethics Committee on the Use of Animals of the State University of Rio Grande do Norte (UERN), through a substantiated opinion of number 015/2016, and the international standard governing the research was based on the guide for the care and use of laboratory animals and according to the principle of 3Rs.

Extraction And Cultivation Of Cells Of The Prefrontal Cortex

The cells of the prefrontal cortex were collected from 2 Wistar rats with 2 days of life. The neonates were sedate with inhalational anesthetic (halothane) at maximum dose and sacrificed, respecting the principles of the use of animals in the laboratory, being submitted to incision in the facial region, being folded the integumentary tissue. Then, section was performed in the anterior region of the skull and cut laterally to the skull cap, resulting in exposure of the brain. After that, under the laminar flow with the aid of scissors, tweezers and spatula, the brain tissue was folded down and exposed to the prefrontal cortex, thus allowing its removal and inclusion in a petri dish containing 5mL of Leibovitz-15 medium (L-15: GIBCO Invitrogen Corporation).

We used new 15mL Falcon conical tubes with 5mL of Low Knockout DMEM medium (Dulbecco's modified Eagle's medium), supplemented with 10% fetal bovine serum and 100 µl of gentamicin obtained from Cultilab® (Medium called D-10) were prepared and received the prefrontal cortex extracted to perform the cell suspension. The suspension was centrifuged at 1500 rpm for five minutes at a temperature of 37oC, after which the sobrentan was discarded and the cells resuspended in 5mL of medium, a procedure repeated three times.

Plates for culture of 24 wells for plating were prepared with 2.0mL of

D-10, then dripping the newly extracted cells, kept in a humid oven at 37oC with 5% CO2 and 95% air. Inverted phase contrast light microscopy was used to observe cell adhering to the bottom of the plates.

Subcultures Of Cells Of The Prefrontal Cortex And The Experimental Groups

When the cells of the prefrontal cortex reached 70-90% confluence at the bottom of the plate, the basic medium was removed and added to the wells 2 ml trypsin/EDTA (0.25% trypsin containing 1 mM of EDTA-Cutilab/Brazil®). The cell suspension was placed in a Falcon-type conical tube with the same volume of D-10 medium for 5 minutes, with the objective of inactivating trypsin. The suspension was centrifuged at 1500 rpm for five minutes three times, after which the sobrenatant was discarded and the cells were resuspended in 5 mL of medium.

These cells were deposited in plates of 16 wells, 4 wells for each group, and sterile round lamínulas of 13 mm were added, so the cells were observed in four time periods: 24, 48 and photographed after 72 hours. With this procedure it was possible to evaluate the adhesion and plasticity of the cells of the prefrontal cortex, in the following groups:

Group 1 (G1): Prefrontal cortex + D-10 medium;

Group 2 (G2): Prefrontal cortex + Phosphatidylserine 1µg;

Group 3 (G3): Prefrontal cortex + Phosphatidylserine 2µg;

Group 4 (G4): Prefrontal cortex + Phosphatidylserine 3µg.

FS dry powder extract was obtained from Evidence Soluções Farmacêuticas Ltda®. The concentration of the solution was prepared using 500 µg of FS, dissolved in 500 µl of bidistilled water (ABD), thus obtaining a concentration of 1 µg per 1 µl.

For cell observation, an inverted microscope with ckx41 phase contrast (Olympus®) with moticam 3.0 digital camera (Motic®) was used.

Regarding cell morphometry, phase microscopy was performed in 4 non-overlapping fields at the 20x increase. Microphotographs of the 4 groups were taken in 24 hours, 48 and 72 hours. After 72 hours of cellular observation, immunocytochemistry was carried out.

Electronic Microscopy

After a period of 72 hours of cell observation, the samples were fixed through the stabilization of the shape, which consists of the process of preservation of the original state of such samples in culture, avoiding the introduction of artifacts as much as possible. For this, the hippocampus cells of each studied group were fixed in glutaraldehyde at 2.5% buffered with sodium phosphate 0.1M and pH 7.4 and washed in sodium

phosphate buffer 0.1 M and pH 7.4.

Then, the postfixation was performed in 1% Osmium Tetroxide buffered with Sodium Phosphate 0.1 M and pH 7.4 for two hours. After postfixation, the samples were submitted to three washes in the buffer solution, previously mentioned, and to two washes, only with distilled water. Subsequently, a series of alcohols (ethanol) was performed with different concentrations (50%, 70%, 90% and 100%). The final stages of this processing consisted of the assembly of the material in support of the sample point (Stub) and metallic coating with gold by "sputtring", for observation in SEM (LEO VP® 435 – Carl-Zeis, Oberkochen, Germany and TESCAN® vega 3 LMU) at UFERSA, being the most representative images, electromicrographed.

The analyses were carried out in the electron microscopy laboratory of the Plant Science Research Center of the Semi-arid Northeast (CPVSA) of the Federal Rural University of the Semi-Arid (UFERSA).

Data Analysis

We used two independent investigators, previously calibrated (kappa = 0.94), assigned the perimeter (μm) and area (μm²) in visual fields not randomly overlapping, using cell culture of at least 3 different experiments, with an increase of 20x. The Software Motic Images Plus 2.0 (Motic®) was used for morphological observation, and the Adobe Photshop CS 6.0 software (Registry: PWW600R7105467-948 Adobe®), for minimal correction of brightness and contrast of photomicrographies.

The database for cell expansion (area and perimeter) after 72 hours was built on the Statistical Package for Social Sciences (SPSS) version 20.0 platform. After verifying the consistency of the data and finalizing the database, tests were performed to verify normality through the Kolmogorov-Smirnov and Shapiro-Wilk procedures, in which it was found that the data are normal and, therefore, parametric tests can be used. For this, the analysis of variance (ANOVA) was chosen, and for the corroboration of the results two post-tests were made - Tukey and Bonferroni - being statistically significant the results of difference with $p<0.05$.

Results

Morphological changes and expansion of neuronal cells

Regarding the observation of morphological tropism of neuronal cells at the end of 72 hours, it was found that the cell populations presented expansion in perimeter and area progressively. However, its greater expressiveness arises from the expansion of cells in the treatment group with 3 microgram conditioning medium, inherent to group 4 (panels D1, D2, D3, D4), as shown in the graph.

Thus, specifying the differences between the experimental groups after

72 hours of cell treatment, a statistical difference was observed between G4 (P=1.000) and G1 (P=0.043), which showed superior when compared to group G2 and G3, as highlighted in Figure 01:

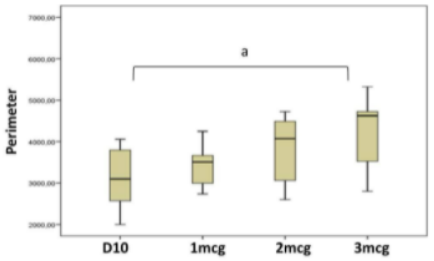


Figure 01: Perimeter of neuronal cells observed in each experimental group after 72 hours. P values: (a= 0.000).

Then, when observing the neuronal area after 72 hours, differences were identified, especially in G4 (p= 0.000), which showed morphological growth when compared to the other groups: G1 (p= 0.003), G3 (p= 0.003) and G2 not presenting neuronal growth. Specifying, statistical similarities were observed between the groups between G1 and G3 (p= 0.003), between G2 and G4 (p=0.000) and G1 and G4 (p=0.003) demonstrating that sF provided for the maintenance of morphological properties (Figure 02).

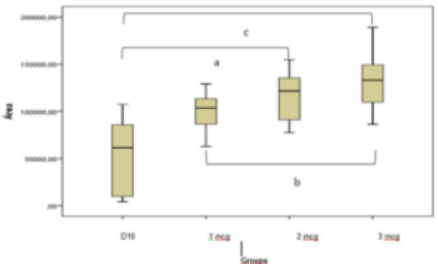
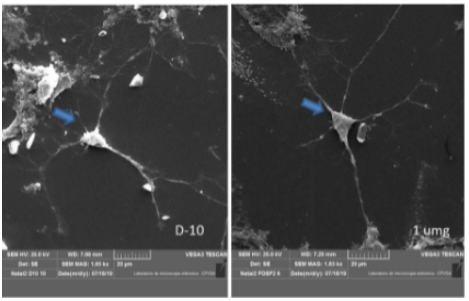


Figure 02: Area of neuronal cells observed in each experimental group after 72 hours. P values: (a= 0.003; b= 0.000; c= 0.003)

Phenotype Of Neuronal And Glial Cells

In this context, after a period of 72 hours of cell observation, the 4 groups of the experiment were processed for Observation in SEM.

Regarding the phenotypic alterations observed, it was found that the groups treated with Phosphatidylserine presented expansion in perimeter and area of cellular populations, especially in the treatment group with 3 microgram conditioning medium, inherent to group 4 (Figure 3), inferring the results previously observed through phase contrast microscopy.



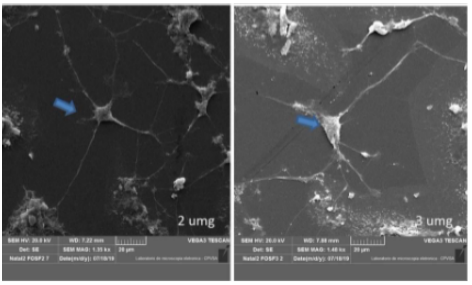


Figure 03: Neural and Glial cells of G1 (D-10), G2 (1µg), G3 (2µg), G4 (3µg) submitted to SME. neuronal cells (arrow).

Discussion

Worldwide, millions of people are affected by neurological disorders. A global average of 47.5 million people live with dementia and annually about 7.7 million new cases are recorded, generating an economic impact of high patient care, with disproportionately scarce neurological services and resources, which can influence their survival (WHO, 2016).

The clinical and social results of pathologies involving the cerebral cortex and its pathophysiological bases reveal an important theme of study and commitment to structure strategies that can contribute to the quality of life of individuals, reverting to consequent benefit to society.

Scientific research has incessantly sought alternatives of therapy and prevention that are capable of slowing or preventing neurodegeneration, and substances that can contribute to cellular plasticity and neuroprotection (DARAKHSHANA *et al*., 2015).

Based on this, numerous studies have investigated therapeutic strategies with the purpose of reducing neurodegeneration, in addition to stimuli and their respective substances that contribute to cellular plasticity. The cerebral cortex is one of the most researched structures in the brain and attracts the attention of neuroscientists, as well as its elementary role in the consolidation of memories, learning and cognition.

In this perspective, the various physiological and structural changes that occur throughout life contribute to the impairment of central nervous system (CNS) functions, compromising the prefrontal cortex and being the target of neuronal cell death. (ESQUENAZI; SILVA, U.S.; GUIMARÃES, 2014).

Thus, scientific research has incessantly sought alternatives of therapy and prevention that are capable of slowing or preventing neurodegeneration, and substances that can contribute to cellular plasticity and neuroprotection (DARAKHSHANA *et al*., 2015).

It is notorious that pharmacological therapies constitute a usual option presented by conventional medicine. However, in recent decades, interest in natural products has grown significantly, reflecting with the side effects of conventional medicine and the efficiency of drugs derived from new herbs, and substances have contributed to the increased use of natural products as a substitute for synthetic drugs (DARAKHSHANA *et al*., 2015).

From such a prism, Saki; Bahmani; Rafieian-Kopaei (2014), report that

about 25% of all drugs prescribed by physicians in current medicine are obtained from herbs in different forms, demonstrating efficacy and promising results in the treatment of various pathologies.

For this purpose, a conditioned medium with SF was developed for this study, with the objective of observing the behavior of CPF cells supplemented with identical concentrations and different Amounts of SF. In this approach, it was expected that the cells would become reactive and naturally secrete factors that would enable an environment favorable to neuroprotection and neural plasticity.

Moreover, by evaluating the parameters, relevant points in our study indicated a higher number of cells and morphological changes more evident in group 4 (3 µg - higher dose group), which theoretically are reflexes of the conditioned medium.

Regarding the D-10 / DMEM (Dulbecco's modified Eagle's medium) medium, used as a control group, this was very important to ascertain whether cpf cells acquired different phenotypes during the observation time. As the properties of SF are widely discussed and there were more visible morphological alterations when compared with cultures devoid of this treatment, especially in group 4, it is believed that the use of SF promoted the maintenance of cell expansion in the experimental groups, thus corroborating previous studies.

Starting for the peculiarities of the action of SF, the significant neuroprotective effect of this substance is notorious. The proposed mechanisms of its neuroprotective function include anti-inflammatory, antioxidant, antiapoptotic, anti-ischemic and restorative response, when mediated microenvironment conducive.

The most important aspect for the conservation of these cells in a receptor tissue is the microenvironment. The nature of the cellular microenvironment is dynamic and changes constantly with lesions, infections and other factors that can directly affect the conditions around a cell or group of cells, compromising cell behavior and, consequently, the physiologist integrity of the host, thus affecting the essential conditions for cell survival.

The present study analyzed the neuroprotective behavior of glial and neuronal cells after 72 hours of Treatment with SF, comtnating these findings between the groups treated with different concentrations and with the control group (established with DMEM medium). Previous studies have pointed to some important functions of neuroglial cells in the nervous system, such as the maintenance of a chemical environment suitable for neuronal signaling, modulation of the rate of propagation of nerve signal through myelin, sinatic action through the control of neurotransmitter absorption, destruction of pathogens, removal of dead neurons and maintenance of homeotase, exercising, thus, relevant functions in neuroprotection (JÄKEL; DIMOU, 2017; Purves *et al*., 2001).

Glial cells (astrocytes, microglia

and oligodendrocytes) are more numerous than nerve cells in the brain, constitute, depending on the mammalian species, between 33 and 66% of the total brain mass. Currently, several studies have revealed the multifaceted role of astrocytes in the homeotase of the cerebral parenchyma. They are the most abundant glial cells in the brain and have an extremely important structure for the survival and maintenance of the neurovascular unit (BECERRA-CALIXTO; CARDONA-GÓMEZ, 2017).

According to Ullian et al., (2001), astrocytes increase the number of functional synapses in CNS neurons by seven times, and are necessary for in vitro synaptic maintenance. Moreover, the study also showed that most synapses are generated, concomitantly, with the development of glia in vivo.

On the other hand, cumulative evidence indicates that hyperactivation of microglia exacerbates the progression of neurodegenerative diseases, including AD. Microglial dysfunction favors neurodegeneration via elevated levels of pro-inflammatory mediators, oxidative stress, altered neurotransmitter levels, as well as sinatic impairment, cognitive decline, neuronal death, reduced memory and malfunction of the neocortex and hippocampus (CHUN *et al*., 2018).

In their review, Chun *et al*. 2018 analyzed the newly developed experimental models and evaluated the roles of astrocyte-microglial interactions in neurodegeneration in the context of AD, allowing a more detailed analysis of glial versus AD interaction. However, it is not clear how this degrading function results in reactivated or hyperactivated astrocytes and microglia, and it is necessary to understand the roles of glia and the development of new interventions and exploratory tools. Therefore, it is reasonable to highlight that, under physiological conditions, its activation is essential, as it is associated with a reparative inflammatory response, which suppresses the pro-inflammatory response (CHUN *et al*., CUNNINGHAM; HENNESSY, 2015; HOLMES, 2013).

Thus, in view of the promising functions of glial cells and knowing their efficacy in the sinatic transmission, it was observed, in the different treatment groups with FS conditioning medium, which groups in which these cells presented greater development over the observation time. In this context, the group of 3 micrograms presented a superior response, both when the area and perimeter of glial cells were evaluated, and when the area and perimeter of neurons were evaluated. This finding can probably be explained by the modulation of the cellular microenvironment promoted by the conditioned environment, as well as by the action of glial cells in the modulation of neuronal microenvironment.

As previously described, the area, glial and neuronal perimeter are important parameters listed in the research, both were evaluated primarily for morphological description of cells throughout the days, with relevance in statistical analysis during the 72 hours of observation. The size of the cell depends on the cell type and its stage in the cell

cycle. In cultivation, changes in cell size and area occur during growth (KHARITONOVA; VASILIEV, 2008).

In the present study, it was found that the cell populations presented expansion in perimeter and area, progressively, through differentiated dosages of SF. However, it was observed that, in the lower dose groups (cells treated with a conditioned medium of 1µg and 2µg of SF), there was a lower expressiveness of growth and expansion of glial and neuronal cells when compared to the group with higher dosage (3 µg).

One of the techniques used in this research to accurately elucidate the results was SSING. A tool that allowed identifying and analyzing, with greater accuracy and quality, the cellular microstructures of the hippocampus of each studied group. At this juncture, a series of steps were followed to preserve the original state of the samples, avoiding the introduction of artifacts as much as possible. (SANAGUSTIN *et al*,, 2009).

SEM has been shown to be an important tool for the study of morphological characters of different species, allowing to correctly observe the phenomena that occur in the micrometric or submicrometric scale (SANAGUSTIN *et al*,, 2009).

As an example, we have the work of FU *et al*., 2008, who used this technique as a tool to investigate whether kavalactona kavain induces ultrastructural changes and impaired liver function, since there is evidence in the literature that kava can produce general adverse effects on the liver. Morphometric analysis of liver tissue was performed in digitized SEM images, and the images were more representative, electromicrographed. In this context, SME is an excellent method for visualizing the surface of cells and organ, providing exquisite details of surface projections.

Thus, important aspects in the present study corroborate the findings of the above-mentioned studies, noting that the groups treated with SF presented neural and glial area and perimeter superior to the other experimental groups, acting synergistically on the morphology of hippocampus cells, proposed mechanisms of their neuroprotective function.

Conclusion

The cell group that composes the cerebral cortex, when conditioned to a medium immersed in SF, showed significant morphological growth when compared to the control group, suggesting that this phospholipid acts promoting survival and maintenance of neurons of the field in culture.

The results presented in this study showed the feasibility of a stimulation method using SF at doses of 3 micrograms, which were able to guarantee the neuroplasticity that is the ability presented by the nervous system to modify and adapt to stimuli.

In addition, we envision cellular expandability observed in group 4 (higher dose = 3 micrograms), at short intervals of exposure to adequate stimuli, which we observed in vitro maintenance. Therefore, the fs-conditioned medium,

plus the cultivation of cells of the cerebral cortex promoted the maintenance of neuronal and glial cells, the latter being responsible for providing the necessary basis for neurons to exercise functionality in the sinatic conduction. This modality reinforces the capacity exercised by the SF component, facilitating the occurrence of morphological changes and causing neuroprotection and neuronal plasticity.

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