ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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MORPHOMETRIC AND FLUORESCENCE ANALYSIS OF NERVE GROWTH FACTOR-DIFFERENTIATED PC12 CELLS

SİNİR BÜYÜME FAKTÖRÜ İLE FARKLILAŞTIRILMIŞ PC12 HÜCRELERİNİN MORFOMETRİK VE FLORESANS ANALİZİ

Simge ÜNAY[1], Ferhat ŞİRİNYILDIZ[2]
[1] Lokman Hekim Üniversitesi, Tıp Fakültesi, Biyofizik Ana Bilim Dalı, Ankara, TÜRKİYE
[2] Aydın Adnan Menderes Üniversitesi, Tıp Fakültesi, Fizyoloji Ana Bilim Dalı, Aydın, TÜRKİYE

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Öz

Amaç
PC12 bir sıçan feokromositoma hücre hattıdır. Bu hücreler, sinir büyüme faktörü (NGF) ile kültürlendiğinde karakteristik olarak farklılaşmaya uğramaktadır. NGF dozuna bağlı olarak, nörit uzantılarının uzunlukları değişir. Bu farklılaşma özelliği sayesinde hücreler nörobilimde ve Alzheimer, Parkinson, Amyotrofik Lateral Skleroz gibi patofizyolojik hastalıkların modellenmesinde kullanılmaktadır. Ancak NGF'nin PC12 hücrelerinde oluşan nörit uzantıları üzerine olan etkisini gösteren literatür çalışmaları oldukça kısıtlıdır. Bu çalışmanın amacı NGF'nin doza ve inkübasyon süresine bağlı olarak nörit uzantıları ve hücre canlılığı üzerine olan etkisini araştırmaktır.

Gereç ve Yöntem
Bu çalışmada PC12 hücreleri 50 ng/ml ve 100 ng/ml NGF ile 3, 6 ve 7 gün inkübe edilmiştir. İnkübe edilen hücrelerde nörit büyümelerinin uzunlukları ve ölü hücre oranları hesaplanmıştır.

Bulgular
Elde edilen bulgular ile NGF dozlarına ve inkübasyon süresine bağlı olarak nörit uzantılarının uzunluğunun ve ölü hücre oranının arttığı gösterilmiştir. NGF inkübasyon süreleri karşılaştırıldığında 50 ng/ml NGF 6 gün ve 100 ng/ml NGF 3 gün grupları arasında fark olmadığı bulunmuştur.

Sonuç
Deney gruplarında ölü hücre oranları ve nörit uzantılarının boyutları değerlendirildiğinde 100 ng/ml NGF ve 3 gün inkübasyon süresi parametrelerinin PC12 hücre farklılaşması için ideal olduğu düşünülmektedir.

Anahtar Kelimeler: Nörit uzunluğu, PC12 hücre hattı, Sinir büyüme faktörü

Abstract

Objective
PC12 is a rat pheochromocytoma cell line. These cells characteristically undergo differentiation when cultured with nerve growth factor (NGF). Depending on the dose of NGF, the length of neurite extensions changes. Thanks to this differentiation property, the cells are used in neuroscience and in modeling pathophysiological diseases such as Alzheimer's, Parkinson's, and Amyotrophic Lateral Sclerosis. However, literature studies showing the effect of NGF

Sorumlu yazar ve iletişim adresi / Corresponding author and contact address: F.Ş. / ferhat.sirinyildiz@adu.edu.tr
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ORCID IDs of the authors: S.Ü: 0000-0002-5582-5574; F.Ş: 0000-0001-8800-9787

on neurite extensions formed in PC12 cells are very limited. This study aimed to investigate the effect of NGF on neurite extensions and cell viability depending on dose and incubation time.

Materials and Methods
In this study, PC12 cells were incubated with 50 ng/ml and 100 ng/ml NGF for 3, 6 and 7 days. The lengths of neurite outgrowths and dead cell ratios were calculated in incubated cells.

Results
The results showed that the length of neurite extensions and dead cell ratio increased depending on NGF doses and incubation time. When NGF incubation times were compared, no difference was found between 50 ng/ml NGF 6 days and 100 ng/ml NGF 3 days groups.

Conclusion
When the dead cell ratios and sizes of neurite extensions in the experimental groups are evaluated, it is thought that 100 ng/ml NGF and 3 days incubation time parameters are ideal for PC12 cell differentiation.

Keywords: Nerve growth factor, Neurite length, PC12 cell line

Introduction

In vitro studies to examine neurotoxicity have gained more momentum in recent years compared to *in vivo* studies. Because of *in vitro* studies, the possibility of repeating the study increases. Appropriate design of the experiment and selection of the appropriate cell line is extremely important in order to respond to the determined hypothesis. Incorrect selection of cell lines can lead to misevaluation of neurotoxins (1-5). For this reason, cell lines such as dorsal root ganglion (DRG), Schwann cell, and PC12 are used both for mechanistic studies and to determine the effect of potential neurotoxic substances (1).

RatPheochromocytomacell(PC12)isusedinneurotoxic studies, neuroprotective, neuroinflammation, and synapse development processes, as well as in models of chemotherapy-induced peripheral neuropathy, Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis, and cancer as well as nerve injury-induced neuropathic pain, nitric oxide-induced neurotoxicity model (1-5). According to the American Type Culture Collection (ATCC), there are 2 different types, PC12 (CRL-1721) and PC12 Adh (CRL-1721.1). PC12 cells grow as small, irregularly shaped, floating cell clusters or as a few scattered lightly attached cells. In addition, agglomeration can be seen on surfaces that are not coated with suitable materials. They also tend to stick badly. On the other hand, PC12 Adh-type cells show good adhesion to plastic surfaces (6).

PC12 cells can differentiate with nerve growth factor (NGF) (6). When NGF is applied to PC12 cells, it triggers dopamine synthesis. Triggered cells transform into neuron phenotype. Cells that transform into neuron form stop growing and become electrically excitable. The neuronal differentiation process begins with the binding of NGF to the receptor tyrosine kinase TrkA. TrkA is stimulated with NGF and activates 3 different intracellular signal transduction pathways, namely PLCy, Ras/MAPK, and PI3K/Akt (7). PLCy, Ras/MAPK, and PI3K/Akt pathways; Identification of neuron-specific proteins, activation of various transcriptions, and triggering of the GAP-43 gene provide the formation of dendritic extensions (8). NGF-differentiated PC12 cells express the protein Synapsin I, a marker of synaptic communication. Moreover, the level of Synapsin I protein expression has proven to be dependent on the level of cell differentiation (9). Synapsin 1 is an important factor in synaptogenesis and neuronal plasticity, as well as playing a role in the release of neurotransmitters at synapses (10). Due to the expression increases of GAP43 and Synapsin I genes, neurite extensions are formed in PC12 cells.

Differentiation of PC12 cells with NGF can be evaluated by quantitative morphological methods such as cell size, neurite count, and neurite length measurement programs such as Matlab and ImageJ are preferred (16).

Although it is known that NGF and PC12 cells can be differentiated, literature studies that determine the relationship between NGF dose and incubation time are very limited. So, this paper aimed to determine the effect of NGF on neurite outgrowth and cell viability in PC12 cells. The neurite length and dead cell ratios were determined in PC12 cells applied at different doses and different incubation times.

Material and Method

Materials

Donor horse serum (DHS; H1138), fetal bovine serum (FBS; F2442), RPMI-1640 (R8758), nerve growth factor B (NGF; N2513), penicillin-streptomycin (P/S; P4333), L-glutamine (G7513) were purchased from

Sigma-Aldrich (Taufkirchen, Germany). PC12 cell line (CRL-1721, Manassas, VA, USA) was purchased from ATCC. Hoechst 33342/Propidium Iodide (PI) kits were purchased from BestBio (Shanghai, China).

Cell Culture

PC12 (CRL-1721) cells were cultured in RPMI-1640 medium with %5 fetal bovine serum, % 10 donor horse serum, %1 L-glutamine, and %1 Penicillin/ Streptomycin in 5% CO2 at 37 °C until 80–90% confluence. PC12 cells were seeded at 10x104 cells/well at matrigel-coated 24-well plate and then incubated with 50 ng/ml or 100 ng/ml NGF for 3, 6, and 7 days in 5% CO2 at 37 °C (6).

Morphometric Analysis of Neurite Outgrowth

PC12 cells were incubated with 50 ng/ml or 100 ng/ml NGF for 3, 6, and 7 days. Cells were examined under a ZEISS Axio Vert.A1 Inverted Microscope equipped with an Axiocam 208 at the end of NGF incubation. NGF-PC12 cells with greater than two cells were chosen from random cells to measure the neurite length. Zen blue and Axiovision software (Carl Zeiss) was used to measure cells' neurites' length (11).

HO/PI Staining

PC12 cells were treated with 50 ng/ml or 100 ng/ml NGF for 3, 6, and 7 days to determine the live and death of cells. Firstly, cells were washed with PBS and then incubated with a mixed solution of 1 mL staining buffer, 1 µl Hoechst 33342, and 1 µl PI for 5 min at room temperature in the dark. The cells were captured using a ZEISS Axio Vert.A1 Inverted Microscope equipped with an Axiocam 208 at x10 magnification. Image J program was used to merge fluorescence images (12). The death ratio was calculated according to below:

Death ratio: (Death cells/total cells number) *100

Statistical Analysis

Analyzes were performed with at least three replications and were statistically calculated as standard mean and standard deviation (mean ± SD). GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Tukey's multiple comparison test is used to compare the means of groups. Statistically, data with a $P < 0.05$ confidence interval are significant.

Results

Assessment of Cell Death in NGF-PC12 Cells

PC12 cells were treated with different doses of NGF (50 and 100 ng/ml) for 3, 6, and 7 days. HO/PI staining evaluated the death ratio of groups (Fig.1). The rate of dead cells in the experimental groups was calculated with the Image J program. The results showed that the highest dead cell rate was 100 ng/ml for 7 days, and the lowest dead cell rate was 50 ng/ml for 3 days (Fig.2). There was a significant difference in all experimental groups. As the incubation time increased in the same NGF dose groups, the rate of dead cells in the cells increased. Similarly, the rate of dead cells in the experimental groups was observed to be boosted with the increase in NGF dose.

NGF-induced Neurite Outgrowth

PC12 cells incubated at different NGF doses and times were examined under a light microscope (Fig.3). The neurite lengths in the experimental groups were calculated with the Zen blue and Axiovision software. We found that NGF-induced differentiation is correlated with neurite lengths. A significant difference was found between the same NGF dose groups (Fig 4). The maximum neurite length was observed at 100 ng/ml in 7 days (Fig 4). There was no statistical difference between 50 ng/ml groups for 6 days and 100 ng/ml for 3 days (Fig 4).

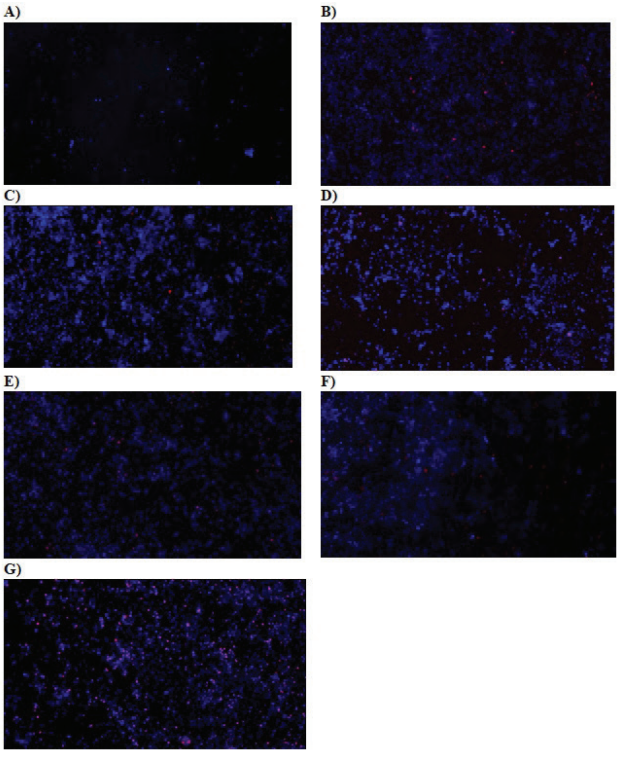

Figure 1

Fluorescence Images of NGF-PC12 cells (10x magnification). Red color cells: Dead cells, Blue color cells: Live cells A) Control B) 50 ng/ml NGF for 3 days C) 50 ng/ml for 6 days D) 50 ng/ml NGF for 7 days E) 100 ng/ml for 3 days F) 100 ng/ml for 6 days G) 100 ng/ml NGF for 7 days

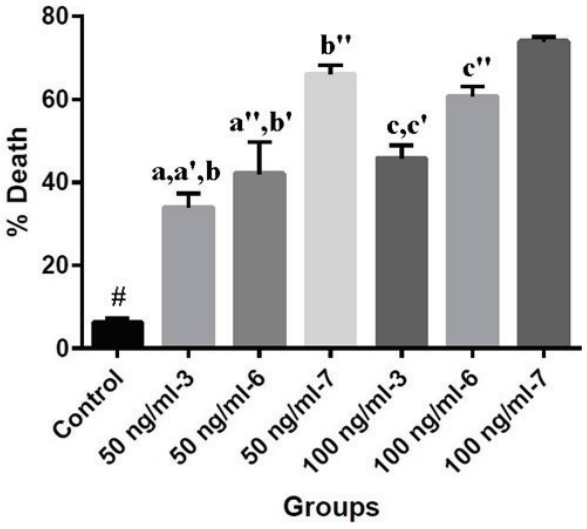

Figure 2
Fluorescence Images and % death ratio of groups in PC12. #: Control vs All the other treatment groups: p<0,001; a: 50 ng/ml-3 vs 50 ng/ml-6: p<0,05; a': 50 ng/ml-3 vs 50 ng/ml-7: p<0,001; a'': 50 ng/ml-6 vs 50 ng/ml-7: p<0,001; b: 50 ng/ml-3 vs 100 ng/ml-3: p<0,005; b': 50 ng/ml-6 vs 100 ng/ml-6: p<0,001; b'': 50 ng/ml-7 vs 100 ng/ml-7: p<0,05; c: 100 ng/ml-3 vs 100 ng/ml-6: p<0,001; c': 100 ng/ml-3 vs 100 ng/ml-7: p<0,001; c'': 100 ng/ml-6 vs 100 ng/ml-7: p<0,001.

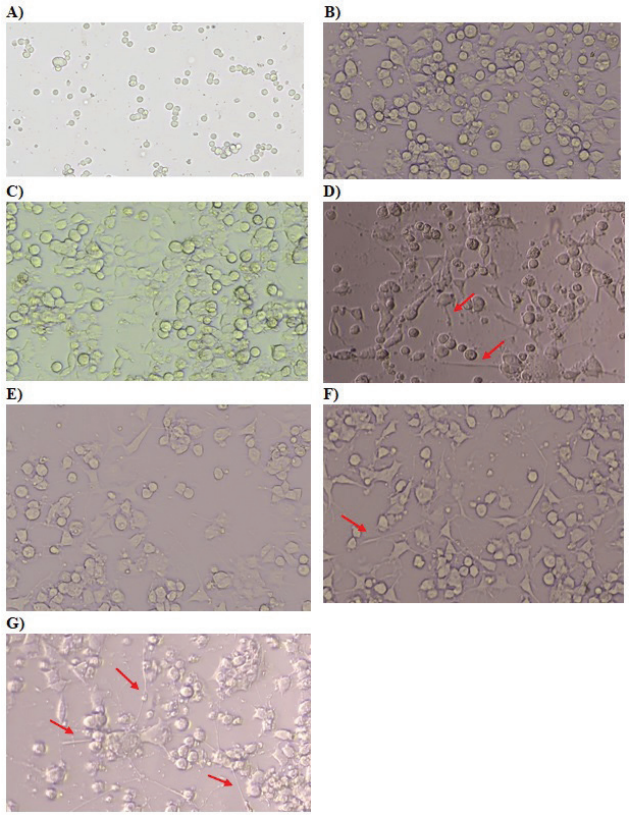

Figure 3
Neurite extensions in NGF-PC12 cells (20x magnification). Red arrows: neurite extensions. A) Control B) 50 ng/ml NGF for 3 days C) 50 ng/ml for 6 days D) 50 ng/ml NGF for 7 days E) 100 ng/ml for 3 days F) 100 ng/ml for 6 days G) 100 ng/ml NGF for 7 days

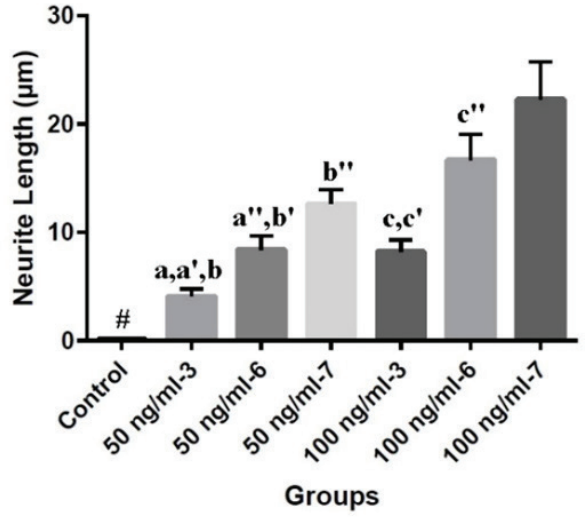

Figure 4
Neurite Length of NGF-PC12 cells. #: Control vs All the other treatment groups: p<0,05; a: 50 ng/ml-3 vs 50 ng/ml-6: p<0,05; a': 50 ng/ml-3 vs 50 ng/ml-7: p<0,001; a'': 50 ng/ml-6 vs 50 ng/ml-7: p<0,05; b: 50 ng/ml-3 vs 100 ng/ml-3: p<0,05; b': 50 ng/ml-6 vs 100 ng/ml-6: p<0,001; b'': 50 ng/ml-7 vs 100 ng/ml-7: p<0,001; c: 100 ng/ml-3 vs 100 ng/ml-6: p<0,001; c': 100 ng/ml-3 vs 100 ng/ml-7: p<0,001; c'': 100 ng/ml-6 vs 100 ng/ml-7: p<0,005.

Discussion

The PC12 line produced from the rat pheochromocytoma is analogous to the fetal neuron primary cell line. It is used in disease models such as pharmacology, neurodegenerative, and peripheral neuropathy due to its easy passage and growth. Upon exposure to NGF, rat adrenal PC12 cell lines can differentiate, and the cells are like primary fetal neurons. PC12 cells have been used in neurophysiologic disease

models such as Alzheimer's, Parkinson's, and peripheral neuropathy (13). Neurite extensions occur in NGF-PC12 cells. Studies showed that biochemical, electrophysiological, and morphological changes could occur by NGF in PC12 cells. These changes depend on NGF doses and incubation time. However, the studies are very limited to the effects of NGF on apoptosis or cell viability.

In the present study, we investigated the effects of different NGF doses and incubation time on neurite length in PC12 cells. PC12 cells were applied with 50 and 100 ng/ml NGF for 3, 6, and 7 days. We observed an increase in neurite length at 100 ng/ml for 3 days and 50 ng/ml for 6 days. We indicated that the dead cells ratio increases more and more NGF doses. Our results are consistent with the other studies of NGF-PC12 cells (14,15). Shilo et al. (2019) indicated that cells were incubated 50 ng/ml NGF for 24, 48, 72, 94, and 144 hours, and there was a decrease in cell viability of approximately 20% after 72 hours in NGF-applied cells (14). Orlowska et al. (2017) determined that cells with 100 ng/ml NGF were found to cause the highest levels of binding to the cell culture dish surface, early stimulation of cell differentiation, and neurite outgrowth in PC12 cells treated with 0, 25, 50, and 100 ng/mL NGF (15).

Wiatrak et al. (2020) showed morphological changes in 50 ng/ml and 100 ng/ml NGF and different incubation times between 3 and 7 days (6). As a result of the analysis, they determined that the longer neurite extension was in cells with 100 ng/ml NGF. Also, they demonstrated that differentiation started from the 3rd day of 100 ng/ml NGF application and was completed on the 7th day. Similarly, some studies determined that neurite length depends on NGF doses. Hu et al (2018) applied different NGF doses (0, 25, 50, 100 ng/ml) in PC12 cells and measured the neurite length of cells (16). They indicated that cells' neurite length and expressions of GAP-43 and synapsis-1 genes increase depending on the NGF dose. Also, we showed that the expression of GAP-43 and synapsin-1 genes was boosted by NGF in the previous study (17).

Sun et al (2021) reported that when 100 ng/ml NGF was applied to PC12 cells, neuronal growth occurred not only in 2D cell culture but also in 3D microenvironments (18). They also showed that neurite extensions were more common in 3D environments in PC12 cells treated with 100 ng/ml NGF. They recommended that PC12 cells with 100 ng/ml NGF could play an important role in future neurophysiologic disease studies.

In this study, PC12 cells were treated with 50 ng/ml, and 100 ng/ml NGF for between 3 and 7 days. According to the viability analysis, differentiation started on the 3rd day of 100 ng/ml NGF application, and the death ratio was lower compared to the other days. It was determined that differentiation took more time and neurite extensions were shorter in cells treated with 50 ng/ml NGF. We believe that the results we have obtained will lay the groundwork for further studies and will be pioneering.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
This article does not contain any studies with human or animal subjects.

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Availability of Data and Materials
Data available on request from the authors.

Authors Contributions
SÜ: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft.

FŞ: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-review & editing.

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