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Research article

Effects of ethanol and deferoxamine on rat primary glial cell cultures, in regard with ischemia induced by traumatic spinal cord injury

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ABSTRACT: Although they have been regarded, in the past, as passive support cells, many experimental data have shown that glial cells play a critical role in the development and functioning of the nervous system. Despite the advances that have been made in understanding astrocytes' role in the nervous system's development and function, our knowledge of their interactions with other cells is still limited, albeit neurons are dependent on the trophic support provided by astrocytes release. **Materials and Methods**. The use of the McCarthy and de Vellis methods for isolating glial cells has been regarded as an essential tool for studying their function. This study aims to evaluate the effects of ethanol and deferoxamine on primary rat glial cell cultures and try to explain, as far as possible, the relevance of such effects for patients with chronic alcoholism and traumatic spinal cord injuries. **Discussion.** Because glial cells are very important in the functioning of the central nervous system and experiments cannot be performed on human primary nerve cell cultures, we performed an experiment on glial cells harvested from the newborn rat, analyzing the dynamics of IL-6 and TNF alpha on models of suffering in spinal cord injury (hypoxia and thermally stress). **Conclusion.** Inhibition of TNF alpha synthesis was more important at 7 days posttraumatic in cells with prolonged ethanolic exposure, even if protein levels of IL-6 were elevated (under similar experimental conditions). Thus, we can say that long-term exposure to ethanol of nerve cells can ensure a favorable evolution of medical recovery (by increasing TNF alpha), even if the inflammatory process remains active (shown by elevated IL-6 values).

Keywords: ethyl alcohol, deferoxamine, primary glial cells cultures, traumatic Spinal Cord Injury

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INTRODUCTION

Traumatic spinal cord injury (tSCI) is an acute injury event that occurs in the spinal cord and its adjacent structures (1). The molecular consequences of SCI influence the inflammatory processes (inflammasome), those of survival, respectively cell death (necrosis and apoptosis) at the loco-regional level.

In addition to primary cell destruction, with necrosis phenomena, the tumor necrosis factor alpha (TNF-α) family of proteins triggers programmed cell death (apoptosis) by acting on the complex death-inducing signaling complex (DISC) that causes activation of specific cysteine-aspartic proteases (caspases) cascade (2).

The inflammatory complex (inflammasome) is located in neurons, oligodendroglias, and astrocytes, and its neuraxial components are in the pre-assembled state, which causes the rapid post-traumatic activation of the innate immune response; inflammasome also participates in the acquired inflammatory response (3,4).

In the acute phase immediately after SCI, microglia are activated (5), which release TNF-α and 1-beta interleukin (IL-1β), producing glutamate in cytotoxic concentrations (6). The expression of the wingless-related integration site (wnt) gene is increased in SCI (negatively influencing the regeneration of the cortico-spinal tracts); having some of the receptors located at the level of axons, neurons, astrocytes, oligodendroglia, microglia in

the injured area (with possible involvement of Wnt in neurodegeneration and neuroinflammation) (7).

After (t)SCI, neural stem cells in the ependymal canal migrate to the injured area and differentiate into glial cells (astrocytes and oligodendroglias) (8). On the other hand, p38α protein is also activated with a role in inflammatory processes triggered after SCI in neurons, astrocytes, microglia, and oligodendroglias by stimulating Inducible Nitric Oxide Synthase (iNOS), creating an additional inflammatory neurotoxic effect (causing neuronal death) (9). Injury-activated astrocytes (see above for inflammasome), however, synthesize anti-inflammatory molecules: interleukin-10 (IL-10), transforming growth factor beta (TGF-β) (10). Is also synthesized Lipocalin 2, a protein with a proinflammatory role in SCI, sensitizing (in vitro) microglia and astrocytes to the effect of nitric oxide (11,12). Thus, the appearance of glial fibrotic scars formed in the area of the spinal lesion – a hallmark of its irreversibility – is favored, consisting of reactive astrocytes and intermediate filaments: glial fibrillary acidic protein -GFAP) and vimentin, proteoglycans (keratan sulfate, heparan sulfate, chondroitin sulfate), type IV collagen, laminin, fibronectin, thrombospondin (10,13–17). Cadherin 2 is a protein that forms scars in the subacute and chronic post-SCI phases, with reactive astrocytosis and increased expression of type I collagen (18).

Ethanol ontogenetically affects the development of the body, starting with intrauterine life, and continuing with: all age / sex / lifestyle / comorbidities. Thus, the chronic ethanolic consumption of the mother disrupts the development of nerve cells [especially in the postmitotic phase of the cell cycle, by decreasing the expression of the enzyme cyclin-dependent kinase 2 (cdk2), cyclin A and D] and continuing to affect even adult nerve tissue cells (by reducing the expression of cdk2 and cells A and D, by slowing the development of the cell cycle and promoting apoptosis similar to the situation in intrauterine life) (19) Ethanol, consumed acutely and chronically [after prolonged exposure (greater than 7-14 days) to high doses of ethanol: more than 8 standard ethanol units per day (standard drink per day unit = 10 g ethanol)] has a toxic effect on glial cells and neurons (in humans and animal models) including decreased cdk2, increased proinflammatory interleukin IL-6 in nerve tissue (20) (21) (22) (23) (24). Peripheral nervous system damage, and polyneuropathy, (which may be favored by ethanolic consumption) can positively influence axonal regeneration of neural axial lesions with the help of IL-6, CREB transcription factors (cAMP response element-binding protein), and ATF3 (Cyclic AMP-dependent transcription factor)(25)(26). There is also a special dynamic of glutamate compared to ethanolic ingestion (27). Glutamate is an essential amino acid (for memory and learning processes) and can bind to ionic gate receptors: AMPA (α amino-3 hydroxy-5-methyl-4-isoxazole propionate), kainate, and NMDA (N-Methyl D-Aspartate) (28). In the acute phase of ethanolic consumption, the GABA-ergic systems are stimulated (having sedative effects)(27). In the chronic phase (when ethanol dependence occurs), the nervous system decreases its receptivity to glutamate, resulting in conformational changes in the receptors for the amino acid gamma-aminobutyric acid (GABA) and N-Methyl-D-Aspartate (NMDA) (27). Due to the reduced representation of NMDA receptors in the neuronal membrane, chronic alcoholism causes excessive compensatory glutamate production (leading to morpho-functional changes in ethanolic dependence syndrome). Excitotoxicity NMDA receptor-mediated occurs after exposure to large amounts of ethanol or after significant overstimulation of membrane receptors (27). On the other hand, increased spinal receptivity to glutamate may accentuate the learning/memory processes of gray matter neurons, helping to support local connectivity.

Therefore, the relationship of the human body with alcohol (including traumatic spinal cord injuries) seems to be complex, as our clinical and some experimental findings reveal a somewhat paradoxical aspect: in this sense, in the acute phases (in the first 2 days) and subacute (within the next 14 days) after SCI we observed a favorable clinical evolution in patients known to have chronic alcoholism, compared to patients without a history of

ethanolic abuse (29) which led us to look for an etiology for the (apparently) contradictory response of chronic ethanol users in acute and subacute spinal cord injury situations.

Thus, in the present study, we investigated aspects of the molecular biology response in newborn rat cells exposed and not exposed for a long time to ethanol and then to acute hypoxemic treatment (see Materials and methods). We tried to reproduce according to human/rat life reports comparable periods of ethanolic tissue impregnation (26.7 human days being corresponding to 1 day of rat life and 13.8 days of life of a rat is equivalent to 1 human year) and, taking into account of the experimental limits on the survival of the cells used in this paper, we emphasize that acute lesion events also produce ischemic phenomena (vascular lesions, edema, dynorphin, opiate receptor activation) that are manifested intracellularly by activating the response to hypoxia(30) (31) (32). At the level of neural cell cultures, we used deferoxamine as an inducer of hypoxia (33), and we analyzed the protein synthesis corresponding to apoptosis (by TNF-α dynamics) and inflammasome (by IL-6 variation).

MATERIALS AND METHODS

Primary culture of glial cells

Solutions and reagents: Saline phosphate buffer [1-Hexyl-3-methylimidazolium bis(trifluormethylsulfonyl)imide -TFS with the composition: 0.13M NaCl + 2.6mM KCl + 8mM Na2HPO4 x12 H2O 8mM + 1.4mM KH2PO4]; Dulbecco's Modified Eagle's Medium (DMEM-Sigma); penicillin 100 U / ml (Antibiotice S. C. Iaşi); streptomycin 100◎g / ml (Antibiotice S. C. Iaşi); neomycin 50g / ml (Sigma); fetal calf serum (Sigma); lithium chloride (Sigma).

Procedure. **:** To obtain the primary culture of glial cells, 7 Wistar rat pups were used, aged 1-4 days, taken from the Biobase of the National Institute for Research and Development in the Field of Pathology and Biomedical Sciences "Victor Babeș". After decapitation, the brain and the cervical spine was placed in phosphate buffered saline (PBS) on ice. After removal of the meninges, the central nervous tissue is placed in 10 ml tubes in DMEM. The mechanical dispersion of the cells was performed with the help of an automatic pipette, aspirating and rapidly releasing a volume of 1 ml in the tube in which the nervous tissue is located. Cells were grown in DMEM medium with 4500mg / l glucose, 25 mM HEPES, 100 U / ml penicillin, 50 μg / ml neomycin, and 100 μg / ml streptomycin. The medium was supplemented with 15% fetal calf serum. The cell culture dishes were incubated at 37°C, 5% CO_2, and 90% humidity. After 24 hours, the culture medium was changed to remove dead cells and cell debris. After the first medium change, the medium was replaced with an equal volume of fresh, pre-warmed DMEM medium every 3 days. Phase contrast microscopy allows the study of living, unfixed and uncolored cells.

Deferoxamine acts as a "scavenger" for free radicals (by breaking their chains), being a chelator of iron molecules (34) (33), improving the activity of antioxidant molecules (35). The administration of deferoxamine in treating neuronal lesions produced in conditions of ischemia/hypoxia prevents the formation of peroxynitrite anion, hydroxyl radicals, and caspase-3; by preventing the oxidation of polyunsaturated lipids (36) (35).

Ethanol and deferoxamine glial cells cultures treatment

Starting from the 17-th day of glial cell cultivation it has been initiated the ethanol treatment using a 50 mM concentration of absolute ethyl alcohol. After this treatment, from the 21st day of glial cell cultivation, was administred the second treatment**:** with deferoxamine (100 μM). In parallel with ethanol and deferoxamine treatment dishes.

Proteins. Electrophoresis allows the separation of serum, urine, and liquor (Cerebrospinal fluid – CSF – in our study) proteins on agarose gel plates. Separated proteins in the electrophoretic pattern are subjected to visual inspection to identify the pathological profiles, which include both the qualitative variations of the bands and the appearance of new additional bands in the pattern. The multiple application method substantially improves sensitivity (1.5mg/dl per band) and hence identifies small bands. The kits have been designed for Pretty Interlab instruments.

Rat Interleukin 6 (IL-6) Immunoassay ELISA Kit

This kit employs the "Double Antibody Sandwich" technique. The pre-coated antibody is an anti-Rat IL-6 monoclonal antibody, while the detection antibody is a biotinylated polyclonal antibody. Samples and biotinylated antibodies are added into ELISA plate wells and washed out with Phosphate buffered saline (PBS) or TRIS buffered saline (TBS) after their respective additions to the wells. Then Avidin-peroxidase conjugates are added to the wells. TMB substrate is used for coloration after the enzyme conjugate has already been thoroughly washed out of the wells by PBS. TMB reacts to form a blue product from the peroxidase activity and finally turns to yellow after the addition of the stop solution. The color intensity and quantity of target analyte in the sample are positively correlated.

Rat TNF-α Immunoassay ELISA Kit

Tumor necrosis factor alpha (TNF-α) is a molecule with important role in inflammation, immune system function, apoptosis and lipid metabolism (37) (38). Human TNF-α exists in 2 forms: soluble and membrane-bound (38). TNF-α is bound to specific receptors (39) (38). Rat TNF-α is a 26 kDa type II transmembrane protein with 35 amino acid cytoplasmic domain, a 21 aa transmembrane segment, and a 156 aa extracellular domain (ECD) (40) (41) (42). TNFR-I shares 95% aa sequence identity "showed 75.8, 62.5, 60.9 and 72.1% similarity with the human, mouse, rat and pig" (43) (42). It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF-α is assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface (39).

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells, and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is proportional to the amount of TNF-α bound in the initial step. The sample values are then read off the standard curve.

Figure 1 Wistar rat pups (aged 3-4 days) and the related research infrastructure used experimentally (presented setup) to obtain primary glial cells cultures.

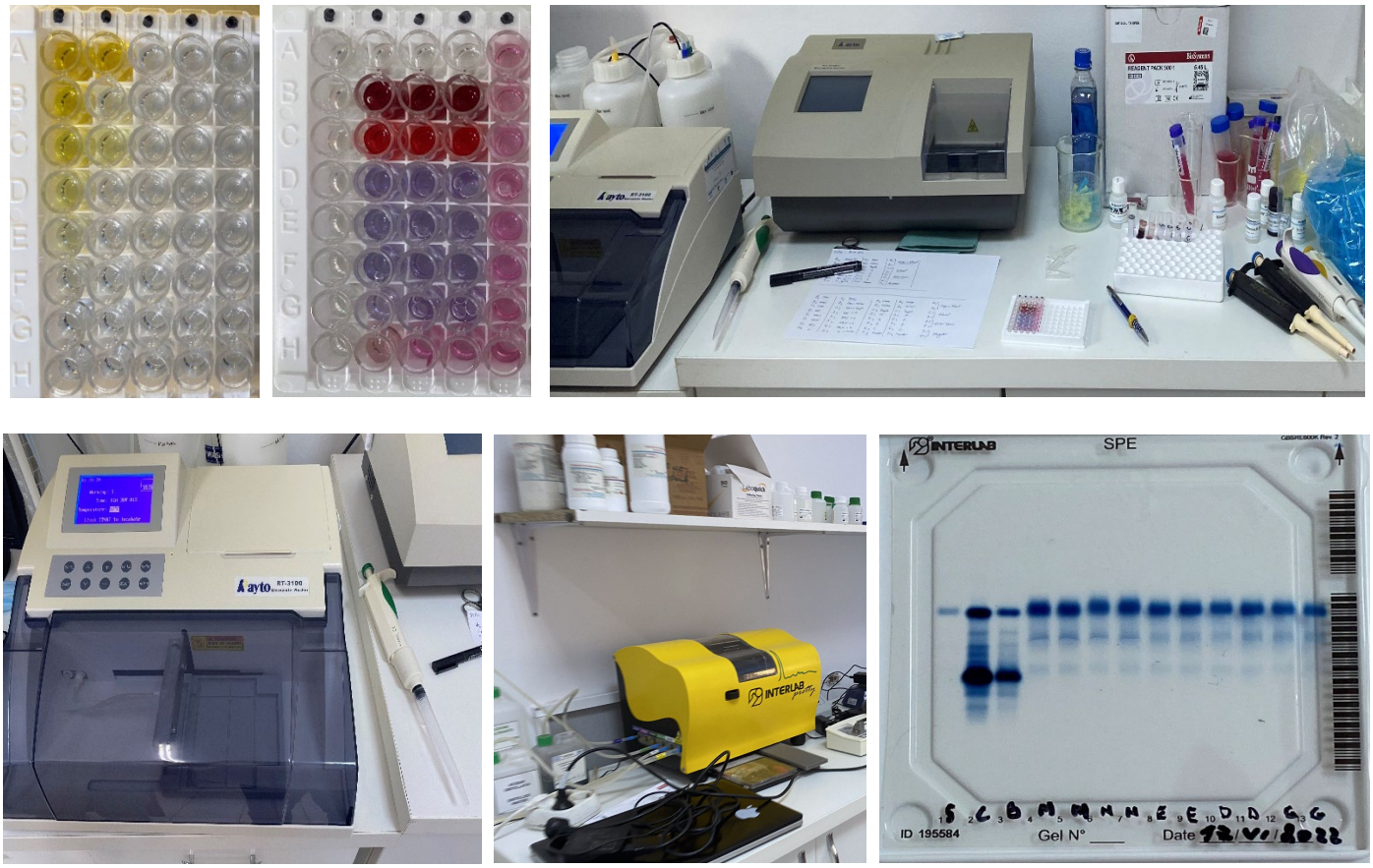
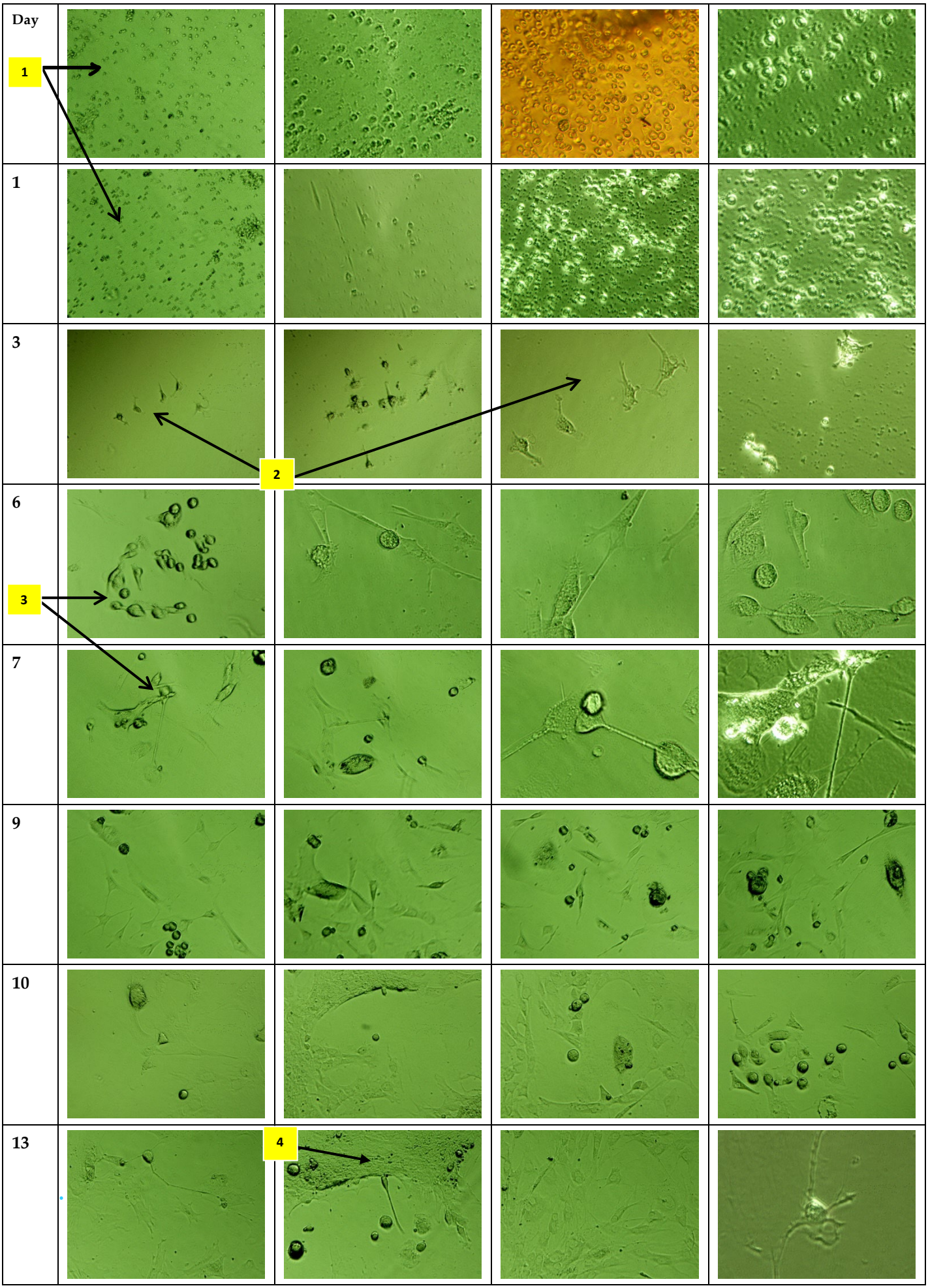


Figure 2 Experimental setup used for electrophoresis and ELISA immunodetection of IL6 and TNFα

RESULTS

Morphological characterization of primary glial cells cultures.

During the first 24 hours of culture, the cells in the inoculum are placed on the culture surface in the direction of gravity, but only viable elements adhere to this surface and flatten. After 48 hours of culturing, many cells begin a process of cell differentiation, with the cells acquiring specific cellular processes and flattening on the culturing surface. No processes of cell division were observed, which means the cells came only from the initial inoculum. After a lag period (during which multiplication is inhibited) of about 48-72 hours, a gradual increase in cell density was observed, and a large number of cell divisions as well. After five days, the period of cell differentiation begins, characterized by the gradual appearance of cell extensions (Figure 3). An increasing number of cells have 1-5 extensions, some of which are 2-4 times the diameter of the cell body from which they emerge. Numerous cell divisions developed from the sixth day of cultivation, resulting in the formation of cell islands consisting of several thousand cells. Cells have multiple connections through cellular processes and form tissue-like structures. The cell islands that appeared on the fifth day are expanding, occupying an increasingly significant cultural space. In the area between the cellular islands, it is observed the establishment of intercellular connections by means of the cellular processes similar to the axon. The level of cell division is high, observing microscopically many cells in different mitotic stages. Starting with the fifteenth day of cultivation, the islands tend to form folds, generating super structured shapes of cellular architecture, similar to a gyrus in the cerebral cortex.


Day
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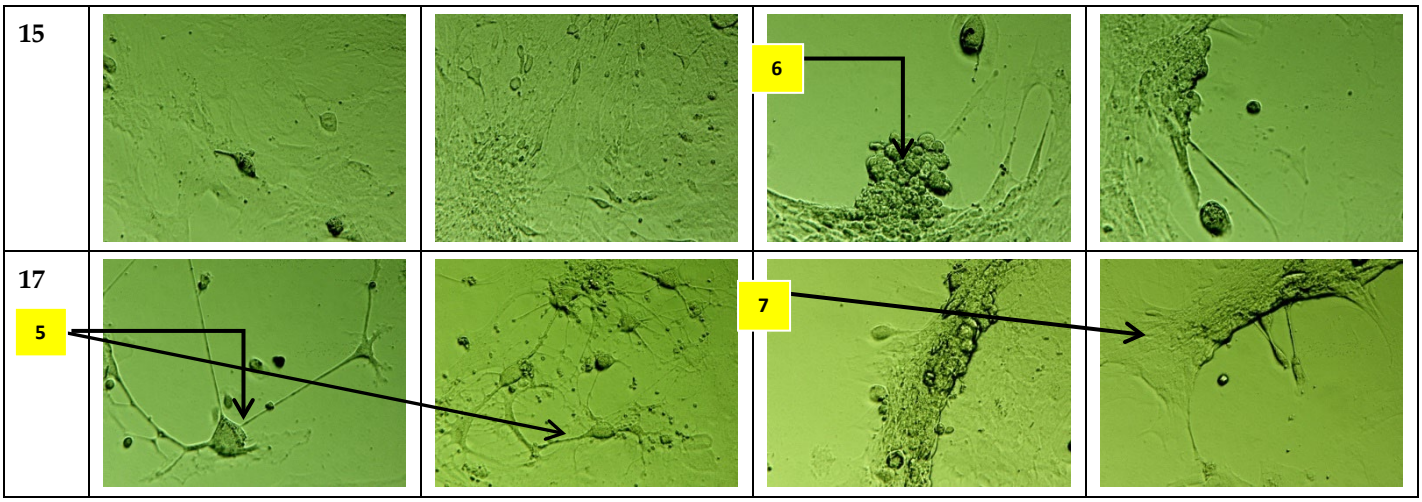


Figure 3 Aspects of phase contrast optical microscopy of control glial cell cultures (from initiation to day 17). Arrows: 1 – cells obtained from inoculum; 2 – differentiated cells from initial inoculum; 3 – cells resulted from initial multiplications; 4 – cellular connections and synapses; 5 – cell differentiation from multiplied cells in culture; 6 – multilayered cells in the culture; 7 – cellular clusters

Phase microscopy was used to observe the morphology of isolated glial cell cultures after 3, 6, 7, 9, 10, 13, 15, and 17 days (Figure 3). These observations allowed interpretation of the morphological development as a consequence of time in culture (up to 17 days). Exploiting glial cells' ability to adhere to uncoated culture flasks, these were isolated from neurons and other components of the nervous tissue. After 3 days in culture, cells were scattered over the bottom of the flasks, as individual cells or small groups, with thin or larger-flat-fan-like patterns. Depending on the homogeneity of the cell suspension and the isolation step after 3 h and 24 h in culture, single neurons were sometimes observed in this step. After 7 days in culture, an evident proliferation of cells was seen, where they gathered in clusters across the bottom of the flasks. Several of these cells had lost their larger-flat-fan-like processes in favor of long, thinner processes. At 15 days in culture, most of the cells had gained a spindle-shaped appearance and covered the bottom of the culture flasks. After 21 days in culture, the cells were multilayered and, in some areas, began to form clusters, and some of them had processes that were aligned and orientated in the same direction (Figure 3).

The morphology of control cell cultures versus those treated with 50 mM ethanol does not differ substantially after 3, 5, 6, or 7 days of culture under these conditions, as can be observed in Figures 4-8. , i.e., days 21, 23, 24, 25, and 27 from the initiation of the culture. This indicates that the 50 mM ethanol concentration is not toxic/lethal to the cells.

In the case of the combined treatment of 50 mM ethanol and 100 μM deferoxamine starting from day 21 after the initiation of cell cultures, we observe an accentuated degradation of the glial cell culture after 5 days of treatment, on the 27th day of initiation, the glial cell culture showing marked aspects of degeneration and necrotic or programmed cell death: an evidence that this treatment is damaging and lethal to the cells in culture. The morphological aspect found by phase contrast microscopy required stopping the cultures 27 days after initiation, sampling the cell culture medium at 48 hours, and obtaining the cell lysates of the cultures for 4 categories: control, ethanol, ethanol + deferoxamine, and respectively, the culture in which the formation of glial scars was thermally induced (related to the emission of the microscope), as presented in the last column of the tables with the morphology of the cultures observed under a microscope by phase contrast figures 4-8*.

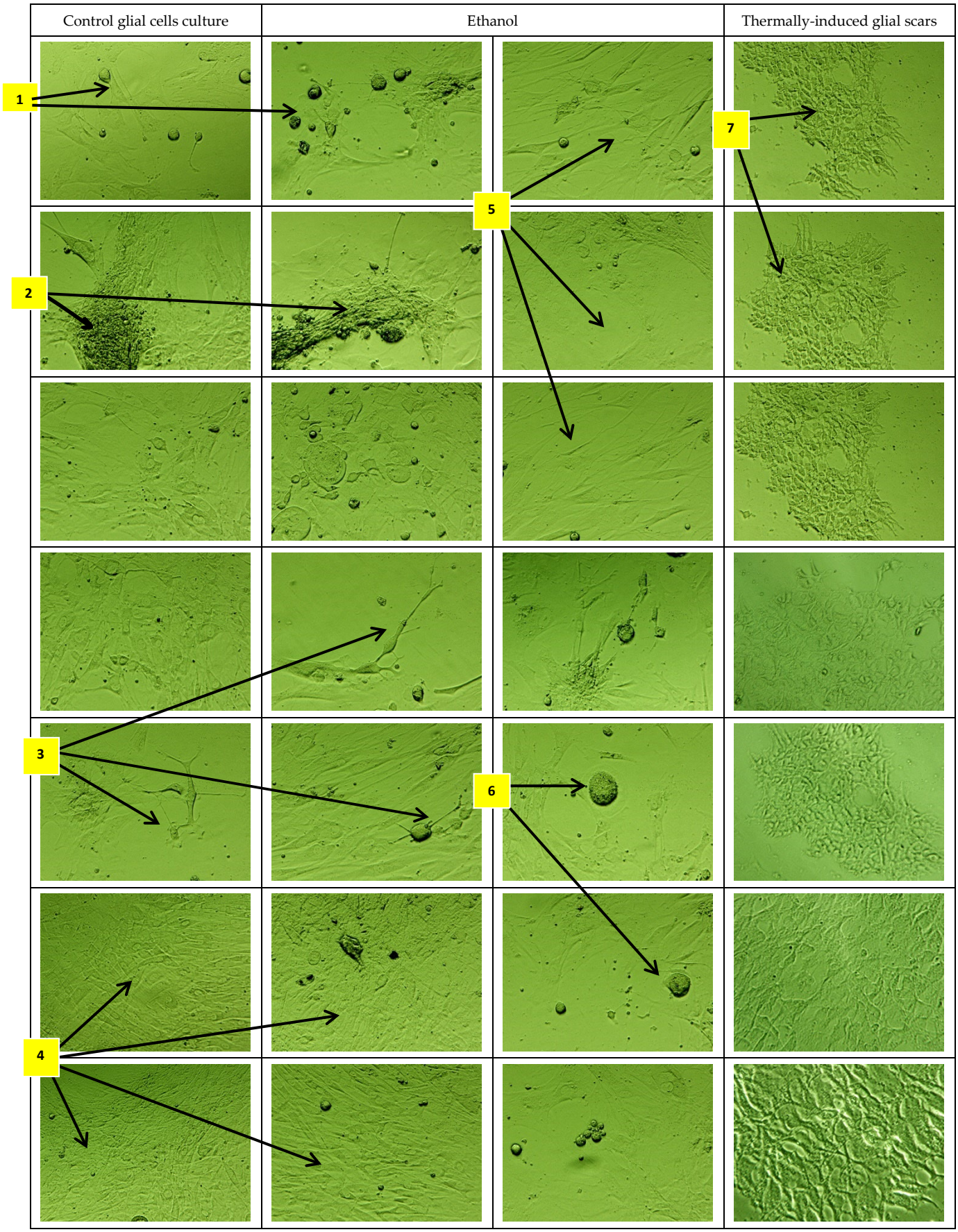


Figure 4 Aspects of phase contrast optical microscopy of control glial cell cultures (**21 days**): the first column presents morphological aspects of control glial cell culture, the second and third columns present morphological aspects of ethanol-treated glial cell culture and the last column shows cellular morphological characteristics of thermally induced glial scars. Arrows: 1 – cells differentiation in the culture monolayer; 2 – multilayered cells in the culture 3 – cellular connections and synapses; 4 – cellular monolayer of differentiated cells; 5 – the larger space between cells in case of ethanol treated culture; 6 – cellular death process; 7 – glial cells scar

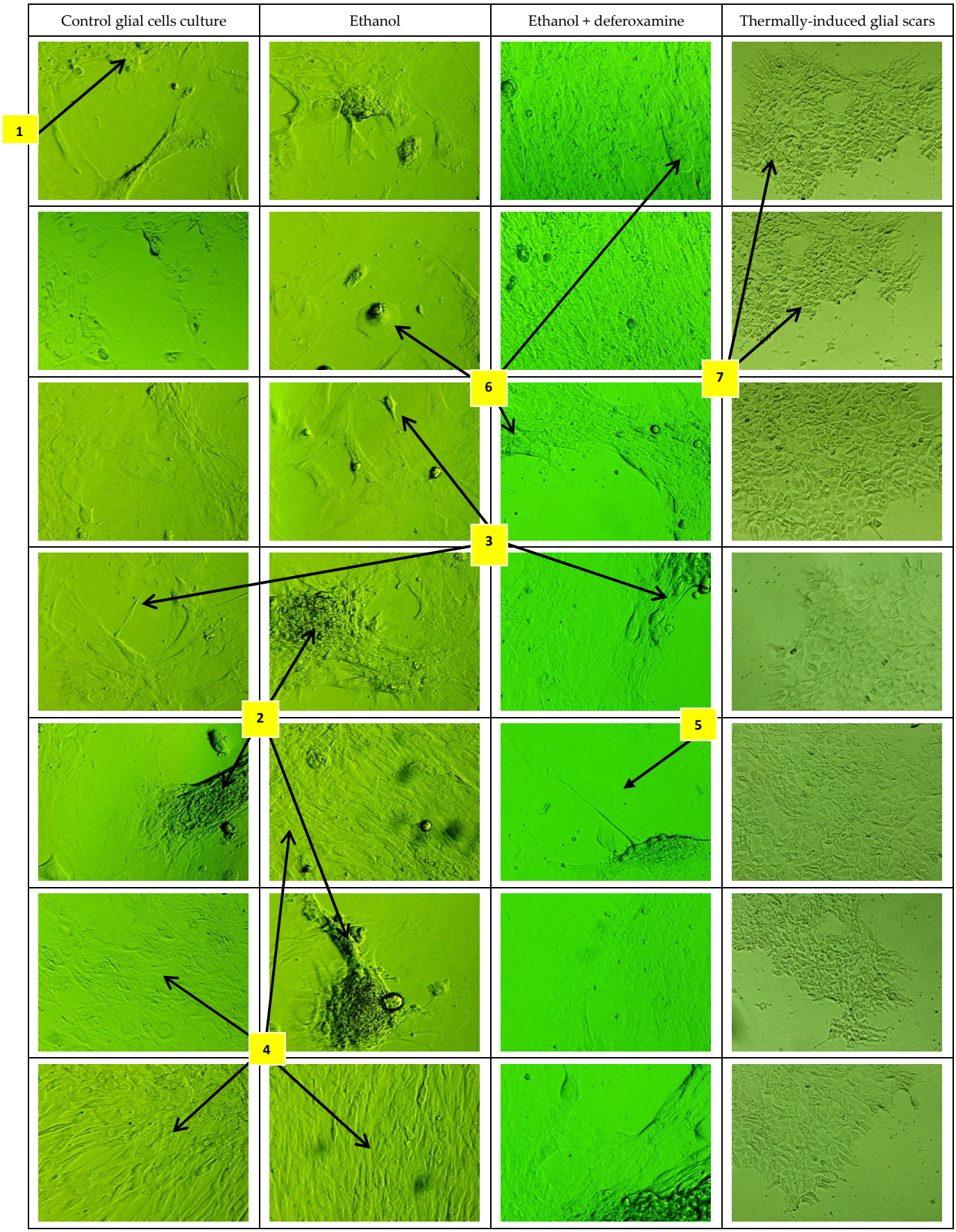


Figure 5 Aspects of phase contrast optical microscopy of control glial cell cultures (**23 days**): the first column presents morphological aspects of control glial cell culture, the second presents aspects of ethanol-treated glial cell culture, the third presents aspects of combined ethanol and deferoxamine treated culture, and the last column shows glial scars. Arrows: 1 – cells differentiation in the culture monolayer; 2 – multilayered cells in the culture 3 – cellular connections and synapses; 4 – cellular monolayer of differentiated cells; 5 – the larger space between cells in case of ethanol + deferoxamine treated culture; 6 – cellular death process; 7 – glial cells scar

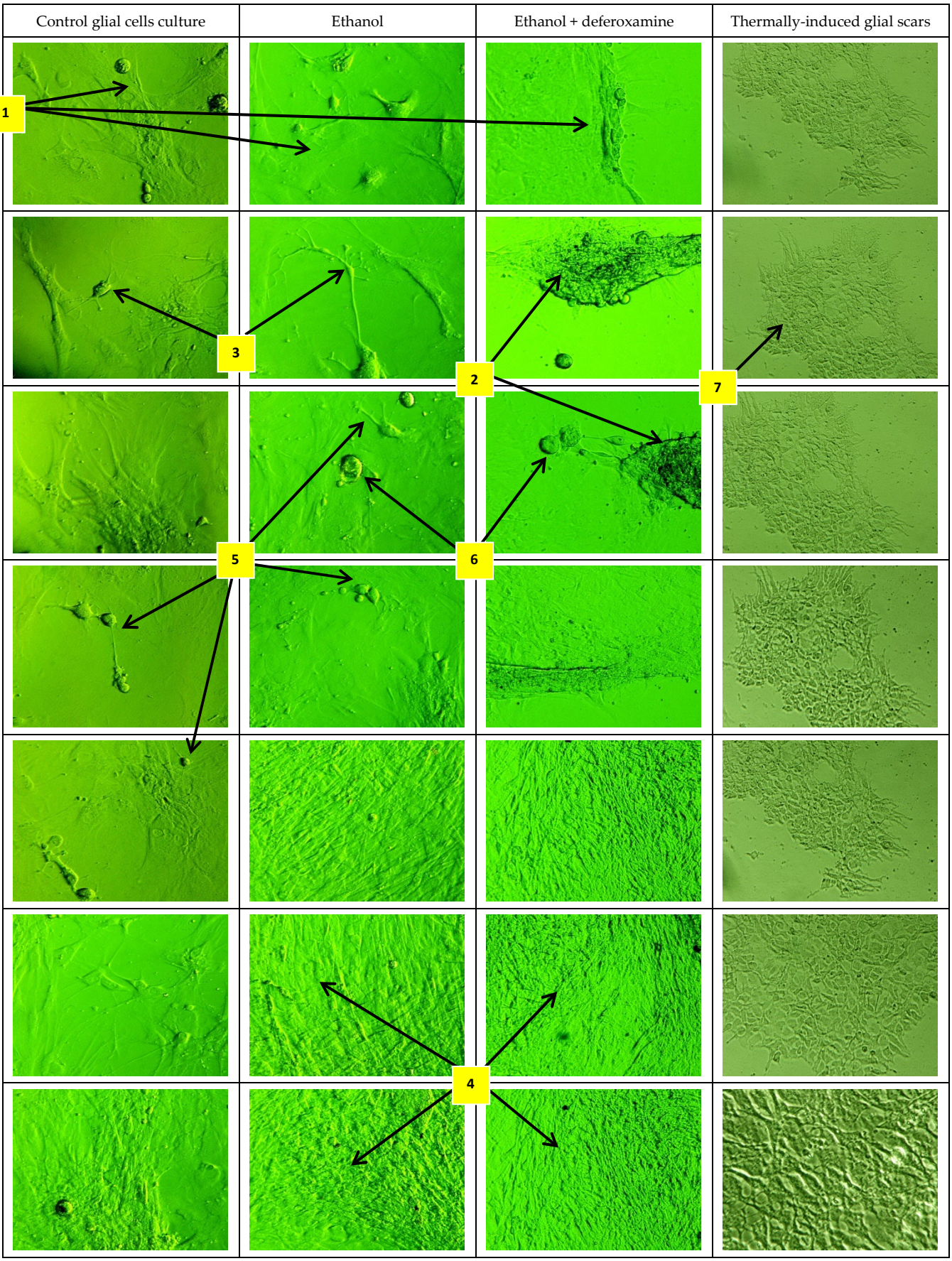


Figure 6 Aspects of phase contrast optical microscopy of control glial cell cultures (**24 days**) : the first column presents morphological aspects of control glial cell culture, the second presents aspects of ethanol-treated glial cell culture, the third presents aspects of combined ethanol and deferoxamine treated culture, and the last column shows glial scars. Arrows: 1 – cells differentiation in the culture monolayer; 2 – multilayered cells in the culture 3 – cellular connections and synapses; 4 – cellular monolayer of differentiated cells; 5 – cells differentiation in the culture monolayer; 6 – cellular death process; 7 – glial cells scar

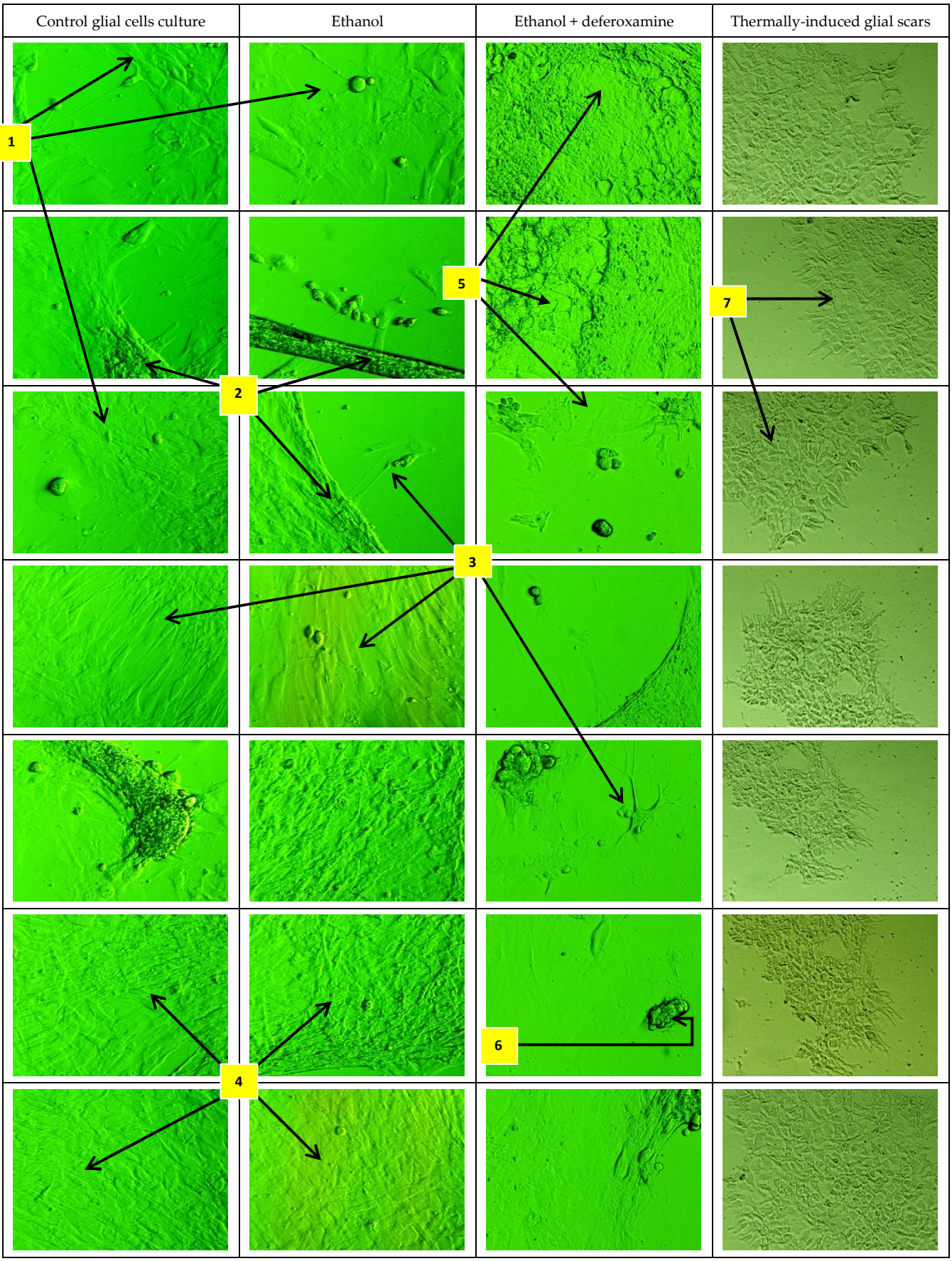


Figure 7 Aspects of phase contrast optical microscopy of control glial cell cultures (**25 days**): the first column presents morphological aspects of control glial cell culture, the second presents aspects of ethanol-treated glial cell culture, the third presents aspects of combined ethanol and deferoxamine treated culture, and the last column shows glial scars. Arrows: 1 – cells differentiation in the culture monolayer; 2 – multilayered cells in the culture 3 – cellular connections and synapses; 4 – cellular monolayer of differentiated cells; 5 – the larger space between cells in case of ethanol + deferoxamine treated culture; 6 – cellular death process; 7 – glial cells scar

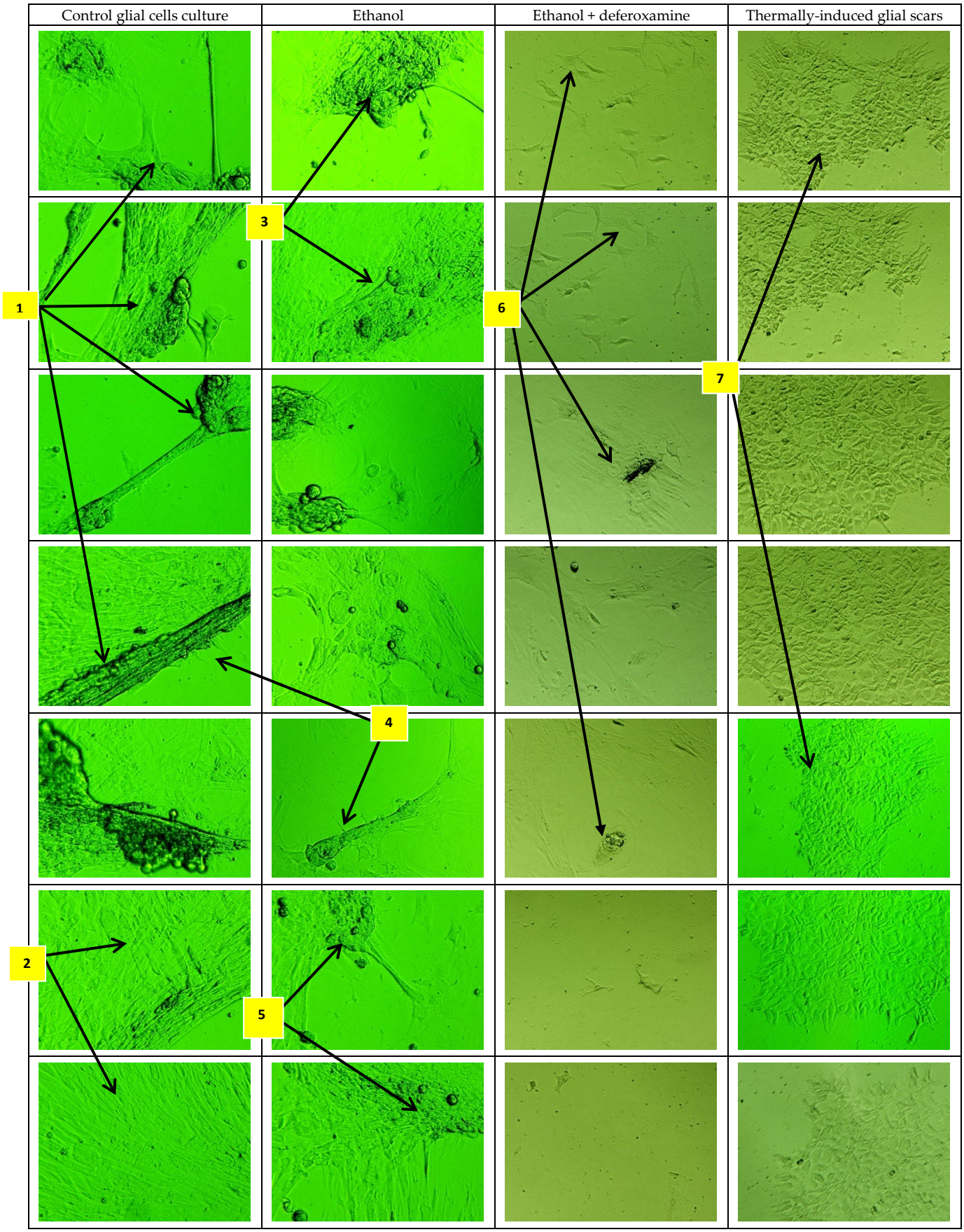
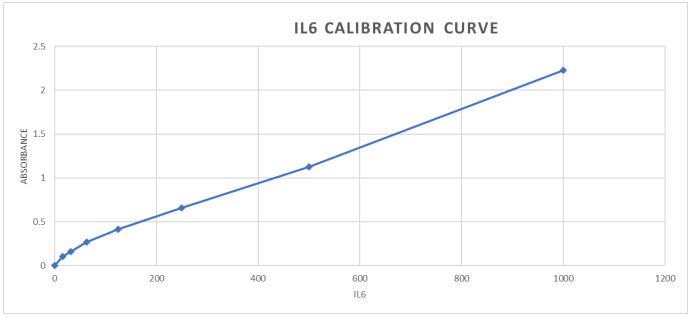
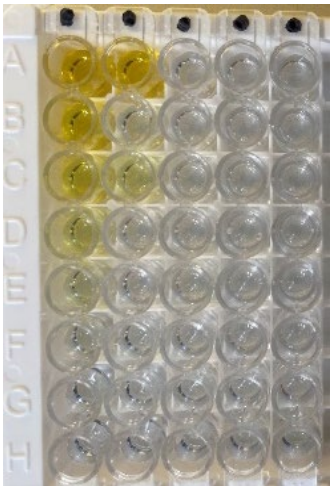


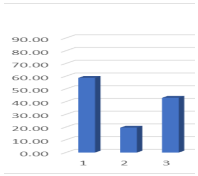
Figure 8 Aspects of phase contrast optical microscopy of control glial cell cultures (**27 days**) : the first column presents morphological aspects of control glial cell culture, the second presents aspects of ethanol-treated glial cell culture, the third presents aspects of combined ethanol and deferoxamine treated culture, and the last column shows glial scars. Arrows: 1 – cellular clusters; 2 – cells differentiation in the culture monolayer; 3 – multilayered cells in the culture; 4 – cellular connections and synapses; 5 – cell differentiation from multiplied cells in culture; 5 – multilayered cells in the culture; 6 – marked aspects of degeneration and necrotic or programmed cell death 7 – glial cells scar

IL6 µg	Abs	Abs - Blank
1000	2.313	2.228
500	1.2111	1.1261
250	0.745	0.66
125	0.504	0.419
62.5	0.355	0.27
31.25	0.248	0.163
15.6	0.191	0.106
0	0.085	0





Sample	Abs	Abs - Blank	IL6 µg
Patient 1 CSF – non alcohoolic, non SCI	0.22	0.135	58.70
Patient 2 CSF – non chronic alcohoolic	0.13	0.045	19.57
Patient 3 CSF – chronic alcohoolic	0.184	0.099	43.04



Sample	Abs	Abs - Blank	IL6 µg
Control – cell lysate	0.187	0.102	44.35
Deferoxamine-Ethanol - cell lysate	0.291	0.206	89.57
Ethanol – cell lysate	0.22	0.135	58.70
Glial Scars – cell lysate	0.2	0.115	50.00
DMEM - medium	0.202	0.117	50.87
Control - medium	0.212	0.127	55.22
Deferoxamine-Ethanol - medium	0.228	0.143	62.17
Ethanol medium	0.268	0.183	79.57
Glial Scars medium	0.257	0.172	74.78

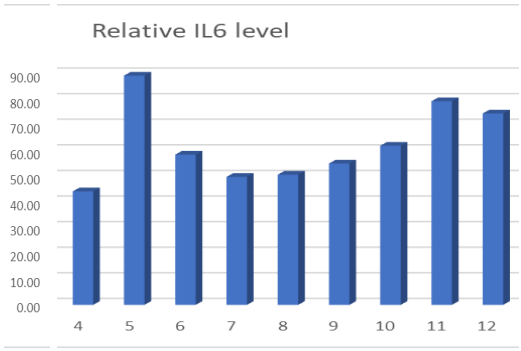
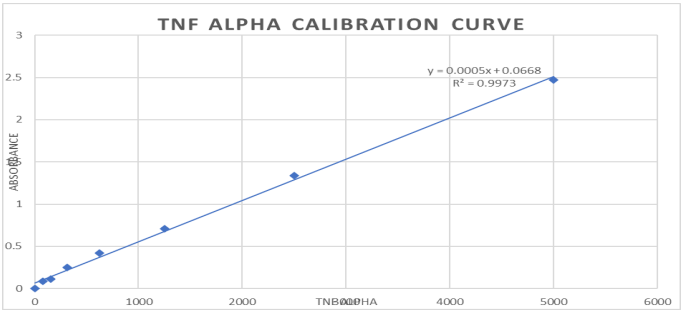
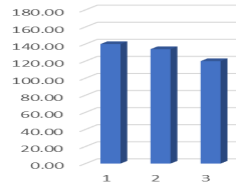


Figure 9 IL6 levels in cerebrospinal fluid (CSF) of three patients with SCI, cell lysate from control, ethanol and ethanol + deferoxamine treated cell cultures and thermally induced glial scars, respectively

TNFα µg	Abs	Abs - Blank
5000	2.496	2.474
2500	1.358	1.336
1250	0.729	0.707
625	0.443	0.421
312	0.274	0.252
156	0.132	0.11
78	0.107	0.085
0	0.022	0



Sample	Abs	Abs - Blank	TNFα µg
Patient 1 CSF – non alcohoolic, non SCI	0.092	0.07	140.00
Patient 2 CSF – non chronic alcohoolic	0.089	0.067	134.00
Patient 3 CSF – chronic alcohoolic	0.082	0.06	120.00



Sample	Abs	Abs - Blank	TNFα µg
Control – cell lysate	0.092	0.07	140.00
Deferoxamine-Ethanol - cell lysate	0.11	0.088	176.00
Ethanol – cell lysate	0.047	0.025	50.00
Glial Scars – cell lysate	0.049	0.027	54.00
DMEM - medium	0.038	0	0.00
Control - medium	0.038	0	0.00
Deferoxamine-Ethanol - medium	0.078	0.04	80.00
Ethanol medium	0.051	0.013	26.00
Glial Scars medium	0.054	0.016	32.00

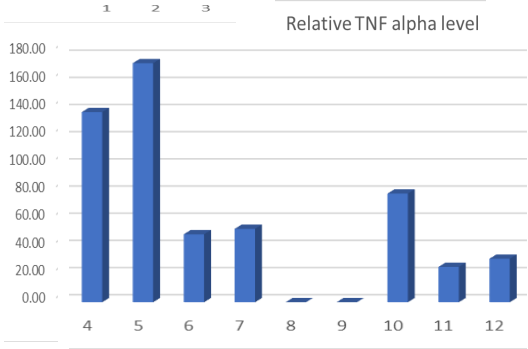


Figure 10 TNFα levels in CSF of three patients with SCI, cell lysate from control, ethanol and ethanol + deferoxamine treated cell cultures and thermically induced glial scars, respectively from collected medium after 48 h

The results obtained show a substantial increase in the level of IL6, in the cell lysate after 27th days after the initiation of cell cultures and 5 days of combined treatment with 50 mM ethanol and 100 μM deferoxamine. By comparison, the level of IL6 in ethanol-treated cultures and in the heat-induced glial scars, respectively, showed only a slight increase in cell lysate, but a higher level in the case of culture medium taken after 48 hours.
Regarding TNFα, we noticed an increase in its level after the combined treatment of deferoxamine and ethanol, but also a consistent reduction compared to the cell lysate of the control culture, after simple treatment with ethanol or after thermal induction of glial scars. Its detection in the medium with a higher concentration, taken after 48 hours after the combined treatment of ethanol and deferoxamine, shows the release of this cytokine from the destroyed cells and maybe its "sequestration" in cells from the glial scars.
To be noted that regarding the analysis of the CSF of the three samples investigated (only three because harvesting such a biological product is very demanding, especially from an ethical perspective: it is acceptable only if a patient with tSCI has the dura mater broken and thus CSF is flowing anyway; this makes the possibility to obtain such samples to be very rare because, well known, most of the tSCI pathogenic mechanisms are by just cord contusion) however of the three patient whose CSF samples have been analyzed, two of them were nonethanol chronic consumers and Patient 2 was regular alcohol drinker. Under these conditions, it is to be remarked the obvious lower level of IL6. This is in line with the physiopathological discussion regarding the (including) spinal cord ethanolic impregnation in chronic alcohol consumers, but obviously, more related samples will be necessary in order to draw factual findings and conclusions on this subject.

DISCUSSION

Glial cells: astrocytes, microglia, oligodendrocytes, and ependymal cells, play several essential roles in the central nervous system (CNS), such as maintaining homeostasis, actively involvement in synaptogenesis, including with the fact that astrocytes are known to secrete many proteins which have been shown to be important in the function of this system (44) (45). Therefore, recently is now recognized that glial cultures are an important instrument for basic and translational research, including with the use of primary glial cell cultures being considered more relevant for investigating human neuro-pathology. The McCarthy and de Vellis (1980) method is considered an "invaluable method for isolation of neonatal astrocyte-like cells" (46).
Glial cells play detrimental roles after (t)SCI –very briefly summarized in this article – but possibly also some beneficial ones "particularly as regards neural protection and repair, and regulation of CNS inflammation" (47) thus warranting their more in-depth quest (48).
During the first few days of culture, the images show that there is a mixed population. The amount of astrocytes in the central nervous system is known to be significant. To be noted that "The ratio of astrocytes to neurons is 1:3 in the cortex of mice and rats, whereas there are 1.4 astrocytes per neuron in the human cortex" (49).
"After SCI, the injured tissue secretes cytokines and chemokines into the circulatory system, recruiting monocytes to the injury site. These monocytes become macrophages … upon reaching the injury site. These macrophages play various roles in wound healing"
During inflammation, the CNS can release large numbers of inflammatory mediators, including neurotransmitters, cytokines, chemokines, and active oxygen free radicals (reactive oxygen species – ROS – o. n.). … Pro-inflammatory cytokines, such as TNF-α, and IL-6, have multiple roles in both neurodegeneration and protection", being involved in "development and resolution of acute and chronic inflammation". (43).
For instance – related including to a focus of this work – "inflammatory cytokines like TNF-α activate NADPH oxidase to trigger the release of ROS" (Anwar et al., 2016).
Regarding ethanol: "Long-term exposure (7 days) … more toxic than an acute (24 hours) exposure, showing a biphasic, hormetic effect on the IL-6 secretion … maximum

stimulation was reached for 50-mM ethanol. In contrast, ethanol reduced the TNF-alpha secretion" (52).

We found in our study that ethanol in concentrations up to 100 mM strongly inhibits the TNF-alpha secretion from cultured astrocytes after 7 days of exposure, whereas 24 hours of exposure did not significantly influence the TNF-alpha secretion.

"IL-6 can down-regulate the expression of TNF-alpha"(24). This is in line with our results, where we found the first significant decrease in the TNF-alpha level at the highest level of IL-6 after long-term exposure to ethanol. Therefore, we could speculate – since, as known in the literature and confirmed by our related studies too: the CNS lesions, including (t)SCI, are highly complex, with a very high/ deep intermingle between detrimental and protective/ beneficial processes, and actually there is no intervention able to cure them – that chronic ethanolic impregnation of this kind of tissue might exert, at intimate biologic level, a sort of mitohormesis (https://www.news-medical.net/life-sciences/What-is-Mitohormesis.aspx) effect, including a possible stimulation of the antioxidant response element (ARE) (53).

Long-term exposure to ethanol and acetaldehyde results in more pronounced effects. "Since the obtained dose-response relationship is non-linear and non-threshold but hormestic, it seems that the concentration of ethanol or acetaldehyde (the metabolite of ethanol)" (54) results in effects that can be opposide: neurodegenerative and/or neuroregenerative"/ neuroprotective ("neurosurvival") pending on the length of action concentration and detrimental inflammatory action produced (24).

LIMITATIONS

Our study has several limitations. First, due to technical limitations, we could obtain only one culture flask for each kind of presented glial cells cultures. Secondly, we think that it was appropriate for a better understanding of differences in the citokines level to have another culture flask for deferoxamine only treatment.

CONCLUSIONS

The experimental data obtained allows us to express the general conclusion that ethanol at a concentration of 50 mM does not cause effects on the survival of cultured glial cells, but instead produces significant changes in the cytokine cocktail they secrete and which influences the inflammatory process. The combination of is deeply harmful to cells in culture and demonstrates that the effect of chronic alcoholism, observed to be protective in spinal cord injuries, is not based on the mechanisms of hypoxia induction that deferoxamine generates (33), nor have been observed actions of deferoxamine as "a chelator in the management of patients with acute or chronic iron toxicity" (55) thereby with reported in the literature of some capabilities to "repair spinal cord injury by inhibiting ferroptosis"(56) – a mechanical traumatic lesional pattern that was not applied in our experimental study.

Our research was carried out respecting the rights of animals, according to the legislation in force (57,58) (59).

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