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ORIGINAL ARTICLE

# Adult human neural cells in culture following traumatic brain injury

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KEYWORDS
Cell Culture Techniques
Fluorescent Antibody Technique
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## ABSTRACT

**Objective:** The present study aimed to evaluate the viability of adult human neural cells in culture obtained from traumatized brain tissues collected during emergency surgery procedures.
**Methods:** Exploratory, descriptive, quantitative and cross-sectional study evaluating samples obtained from patients who underwent traumatic brain injury with extrusion of brain tissue submitted to cell culture in a standardized medium preserved for 168 h. After observation under phase contrast microscopy and immunohistochemical processing for neuronal (MAP-2) and glial (GFAP) markers, morphometric parameters of neural cells (cell body area, cell body perimeter and fractal dimension) were evaluated using ImageJ software, with data obtained after 24, 72 and 168 h being compared using non-parametric Kruskal-Wallis test, followed by Dunn's *post hoc* test.
**Results:** The explant of the nervous tissue revealed a consolidated pattern of cell migration into the culture medium. Upon reaching confluence, cell proliferation presented an aspect of cellular distribution juxtaposed along the culture medium at all time points analyzed. Both neurons and glial cells remained viable after 168 h in culture, with their morphologies not varying significantly throughout the time points evaluated. Immunohistochemistry for MAP-2 showed a relatively well-preserved cytoskeletal organization. GFAP immunoreactivity revealed activated astrocytes, especially at the later time point.
**Conclusions:** Our results indicate the viability of cell culture from traumatized human nervous tissue, opening perspectives for using substances of natural origin that may contribute to neuroprotection and neuronal maintenance in culture, allowing future translational approaches.

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## INTRODUCTION

One of the most common and detrimental events that can impair brain functioning is traumatic brain injury (TBI), which constitutes a severe public health concern[1]. TBI is among the leading causes of incapacities and death worldwide[2], mainly affecting children and young adults[3,4]. The morbidity rate associated with TBI varies between 30% and 70%, ultimately causing severe and occasionally permanent sequelae and impairing the quality of life of affected individuals[2]. According to the Global Burden of Disease (GBD), the incidence of TBI tends to rise until 2050, mainly due to the increasing use of motor vehicles and bicycles as a way of transport[5], affecting the population of both low-and high-income countries[4,6].

Every year, almost 70 million people suffer some type of TBI worldwide[4], which implies a cost estimated to be U$ 400 billion[7]. In the United States (USA), more than 2.8 million people seek medical care for traumatic head or spinal cord injury annually[8], resulting in more than 6,000 deaths in 2017[9]. In Latin America, mortality from severe TBI is high, emerging as a serious concern in this region[10]. In Brazil, TBI accounts for approximately 125,000 hospitalizations every year, with an average cost per admission of around U$ 570 and an estimated average yearly cost reaching U$ 71 thousand[11], affecting especially young and adult males, with the leading causes of TBI being automobile crashes and falls[12]. TBI treatment generally requires prolonged clinical care and often includes functional rehabilitation[13,14], which is often associated with cognitive impairment[15-17].

Among the events that contribute to the exacerbation of injuries caused by TBI, axonal damage, inflammatory response, excitotoxicity, and oxidative stress are crucial[18,19] (Figure 1). Inflammation, in particular, can worsen the patient's general condition when it becomes chronic[20,21].

*In vitro* studies have emerged as an interesting option to the use of experimental animals. The implementation of the nervous cell culture model dates to the beginning of the 20th century, with the pioneering studies of the biologist Ross Harrison, after the publication of his seminal study "Observations on the living developing nerve fiber" in 1907, which evaluated embryonic tissue of frogs[22]. Harrison's studies established a very fruitful field of investigation, which was consolidated over the decades and spread among various research groups around the world, allowing the analysis of cellular behavior outside the organism in a controlled manner, in addition to providing important information not obtained from animal models, especially concerning pathological states of the central nervous system[23].

Cell culture of human brain tissue has emerged as a promising approach[24,25], mainly based on the use of pluripotent stem cells[26] and tissue from adult patients undergoing elective surgeries[27,28]. Nevertheless, the viability of a human nerve cell culture technique based on the collection of biological samples from patients who suffered from TBI and required emergency neurosurgery was not well-characterized so far. Therefore, the present study aims to fill this gap by establishing the viability of cell culture of human tissue following TBI and evaluating some morphological aspects of both neurons and glial cells cultivated by adopting the above-cited approach.

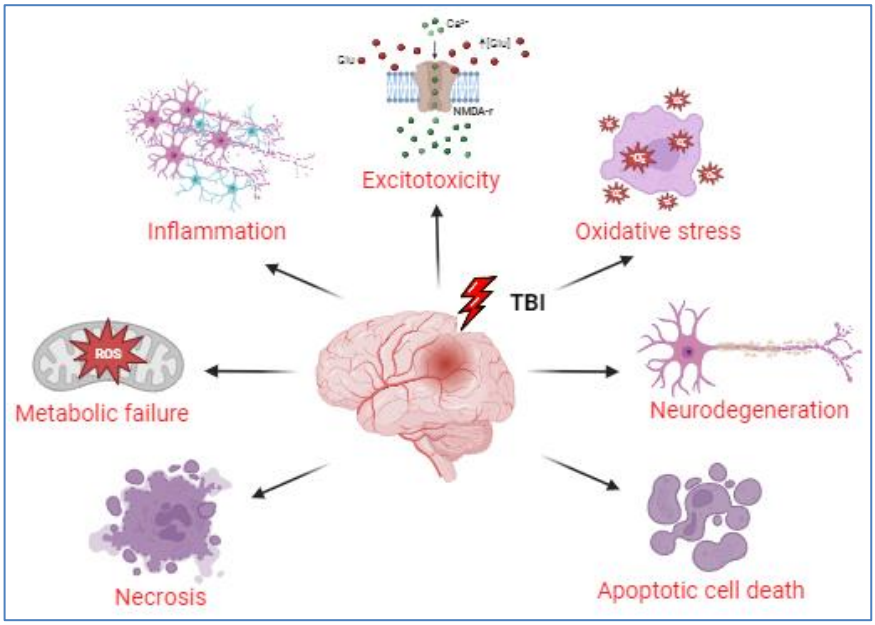


***Figure 1*** — Schematic picture depicting the main events triggered by TBI. The primary lesion provokes a cascade of deleterious events (inflammation, excitotoxicity, oxidative stress, metabolic failure), ultimately resulting in neurodegeneration, necrotic and apoptotic cell death. Figure generated using BioRender.com.

## METHODS

Exploratory, descriptive, quantitative and cross-sectional study evaluating samples of traumatized human brain tissue obtained from three adult male patients collected at the Tarcisio de Vasconcelos Maia Regional Hospital, the main public general hospital of the city of Mossoró/RN, Brazil, under license from the Institutional Human Ethics Committee (CEP/UERN; ID #3.346.997, CAAE: 65640517.0.0000.5294), after agreement of the patient's legally authorized representatives by signing an informed consent, following the guidelines of the Helsinki Declaration - Ethical principles for medical research involving human subjects and under the ethical regulations of the National Research Ethics Committee (CONEP) (resolution 466/12).

Patients were admitted to the emergency room with TBI resulting from an automobile crash, with laceration of peripheral tissues and fracture of the skull bone in the temporal region, resulting in extrusion of nervous tissue, which was collected during the standard procedure for surgery. Tissue was immediately stored in Leibovitz-15 conservative medium (L-15; GIBCO Thermo Fisher Scientific, Waltham, MA, USA) in a sterile Falcon tube and further transferred for treatment and plating with the addition of D-10 culture medium in a $CO_2$ oven.

The human nerve cell culture technique utilized in the present study was adapted from Lucena et al.[29], as follows: under laminar flow, low Knockout Dulbecco's modified Eagle's cell culture medium (DMEM) (GIBCO Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 10% fetal bovine serum (Sigma Company, St Louis, MO, USA) and 10 µg/mL ceftriaxone (Cultilab, Campinas, SP, Brazil) (D-10 medium). The nervous tissue was then incubated in 4 mL of this medium and placed in sterile 15 mL Falcon tubes for cell suspension. The supernatant was discarded, and the cells were resuspended in 1 mL of D-10 medium, with the procedure repeated 3x. Next, 3 mL of D-10 medium was added to a 12-well cell culture plate, followed by dripping the newly extracted cells, which were kept at 37 °C with 5% $CO_2$ and 95% air in an oven. Inverted CKX41 light microscopy with phase contrast (Olympus, Tokyo, Japan) was used to observe cell adhesion at the bottom of the dishes.

When the explant cells reached 70-90% confluence at the bottom of the dish, the basic medium was removed, and 0.25% trypsin containing 1 mM EDTA (Cultilab, Campinas, SP, Brazil) was added. The cell suspension was then placed in a sterile Falcon tube with the same volume of D-10 medium for 10 min to inactivate the trypsin. The suspension was centrifuged at 1500 rpm for 10 min; the supernatant was discarded, and the cells were resuspended in 1 mL of medium.

The cells deposited in the dishes were observed at three time points (24, 72, and 168 h) to evaluate their adhesion, migration and trophism. A CKX41 inverted phase-contrast microscope (Olympus, Tokyo, Japan) was used for cell observation. Photomicrographs were obtained using a Moticam 3.0 digital camera (Motic Instruments Inc., Richmond, BC, Canada) attached to the microscope.

After every time point was investigated, two wells of the plates were randomly selected to perform immunohistochemistry for neuronal (Microtubule-associated protein 2 - MAP-2) and astrocytic (Glial Fibrillary Acidic Protein - GFAP) labeling. The cells were adhered to the plates, the medium was removed, and the culture was dished in 0.1 M phosphate-buffered saline (PBS), pH 7,4, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min and washed in 0.1 M PBS (3x, 5 min each). Cell cultures were incubated in blocking solution (0.2% Triton X-100 and 1% bovine serum albumin in 0.1 M PBS) for 30 min. The blocking solution was removed, and plates were incubated with mouse anti-GFAP (1:500, Sigma Co., St Louis, MO, USA) and rabbit anti-MAP-2 (1:2000, Abcam Inc., Cambridge, MA, USA) primary antibodies diluted in 0.1 M PBS for 2 h, rinsed in 0.1 M PBS for 5 min, and then incubated with fluorescent Alexa Fluor 488-conjugated horse anti-mouse or Alexa Fluor 594-conjugated goat anti-rabbit (1:500, Jackson ImmunoResearch Labs, West Grove, PA, USA) for 1 h at room temperature (~25 °C) in the absence of light. Next, cells were washed with 0.1 M PBS for 5 min and immediately examined under a fluorescence microscope (Zeiss Axioplan DM6-B, Carl Zeiss, Oberkochen, Germany). Photomicrographs were taken using a Moticam 5.0 digital camera (Motic Instruments Inc., Richmond, BC, Canada) attached to the microscope.

Cell evaluation was performed by two independent investigators previously calibrated (kappa = 0,94) in 4 nonoverlapping fields at 10x magnification using a CKX41 microscope (Olympus, Tokyo, Japan). The Motic Images Plus 2.0 software (Motic Instruments Inc., Richmond, BC, Canada) was used for the morphological observations. Morphometric analyzes (cell body area, cell body perimeter and fractal dimension)[30] were performed using the NIH ImageJ software (https://imagej.nih.gov/ij/). The brightness and contrast of the images were adjusted using Adobe Photoshop CS 5.0 software (Adobe Systems Inc., San José, CA, USA), and the pictures were organized using Canvas X software (ACD Systems Inc., Fort Lauderdale, FL, USA).

After verifying normality with the Shapiro-Wilk test, the non-parametric Kruskal-Wallis test was adopted for comparison among morphological measurements, followed by Dunn's *post hoc* test (statistical significance when $p < 0.05$) using Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Average values are referred to as mean ± standard error of the mean (SEM). The values expressed in boxplots are presented as median values with indications of the 25th to 75th percentiles. To minimize bias during quantification, one researcher performed the analyzes. The data obtained were then assessed independently by two other researchers, both unaware of the data's origin (blind analysis). Further, analyzes were compared to identify variations. The experimental design is summarized in Figure 2.

## RESULTS

The explant of the nervous tissue revealed a consolidated pattern of cell migration into the culture

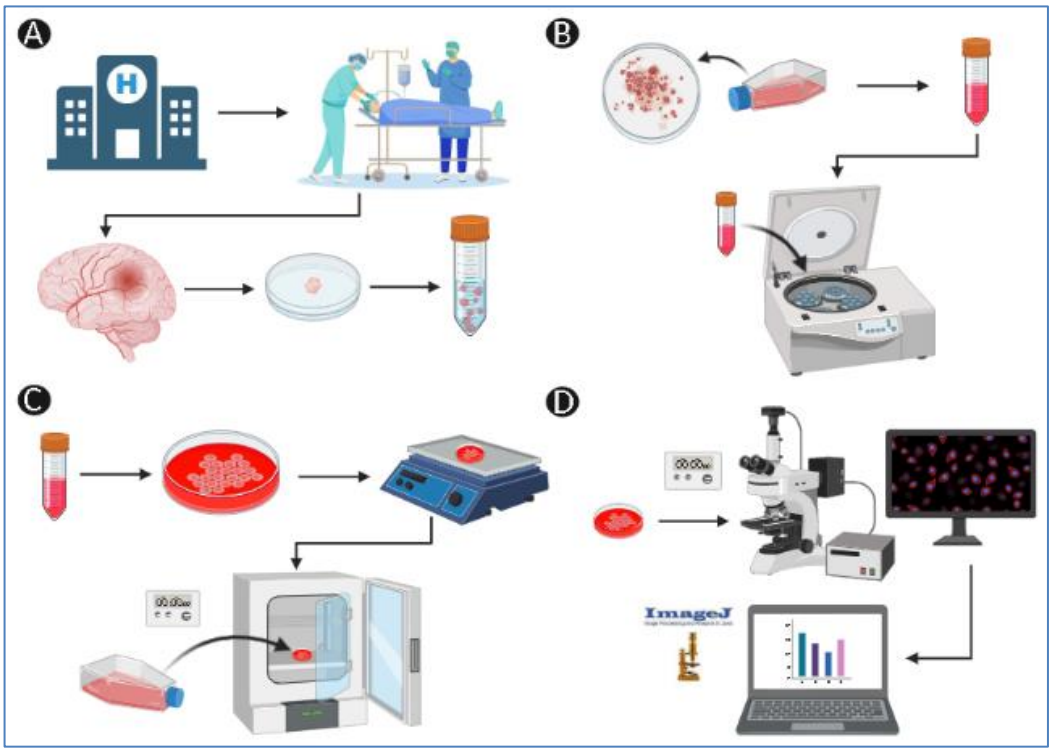


***Figure 2*** — Study design. Brain tissue was collected at the hospital center during emergency surgery (A), immediately incubated in a conservative medium, and transferred to the laboratory for preparation for plating in culture (B). Next, the cell culture was placed in a $CO_2$ oven, with monitoring up to 168 h (C). Throughout the time points evaluated, microscopic analysis of cultures in phase contrast microscopy was performed, followed by immunohistochemical processing for neuron (MAP-2) and astrocyte (GFAP) labeling, with subsequent image capture to evaluate cell morphology in ImageJ software. Figure generated using BioRender.com.

medium (Figure 3A), with the cells adhering to the bottom of the culture dish. Upon reaching confluence, cell migration presented an aspect of cellular distribution juxtaposed along the culture medium at all time points analyzed (24, 72 and 168 h) (Figures 3B, 3C and 3D, respectively).

Following subculture, the cells that adhered to the bottom of the culture dish were evaluated to characterize their morphology. In a qualitative analysis, it was possible to identify the presence of neurons in the culture medium after the time points assessed (Figure 4A). Overall, neurons showed no significant morphological alteration, with the cell body and dendrites well identified (Figure 4A). Such a pattern was corroborated by the quantitative analysis, which indicated no significant difference among cell body areas (24 h: 5.08 ± 0.09 μm²; 72 h: 5.12 ± 0.09 μm²; 168 h: 5.18 ± 0.10 μm²), cell body perimeters (24 h: 3.41 ± 0.05 μm; 72 h: 3.38 ± 0.04 μm; 168 h: 3.46 ± 0.03 μm) and fractal dimension (24 h: 0.49 ± 0.04; 72 h: 0.48 ± 0.03; 168 h: 0.55 ± 0.03) across the above cited time points ($p > 0.05$; Kruskal-Wallis test, Dunn's *post hoc* test) (Figure 4B).

The pattern observed in neurons was also identified in glial cells. It was possible to identify a significant number of glial cells, especially astrocytes, across the culture medium at all time points evaluated. Similar to those described for neuronal cells, there was no qualitative difference in glial cells among the time points investigated (Figure 5A). This observation was supported by the quantitative analysis, which indicated no significant difference among cell body areas (24 h: 4.41 ± 0.05 μm²; 72 h: 4.38 ± 0.04 μm²; 168 h: 4.43 ± 0.14 μm²), cell body perimeters (24 h: 3.25 ± 0.06 μm; 72 h: 3.33 ± 0.06 μm; 168 h: 3.42 ± 0.04 μm) and fractal dimension (24 h: 0.45 ± 0.03; 72 h: 0.46 ± 0.03; 168 h: 0.47 ± 0.02) across the time points evaluated ($p > 0.05$; Kruskal-Wallis test, Dunn's *post hoc* test) (Figure 5B).

MAP-2 expression allowed visualization of the morphology of neuronal cell bodies, revealing a relatively well-preserved cytoskeletal pattern at all times evaluated. However, no dendritic labeling pattern could be clearly discerned (Figure 6A). In turn, GFAP labeling showed clear-cut astrocytosis at all time points investigated, with cells showing an ameboid-like pattern characteristic of an inflammatory response, defined by swelling and shortening of processes, notably at the last time point evaluated (168 h) (Figure 6B).

## DISCUSSION

In the present study, we evaluated the viability of a human nerve cell culture technique obtained from traumatized brain tissues collected during emergency surgery, assessing some morphological aspects of both neuronal and glial cells. Our results indicated that both neurons and glial cells remained viable after 168 h in culture, with their morphologies not varying significantly throughout the time points evaluated.

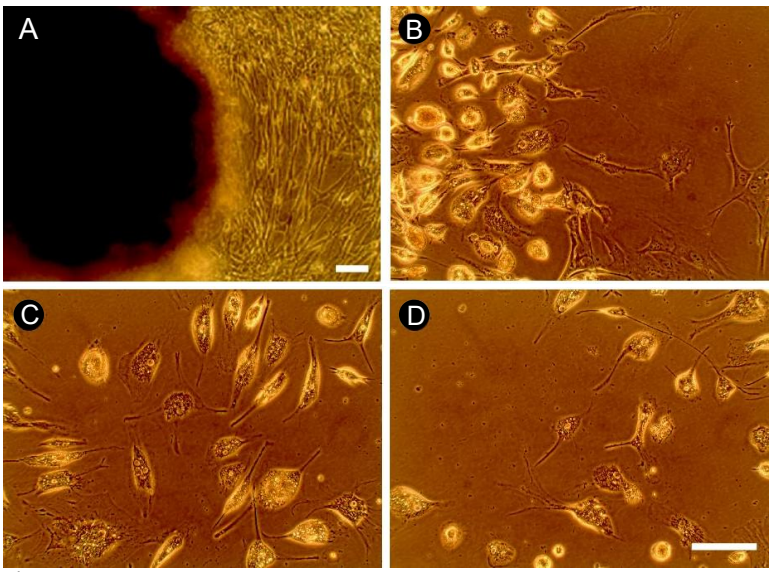


***Figure 3*** — Explant of nervous tissue under phase contrast microscopy, showing cell migration into the culture medium (A). Cell migration was observed 24 (B), 72 (C), and 168 h (D) after explantation. Scale bars: 100 µm.

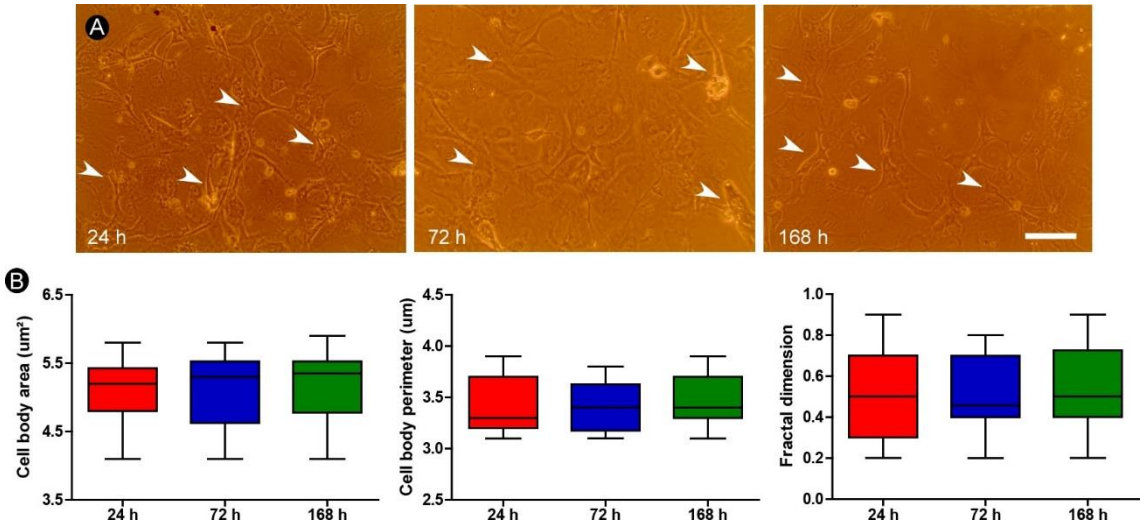


***Figure 4*** — Morphological aspect of proliferating neuronal cells (arrowheads) after cultivation, with morphologically normal cell bodies and dendrites being identified (A). (B) Quantitative analysis revealed no significant difference in morphometric parameters analyzed over the time points investigated ($p > 0.05$; Kruskal-Wallis test, Dunn 's post hoc test). The boxes correspond to the 25th and 75th percentiles (bottom and top of the boxes, respectively), the horizontal line inside each box indicates the median values, and the whiskers indicate the minimum and maximum values. Scale bar: 100 µm.

Our study is based on human tissue collected following a severe TBI, one of the most significant causes of disability worldwide[2], impairing the normal life of its sufferers and burdening both social and healthcare systems[4,7]. On a cellular level, excitotoxicity, inflammatory response, oxidative stress, and metabolic failure are the main morphophysiological alterations caused by TBI, ultimately resulting in cell death[20]. Depending on the severity of TBI, symptoms such as headache, fatigue, anxiety, irritability, memory loss, attention disturbances and depression are observed, as well as a long-lasting and progressive cognitive decline, often requiring a very difficult rehabilitation process for sufferers and their relatives[31,32]. In light of the impact caused by TBI, both *in vivo* and *in vitro* models are critical for an in-depth understanding of the events underlying the primary injury[33,34]. In this study, we evaluated both neurons and glial cells, demonstrating the viability of cell culture using adult injured tissue.

Cell culture models using human nerve tissue have emerged in recent years, mainly using induced pluripotent stem cells[26] and tissue obtained from elective neurosurgeries in adult subjects[27,28,35,36], opening an important field of research. However, contrary to the present work, the aforementioned studies did not evaluate traumatized tissue, which

motivated us to assess the viability of this type of tissue in culture. Our study collected brain tissue immediately after the patient entered the operating room. Notwithstanding, the tissue was not surgically removed since it was already separated from the brain due to the TBI suffered by the subject. Thus, the nervous tissue, in addition to the mechanical trauma that triggers a myriad of disturbances at the cellular level, was also subjected to oxygen and nutrient deprivation, presumably increasing levels of oxidative stress and mitochondrial dysfunction, ultimately resulting in cell failure. Future studies evaluating oxidative stress levels in samples following severe human TBI are critical for adequately characterizing this event in the impairment of the nervous system.

Our results indicate a distinct inflammatory process in the nervous tissue, characterized by an

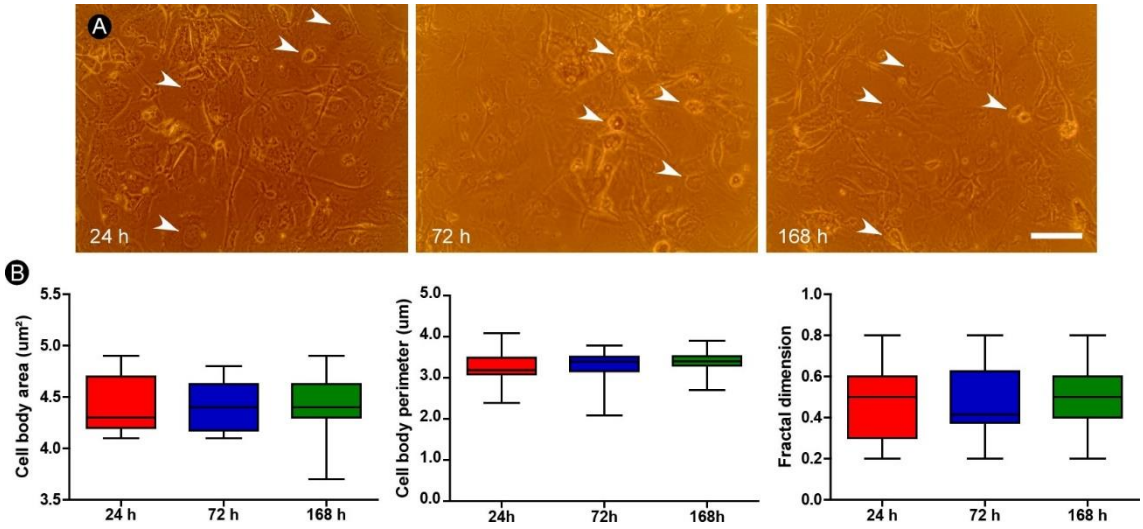


***Figure 5*** — Morphological aspect of proliferating glial cells (arrowheads) after cultivation under phase contrast microscopy (A), with morphologically normal cell bodies and processes identified. Quantitative analysis revealed no significant difference in the morphometric parameters analyzed over the time points investigated ($p > 0.05$; Kruskal–Wallis test, Dunn's *post hoc* test) (B). The boxes correspond to the 25$^{th}$ and 75$^{th}$ percentiles (bottom and top of the boxes, respectively), the horizontal line inside each box indicates the median values, and the whiskers indicate the minimum and maximum values. Scale bar: 100 µm.

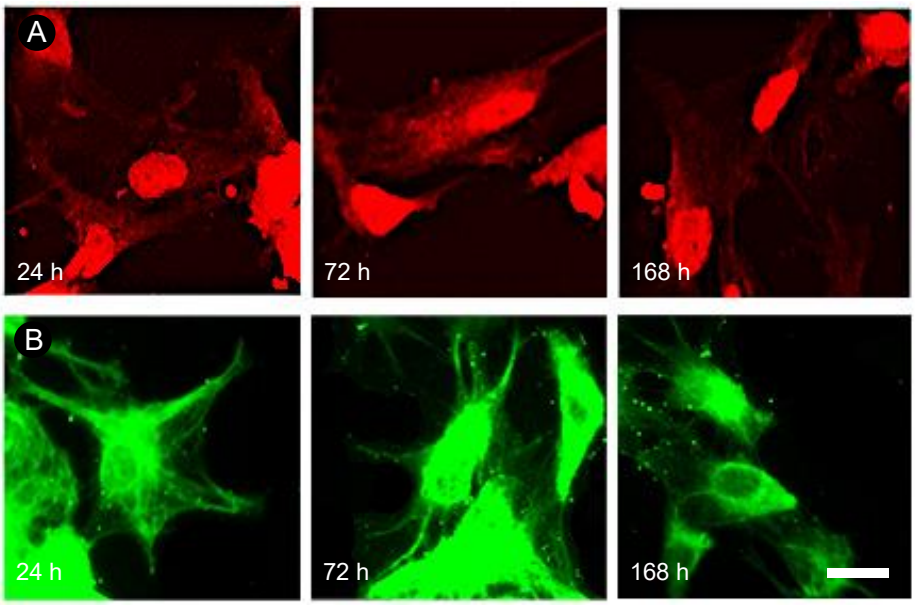


***Figure 6*** — Morphological aspects of neuronal cells and astrocytes revealed by immunostaining for MAP-2 (A) and GFAP (B), respectively, over the evaluated survival times. It is possible to notice the preservation of the structural aspects of the cytoskeleton of the neurons, as well as the progressive morphological alteration of the astrocytes, characterized by an ameboid-like pattern typical of an inflammatory response, defined by swelling of the cell bodies and shortening of processes. Scale bar: 50 µm.

evident astrocytic process across. This result is expected since TBI triggers several detrimental events, such as inflammation, excitotoxicity and oxidative stress. Inflammation has a dual role during a harmful insult, initially acting as a protective element by controlling the spread of the lesion by releasing cytokines, chemokines and inflammatory mediators, and phagocytosing debris[18,20]. However, when it becomes chronic, inflammation increases the injurious process by releasing chronic proinflammatory substances such as TNF-α and IL-1β[18]. Because the tissue used in the present study was obtained from a massive cortical lesion that resulted in tissue extrusion, harmful processes such as hypoxia, excitotoxicity and inflammation are expected, with inflammation being corroborated by the morphological modification observed in astrocytes.

MAP-2, a protein constitutively expressed in the neuronal cytoskeleton, is directly involved in many microtubule-related processes, including assembly, stabilization and cross-linking[37]. In the present study, MAP-2 immunohistochemistry revealed a well-preserved cell structure, indicating that, despite the impaired state of the nervous tissue sampled, it was possible to identify structurally viable cells with a relative state of cytoskeletal preservation.

As previously mentioned, despite its altered condition, we could attest to the viability of traumatized nerve tissue in our culture protocol, as confirmed by qualitative and quantitative analyzes of both neurons and glial cells. This opens interesting scenarios for the future therapeutic use of compounds of natural origin that can increase the functional period of human cells in culture, seeking a translational approach. In addition, substances with anti-inflammatory and antioxidant properties[38-40] may become the cellular environment less deleterious for cells that have not been or even were only marginally affected by the inflammatory process and oxidative stress resulting from TBI, which may also participate in processes of neuroplasticity and neuroprotection.

One of the limitations of the present study was obtaining tissue for cell culture, especially during the COVID-19 pandemic, given the particularity related to the condition of the patients (traumatic injury with tissue extrusion), which reduced the sample size. Furthermore, it is essential to stress the need for future studies comparing the injured tissue with a sample of non-traumatized tissue obtained through elective surgeries to comparatively evaluate the different cellular structural aspects, as well as the evaluation of other cell markers to draw a complete picture of the evolution of traumatized adult human nervous tissue in culture.

## CONCLUSION

Our results demonstrate the feasibility of cell culture from traumatized human nervous tissue. Future studies employing substances of natural origin that may contribute to neuronal maintenance in culture are required to develop a therapeutic approach for the associated neurological and cognitive deficits following TBI.

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Writing of the manuscript: MAMF, DF, JRLPC
Critical revision of the article: DF
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