anatomy
www.anatomy.org.tr
An International Journal of Experimental and Clinical Anatomy

Original Article

www.anatomy.org.tr
Received: November 30, 2015; Accepted: December 15, 2015
doi:10.2399/ana.15.030

Graphene oxide has a neuroprotective effect against glutamate-induced excitoxicity on B35 neuroblastoma cell line*

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Abstract

Objectives: Graphene is a quasi-two-dimensional material with unique electrical and chemical properties. In terms of biomedical applications of graphene, nervous system would be an ideal breakthrough model because neural cells are electroactive. Extreme glutamate concentrations cause excitotoxicity. In this study, we aimed to investigate if graphene can increase the resistance to glutamate stress in B35 rat neuroblastoma cells as a cultured cell model for central nervous system neurons.

Methods: B35 neuroblastoma cells were grown in DMEM-F12 growth medium containing 10% fetal bovine serum. Graphene oxide (GO) powder was coated onto glass slides with chitosan as a thin film. B35 cells were cultured on GO films. Cells cultivated on glass slides were used as controls. After 24 h of cell culture, L-glutamine induced excitotoxicity was imposed on B35 cells. After 24 h of glutamate-induced stress, cell morphology was examined by scanning electron microscopy. Cell viability was measured with MTT assay.

Results: The effects of glutamate stress on cell viability were visible as early as 1 h. The cell viability on GO films was higher than that on glass slides, and cells recovered from stress within 6 h on GO surfaces. After 24 h, viability on glass surfaces was 54% lower than that on GO surfaces; these findings were supported with cell morphology observations.

Conclusion: The results of this study showed that GO has a protective role in reducing glutamate-induced excitotoxicity in B35 cell culture, indicating a potential use of GO for treatment of excitotoxicity induced neurodegenerative diseases.

Keywords: B35 cell line; excitotoxicity; glutamate stress; graphene oxide

Anatomy 2015;9(3):156–167 ©2015 Turkish Society of Anatomy and Clinical Anatomy (TSACA)

Introduction

Glutamate is one of the main excitatory neurotransmitters in the central nervous system (CNS), involved in neural transmission, development, differentiation and plasticity. It plays a vital role in neural pathways for cognition, memory and learning, and synapse induction and elimination during development, cell migration, differentiation and death. Glutamate is continuously released from neurons and uptaken from extracellular fluid. There are no enzymes in the extracellular environment that can degrade glutamate. Most neurons and glial cells have glutamate receptors in their plasma membranes.

**This study was presented at the XXIV International Symposium on Morphological Sciences (ISMS), 2–6 September 2015, Istanbul, Turkey*

Glutamate concentration regulates the extent of receptor stimulation. Extracellular glutamate concentration should be kept low because excessive stimulation of glutamate receptors may cause excitotoxicity.[1,2] Excitotoxicity is linked with many neurodegenerative conditions such as Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's, stroke and traumatic brain injury.[3]

To investigate the effects of glutamate stress on an in vitro model of CNS, we used B35 cell line, which has been shown useful in the molecular analysis of endocytosis and signaling pathways, in particular, those that guide axonal outgrowth and cell motility.[4] B35 neuroblastoma cells can be induced to differentiate reversibly, extending long neurites even in low serum conditions, so they are particularly useful for CNS neuronal differentiation and neurite outgrowth studies. They provide substantial advantages over primary CNS neurons in cell biology and biochemistry experiments, because of their ease of culture, efficiency of transfection and ability to establish stable cell lines.[5]

Glutamate may be directly toxic to cultured neuronal cells via two different processes that result in production of free radicals. The classical pathway, excitotoxicity, is through the activation of N-methyl-D-aspartate (NMDA) and non-NMDA glutamatergic receptors, and subsequent calcium influx into the cell.[6] The second pathway is oxidative glutamate toxicity pathway, which is a transporter-mediated type of cell death.[7] This pathway requires cellular expression of the cystine /glutamate antiporter system shown in primary neuronal cultures,[7–11] neuronal cell lines[7,11–14] and tissue slices.[15] The competition of glutamate with cystine for the cystine/glutamate antiporter induces an imbalance in the homeostasis of cystine, the precursor of glutathione (GSH). Therefore, the inhibition of cystine uptake by constant and high exposure to glutamate causes an inability to maintain intracellular GSH levels, leading to a reduced protection against oxidative injury and cell death. The accumulation of excess free radicals seems to be responsible for excitotoxicity. This second pathway can be blocked by antioxidants.[11,14]

In CNS, glutamate transporters play the main role in regulating the physiological clearance of neurotransmitters from the synaptic cleft.[16] Under specific circumstances, glutamate transporters may also play a neuroprotective role.[17–20] High and prolonged exposure to glutamate is a critical event for the pathogenesis of numerous neurological disorders,[21,22] either acute, such as stroke[23] and epilepsy,[24] or chronic, such as Alzheimer's disease,[25] Parkinson's disease,[26] and amyotrophic lateral sclerosis.[27]

Neural tissue repair and regeneration research has been at the forefront lately, due to the adverse impact of neurological diseases on patient's life quality. In recent years, the emergence of nanotechnology provided a promising strategy for treatment of neurodegenerative diseases.[28,29] Researchers implanted nanometer-scale scaffolds to support and promote neurite and axonal growth using tissue-engineering approaches.[28] In experimental in vitro studies to test novel treatment strategies, the use of 3D culture constructs can modulate cell behavior and tissue development, and mimic extracellular matrix (ECM) with unique biomaterials to support realistic in vitro tissue and disease models. Biomedical nanotechnology, electrospinning technique and tissue engineering methods give us exciting insights to the design of a 3D construct with good electrical, mechanical and biological properties as well as compliance match closely resembling the native ECM.[30]

Graphene is one of the most attractive nano-structured materials consisting of a monolayer of sp2-bonded carbon atoms. This quasi–two-dimensional (2D) material has high transmittance and excellent conductivity. Graphene and graphene oxide (GO) sheets are biocompatible platforms that have the potential to mediate stem cell lineage specification for tissue regeneration. As neuronal cells are electro-active and their function is based on electrical activity, graphene is suitable as a substrate for neural cells.[31] Graphene and its derivatives (graphene oxide or reduced graphene oxide) are used as scaffolds to support cellular attachment, proliferation and differentiation.[32–34] To grow or communicate with neighboring cells, neurons secrete substances, many of which are adsorbed on graphene surfaces, and subsequently affect cell proliferation and differentiation.[35] Due to its unique electrical properties, graphene and GO are considered as potential treatments for regulating neural cell behavior in degenerative CNS conditions.[31]

GO can be easily dispersed in many solvents and especially well in water, which does not lessen biocompatibility, and also facilitate any subsequent processing.[36] We used GO films prepared on chitosan (CS) coated glass surfaces as culture substrate. CS is produced through deacetylation of chitin, one of the most commonly found natural polymers on earth.[37] It is a biocompatible and biodegradable hydrophilic biopolymer and has multiple functional groups. CS is protonated in acid media, which favor the interaction between polymer

chains and GO sheets, and allow dispersion of GO as a thin film onto CS coating.[38]

In this study, we used B35 neuroblastoma cells as a suitable model for CNS neurons and investigated if GO may have a neuroprotective effect under glutamate stress, and therefore if it may be useful in the treatment of neurodegenerative diseases.

Materials and Methods

Preparation of graphene oxide films

Graphene oxide was purchased from Graphene Chemical Industries Co., 2% (w/v) chitosan (Sigma C3646; Sigma-Aldrich, Taufkirchen, Germany) was prepared by dissolving in 0.2 M acetic acid for a day on magnetic stirrer. 10 mg/mL graphene oxide suspension was prepared in distilled water. After a homogenous dispersion was obtained, chitosan solution was dropped onto glass slides. Immediately after chitosan was spread on glass, graphene oxide suspension was dripped slowly on chitosan films. The resulting coatings were allowed to dry overnight under fume hood. Each coverslip was sterilized by exposing to UV light for 2 hours (**Figure 1**).

Cell culture

B35 rat neuroblastoma cells were obtained from Ege University Bioengineering Department, Animal Cell Culture and Tissue Engineering Laboratories. B35 cells were cultured in Dulbecco's Modified Eagle Medium F12 (DMEM-F12) (F4815; BioChrom AG, Berlin, Germany) growth medium supplemented with 1% L-glutamine (K0282; BioChrom AG, Berlin, Germany), 0.1% gentamycin (BioChrom AG, A2710) and 10% fetal bovine serum (FBS) (S0115; BioChrom AG, Berlin, Germany) at 37 °C in a 5% CO_2 incubator. Cell media were changed every 48 h. Cells were seeded onto GO films and glass slides at a concentration of 105 cells/cm^2 and cultured for 24 h before L-glutamine exposure.[39]

Glutamate excitotoxicity

After 24 h of cell culture, B35 cells on both surfaces were treated with 150 mM L-glutamine to induce excitotoxicity. Different groups of B35 cells were exposed to L-glutamine for durations of 1, 3, 6 and 24 h. After L-glutamine exposure for given durations, culture media on the cells were replaced by fresh growth media, and the culture was continued until total of 24 h. Each experimental and control treatment was applied on five replicates (n=5).

Cell viability assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma M5655; Sigma-Aldrich, Taufkirchen, Germany) test assess cellular metabolic activity on the basis of the ability of mitochondrial succinate-tetrazolium reductase to convert the yellow tetrazole dye (MTT) to a purple-colored formazan in viable cells. The metabolic activity of the cells is proportional to the color density produced. Briefly, following B35 cell incubation for 24 h, the culture medium was aspirated and serum-free DMEM-F12 media containing 10% v/v MTT stock solution (5 mg/mL MTT dissolved in PBS) were added to each sample and they were incubated at 37 °C and 5% CO_2 for 3 h. The supernatant was removed, and the formazan crystals were dissolved in dimethylsulfoxide (DMSO, Sigma 41640; Sigma-Aldrich, Taufkirchen, Germany). Absorbance was recorded at 570 nm and 690 nm using a microplate reader VersaMax; Molecular Devices, Sunnyvale, CA, USA).[40]

Evaluation of cell morphology

To evaluate possible changes in B35 cell morphology induced by L-glutamine, light and scanning electron microscopy (SEM) images were examined. Phase contrast images were captured using an inverted light microscope (Olympus CK 40; Olympus, Tokyo, Japan) after 1, 3, 6 and 24 h.

To prepare the samples for SEM, they were washed with PBS for 30 s and fixed with 5% (v/v) glutaraldehyde (25%, Merck, 8.20603.1000; Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate (Sigma CO250; Sigma-Aldrich, Taufkirchen, Germany) for 30 min at +4 °C. Glutaraldehyde was aspirated and 7% sucrose in 0.1 M sodium cacodylate was applied for 15 min. at +4 °C twice. The samples were post fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate for 30 min. on ice. They were washed three times with distilled water for 5 min., dehydrated in ethanol series (35%, 50%, 70%, 85%, 95% and 100%) and rinsed with hexamethyldisilazane (HMDS) for 5 min. and air dried at room temperature for 30 min. After being completely dried, the samples were mounted on aluminum pins with double-sided carbon tape and sputter coated with gold, and observed by SEM (JEOL JSM-6060; Jeol, Tokyo, Japan).

Actin cytoskeleton staining

Cells on GO and glass surfaces were fixed with a 4% paraformaldehyde solution (USB 19943; USB, Cleveland, OH, USA) for 15 min. Each sample was washed 2 times with PBS without Ca+2 and Mg+2.

Then, NH4 Cl (Sigma A9434; Sigma-Aldrich, Taufkirchen, Germany) solution was added for 7 min. to quench the fixative. Cells were permeabilized with 0.2% PBS-Triton X-100 (A1388; AppliChem, Darmstadt, Germany) for 15 min, and stained with 1:1000 DAPI, 1:100 Alexa Fluor 488 Phalloidin (Invitrogen A12379; Thermo Fisher Scientific, Waltham, MA, USA) for 40 min. Access water was removed, and the coverslip was inverted on a slide on 10 µL Mowiol 4-88 (Calbiochem, Merck 475904; Merck Millipore, Darmstadt, Germany) mounting medium. They were kept overnight at 4 °C to dry, and imaged using a fluorescence microscope (AXI0; Zeiss, Oberkochen, Germany).

Statistical analysis

For cell viability analysis, data were expressed as mean ± standard error (SE) based on five replicate observations from two independent experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A value of $p < 0.05$ was considered statistically significant. All analyses were conducted using the Prism software package (GraphPad Software, La Jolla, CA, USA).

Results

Toxic effects of L-glutamine exposure on B35 cell viability was visible as early as 1 h. Cells that were exposed to L-glutamine showed decreased number of neurites, and visible accumulation compared to control group (**Figures 2a** and **b**).

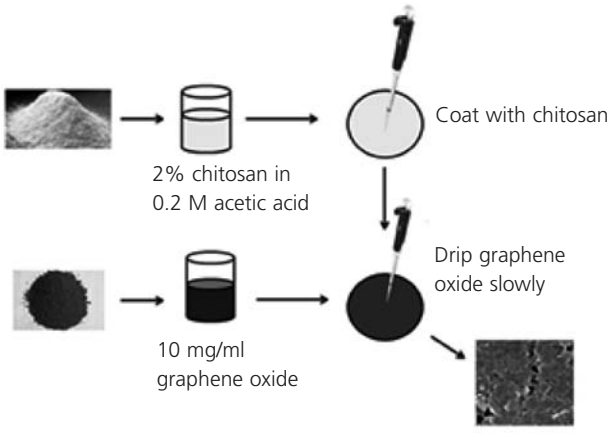

Figure 1. Graphene oxide flakes were coated onto chitosan covered glass slides as a film.

B35 cell viability under no stress condition and after L-glutamine exposure on GO films was measured with MTT assay after 1, 3, 6 and 24 h. On GO, cell viability decreased after 1 h L-glutamine exposure, but this trend did not continue at further time points, and significant cell recovery was observed after 3h (**Figure 3a**).

On glass slides, highest level of excitotoxicity was observed after 3 h L-glutamine exposure. On GO surfaces, cells fully recovered from stress within 6h while cell viability on glass surfaces was still lower than 54% after 24h. The culture on GO increased the resistance to glutamate stress of B35 cells, and helped fast recovery.

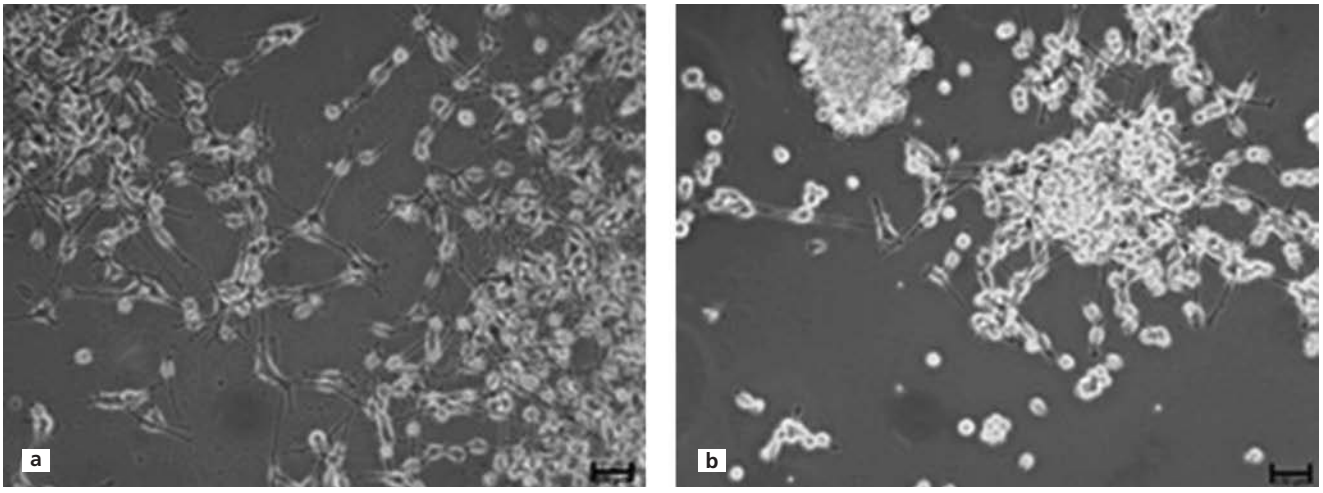

Figure 2. B35 neuroblastoma cells under light microscopy before and after addition of 150 mM L-glutamine. (**a**) Control B35 cells, (**b**) B35 cells treated with L-glutamine for 1 h. Scale bar = 100 µm.

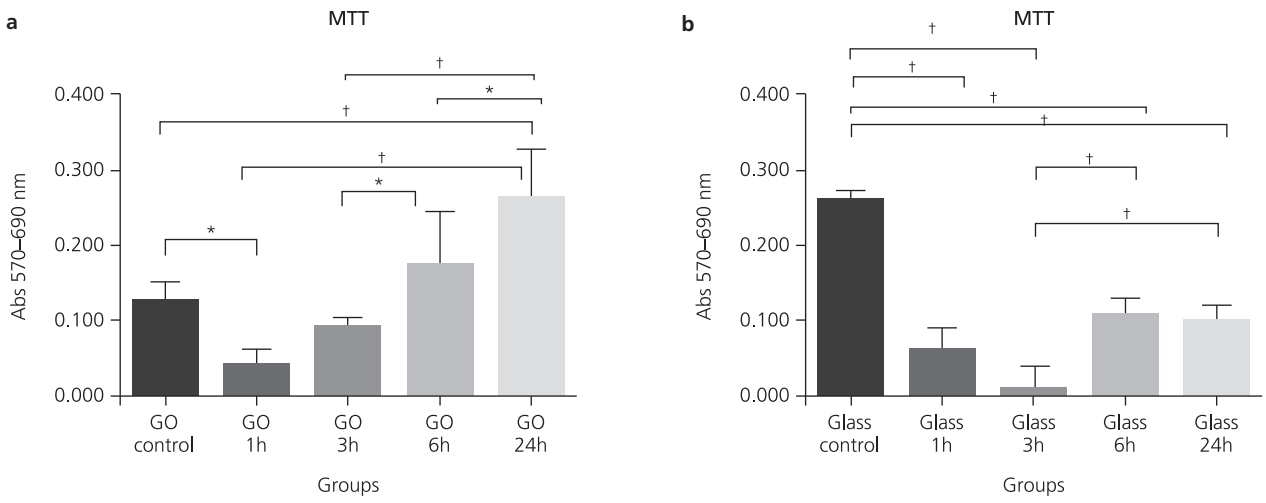

Figure 3. Cell viability after glutamate stress (**a**, **b**). *p<0.02, †p<0.0002.

Fluorescence microscopy images showed that the cell number and viability started to be affected after 1 h L-glutamine exposure, (**Figure 4b**). After 6h L-glutamine exposure, B35 cells were adapted to glutamate stress on GO surfaces (**Figure 4d**). This observation is parallel with MTT assay results. We could not take clear fluorescent images on GO films, because the neurites are overlapped and chitosan has auto-fluorescence (**Figure 5**).

SEM images show the decreased cell number after 1 h L-glutamine exposure on both surfaces (**Figures 6b** and **7b**). Higher cell numbers are visible on GO surface compared to glass slides after 24 h L- glutamine exposure (**Figures 6e** and **7e**). The MTT assay supported this observation.

The effects of glutamate stress on cell viability were visible as early as 1 hour on both surfaces. The cell viability of B35 cells on GO sheets was higher than on glass slides, and cells recovered from the stress within 6 hours on GO surfaces while viability on glass surfaces was 54% lower than that on GO surfaces after 24 hours. Cell morphology and toxicity measurements also supported these observations.

Discussion

In this study, we investigated whether GO may have a neuroprotective effect on B35 cell culture, a model for CNS neurons, under glutamate stress and therefore, if it may be useful in the treatment of neurodegenerative diseases linked to glutamate stress. We have demonstrated that glutamate is toxic to B35 cells above the concentration of 100 mM. The culture on GO films increased the resistance of B35 cells to glutamate stress, and helped fast recovery as shown in our results with MTT (**Figure 3**), fluorescence microscopy (**Figures 4** and **5**) and SEM microscopy (**Figures 6** and **7**).

Neuronal cells are electro-active and their function is based on electrical activities. Therefore graphene, being electrically conductive, has been shown suitable for neural cell adhesion.[31] Li et al.[41] also showed that graphene exhibits excellent biocompatibility during the development and maturation in a mouse hippocampal culture model. This study demonstrated that viability of the neural cells and average of neurite lengths on graphene substrates were significantly enhanced compared to the conventional polystyrene tissue culture substrates, indicating that graphene is a neuron-favorable material.

Due to their unique electrical properties, graphene and graphene oxide can also be considered in potential treatment strategies for regulating neural cell behavior in CNS degeneration. Park et al.[42] showed that graphene induced differentiation of human neural stem cells (hNSCs) to neurons more effectively compared to glass substrates. Weaver et al.[43] also explored the potential of a GO/PEDOT (poly 3,4-ethylenedioxythiophene) nanocomposite films for NSCs differentiation in vitro and showed that they GO composites promoted neuronal differentiation while only PEDOT surfaces lead more toward oligodendrocyte differentiation. On the contrary, Shah et al.[44] used nanofibrous scaffolds with graphene to guide NSC differentiation to oligodendrocytes without any differentiating agent. They observed that coating with a higher concentration of GO promoted differentiation toward mature oligodendrocytes.

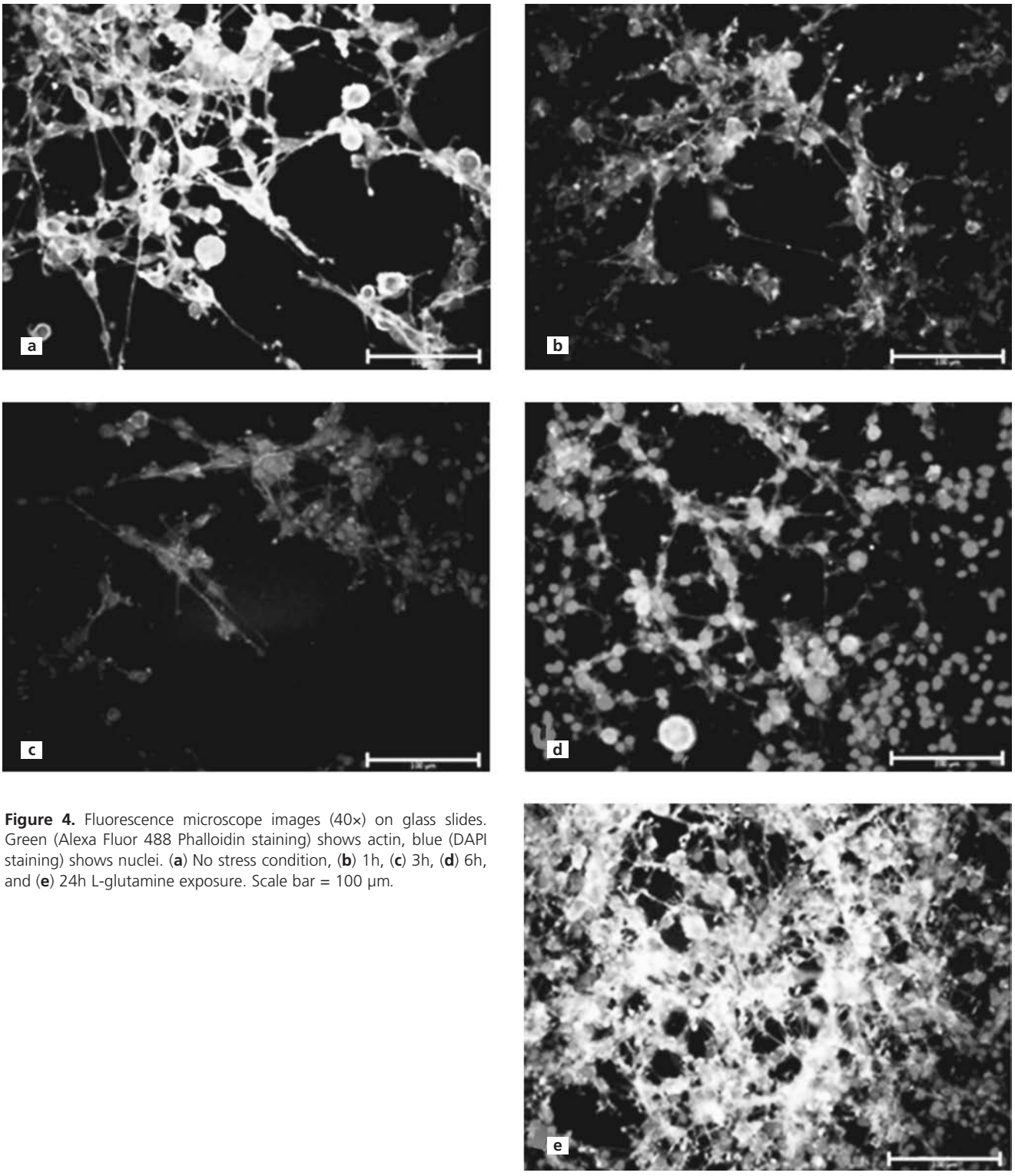

Figure 4. Fluorescence microscope images (40×) on glass slides. Green (Alexa Fluor 488 Phalloidin staining) shows actin, blue (DAPI staining) shows nuclei. (**a**) No stress condition, (**b**) 1h, (**c**) 3h, (**d**) 6h, and (**e**) 24h L-glutamine exposure. Scale bar = 100 μm.

Wang et al.[45] demonstrated that fluorinated graphene enhanced cell adhesion and proliferation of mesenchymal stem cells (MSCs), induced cell polarization and promoted differentiation to neuronal lineage. Lee et al.[46] showed that MSCs exhibited different morphologies on graphene, graphene oxide and PMDS (polydimethylsiloxane) substrates. On PMDS, cells appeared round and lacked filopodia extensions; but on

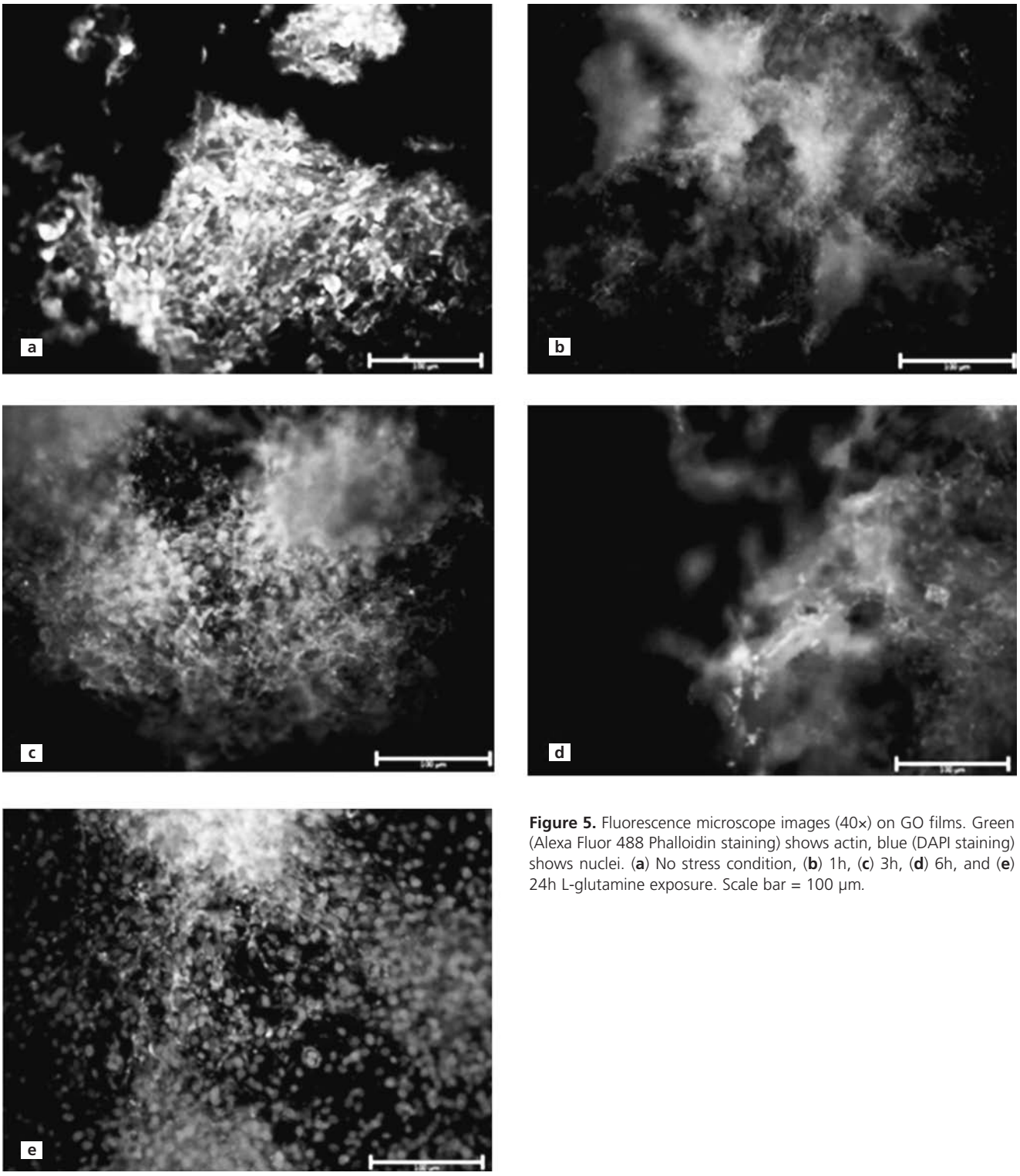

Figure 5. Fluorescence microscope images (40×) on GO films. Green (Alexa Fluor 488 Phalloidin staining) shows actin, blue (DAPI staining) shows nuclei. (**a**) No stress condition, (**b**) 1h, (**c**) 3h, (**d**) 6h, and (**e**) 24h L-glutamine exposure. Scale bar = 100 μm.

graphene films, they had spindle shaped morphology, whereas on GO films, they were widespread and larger. These studies suggest that graphene can be used as a nanostructured scaffold for controlling the differentiation of stem cells in a concentration dependent manner for neural regenerative medicine.

In this study, we used chitosan as a vehicle to hold GO flakes as a film on the surface. Chitosan, itself, is a

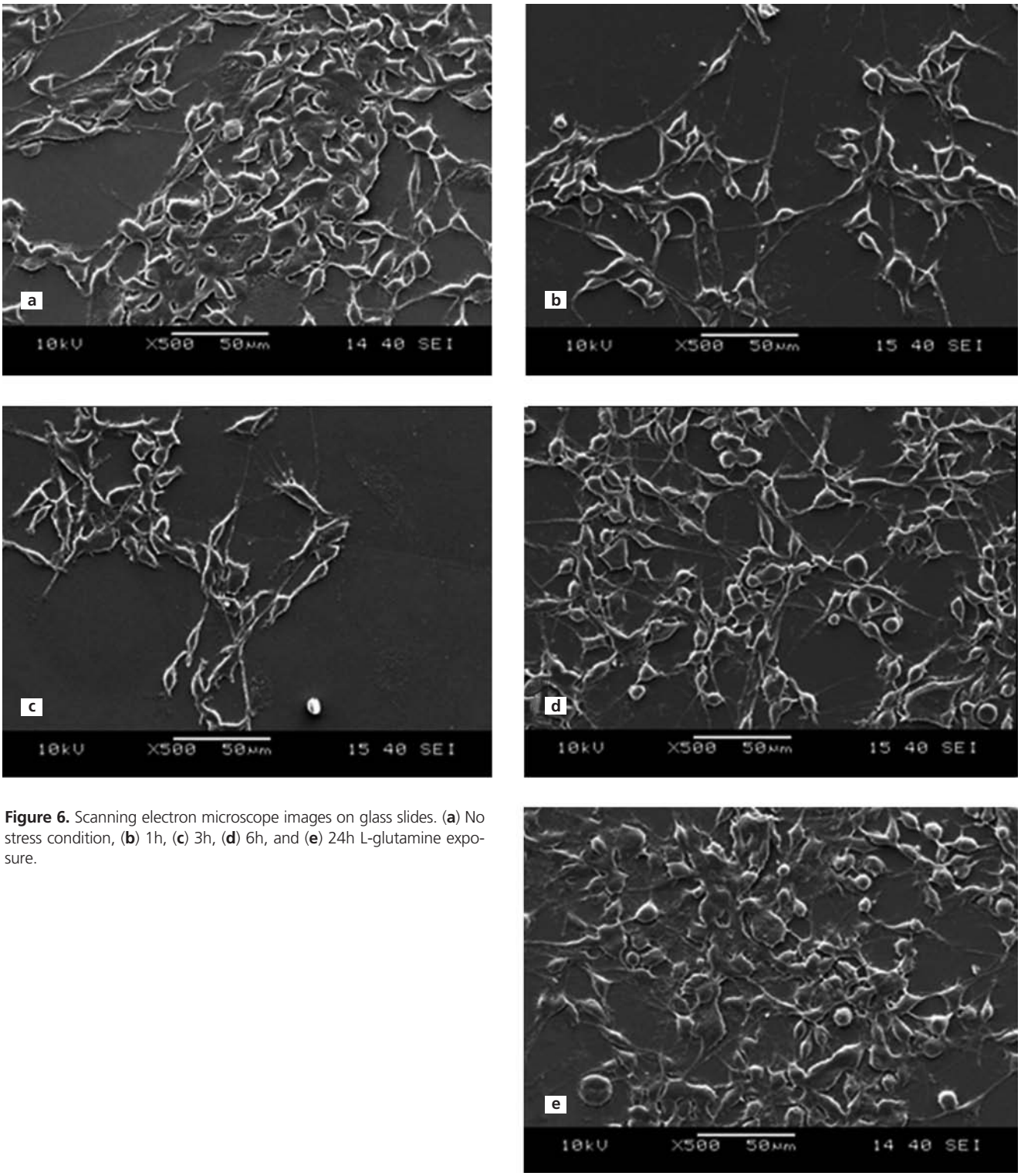

Figure 6. Scanning electron microscope images on glass slides. (**a**) No stress condition, (**b**) 1h, (**c**) 3h, (**d**) 6h, and (**e**) 24h L-glutamine exposure.

biocompatible material. Depan et al.[47] used chitosan-graphene network structured scaffolds to cultivate MC3T3-E1 mouse pre-osteoblast cell line, and also observed that chitosan-graphene film facilitated cell attachment, proliferation and growth. Zuo et al.[48] used three different films, chitosan, GO-chitosan and GO/chitosan mix to investigate the potential application of C3H10T1/2 cells in growth of articular carti-

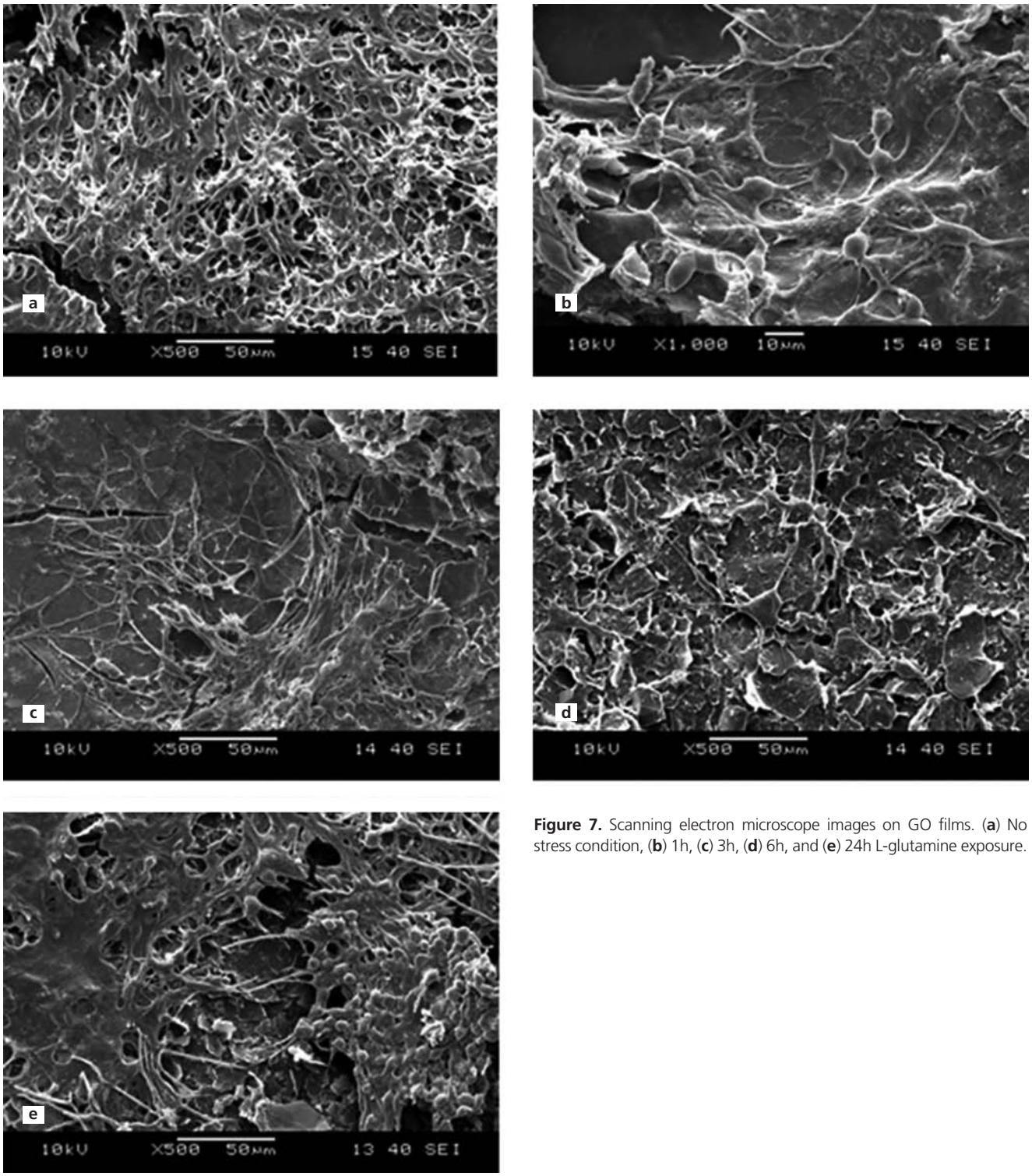

Figure 7. Scanning electron microscope images on GO films. (**a**) No stress condition, (**b**) 1h, (**c**) 3h, (**d**) 6h, and (**e**) 24h L-glutamine exposure.

lage. They observed that GO-chitosan film had good biocompatibility, biodegradability, good solubility in aqueous medium and was suitable for cell proliferation. Pan et al.[49] also showed green production of nanocomposite GO/chitosan films by dissolving chitosan in aqueous acetic acid solution and adding GO suspension into the chitosan solution. To the authors' knowledge, there are no other studies in the literature investigating

the effects of chitosan assisted GO films on neural cell behavior.

Rapid removal of glutamate from the extracellular space is required for the survival and normal function of neurons; excessive concentrations cause excitotoxicity.[50] Greene and Tischler[51] studied with PC12 cells with induced glutamate stress. They showed that toxic effects of glutamate on PC12 occur in a dose and time dependent manner. Toxic concentration of glutamate varies between 0.01 and 10 mM,[52–57] while time varies from 30 min to 3, 12, 24, and 48 h of incubation. There are also studies reporting that excitotoxic cell death in PC12 cells can be effected by apoptosis and necrosis.[58–60] We have demonstrated that glutamate is toxic to B35 cells above the concentration of 100 mM and chose the concentration of 150 mM.

Our results indicate that GO film substrates support CNS cell attachment and proliferation, as well as increase their resistance toward glutamate-induced excitotoxicity. It is possible that GO, with its electrical conductivity, is providing biomimetic cues to the cells, and implementing a suitable niche for cell-cell and cell-extracellular matrix interactions for improved cellular homeostasis to resist glutamate mediated excitotoxicity. This observation suggests that GO based biomaterials may find future applications in neural interfacing and in novel therapeutic approaches for neurodegenerative disorders. Using aqueous chitosan solutions to assist GO film production was successfully achieved, and showed no detrimental effect on cellular viability; but we were not able to take clear fluorescent images because chitosan has auto-fluorescence (**Figure 5**). Therefore, another method for GO film production might be more suitable for easier observation of cell behavior.

Conclusion

The results of this study showed that culture of CNS model cell line (B35) on GO films increases the resistance of the cells against glutamate stress. This attribute of the material suggests that there may be a potential use of GO as a therapeutic biomaterial for neurodegenerative diseases that are linked to glutamate-induced excitotoxicity.

Acknowledgements

This study was supported by TUBITAK (The Scientific and Technological Research Council of Turkey), Grant number: 115S066.

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Online available at:
www.anatomy.org.tr
doi:10.2399/ana.15.030
QR code:

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Conflict of interest statement: No conflicts declared.

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