Human pluripotent NT2/D1 cells immobilized in alginate microfibers: a 3D system for testing the effects of bioactive compounds

Jelena Lj. Pejić[1], Milena C. Milivojević[1], Marija L. Schwirtlich[1], Jelena S. Petrović,[2,3] Jasmina J. Stojkovska,[2] Milena J. Stevanović[1,4,5] and Marija M. Mojsin[1]

[1]*University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia*
[2]*University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia*
[3]*Innovation Centre of the Faculty of Technology and Metallurgy, Belgrade, Serbia*
[4]*University of Belgrade, Faculty of Biology, Belgrade, Serbia*
[5]*Serbian Academy of Sciences and Arts, Belgrade, Serbia*

Abstract

Extensive consumption of energy drinks (ED) and alcohol mixed with energy drinks (AmED) has become a prevalent practice among young people. Marked as enhancers of physical and mental performance due to their high levels of stimulant ingredients, such as caffeine and taurine, ED and AmED consumption can potentially cause adverse effects on the central nervous system (CNS) and cardiovascular system. Despite the role of stem cells in development and tissue renewal, studies examining the potential effects of consumption on these cells are lacking. In this study, we established a 3D system based on alginate microfibers to test the effects of bioactive compounds on human NT2/D1 embryonal carcinoma cells, a widely used malignant counterpart of human stem cells. We also assessed the effects of simulated acute ED and AmED consumption on the viability of pluripotent cells and evaluated the efficiency of mass transport to the cells using mathematical modeling. The obtained results show that the 3D system enables undisturbed growth and proliferation of NT2/D1 cells and uniform distribution of the tested compounds to all cells within the microfiber. Simulated acute ED and AmED consumption in the 3D culture did not affect the viability of NT2/D1 cells, compared to a 2D culture, where caffeine induced a significant, yet small, decrease in cell viability.

Keywords: Human embryonal carcinoma cells; alginate hydrogels; cell viability; mass transport; energy drinks; caffeine.

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

ORIGINAL SCIENTIFIC PAPER

UDC: 616-089.22:677.469:576.5

Hem. Ind. **00(0)** 000-000 (2025)

1. INTRODUCTION

Energy drinks (ED) are non-alcoholic beverages widely consumed by children, adolescents, and young people [1,2]. The major constituents of ED, caffeine and taurine, present in high concentrations, have been shown to enhance mental and physical performance [1]. On the other hand, excessive consumption of ED can cause significant health issues, primarily in the cardiovascular and neurological systems, raising health concerns and the necessity for regulatory guidelines addressing the safe consumption of ED [3,4]. Consumption of ED in combination with alcohol (alcohol mixed with energy drinks - AmED), an increasingly popular trend among adolescents, leads to altered and risk-taking behavior and makes them more prone to intoxication than consuming ED alone [5]. Studies addressing this issue were mainly focused on the effects of ED on mature cells of neural and cardiovascular origins [6-8]. Research, which analyzed the impact of ED constituents on human induced pluripotent stem cell (iPSC)-derived cardiomyocytes, shed light on the potential adverse effects these components may exert on the cardiovascular system [6]. The negative effect of caffeine and taurine, used in concentrations labeled on ED, was also confirmed in immature oligodendrocytes and hippocampal neurons derived from P0-P1 Wistar rats, causing a disturbance in the development capacity of oligodendrocytes and in the morphology of neurons [7]. In addition, research on human neuronal cell line SH-SY5Y showed that major ED constituents, caffeine, taurine, and guarana, caused the excessive removal of antioxidant species in cells, thus causing antioxidative stress, which disrupts cellular homeostasis [8]. Despite the importance of stem cells and progenitor cells in both embryonal and adult development,

Corresponding author: Marija M. Mojsin, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade
E-mail: mojsin@imgge.bg.ac.rs
Paper received: 24 July 2024; Paper accepted: 30 July 2025; Paper published: 5 September 2025.
https://doi.org/10.2298/HEMIND240724011P

research regarding the effects of ED on these cell types is missing. Therefore, there is a need for the development of adequate and relevant models based on stem cells and progenitor cells.

NT2/D1 cell line, a pluripotent embryonal carcinoma (EC) cells [9], could be used to create such models since it is considered the malignant counterpart of human embryonic stem (ES) cells [10]. Previous studies demonstrated that human EC cells and human ES cells share significant genetic similarities. Microarray analysis identified 330 genes as highly expressed in both cell types, particularly pluripotency-related genes such as POU5F1 (Oct4), suggesting a common molecular basis underlying their pluripotent phenotype [11]. Additionally, it was shown that EC cells and ES cells exhibit genetic similarities in the expression of pluripotency markers such as SSEA3, SSEA4, and Oct4, also in class 1 MHC antigens and Thy1, reflecting their common embryonic origins [9]. EC cells, such as NT2/D1 cell line, despite originating from tumors, offer a simplified model compared to ES cells, thus facilitating the study of the effect of diverse compounds on the developmental mechanisms of humans without ethical concerns and providing complementary insights alongside ES cells [9].

2D cell culture is the most suitable, cost-effective, and widely used approach for *in vitro* monitoring of cell growth and analyzing the cell response upon drug treatment [12,13]. Despite being the effective approach for the first step in drug screening, it has many limitations due to the absence or changed cell morphology, polarity, cell-to-cell, and cell-to-ECM interactions present in native tissues in the human body, important for maintaining a physiologically relevant microenvironment [13,14]. These limitations could be addressed using experimental animals as *in vivo* models. Although widely used in research, significant differences between animals and humans prevent the extrapolation of results obtained in animal models to clinical trials [15]. Studies also showed large differences between human and mouse embryonic stem cells in morphology, tempo of differentiation, expression profiles of genes involved in the regulation of cell cycle, cell death, differentiation, etc [15-18]. Consequently, certain stimuli, e.g, signaling molecules, compounds, that affect some biological processes in mouse cells could have either negligible effect or the opposite effect in human cells . Therefore, 3D cell culture models emerged as necessary to bridge the gap between two widely used approaches for drug screening - 2D cell culture and animal models [13].These challenges are recognised by EU Directive 2010/63, which encourages the 3Rs principle (refine, reduce and replace) to develop alternative three-dimensional (3D) models that can mimic human tissues and disease complexity for research and drug testing [19]. NT2/D1 cells were successfully differentiated into mature neurons in a 3D model of sphere formation [20] and alginate hydrogels [21]. Cell immobilization in alginate hydrogels shows great potential due to the natural origin of alginate, enabling cells to grow and interact within, and allowing the exchange of metabolites, oxygen, and stimuli between cells in fibers and the surrounding medium [22,23].

The aim of this research was to establish a 3D model system of pluripotent NT2/D1 cells immobilized in alginate microfibers and analyze the potential effects of ED and AmED consumption on the viability of human pluripotent cells.

2. EXPERIMENTAL

2. 1. Cell culture

NT2/D1 cells, a kind gift from Prof. Peter W. Andrews (University of Sheffield, UK), were grown in Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4.5 g dm^{-3} glucose) supplemented with 10 % Fetal Bovine Serum (FBS) and Antibiotic-Antimycotic solution (10,000 units cm^{-3} of penicillin, 10,000 μg cm^{-3} of streptomycin, and 25 μg cm^{-3} of Amphotericin B), all purchased from Thermo Fisher Scientific (MA, USD). Cells were maintained in cell incubators at 37 °C, 10 % CO_2 and high humidity atmosphere. A 2D model system of NT2/D1 cells was maintained by propagating cells in standard adherent culture dishes where these cells grow in a monolayer. A 3D culture system was obtained by immobilization of NT2/D1 cells in alginate microfibers as previously described [22]. Briefly, sodium alginate (A3249, low viscosity, AppliChem, Germany) solution (1.5 wt.%) with cells (5×10^6 cells cm^{-3} alginate) was manually extruded through a blunt edge stainless steel needle (25 gauge, Small Parts Inc., USA) into a gelling bath containing 0.18 M Ca^{2+} ions ($CaCl_2{\cdot}2H_2O$, Sigma-Aldrich, USA). After complete gelling (15 min incubation in the gelling bath), microfibers were washed in a cell culture medium without FBS and finally maintained in the cell culture conditions described above. Alginate microfibers were visualized by light microscopy and diameters were measured using Leica LAS v4.12 Software. Measurements were performed in three biological replicates, with three independent average diameter calculations for each alginate microfiber.

2. 2. Cell recovery

Immobilized NT2/D1 cells were released by dissolving alginate microfibers in 2 % w/v sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, Sigma-Aldrich, USA) solution (0.1 g of microfibers in 1 cm^3 of the solution) for 10 min at 37 °C. Cell suspension was pelleted by centrifugation at 1300 rpm for 5 min. The supernatant was discarded and the cell pellet was washed two times with the cell culture medium. Cell number was determined using the Trypan blue exclusion test of cell viability. Retrieved cells were cultivated in adherent culture dishes for 72 h and analyzed for viability, adhesion, and proliferative capacity.

2. 3. Cell treatments

NT2/D1 cells in 2D and 3D cell cultures were treated with the main components of energy drinks, caffeine (CAF) and taurine (TAU), individually, together (CAF+TAU) and in combination with alcohol (CAF+TAU+EtOH); energy drink alone (ED) or in combination with alcohol (ED+EtOH). Components were dissolved in a cell culture medium in concentrations corresponding to their actual amounts in the energy drink, *i.e.* 0.32 mg cm^{-3} caffeine and 4 mg cm^{-3} taurine. Energy drink samples (500 mm^3 each) were dried in a vacuum concentrator (Concentrator 5301, Eppendorf). Then, dry pellets were dissolved in initial volumes (500 mm^3) of cell culture medium to preserve the original concentrations of all components and pH was adjusted to 7.4 using 0.1 N NaOH/HCl [6]. Final concentrations of all components in the cell culture medium during treatments were ten times lower than the original concentrations in energy drinks to simulate the real consumption *in vivo* [6].

Ethanol, in a concentration of 20 mM, was used with caffeine and taurine or ED to simulate AmED consumption. This concentration corresponds to the physiologically relevant blood alcohol content in adults after quick consumption of a few drinks [24]. For treatments, NT2/D1 cells were cultured in 96-well plates, either seeded in 2D monolayer culture (10^4 cells per well) or in 3D culture immobilized in alginate microfibers (cut to 1 cm in length per well). Treatment was initiated 24 h after cell seeding in both cell culture conditions - 2D monolayer and 3D microfiber culture, and it lasted for 24 h to simulate acute consumption, as described previously [7,25]. Untreated NT2/D1 cells were used as a control since all components were dissolved in the cell culture medium.

2. 4. Fixation of NT2/D1 cells immobilized in alginate microfibers

NT2/D1 cells immobilized and propagated within alginate microfibers for 7 days were washed once in Phosphate Buffered Saline (PBS) and incubated in Fixative Solution (Cell Signaling Technology, Inc., Danvers, MA, USA) for 15 min at room temperature. After incubation, cells were rinsed two times in PBS and visually inspected using a Leica DM IL LED Inverted Microscope (Leica Microsystems, Leica Microsystems GmbH, Germany). During fixation and washing in PBS, alginate microfibers were disintegrated, but cell clusters remained intact.

2. 5. MTT assay

Viability of NT2/D1 cells was assessed using a colorimetric MTT test. This assay measures cell viability by assessment of the mitochondrial ability to metabolize MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Merck & Co., Inc., USA). In viable, metabolically active cells, MTT is reduced into purple formazan crystals, which can be visually confirmed by light microscopy or spectrophotometrically measured upon solubilization in DMSO (dimethyl sulfoxide) [26]. For the visual inspection of NT2/D1 cells during the 7 day propagation in alginate microfibers, cells in 3D culture were incubated with MTT solution (MTT dissolved in cell culture medium at a concentration 0.5 mg cm^{-3}) for 24 h, at four time points – after 1, 2, 3 and 7 days at 37°C in cell incubator. Images were taken using a Leica DM IL LED Inverted Microscope (Leica Microsystems, Leica Microsystems GmbH, Germany). Colorimetric measurements were performed as previously described [26,27]. NT2/D1 cells grown in 96-well plates, in 2D and 3D cell cultures, were incubated with MTT solution (MTT dissolved in cell culture medium at a concentration of 0.5 mg cm^{-3}) for 4 h at 37 °C in a cell incubator. After incubation, the MTT solution was discarded, and water-insoluble formazan crystals were dissolved by incubation in DMSO for 5 min with agitation at room temperature. Solubilization of formazan was monitored by visual inspection of the alginate microfibers using light microscopy. Absorbances of the dissolved formazan solutions were measured at 570 nm using a microplate reader, Tecan Infinite 200 PRO (Tecan Group Ltd., Switzerland). Statistical analysis was performed with SPSS statistical

software package (SPSS Inc. Released 1999. SPSS for Windows, Version 10.0., USA). Cell viability was calculated and presented as a percentage of the corresponding untreated control NT2/D1 cells (either 2D or 3D) expressed as 100 %. Data were expressed as means ± SEM (Standard Error of the Means).

2. 6. Live/dead assay

Effects of ED and AmED treatments on the viability of NT2/D1 cells in alginate microfibers were evaluated using LIVE/DEAD™ Cell Imaging Kit (488/570) (Invitrogen, ThermoFisher Scientific, USA). Live/dead assay is based on two distinctive characteristics of live cells - membrane integrity and intracellular esterase activity. In this two-color assay, cells are simultaneously stained with green-fluorescent calcein-AM (indicating live cells with intracellular esterase activity) and red-fluorescent BOBO-3 Iodide (indicating dead cells that lost membrane integrity). NT2/D1 cells immobilized in alginate microfibers were propagated for 48 h in the cell culture (24 h without treatment and 24 h with/without treatment) and stained according to the manufacturer's instructions. Images of the stained NT2/D1 cells immobilized in alginate microfibers were taken using a Leica TCS SP7 confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). Sequence of 40 cross section images (5 µm each) of alginate microfibers were analyzed. Z stacks were overlapped by Maximal intensity processing tool of LAS AF software (Leica Microsystems, Leica Microsystems GmbH, Germany).

3. RESULTS AND DISCUSSION

3. 1. Immobilization of NT2/D1 cells in alginate microfibers

NT2/D1 cells were successfully immobilized in alginate microfibers with consistent and uniform size (average diameter of 190 ± 10 µm, Fig. 1) using manual extrusion of cell/alginate suspension (5×10^6 cells cm^{-3}, 1.5 % w/w alginate) into the gelling bath containing Ca^{2+} ions. The obtained alginate microfibers with immobilized cells were further cultivated in standard cell culture conditions for up to 7 days.

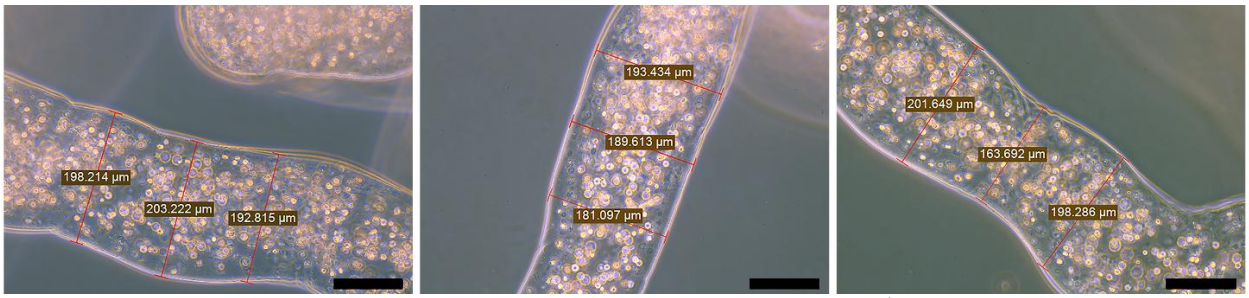

Figure 1. *Representative optical micrographs of alginate microfibers with immobilized NT2/D1 cells showing diameter measurements. Scale bar: 100µm*

Morphology of NT2/D1 cells and their growth in alginate microfibers were analyzed by light microscopy (Fig. 2, Panel A). Representative images of cells captured during the propagation have shown uniform cell distribution in microfibers, as well as a continuous increase in cell number during the 7-day course of cultivation (Fig. 2, Panel A). In addition, cells spontaneously form cellular aggregates and clusters of cells (large cellular aggregates) (Fig. 2, Panel A). At the first time point, day 1, we observed a high density of single cells within microfibers (Fig. 2, Panel A, day1), while increased number of cells during prolonged growth in alginate microfibers resulted in cell aggregation (Fig. 2, Panel A, days 2 and 3) and the formation of cell clusters at day 7 (Fig. 2, Panel A). To confirm cellular aggregation and clustering, we performed chemical fixation of the NT2/D1 cells after 7 days of propagation in alginate microfibers (Fig. 2, Panel B). Cell fixation ensured the preservation of the original state, shape and structures of cellular aggregates and clusters (Fig. 2, Panel B) and corroborate previous observation that NT2/D1 cells in alginate microfibers proliferate and establish cell-to-cell contacts to enhance cell viability and functionality within cellular aggregates [28].

These results were also confirmed using MTT assay for visualization of viable cells within alginate fibers. As presented in Figure 2, Panel C, the intensity of formazan dye, indicator of the presence of live cells, markedly increased during

7 days of propagation. MTT assay also showed cell aggregations at day 2 and 3 and cell cluster formation at day 7 (Fig. 2, Panel C, Images g-i).

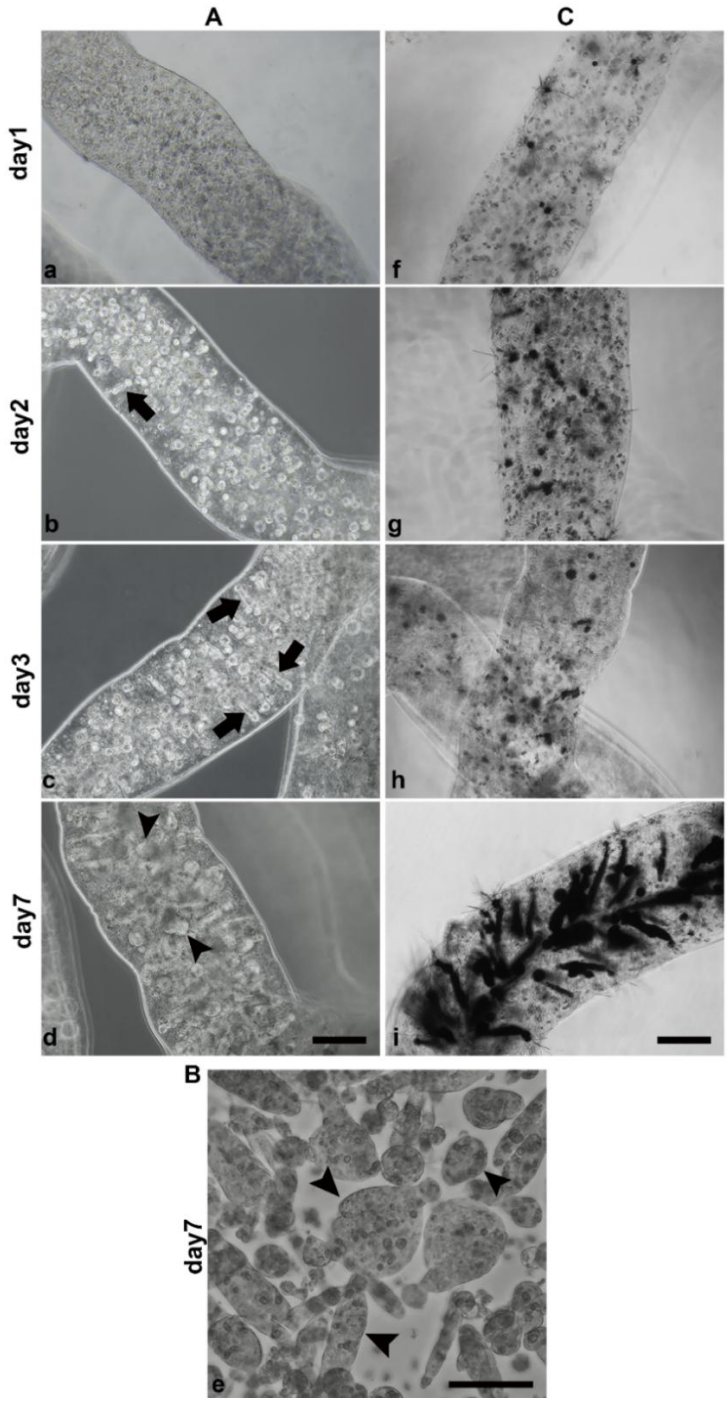

Figure 2. *NT2/D1 cells during a 7-day course of cells growing in alginate microfibers. Light microscopy of A) NT2/D1 cells in alginate microfibers at indicated time points. Black arrows indicate cell aggregates at days 2 and 3 and cell clusters at day 7. Scale bar: 100 µm. B) Fixation of NT2/D1 cells after 7 day propagation showing cellular clusters (black arrowheads) in alginate microfibers. Scale bar-50µm. C) Formazan dyed NT2/D1 cells in alginate microfibers at indicated time points. Scale bar: 100 µm.*

We also wanted to investigate the effect of cell retrieving procedure on the viability, proliferation capacity and adhesion potential of immobilized cells. Immobilized NT2/D1, cells were retrieved from microfibers 24 h after immobilization, counted and seeded in adherent culture dishes for growth in 2D culture, Figure 3. Concentration of

retrieved NT2/D1 cells was 1.5×10^6 cells per cm^3. Compared to $5x10^6$ cells per cm^3, initially immobilized in alginate microfibers, the observed cell loss was significant (~3 fold).

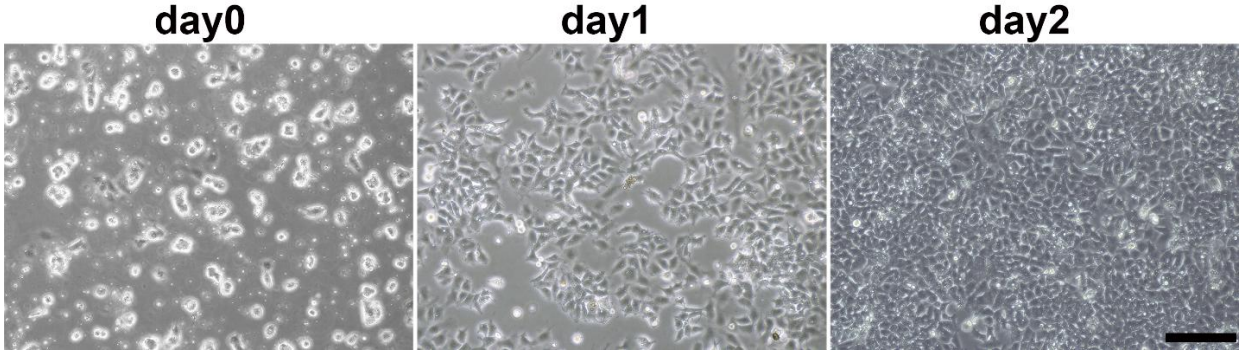

Figure 3. *Light microscopy of NT2/D1 cells retrieved from alginate microfibers and propagated in 2D cell culture for the indicated periods of time. Scale bar-100 μm*

The observed result is comparable to the cell number reduction during retrieval from alginate microfiber previously described in similar studies [29,30] and it is due to cytotoxicity of Na-citrate [31]. Sodium citrate inhibits proliferation and induces apoptosis of hepatocellular carcinoma cells [31,32]. In addition, increased sensitivity of NT2/D1 cells to cytotoxic agents was previously established [33]. Nevertheless, retrieved NT2/D1 cells seeded in monolayer 2D culture retained viability, ability to attach to a culture dish surface and capacity to proliferate (compare images at day 0, day 1, and day 2 in Figure 3).

NT2/D1 cells were previously maintained in both 2D (adherent monolayer culture) and 3D model systems (cultivation in non-adherent conditions that promote spheres formation) [34]. That study showed remarkable differences in NT2/D1 cells growth under 2D and 3D culturing conditions [34]. Cells in the 2D culture had 5 times higher proliferative capacity then spheroid culture that was limited by the gradual decrease in oxygen concentration from the periphery to the center of spheroid [34]. Hypoxic condition in the center of spheroid induced very low cell proliferation rate in that zone [34]. Limitations in oxygen and nutrients diffusion, accumulation of metabolic waste and consequent cell necrosis in spheroids [35,36] could be overcome using alginate 3D cell culture models. As a matter of fact, NT2/D1 cells were successfully encapsulated in alginate beads [21]. In order to create a 3D model for efficient differentiation of NT2/D1 cells in dopaminergic neurons authors tested optimal conditions (cell density and permeability of the beads) for encapsulation of cells in alginate beads [21].They showed that 1.0×10^6 cells cm^{-3} encapsulated in the highly permeable alginate beads (obtained by 0.1 M $CaCl_2$ in the gelling bath) promoted high exchange between cells and medium and consequently increased viability, proliferation, and metabolic activity of NT2/D1 cells [21]. Presented results confirm that biocompatible, immunogenic, and non-toxic alginate hydrogels are highly suitable for supporting the growth and propagation of human pluripotent NT2/D1 cells in a 3D environment.

We also detected differences in morphology of NT2/D1 cells grown in alginate microfibers and 2D monolayer cultures, which is in concordance with previous observations [37-39]. This result could be explained by the fact that cells in the 3D cell culture gained a round shape, due to a single growth direction and the absence of cell attachment to the cultureware. This is in contrast to the flattened shape of cells in a 2D monolayer, which is in agreement with results from previous studies. [40]

3. 2. The effects of ED and AmED treatments on the viability of NT2/D1 cells in 2D and 3D model systems

In order to assess the effects of treatments on cells in 2D and 3D cell cultures, the cells were exposed for 24 h to treatments that simulated the consumption of energy drinks and their major components (caffeine and taurine) alone or in combination with alcohol. Experimental settings, duration and concentrations of tested compounds were in concordance with the previously conducted studies [7,25]. Accordingly, the following treatments were applied: caffeine (CAF); taurine (TAU); the combination of caffeine and taurine (CAF+TAU); caffeine and taurine in combination with alcohol (CAF+TAU+EtOH); energy drink (ED); ED in combination with alcohol (ED+EtOH).

After 24 h of treatment, microfibers in all treated groups preserved their integrity, while NT2/D1 cells preserved their morphology compared to the untreated control (Fig. 4, Panel A). To assess the potential effects of ED and AmED treatments on the viability of NT2/D1 cells in 2D and 3D cell cultures MTT assay was used (Fig. 4, Panel B).

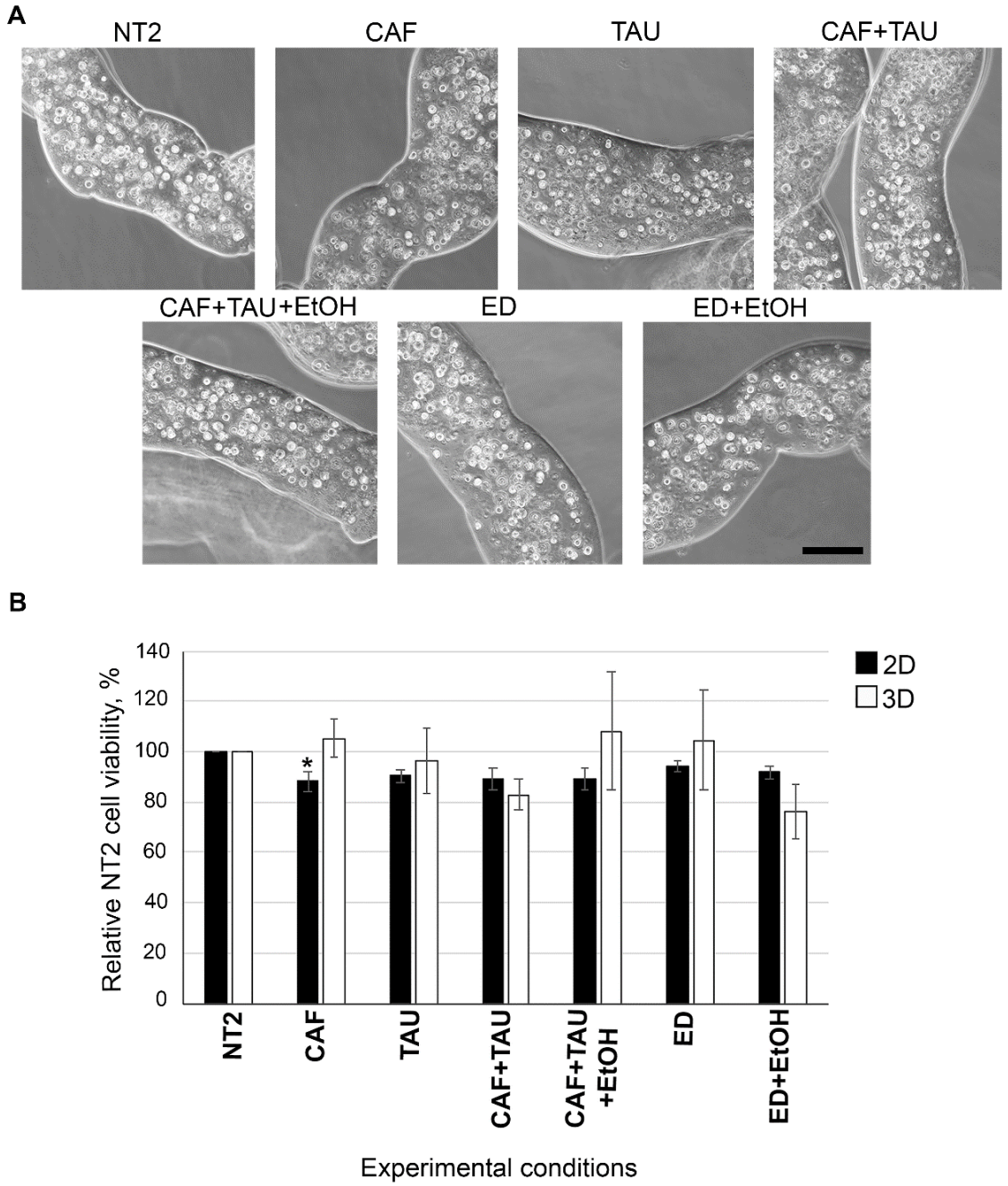

Figure 4: *NT2/D1 cells immobilized in alginate microfibers cultivated for 24 h and then treated for 24 h with the indicated treatments. A) Light microscopy of treated NT2/D1 cells in alginate microfibers. NT2-control untreated cells. Scale bar: 100 µm. B) Viability of NT2/D1 cells assessed by MTT assay. NT2/D1 cells were cultivated for 24 h in monolayer cultures (2D) or immobilized in alginate microfibers (3D) and then exposed for 24 h to the treatments listed above. Values are normalized to the values of corresponding controls, either 2D NT2/D1 or 3D NT2/D1, untreated cells expressed as 100 %. Data are represented as means ± SEM of three independent experiments performed in triplicate.* indicates $p < 0.05$. NT2-control untreated cells.*
SEM - standard error of the means.

The obtained results indicate a slight difference in cell viability between 2D and 3D cell cultures in response to treatments. The slight reduction in cell viability, although statistically nonsignificant, in response to all treatments in the 2D model system can be observed, except for caffeine, where a statistically significant but low decrease (12 %) in cell

viability is observed (Fig. 4, Panel B). On the other hand, none of the treatments significantly affect NT2/D1 cell viability in a 3D model system (Fig. 4, Panel B). Noticeable, although statistically insignificant, decrease in cell viability after treatments with caffeine/taurine and ED/EtOH in 3D probably reflects the fact that the 3D cell culture had higher deviations than 2D cultures (Fig. 4, Panel B). This is one of the well-established properties of 3D cultures, caused by the variation in the initial culture conditions, such as cell density and distribution in the 3D matrix [41-43].

In addition, cell viability after treatments with ED and AmED was evaluated by LIVE/DEAD assay (Fig. 5).

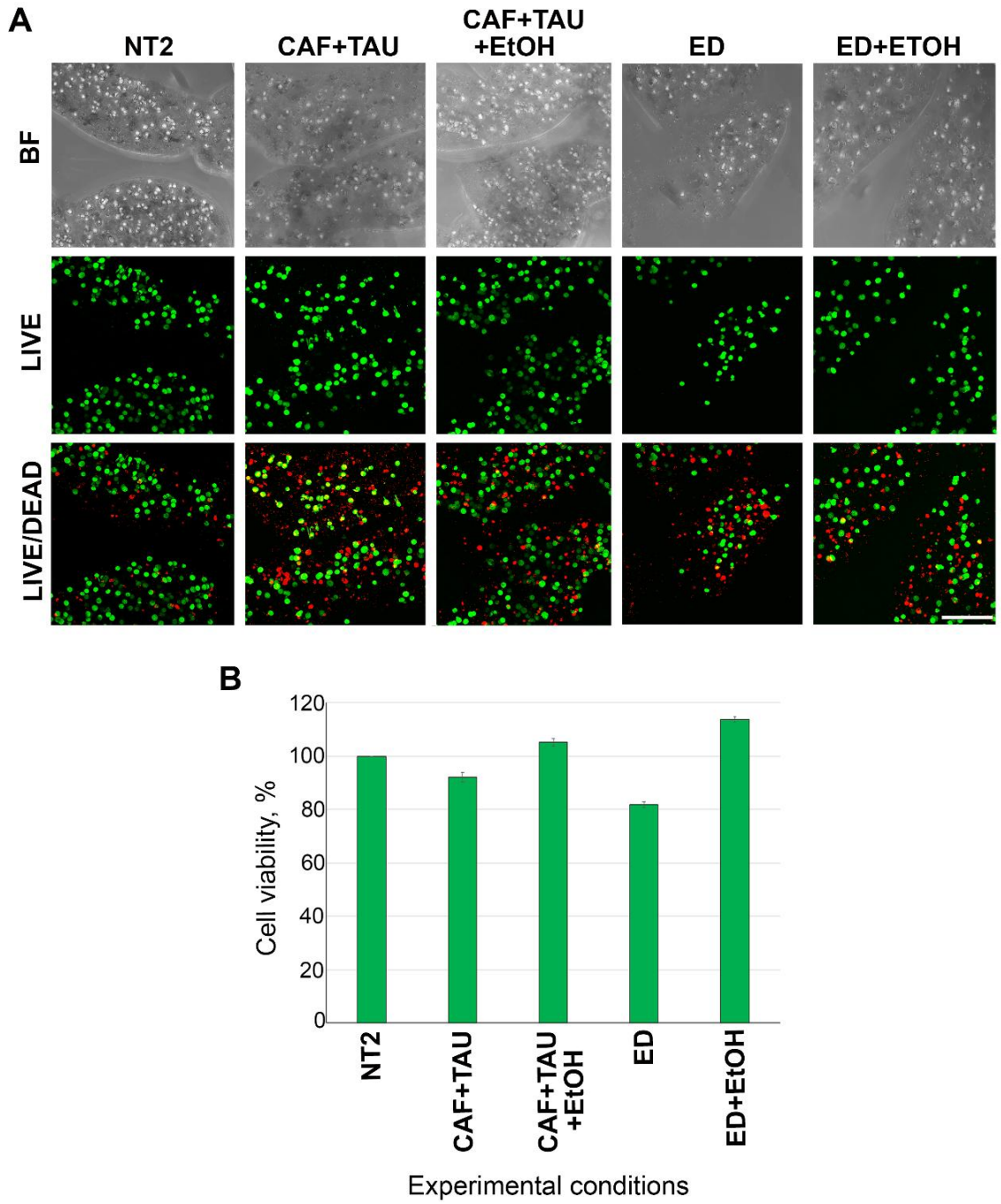

Figure 5. *Live/dead cell viability assay of NT2/D1 cells in alginate microfibers after ED and AmED treatments. A) Representative images of NT2/D1 cells in alginate microfibers after 24 h of indicated treatments. A representative Z stack of bright-field (BF), green fluorescence (LIVE), and merged fluorescence (LIVE/DEAD) that was overlapped (40 cross-sections, 5 µm each) by the Maximal intensity processing tool of LAS AF Software. Scale bar: 200 µm. B) Quantification of viable cells from the LIVE/DEAD assay using ImageJ software. Values are presented as a cell viability compared to control cells, which is set to 100 %. Data are presented as mean ± SEM, * indicates $p < 0.05$. SEM - standard error of the means*

This assay discriminates viable and non-viable cells using a green fluorescent dye, calcein-AM for live cells and red fluorescent BOBO-3 Iodide dye to visualize dead cells. As presented in Figure 5, Panel A, vast majority of cells entrapped in alginate microfibers retained viability with respect to the limited and moderate mortality. Viable cells maintained equal and uniform distribution within the microfiber (Fig. 5, Panel A). Apparently, the differences in distributions between central and peripheral regions of the microfibers could not be distinguished (Fig. 5, Panel A). Quantification of the LIVE/DEAD assay showed that indicated treatments did not significantly affect the NT2/D1 cell viability (Fig. 5, Panel B). Statistically insignificant but detectable decrease in cell viability after CAF/TAU and ED treatments is probably due to high deviations caused by the repeatability and reproducibility issues of 3D cultures [41-43].

The obtained results suggest that 24 h treatments that simulate ED and AmED consumption in both 2D and 3D cell culture models did not affect the viability of pluripotent NT2/D1 cells. In the case of treatments with ED components, caffeine induced a 12 % decrease in cell viability in the 2D model, while in the 3D model, the effect on NT2/D1 viability was non-detectable. We speculate that the alginate 3D system may provide a protective microenvironment for pluripotent cells with respect to the 2D monolayer culture. It has been shown previously that alginate oligosaccharides showed antioxidant and neuroprotective effects on NT2-derived mature neurons against H_2O_2-induced neurotoxicity [44]. The same protective effects were described in chromium-induced brain damage in rats and H_2O_2-induced senescence in H9C2 cardiomyocytes [45,46].

Effects of ED and AmED were extensively investigated in correlation with mental health and behavioral control in human volunteers and animal models [47,48]. Considering that ED and AmED primarily affect the CNS and cardiovascular system, physiological effects were mostly examined in animal models, animal primary cell cultures, and mature cells from these two organic systems [7,47,49,50]. Investigations of the effects of ED and AmED on pluripotent cells, stem cells and induced pluripotent stem cells (iPSc) are missing. Further studies should be focused on the effects of ED and AmED on the populations of stem cells and progenitor cells since consumption has increased among children, adolescents (vulnerable groups with ongoing neurodevelopment) and young people (potential association of ED intake before and during pregnancy with health risks) [4,7]. These results show, for the first time, that NT2/D1 cells immobilized in alginate microfibers represent a valuable 3D model system for studying the potential cytotoxic effects of bioactive compounds, components of food and beverages, on the pluripotent cells.

3. 3. Mathematical modeling of mass transport in 3D cell culture

In order to assess the efficiency of substance transport, specifically caffeine, taurine, and ethanol, within alginate microfibers, and to evaluate whether the hydrogel imposes any mass transport limitations, which could contribute to its protective effects, mathematical modeling was applied. Namely, the aim was to adopt a diffusion-reaction mass transport model previously developed [22] to predict concentration profiles of caffeine, taurine and ethanol within the single microfiber under cell culture conditions. Briefly, the model was based on the assumption that the external mass transfer resistance on the microfiber surface could be neglected and that the mass transport within the microfibers was the rate-limiting factor. Additionally, the following assumptions were made [22]: 1) the microfiber is cylindrical; 2) the diameter of the microfiber is constant; 3) the modeled substance is consumed at a constant rate per cell, following a zero-order chemical reaction; 4) the concentration of the modeled substance in the medium, and consequently at the microfiber surface, is constant; 5) mass transport occurs solely in the radial direction. Hence, the internal mass transport through the microfiber was described by the subsequent diffusion-reaction equation [22]:

$$\frac{\partial c}{\partial t} = \frac{D}{r}\frac{\partial}{\partial r}\left(r\frac{\partial c}{\partial r}\right) - \rho q \qquad (1)$$

where D represents the diffusion coefficient of the modeled substance, r denotes the radial coordinate from the center of the microfiber, q is the consumption rate of the substance per cell and ρ indicates the cell concentration per unit volume of the microfiber.

Solution of the partial differential Equation (1) was achieved numerically, by using the 'pdepe' function in MATLAB R2018a programming platform (MathWorks Inc.) [51].

3. 3. 1. Initial and boundary conditions

For all three modeled substances (caffeine, taurine and ethanol), the starting concentration in the microfiber was equal to zero, giving the following initial condition, Equation (2):

$$t = 0 \qquad 0 \le r \le R \qquad c = 0 \tag{2}$$

where R is the radius of the microfiber.

In accordance with the assumption about cylindrical geometry of the microfibers, the symmetry boundary condition was set along the microfiber axis, Equation (3):

$$r = 0 \qquad \frac{\partial c}{\partial r} = 0 \tag{3}$$

A constant concentration, equal to that in the medium (c_m), was assumed at the outer boundary of the microfiber, Equation (4):

$$r = R \qquad c = c_m \tag{4}$$

3. 3. 2. Model parameters

The diameter of the microfibers was specified as 190 μm, based on the average value obtained from at least 15 diameter measurements. The cell concentration per unit volume was specified as 5×10^6 cells cm^{-3}, based on the initial cell density.

3. 3. 3. Modeling of caffeine transport

The caffeine diffusion coefficient was adopted as 5.01×10^{-7} cm^2 s^{-1}, as reported in literature for caffeine diffusion in anionic hydrogels [52], while the caffeine concentration in the culture medium was 0.16 mol m^{-3} after treatment with either pure caffeine or the energy drink. It should be noted that in this study, NT2/D1 cells were used as *in vitro* models of embryonic neurogenesis and formation of different neuronal phenotypes, and it is well known that caffeine binds to the adenosine receptors of neurons and glial cells, as an antagonist, while it is metabolized in hepatocytes [53]. Therefore, the consumption term in Equation (1) was omitted. The mathematical model was solved using a spatial step size of Δr = 0.96 μm and a time step of Δt = 0.25 s.

The modeling results showed that the steady state was reached after 343 s with the caffeine concentration in the microfiber center reaching the concentration in the culture medium. This result implies efficient mass transport of caffeine throughout the whole microfiber under culture conditions.

3. 3. 4. Modeling of taurine transport

The taurine diffusion coefficient was adopted as 5.1×10^{-6} cm^2 s^{-1}, as reported in literature for glucose [54]. Glucose (M = 180.16 g mol^{-1}) is a small, polar molecule, with similar molecular weight to that of taurine (M=125.14 g mol^{-1}). The taurine concentration in the culture medium was 3.2 mol m^{-3} after treatment with either pure taurine or the energy drink. Additionally, the taurine consumption rate per cell for SF-295 human glioblastoma cell line, the most similar cell line with NT2/D1 cells, was reported to be 0.150 fmol $cell^{-1}$ h^{-1} (the mean value of two measurements: 0.097 and 0.203 fmol $cell^{-1}$ h^{-1}, taken from Supplement Database_S1, Jain *et al.* [55]. It is important to note that the assumption of a constant concentration of taurine in the medium was based on calculations demonstrating that the time required for complete consumption of taurine by the cells was vastly longer than the duration of the experiment, and thus the consumption was negligible within the experimental timeframe. The mathematical model was solved using a spatial step size of Δr = 0.96 μm and a time step of Δt = 0.25 s. The taurine concentration profile was plotted as a function of normalized radial distance (Fig. 6).

The simulation demonstrates that the steady state is reached after 33 s with the taurine concentration in the center of the microfiber practically reaching the value of taurine concentration in the culture medium. Further modeling of taurine transport, taking into account the experimental setup and the 24-hour exposure period, demonstrated that after 24 h, the taurine concentration throughout the entire microfiber remained equal to that in the surrounding medium.

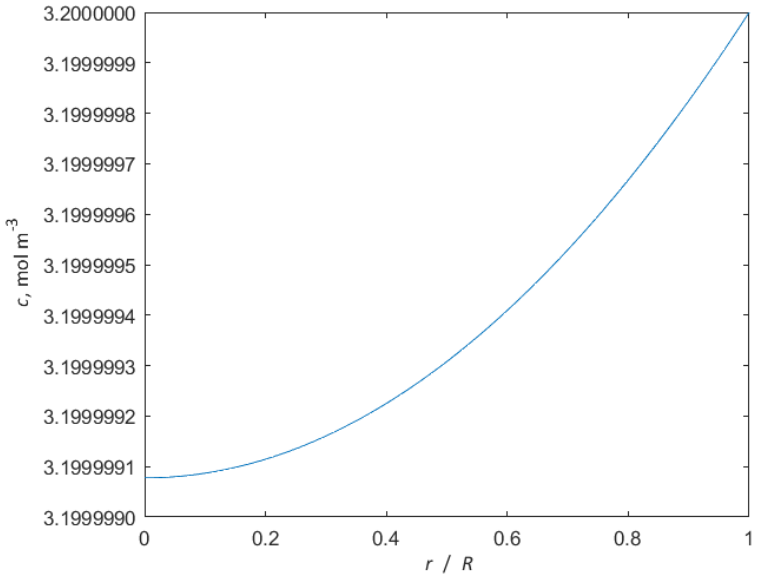

Figure 6: *Taurine concentration is represented in relation to the normalized radial distance within a microfiber (r/R) at steady state (R represents the microfiber radius, r/R=0 indicates the central axis of the microfiber and r/R=1 indicates its outer surface).*

3. 3. 5. Modeling of ethanol transport

For modeling of ethanol transport through the alginate microfibers, the diffusion coefficient was set to 9.6×10^{-6} cm^2 s^{-1}, as reported in literature for ethanol in 2 wt.% alginate hydrogel [54]. The ethanol concentration was 20 mol m^{-3} in the culture medium after addition of ethanol in the combination with caffeine and taurine, as well as in the combination with the energy drink. On the other hand, there is a significant lack of literature data regarding ethanol consumption by stem cells. Therefore, we adopted the highest calculated value of consumption rate of oxygen by human mesenchymal stem cells (hMSC) as 0.012 µmol 10^{-6} cells^{-1} h^{-1}, reported in literature [56]. As in the case of taurine, the assumption of a constant ethanol concentration in the medium was based on calculations demonstrating that the time required for complete consumption by the cells was significantly longer than the duration of the experiment. The mathematical model was solved using a spatial step size of Δr = 0.96 µm and a time step of Δt = 0.25 s. The ethanol concentration profile in the microfiber was plotted as a function of normalized radial distance (Fig. 7).

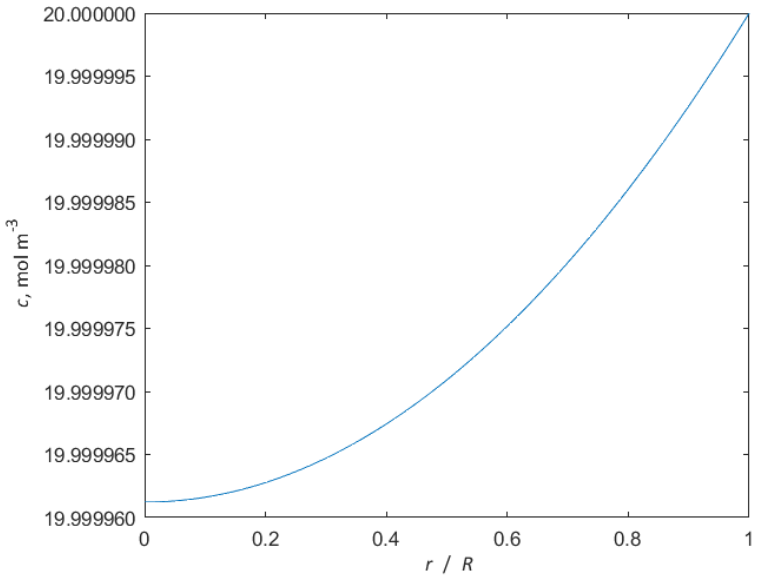

Figure 7: *The profile of ethanol concentration is represented in relation to the normalized radial distance within a microfiber (r/R) at a steady state (R represents the microfiber radius, r/R=0 indicates the central axis of the microfiber and r/R=1 indicates its outer surface)*

The ethanol concentration in the center of the microfiber reaches that in the medium in only 22 s, demonstrating highly efficient transport of ethanol throughout the whole microfiber. The steady state is achieved almost immediately, with the ethanol concentration in the centre of the microfiber equal to that in the culture medium. Upon modeling ethanol transport over a 24-hour period, it was found that the concentration within the microfiber closely matched that of the surrounding medium.

Overall, the modeling results demonstrated efficient mass transport of all three investigated substances within the 3D cell culture model. The model supported growth and proliferation of pluripotent NT2/D1 cells making it possible to mimic the certain behavior and structure characteristics of cells *in vivo* - round shaped single cells that form 3D aggregates with efficient supply of nutrients and active molecules throughout the microfibers. This 3D model could be used for the evaluation of substance effects on individual cells at the beginning of cultivation, as well as on cell aggregates at day 2, and on multilayered cell cluster at day 7. It is anticipated that cellular responses to a given substance may differ between single cells and those within aggregates, highlighting the importance of considering cell organization during experimental data analysis. In contrast to 2D models, 3D models provide gradients of nutrients, oxygen, metabolites, and tested compounds to cells which make them more accurate and physiologically relevant [57].

Retinoic acid induced neural differentiation of NT2/D1 cells in 2D culture is a widely used model of human neurogenesis [58]. After four weeks of induction NT2/D1 cells differentiate into mature neurons and astrocytes [58]. In addition, NT2/D1 cells encapsulation in alginate microbeads enabled low-rate spontaneous dopaminergic neuronal differentiation in a 3-week period [21]. Our future goal will be to establish 3D culture of alginate microfibers for 4 weeks long RA-induced neural differentiation of NT2/D1 cells to test the effects of acute and chronic consumption of ED and AmED on different stages of human neurogenesis.

4. CONCLUSION

In this study, we established a 3D model of human pluripotent NT2/D1 cells immobilized in alginate microfibers. The immobilized NT2/D1 cells preserved viability, proliferative capacity and potential to form aggregates during a 7-day course of propagation. To validate the 3D NT2/D1 cell model system for bioactive compounds testing, we performed mathematical modeling of mass transport in the 3D cell cultures. Obtained results showed that all tested compounds were uniformly distributed to all cells inside the microfiber, similar to the uniform distribution of compounds to cells growing in a monolayer. Acute 24-hour treatment with ED and the contained compounds, alone or in combination with alcohol, did not affect the viability of immobilized NT2/D1 cells in the 3D model. Correlation with the same treatments in 2D culture showed differences in the effect of caffeine on cell viability. The caffeine treatment resulted in a low but statistically significant decrease in cell viability of 12 % in 2D monolayer culture. NT2/D1 cells immobilized in alginate microfibers represent a potential 3D platform for testing the effects of different compounds from food and beverages on human pluripotent cells.

Acknowledgement: This study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (contract numbers: 451-03-66/2024-03/200042; 451-03-65/2024-03/200135; 451-03-66/2024-03/200287).

REFERENCES

[1] Alsunni AA. Energy Drink Consumption: Beneficial and Adverse Health Effects. *Int J Health Sci (Qassim)*. 2015; 9(4): 468-474. https://ijhs.qu.edu.sa/index.php/journal/article/view/1244

[2] Nadeem IM, Shanmugaraj A, Sakha S, Horner NS, Ayeni OR, Khan M. Energy Drinks and Their Adverse Health Effects: A Systematic Review and Meta-analysis. *Sports Health*. 2021; 13(3): 265-277. https://doi.org/10.1177/1941738120949181PMC8083152

[3] De Sanctis V, Soliman N, Soliman AT, Elsedfy H, Di Maio S, El Kholy M, Fiscina B. Caffeinated energy drink consumption among adolescents and potential health consequences associated with their use: a significant public health hazard. *Acta Biomed*. 2017; 88(2): 222-231. https://doi.org/10.23750/abm.v88i2.6664PMC6166148

[4] Ding M, Markon AO, Jones-Dominic OE, Purdue-Smithe AC, Rich-Edwards JW, Wolpert BJ, Chavarro JE. Intake of Energy Drinks Before and During Pregnancy and Adverse Pregnancy Outcomes. *JAMA Netw Open*. 2023; 6(11): e2344023. https://doi.org/10.1001/jamanetworkopen.2023.44023PMC10660164

[5] Oteri A, Salvo F, Caputi AP, Calapai G. Intake of energy drinks in association with alcoholic beverages in a cohort of students of the School of Medicine of the University of Messina. *Alcohol Clin Exp Res*. 2007; 31(10): 1677-1680. https://doi.org/10.1111/j.1530-0277.2007.00464.x

[6] Luo YS, Chen Z, Blanchette AD, Zhou YH, Wright FA, Baker ES, Chiu WA, Rusyn I. Relationships between constituents of energy drinks and beating parameters in human induced pluripotent stem cell (iPSC)-Derived cardiomyocytes. *Food Chem Toxicol*. 2021; 149: 111979. https://doi.org/10.1016/j.fct.2021.111979PMC8286543

[7] Serdar M, Mordelt A, Müser K, Kempe K, Felderhoff-Müser U, Herz J, Bendix I. Detrimental Impact of Energy Drink Compounds on Developing Oligodendrocytes and Neurons. *Cells*. 2019; 8(11): 1381. https://doi.org/10.3390/cells8111381PMC6912672

[8] Zeidán-Chuliá F, Gelain DP, Kolling EA, Rybarczyk-Filho JL, Ambrosi P, Terra SR, Pires AS, da Rocha JB, Behr GA, Moreira JC. Major components of energy drinks (caffeine, taurine, and guarana) exert cytotoxic effects on human neuronal SH-SY5Y cells by decreasing reactive oxygen species production. *Oxid Med Cell Longev*. 2013; 2013: 791795. https://doi.org/10.1155/2013/791795PMC3674721

[9] Andrews PW. From teratocarcinomas to embryonic stem cells. *Philos Trans R Soc Lond B Biol Sci*. 2002; 357(1420): 405-417. https://doi.org/10.1098/rstb.2002.1058PMC1692959

[10] Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Føgh J. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation *in vivo* and *in vitro*. *Lab Invest*. 1984; 50(2): 147-162. https://pubmed.ncbi.nlm.nih.gov/6694356/

[11] Sperger JM, Chen X, Draper JS, Antosiewicz JE, Chon CH, Jones SB, Brooks JD, Andrews PW, Brown PO, Thomson JA. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc Natl Acad Sci U S A*. 2003; 100(23): 13350-13355. https://doi.org/10.1073/pnas.2235735100PMC263817

[12] Foglizzo V, Cocco E, Marchiò S. Advanced Cellular Models for Preclinical Drug Testing: From 2D Cultures to Organ-on-a-Chip Technology. *Cancers (Basel)*. 2022; 14(15): 3692. https://doi.org/10.3390/cancers14153692PMC9367322

[13] Kapałczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, Ibbs M, Bliźniak R, Łuczewski Ł, Lamperska K. 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. *Arch Med Sci*. 2018; 14(4): 910-919. https://doi.org/10.5114/aoms.2016.63743PMC6040128

[14] Habanjar O, Diab-Assaf M, Caldefie-Chezet F, Delort L. 3D Cell Culture Systems: Tumor Application, Advantages, and Disadvantages. *Int J Mol Sci*. 2021; 22(22): 12200. https://doi.org/10.3390/ijms222212200PMC8618305

[15] Zhao X, Bhattacharyya A. Human Models Are Needed for Studying Human Neurodevelopmental Disorders. *Am J Hum Genet*. 2018; 103(6): 829-857. https://doi.org/10.1016/j.ajhg.2018.10.009PMC6288051

[16] Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao MS. Differences between human and mouse embryonic stem cells. *Dev Biol*. 2004; 269(2): 360-380. https://doi.org/10.1016/j.ydbio.2003.12.034

[17] Greber B, Wu G, Bernemann C, Joo JY, Han DW, Ko K, Tapia N, Sabour D, Sterneckert J, Tesar P, Schöler HR. Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell*. 2010; 6(3): 215-226. https://doi.org/10.1016/j.stem.2010.01.003

[18] Matsuda M, Hayashi H, Garcia-Ojalvo J, Yoshioka-Kobayashi K, Kageyama R, Yamanaka Y, Ikeya M, Toguchida J, Alev C, Ebisuya M. Species-specific segmentation clock periods are due to differential biochemical reaction speeds. *Science*. 2020; 369(6510): 1450-1455. https://doi.org/doi:10.1126/science.aba7668

[19] European Parliament and of the Council. Consolidated text: Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Off J Eur Union. 2010; L 276: 233–279. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02010L0063-20190626

[20] Hill EJ, Woehrling EK, Prince M, Coleman MD. Differentiating human NT2/D1 neurospheres as a versatile in vitro 3D model system for developmental neurotoxicity testing. *Toxicology*. 2008; 249 (2-3): 243-250. https://doi.org/10.1016/j.tox.2008.05.014

[21] Cacciotti I, Ceci C, Bianco A, Pistritto G. Neuro-differentiated Ntera2 cancer stem cells encapsulated in alginate beads: First evidence of biological functionality. *Mater Sci Eng C Mater Biol Appl*. 2017; 81: 32-38. https://doi.org/10.1016/j.msec.2017.07.033

[22] Radonjić M, Petrović J, Milivojević M, Stevanović M, Stojkovska J, Obradović B. Chemical engineering methods in analyses of 3D cancer cell cultures: Hydrodinamic and mass transport considerations. *Chem Ind Chem Eng Q*. 2022; 28(3): 211-223. https://doi.org/10.2298/CICEQ210607033R

[23] Sun J, Tan H. Alginate-Based Biomaterials for Regenerative Medicine Applications. *Materials (Basel)*. 2013; 6(4): 1285-1309. https://doi.org/10.3390/ma6041285PMC5452316

[24] Mead EA, Wang Y, Patel S, Thekkumthala AP, Kepich R, Benn-Hirsch E, Lee V, Basaly A, Bergeson S, Siegelmann HT, Pietrzykowski AZ. miR-9 utilizes precursor pathways in adaptation to alcohol in mouse striatal neurons. *Adv Drug Alcohol Res*. 2023; 3: 11323. https://doi.org/10.3389/adar.2023.11323PMC10730111

[25] Doyle W, Shide E, Thapa S, Chandrasekaran V. The effects of energy beverages on cultured cells. *Food Chem Toxicol*. 2012; 50(10): 3759-3768. https://doi.org/10.1016/j.fct.2012.07.008

[26] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65(1-2): 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

[27] Stojkovska JJ, Zvicer J, Milivojevic M, Petrovic I, Stevanovic M, Obradovic B. Validation of a novel perfusion bioreactor system in cancer research. *Hem Ind*. 2020; 74(3): 187-196. https://doi.org/10.2298/HEMIND200329015S

[28] Saltzman WM, Kyriakides TR. Cell interactions with polymers. In: Lanza R, Langer R, Vacanti JP, Atala A, eds. *Principles of Tissue Engineering* 5th ed. Cambridge, MA: Academic Press; 2020: 275-293. https://doi.org/10.1016/B978-0-12-818422-6.00017-4

[29] Nebel S, Lux M, Kuth S, Bider F, Dietrich W, Egger D, Boccaccini AR, Kasper C. Alginate Core-Shell Capsules for 3D Cultivation of Adipose-Derived Mesenchymal Stem Cells. *Bioengineering (Basel)*. 2022; 9(2): 66. https://doi.org/10.3390/bioengineering9020066PMC8869374

[30] Rodriguez S, Lau H, Corrales N, Heng J, Lee S, Stiner R, Alexander M, Lakey JRT. Characterization of chelator-mediated recovery of pancreatic islets from barium-stabilized alginate microcapsules. *Xenotransplantation*. 2020; 27(1): e12554. https://doi.org/10.1111/xen.12554

[31] Vo P, Nguyen S, Minh Do N, Truong K, Pham P. Sodium citrate inhibits proliferation and induces apoptosis of hepatocellular carcinoma cells. *Biomed Res Ther*. 2020; 7: 3659-3666. https://doi.org/10.15419/bmrat.v7i3.592

[32] Wu Y, Jia C, Liu W, Zhan W, Chen Y, Lu J, Bao Y, Wang S, Yu C, Zheng L, Sun L, Song Z. Sodium citrate targeting Ca(2+)/CAMKK2 pathway exhibits anti-tumor activity through inducing apoptosis and ferroptosis in ovarian cancer. *J Adv Res*. 2024; 65: 89-104. https://doi.org/10.1016/j.jare.2024.04.033PMC11518946

[33] Burger H, Nooter K, Boersma AW, Kortland CJ, Stoter G. Expression of p53, Bcl-2 and Bax in cisplatin-induced apoptosis in testicular germ cell tumour cell lines. *Br J Cancer*. 1998; 77(10): 1562-1567. https://doi.org/10.1038/bjc.1998.257PMC2150079

[34] Kitano O, Nakazawa K. Neuronal Differentiation of NT2 Cells in Monolayer and Spheroid Cultures. *MATEC Web Conf*. 2021; 333: 07008. https://doi.org/10.1051/matecconf/202133307008

[35] Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol*. 2010; 148(1): 3-15. https://doi.org/10.1016/j.jbiotec.2010.01.012

[36] Pinto B, Henriques AC, Silva PMA, Bousbaa H. Three-Dimensional Spheroids as In Vitro Preclinical Models for Cancer Research. *Pharmaceutics*. 2020; 12(12): 1186. https://doi.org/10.3390/pharmaceutics12121186PMC7762220

[37] Han YL, Wang S, Zhang X, Li Y, Huang G, Qi H, Pingguan-Murphy B, Li Y, Lu TJ, Xu F. Engineering physical microenvironment for stem cell based regenerative medicine. *Drug Discov Today*. 2014; 19(6): 763-773. https://doi.org/10.1016/j.drudis.2014.01.015

[38] Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. *Tissue Eng Part B Rev*. 2008; 14(2): 149-165. https://doi.org/10.1089/ten.teb.2007.0332PMC2962861

[39] Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng*. 2009; 103(4): 655-663. https://doi.org/10.1002/bit.22361PMC2997742

[40] Vergani L, Grattarola M, Nicolini C. Modifications of chromatin structure and gene expression following induced alterations of cellular shape. *Int J Biochem Cell Biol*. 2004; 36(8): 1447-1461. https://doi.org/10.1016/j.biocel.2003.11.015

[41] Demircan Yalcin Y, Luttge R. Electrical monitoring approaches in 3-dimensional cell culture systems: Toward label-free, high spatiotemporal resolution, and high-content data collection in vitro. *Organs-on-a-Chip*. 2021; 3: 100006. https://doi.org/https://doi.org/10.1016/j.ooc.2021.100006

[42] Hagiwara M, Nobata R, Kawahara T. High repeatability from 3D experimental platform for quantitative analysis of cellular branch pattern formations. *Integr Biol (Camb)*. 2018; 10(5): 306-312. https://doi.org/10.1039/c8ib00032h

[43] Jensen C, Teng Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci*. 2020; 7: 33. https://doi.org/10.3389/fmolb.2020.00033PMC7067892

[44] Eftekharzadeh B, Khodagholi F, Abdi A, Maghsoudi N. Alginate protects NT2 neurons against H2O2-induced neurotoxicity. *Carbohydr Polym*. 2010; 79(4): 1063-1072. https://doi.org/https://doi.org/10.1016/j.carbpol.2009.10.040

[45] Feng WJ, Mou J, Liao PP, Zhou J, Zhang NN, Hu T, Wang S, Zhang SY, Mao YJ. Alginate oligosaccharides exert protective effects on hydrogen peroxide-induced senescence in H9C2 cardiomyocytes by regulating the redox state of cells. *Food Sci Biotechnol*. 2024; 33(12): 2835-2844. https://doi.org/10.1007/s10068-024-01534-yPMC11339193

[46] Saleh EM, Hamdy GM, Hassan RE. Neuroprotective effect of sodium alginate against chromium-induced brain damage in rats. *PLoS One*. 2022; 17(4): e0266898. https://doi.org/10.1371/journal.pone.0266898PMC9009676

[47] Krahe TE, Filgueiras CC, da Silva Quaresma R, Schibuola HG, Abreu-Villaça Y, Manhães AC, Ribeiro-Carvalho A. Energy drink enhances the behavioral effects of alcohol in adolescent mice. *Neurosci Lett*. 2017; 651: 102-108. https://doi.org/https://doi.org/10.1016/j.neulet.2017.04.050

[48] Richards G, Smith AP. A Review of Energy Drinks and Mental Health, with a Focus on Stress, Anxiety, and Depression. *J Caffeine Res*. 2016; 6(2): 49-63. https://doi.org/10.1089/jcr.2015.0033PMC4892220

[49] Graneri L, Lam V, D'Alonzo Z, Nesbit M, Mamo JCL, Takechi R. The Consumption of Energy Drinks Induces Blood-Brain Barrier Dysfunction in Wild-Type Mice. *Front Nutr*. 2021; 8: 668514. https://doi.org/10.3389/fnut.2021.668514PMC8126614

[50] Petribu BN, Abrahao KP, Souza-Formigoni MLO. Ethanol combined with energy drinks: Two decades of research in rodents. *Front Behav Neurosci*. 2022; 16: 1100608. https://doi.org/10.3389/fnbeh.2022.1100608PMC10017554

[51] *MATLAB Release 2018a* [computer program]. Natick, MA: The MathWorks, Inc.; 2018. https://www.mathworks.com/

[52] Chern J-M, Lee W-F, Hsieh M-Y. Absorption Isotherm of Caffeine and Release Kinetics from Swollen NIPAAm Hydrogels: Experiments and Modeling. *Ind Eng Chem Res*. 2004; 43(19): 6150-6156. https://doi.org/10.1021/ie049616d

[53] Rodak K, Kokot I, Kratz EM. Caffeine as a Factor Influencing the Functioning of the Human Body-Friend or Foe? *Nutrients*. 2021; 13(9): 3088. https://doi.org/10.3390/nu13093088PMC8467199

[54] Estapé D, Gòdia F, Solà C. Determination of glucose and ethanol effective diffusion coefficients in Ca-alginate gel. *Enzyme Microb Technol*. 1992; 14(5): 396-401. https://doi.org/10.1016/0141-0229(92)90009-d

[55] Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza AL, Kafri R, Kirschner MW, Clish CB, Mootha VK. Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science*. 2012; 336(6084): 1040-1044. https://doi.org/10.1126/science.1218595PMC3526189

[56] Zhao F, Pathi P, Grayson W, Xing Q, Locke BR, Ma T. Effects of oxygen transport on 3-d human mesenchymal stem cell metabolic activity in perfusion and static cultures: experiments and mathematical model. *Biotechnol Prog*. 2005; 21(4): 1269-1280. https://doi.org/10.1021/bp0500664

[57] Urzì O, Gasparro R, Costanzo E, De Luca A, Giavaresi G, Fontana S, Alessandro R. Three-Dimensional Cell Cultures: The Bridge between In Vitro and In Vivo Models. *Int J Mol Sci*. 2023; 24(15): 12046. https://doi.org/10.3390/ijms241512046PMC10419178

[58] Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. *Dev Biol*. 1984; 103(2): 285-293. https://doi.org/10.1016/0012-1606(84)90316-6

Humane pluripotentne NT2/D1 ćelije imobilizovane u alginatnim mikrovlaknima: 3D sistem za testiranje uticaja bioaktivnih jedinjenja

Jelena Lj. Pejić[1], Milena C. Milivojević[1], Marija L. Schwirtlich[1], Jelena S. Petrović[2,3], Jasmina J. Stojkovska[2], Milena J. Stevanović[1,4,5] i Marija M. Mojsin[1]

[1]Univerzitet u Beogradu, Institut za molekularnu genetiku i genetičko inženjerstvo, Beograd, Srbija
[2]Univerzitet u Beogradu, Tehnološko-metalurški fakultet, Beograd, Srbija
[3]Inovacioni centar Tehnološko-metalurškog fakulteta, Beograd, Srbija
[4]Univerzitet u Beogradu, Biološki fakultet, Beograd, Srbija
[5]Srpska akademija nauka i umetnosti, Beograd, Srbija

(Naučni rad)

Izvod

Učestala konzumacija energetskih pića (engl. *energy drinks-ED*) i njihova kombinacija sa alkoholom (engl. *energy drinks mixed with alcohol-AmED*) postala je rastući trend među mladim ljudima. Proklamovano povećanje psihičkih i fizičkih performansi usled konzumiranja ED, koji u svom sastavu sadrže visoke doze stimulativnih sastojka - kofeina i taurina, može dovesti do neželjenih posledica, prvenstveno na funkcije centralnog nervnog sistema (CNS) i kardiovaskularnog sistema. Uprkos ključnoj ulozi u razviću i adultnoj homeostazi, istraživanja o potencijalnom efektu konzumacije ED i AmED na matične i progenitorske ćelije su malobrojne. U radu je predstavljena optimizacija 3D model sistema na bazi alginatnih mikrovlakana za testiranje uticaja bioaktivnih jedinjenja na humane NT2/D1 embrionalne karcinomske ćelije, široko korišćeni model sistem kao pandan humanim matičnim ćelijama. Ispitan je uticaj akutne konzumacije ED i AmED na vijabilnost ovih ćelija i uz pomoć matematičkog modelovanja evaluirana je efikasnost prenosa mase ispitivanih komponenti do imobilisanih ćelija. Dobijeni rezultati pokazuju da ovaj model sistem omogućava optimalan rast i proliferaciju NT2/D1 pluripotentnih ćelija i uniformnu distribuciju ispitivanih komponenti kroz alginatna mikrovlakna. Simulirana akutna konzumacija ED i AmED nije uticala na vijabilnost NT2/D1 ćelija u 3D sistemu, za razliku od 2D modela gde je kofein doveo do malog ali statistički značajnog pada vijabilnosti.

Ključne reči: humane embrionalne karcinomske ćelije; alginatni hidrogel; ćelijska vijabilnost; prenos mase; energetska pića; kofein