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Research Article

Investigating the Effect of Stable Glutamine on the Neuronal Differentiation of PC-12 Cells in 2D- and 3D-Culture Media

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Abstract

Glutamine (Gln) is an essential amino acid with a wide range of cellular functions and is necessary for cell proliferation. It is usually added to the culture media in the form of L-glutamine, which is highly unstable and degrades in a temperature-dependent manner during the culture period. Although, Gln is beneficial for the cells, its degradation produces ammonia which is toxic and negatively affect cell culture. Pheochromocytoma cells (PC-12), originating from cancerous cells of the rat adrenal gland, are considered as a suitable model to study the differentiating effects of different factors. Previous studies showed the importance of Gln in the normal growth and differentiation of the cells. Alginate, is one of the biomaterials currently used as a natural scaffold for the induction of neuronal differentiation. In the present experimental research, the effect of stable and elevated levels of Gln on the growth and neuronal differentiation of PC-12 cells was compared under 2D- and 3D- (sodium alginate hydrogel beads) culture conditions. The cells' viabilities were determined and compared between experimental groups using live/dead cell staining by Acridine orange/Propidium iodide (AO/PI), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Furthermore, cells were stained using cresyl violet to detect neuronal Nissl bodies. The induction of differentiation was confirmed using immunocytochemical analysis of Nestin and β-tubulin III proteins and Cells' nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Results showed that high concentration of Gln can induce neuronal differentiation in PC-12 cells under both 2D- and 3D- culture conditions and increases the expression of progenitor and mature neuronal markers Nestin and β-tubulin III, respectively.

Keywords*: *Neural differentiation, Glutamine, PC-12 cells, Alginate

Introduction

Glutamine (Gln) is a conditionally essential amino acid in animals and is the most abundant free amino acid in the body. Gln has a wide range of cellular and physiological functions, including protein synthesis, lipid metabolism, and cell growth. Since Gln is involved in the nucleic acid biosynthesis it is essential for cell proliferation (Sarkadi et al., 2020; Suh et al., 2022). Furthermore, it is the main energy supply substance of mitochondria. The oxidation of Gln can eliminate some strong oxidizing substances in cells. (Zhao et al., 2019). Both intracellular and extracellular (free) Gln are essential for neuronal health considering their significant neuroprotective effects (Wang et al., 2019).

It was determined that Gln has different effects on gene expression profile of intestinal cells based on its concentration (Ban& Kozar, 2010). Many studies showed the impact of Gln on the growth, proliferation, and differentiation of different cell types during tissue engineering studies (Ban& Kozar, 2010; Yousefi Taemeh et al., 2021; Brose et al., 2014). For instance, in a previous study it was confirmed that Gln improves intestinal cell proliferation and stem cell differentiation in mice (Chen et al., 2018). Moreover, it can reduce muscle cells damage and inflammation (Raizel & Tirapegui, 2018).

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Gln is usually added to the culture media in the form of L-glutamine and at the concentration of 2mM. It is highly unstable and degrades in a temperature-dependent manner during the culture period. Although Gln is yet effective at very low concentrations, differentiated cells require much higher concentrations of Gln to survive (Rubin et al., 2019). The degradation of Gln produces ammonia which is toxic for the cells in the culture. Glutamine dipeptides, such as GlutaMAX (Glx), which is commercially available, are more stable and are resistant to the degradation. Hence, Glx can be used at higher concentrations and for long-term culture conditions (Yousefi Taemeh et al., 2021). Despite the numerous studies in the field, the application of the stable form of Gln at high concentrations still requires more research.

Pheochromocytoma cells (PC-12) are originated from rat neuroendocrine tumor pheochromocytoma, which is the tumor of the chromaffin cells of the adrenal medulla. These cells are considered as a suitable model cell line to study the differentiation induction potential of different compounds. When these cells are exposed with proper concentrations of nerve growth factor (NGF), their proliferation slows down, and they adopt the appearance of neurons (Darbinian et al., 2013; Gordon et al., 2013). Alginate, alginic acid, is a polysaccharide obtained from the cell wall of brown algae (Mobini et al., 2019). Alginate is one of the biomaterials with high biocompatibility, high degradability, and non-antigenic properties which is currently used as a natural scaffold for neuro-regeneration (Yi et al., 2019). Alginate can promote the survival and growth of Schwann cells and neurite outgrowth in nerve cells. It was shown that alginate increases axon elongation in the nervous system of mice (Akter et al., 2016).

The present study evaluated the capabilities of PC-12 cells under 2D- and 3D- culture conditions to simulate *in vivo* conditions for the induction of neural differentiation at a high concentration of Gln. Our results showed that high concentration of Gln under both 2D- and 3D- culture conditions can induce neuronal differentiation in PC-12 cells as well as the expression of neuronal markers Nestin (a marker of neural progenitors) and β-tubulin III (a marker of mature neurons). Hence, it could be a novel strategy to generate neural cells for *in vitro* and *in vivo* experiments.

Materials and Methods

Experimental groups

2D-culture; PC-12 cells were cultured in the polystyrene flasks (3×10^4 cell/ml). In the treatment group, cells received Gln at a concentration of 40mM for 7d (GLN), while in the control group they received no Gln (PC-12) (stable glutamine;GlutaMAX, Gibco-USA).

3D culture; Alginate-encapsulated PC-12 cells cultured in a six-well plates containing Gln (40Mm) for 7d (from day 8 to14) (ALG+GLN). No Gln was added to the beads' culture media at control groups (ALGINATE).

PC-12 cell line

PC-12 cell line was purchased from Pasteur Institute cell bank, Tehran, Iran. The Roswell Park Memorial Institute (RPMI1640; Gibco-USA) medium supplemented with 10% fetal bovine serum (FBS; Gibco-USA), 5% horse serum (HS; Sigma-USA), and 1% penicillin/streptomycin (PS; Sigma-USA), was applied as the culture media. Cells were incubated in the presence of 5% carbon dioxide (CO_2), 90% humidity, and a temperature of 37°C (Memmert; Germany). Upon reaching 80% confluency, cells were sub-cultured, and applied for downstream assays (Abe et al., 2015).

Encapsulation of PC-12 cells in sodium alginate hydrogel

To prepare alginate hydrogel, sodium alginic acid (Sigma-USA) (0.3gr) was dissolved in 10ml of deionized water (3% v/w), and sterilized (autoclave; 121°C for 15min). 1ml of cell suspension, containing 3×10^6 cells, was considered per ml of alginate. Alginate beads were prepared by dripping the mixture onto a 6-well plates containing 102mM calcium chloride (Sigma-USA) using a 21-gauge syringe. They were incubated at RT under sterile conditions for 20min. Then, solution was removed from each well and remained beads were washed with Phosphate Buffered Saline (PBS; 3 times) and RPMI1640 culture medium (1 time). Following the washing steps, beads were transferred to another plate with complete RPMI1640 culture medium. To induce neuronal differentiation, the medium was exchanged with the freshly prepared differentiation medium every other day for 14d (Razavi et al., 2015).

Morphological observations

2D- and 3D-cultures were carefully observed by an inverted microscope (Olympus, IX70-Japan) with 200X magnification. Features such as cell appearances, growth rate of the colonies, neurite

formation, and the appearance of alginate beads were monitored.

Cell viability (MTT) assay

PC-12 cells encapsulated in alginate beads were kept inside 6-well plates (CO_2 incubator; 37°C, 14d). After draining the culture medium 10 beads were broken in a microtube and transferred to the 96-well plates on 10 and 14d; Then, MTT solution (Sigma, USA;100µl of 1µg/ml solution) was added per well (dark (CO_2) incubation; 37°C). 3h later, upon the appearance of formazan crystals the supernatant was replaced with 80µl of dimethyl sulfoxide (DMSO, Sigma-USA) (Abnosiet al., 2010). Remained samples were completely removed from DMSO after 30min, and absorbances were measured (wavelength of 570nm) (EPOCH spectrophotometer; Epoch-USA)).

Cell survival evaluation (acridine orange/propidium iodide) staining

To compare the survival of the cells encapsulated in hydrogel scaffolds qualitatively, acridine orange/propidium iodide (AO/PI) staining (Sigma, USA) was performed. In this method, live cells are detected with their green fluorescent, while dead cells are seen in yellow, orange, or red colors (Kim et al., 2017). Beads were exposed with acridine orange solution for 15min at room temperature on day 14. The cells were then washed 3 times with PBS 1X before the addition of PI (Incubation time, 30sec). Samples were rinsed (PBS 1X, 3 times) and subsequent fluorescent microscopy was performed (Olympus-Japan).

Cresyl violet staining

Cresyl violet staining (Sigma Aldrich, USA) of neuronal Nissl bodies was done on 7 and 14d for 2D- and 3D-cultures, respectively. Following one round of washing with PBS 1X, cells were fixed using 70% ethanol for 10min at room temperature, and then incubated in cresyl violet solution (0.25% cresyl violet, 0.8% glacial acetic acid, 0.6mM sodium acetate, 100cm^3 distilled water) for 3 to 10min (light microscopy, Olympus IX70, Japan).

Immunocytochemistry

The supernatant of 2D- and 3D- cultures was removed and the samples were fixed with 4% paraformaldehyde (PFA; Sigma, USA) for 20min after washing with PBS 1X, followed by washing steps (3 times, with a 5-minute interval). Then, cell membranes were permeabilized with 0.3% Triton X-100 (Sigma, USA) for 30min, plus another round of washing with PBS 1X. Afterward, to block the secondary antibody reaction, 10% goat serum (Sigma, USA) was added to the samples for 45min. After removing the goat serum, samples were stained with the primary antibodies Nestin (1:100) (Biorbyt, UK), and β-tubulin III (1:100) (Biorbyt-UK), and were kept in the refrigerator (2-8°C, 24h). Later, washed samples (4times with PBS 1X) were incubated with goat anti-rabbit IgG (H+L) antibody (FITC; 1:150; dark incubation, 37°C, 1.5h) (Biorbyt-UK). At the final step, samples were transferred to the dark room, washed (3times), and stained with DAPI (DAPI (4′,6-diamidino-2-phenylindole; Sigma, USA) just before the fluorescence imaging (Olympus, IX70-Japan) based on the previous protocols (Aamodt and Grainger, 2016; Agarwal et al., 2022; Bernal and Arranz, 2018).

Data analysis

The percentage of visual expression of desired differentiation markers was measured using ImageJ software, version 1.51 and averages were expressed as Mean ± SD. Further statistical analyses were carried out using ANOVA and Tukey's tests (GraphPad PRISM, version 9.4.1., *p*-value <0.05).

Results

Morphological evaluation of PC-12 cells

Preliminary examination of PC-12 cells (2D-culture) showed a round morphology for these cells. Successive divisions were observed with 24-48h intervals. Each individual cell began to form a colony with a low adhesiveness (Figure 1A), whereas in the presence of glutamine (40mM), cells showed cytoplasmic retraction, changes in their spherical morphology, and the appearance of short neurites (Figure 1B). In 3D culture PC-12 cells retained their round appearance and formed colonies. Transparent appearance of colonies indicates the growth and proliferation of PC-12 cells in Glutamine-free 3D cultures (Figure 2C), and culture supplemented with Gln (40Mm) (Figure 2D).

Evaluation of 3D-cultured cell viabilities

The viability of alginate-encapsulated PC-12 cells (in the presence and absence of Gln) was measured on 10 and 14d post encapsulation through MTT assay. As it is shown in Figure 3, although minor increase was observed regarding the viability of the cells from 3D culture groups at 10 and 14d (ALGINATE and ALG+GLN), these changes were not statistically significant. Thus, our results demonstrated that encapsulation the PC-12 cells with alginate does not exert remarkable toxicity (Figure 3).

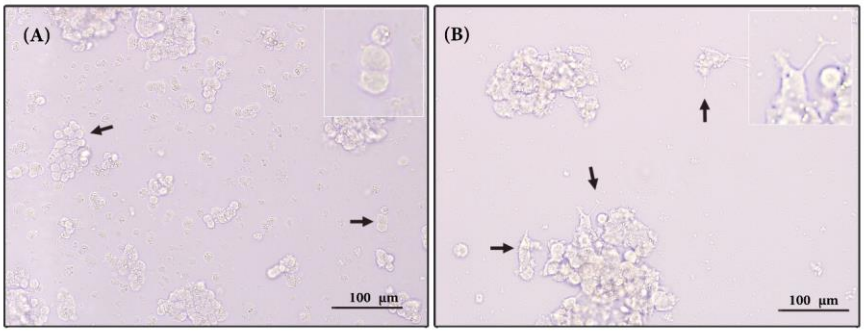

Figure 1. The morphology of PC-12 cells under 2D-culture condition (5d). (A) PC-12 cells' morphologies are shown for Gln-free cultures (arrows indicate dividing rounded cells), and (B) cultures supplemented with Gln (40Mm). Arrows show cytoplasmic retraction and neurite formation (Scale bar 100µm, light microscopy, Olympus IX70, Japan).

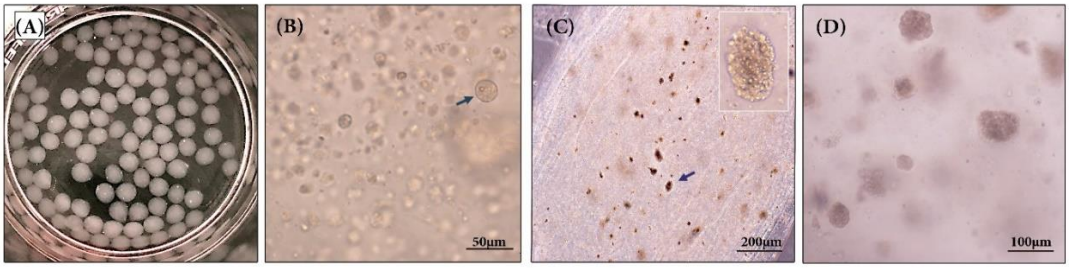

Figure 2. Light microscopy of PC-12 cells encapsulated with alginate beads. (A) Alginate beads, established in the calcium chloride solution, are shown. (B) PC-12 cells (arrows) encapsulated in alginate beads before they start any division or proliferation (1d), (C) following the formation of transparent cell colonies inside the beads in Gln-free culture (6d), and (D) culture supplemented with 40Mm Gln (1d after treatment) (Scale bars=50µm (B), 200µm (C), 100µm(D)).

PC-12 cell survival in alginate beads

Live-dead cell staining of PC-12 cells in alginate beads, indicated their proper survival 2 weeks post encapsulation. Live cells were detected in green (acridine orange dye), while dead ones were observed as red entities (PI dye) (Figure 4).

Cresyl body staining of Nissl bodies

Nissl bodies are rough endoplasmic reticulum (with ribosomes) and are the sites of protein synthesis. The detection of purple Nissl bodies in PC-12 cells indicated their neural differentiation in all investigated groups (Figure 5A). Quantified results showed that the mean number of encapsulated cells differentiated into neuron-like cells (ALG+GLN; 14d, 7d with 40mM Gln) was significantly increased in comparison to the control ALGINATE group. No significant difference was observed for PC-12 cells in 2D-culture in the presence and absence of Gln (7d, 40mM) (Figure 5B).

Immunocytochemistry reveals neuronal differentiation biomarkers in 2D- and 3D cultures of PC-12 cells

To determine the neuronal differentiation potential of PC-12 cells, the protein expression of 2 specific neural markers Nestin (neural progenitor cells) and β-tubulin III (neural mature cells) was investigated via immunohistochemistry analysis. Results from 2D-cultures showed that the mean percentage of Nestin and β-tubulin III in the presence of Gln (7d, 40mM) was increased (31.39 ± 2.71%; 32.83 ± 3.36%) in comparison to the control group (11.87 ± 2.28%;12.88 ± 3.43%) (Figures 6 and 8A).

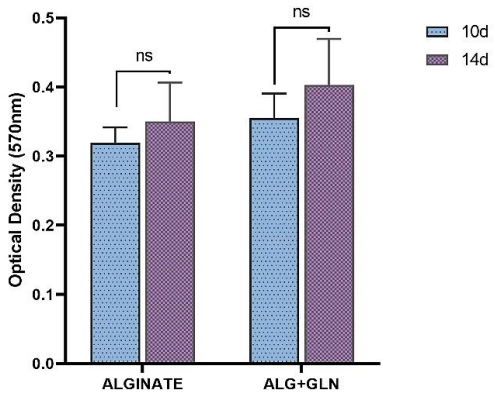

Figure 3. PC-12 cell survival under 3D cultures, obtained by MTT assay. Mean optical densities (570nm) are shown at two investigated time points (10, 14d). Differences were not significant between days 10 and 14 for 2D-cultured PC-12 cells (Gln-free

as control group) (P=0.2195), 3D-cultured cells in the absence [ALGINATE (P=0.8699)], and presence [ALG+GLN (P=0.8951)] of glutamine (Gln). Results are shown as Mean of 3 independent replicates ± SD (p<0.05).

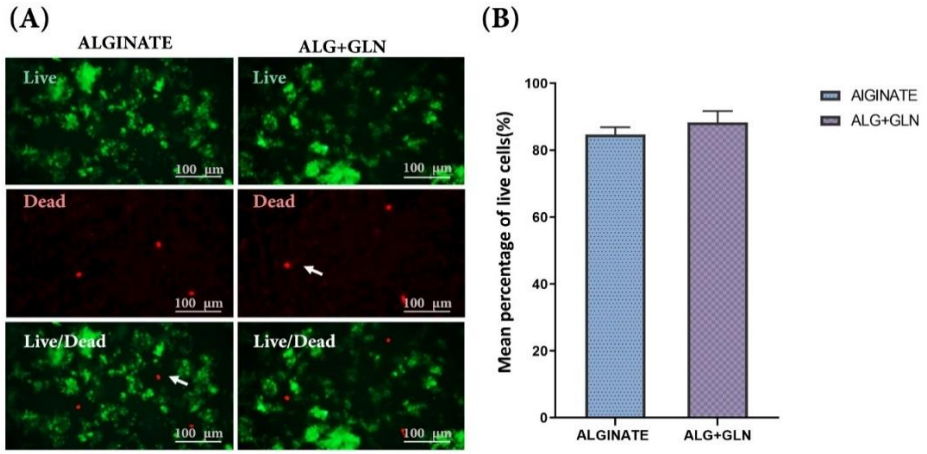

Figure 4. PC-12 cell survival under 3D-culture condition in the absence and presence of Gln (40mM). (A) Acridine orange (AO)/propidium iodide (PI) staining of PC-12 cells in 3D- cultures confirmed proper survival of the cells. It is clear that most of the cells are remained alive (green) 2 weeks following the encapsulation. (B) Images from AO/PI staining of the cells were quantified and reported as the mean percentage of live cells. There was no significant difference between the viability of the cells from ALGINATE (84.67±2.18) and ALG+GLN groups (89.67±2.08) (p<0.05; 3 independent replicates).

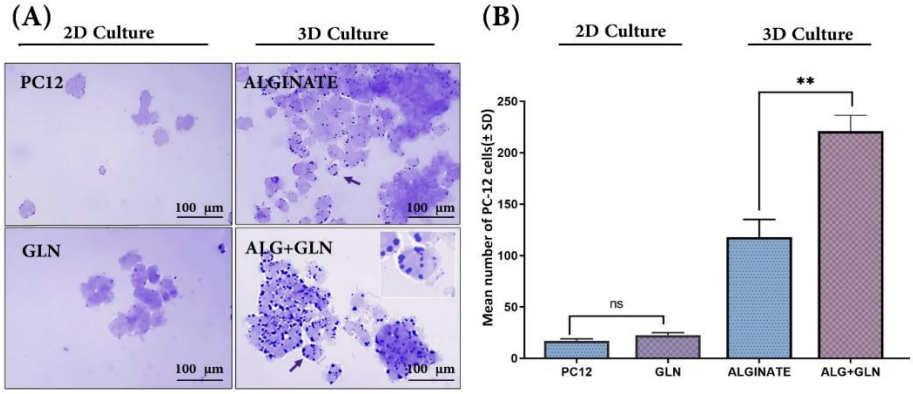

Figure 5. Cresyl violet staining confirmed Nissl bodies' formation. (A) Light microscopic images of Cresyl violet stained PC-12 cells from 2D- and 3D-groups (arrows) (3 replicates, scale bar=100µm). (B) Mean number (±SD) of PC-12 cells, which were positive for Nissl bodies 7d after treatment with 40mM glutamine are shown under 2D- and 3D-culture conditions (p<0.05; 3 replicates).

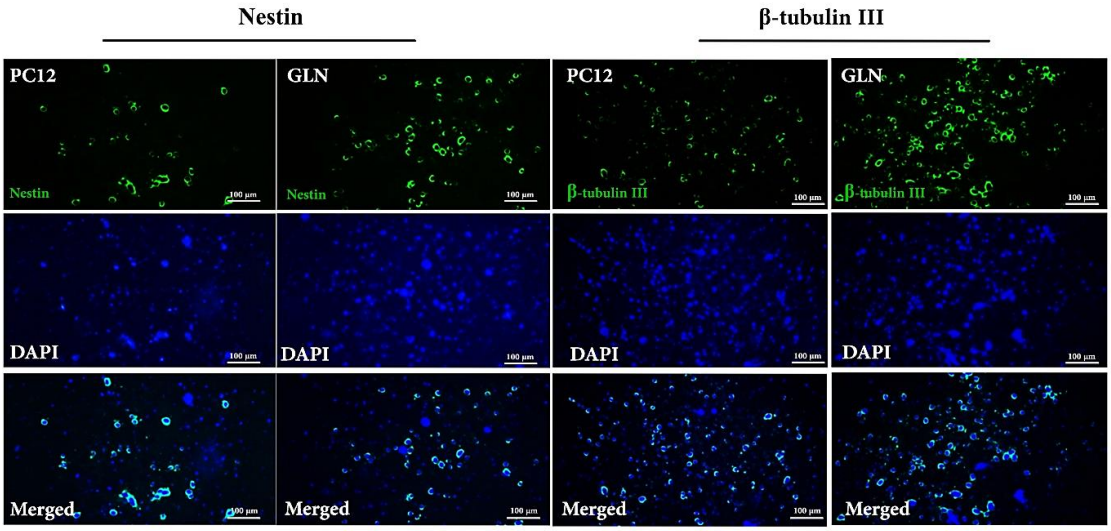

Figure 6. Immunocytochemistry staining for neural markers Nestin and β-tubulin III of PC-12 cells in 2D culture (7d, 40mM Gln). Cells' nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). PC12, control; GLN, the presence of 40mM glutamine (Scale bar=100µm, 3 replicates).

For 3D cultures, immunocytochemistry analysis on 14d (7d after treatment with 40mM Gln) demonstrated the remarkable increase of Nestin (38.40 ± 2.60%) and β-tubulin III (47.68 ± 2.23%) in comparison to the controls (24.67 ± 2.08%; 29.74 ± 2.27%, respectively). Also, a higher level of Nestin and β-tubulin III protein expression was detected for the cells in 3D-culture vs. 2D-cultures (Figures 7 and 8B).

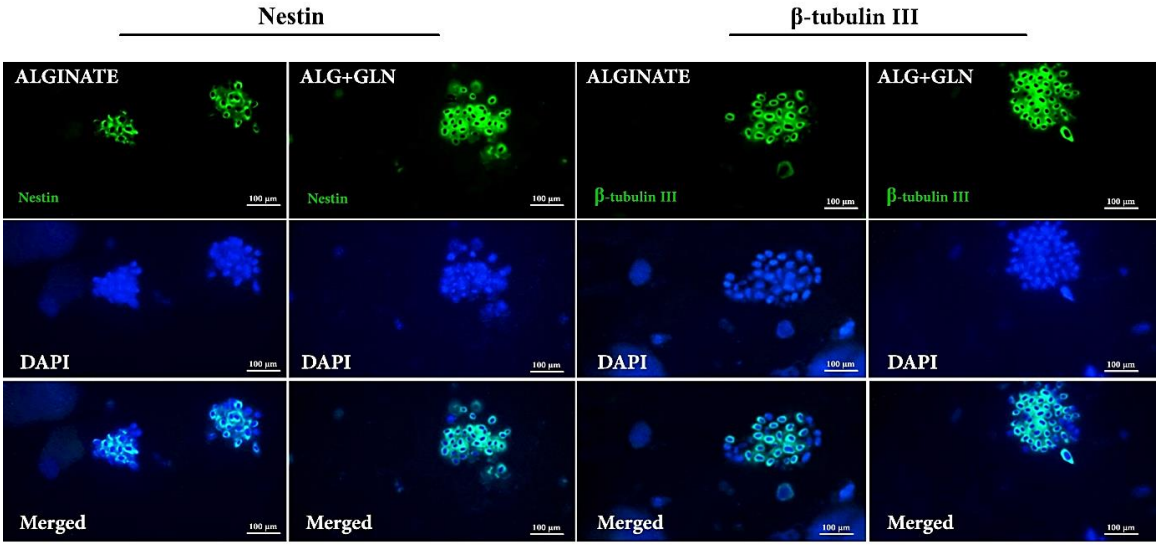

Figure 7. Immunocytochemistry staining for neural markers Nestin and β-tubulin III of PC-12 cells in 3D culture 7d after the treatment (on 14d) in the absence (ALGINATE) or presence (ALG+GLN) of 40mM glutamine. All nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Scale bar=100µm, 3 replicates).

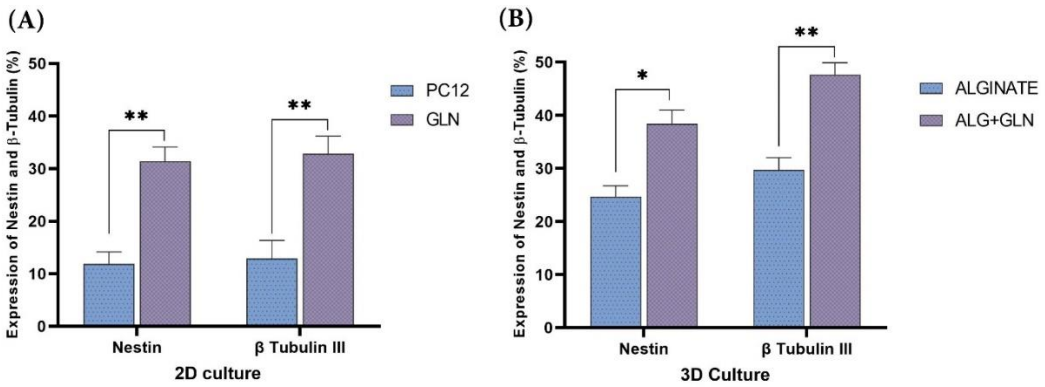

Figure 8. Comparing mean percentages of neuronal markers protein expression 7d after treatment with 40mM glutamine under 2D- and 3D culture conditions (p<0.05; 3 independent replicates). (A) The mean percentage of Nestin and β-tubulin III protein expression in 2D-culture showed a significant increase in GLN group in comparison to the PC-12 group for both markers. (B) The mean percentage of Nestin and β-tubulin III protein expression in 3D-culture showed a significant increase for both markers in ALG+GLN group vs. ALGINATE.

Discussion

The effect of stable Gln on the growth and neuronal differentiation of PC-12 cells in 2D polystyrene culture flasks and 3D alginate hydrogel beads was studied. Morphological changes of neuronal cell lines following the treatment with different chemicals have been the subject of many studies in the field of developmental neurotoxicity. Assessment of neurite growth and density could be considered as an accurate factor (Geranmayeh et al., 2015) and is regarded as an indicator of neuronal differentiation in PC-12 cells (Wiatrak et al., 2020). Our results from 2D-cultures indicated clear cytoplasmic condensation and morphological change of the cells, as well as limited neurite outgrowth with short length.

It was shown in the present study that Gln can increase the survival of PC-12 cells. It may be due to the protective role of Gln against the oxidative stress and its inhibitory effect on the activation of PI3K/Akt signaling pathway (Zhao et al., 2019). It was found that a high concentration of Gln (500mg/kg) can heal hippocampal injuries in rats by the inhibition of apoptosis (Wang et al., 2019). Another *in vitro* study based on pH and ammonia values showed that Gln (2% and 3%) is not toxic (Suh et al., 2022). In various studies, the effects of high concentrations of Gln were studied in the range of 20 to 80 mM (Ban & Kozar, 2010). Results showed that stable Gln (40mM) can act as a differentiation factor for PC-12 cells as it was evidenced by the increased expression of Nestin and β-tubulin III protein markers. This capacity for inducing the neural differentiation of the cells was further confirmed by comparative and quantitative staining of Nissl bodies from PC-12 cells.

It was also shown that Gln concentrations higher than 10mM can significantly increase the mRNA and protein levels of Hsp70 (Wang et al., 2017). Alginate plays a significant role in supporting the proliferation and differentiation of neural/-like cells. Cell encapsulation with alginate-based hydrogels is a rapid, non-toxic, and complete method to immobilized the cells (Andersen et al., 2015). Alginate hydrogel provides a suitable 3D platform for neural cultures and their proper differentiation (Cheravi et al., 2022; Razavi et al., 2015). The present study showed a significant difference (p<0.05) among alginate-encapsulated cells in the presence of a high concentration of Gln in comparison to the control group, based on the increased protein expression of Nestin and β-tubulin III .These differences were also significant for 3D-cultured cells in comparison to the cells in 2D culture, which could be attributed to the beneficial characteristics of alginate, such as supporting the proper cell adhesion and differentiation (Martins et al., 2018). Also, the results of investigating cell survival in 3D-culture showed that there was no significant difference between days 10 and 14, which is consistent with the results of similar studies (Razavi et al., 2015).

In summary, results from the present study, confirmed that supplementing 2D- and 3D- cultures of PC-12 cells with a high concentration of the stable form of glutamine can induce their differentiation and the acquisition of neuronal-like phenotype, which could be a promising approach in neural tissue engineering and regenerative medicine studies aimed to treat neuronal injuries.

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Conflict of interest

The authors declare that they have no conflict of interest.

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