Advanced Pharmaceutical Bulletin

*Adv Pharm Bull*, 2015, 5(4), 515-521
doi: 10.15171/apb.2015.070
http://apb.tbzmed.ac.ir

TUOMS Publishing Group

*Research Article*

# Paracrine Neuroprotective Effects of Neural Stem Cells on Glutamate-Induced Cortical Neuronal Cell Excitotoxicity

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**Article info**

***Article History:***
Received: 8 January 2015
Revised: 28 June 2015
Accepted: 30 July 2015
ePublished: 30 November 2015

***Keywords:***
· Glutamate
· Neural stem cells
· Neurons
· Primary cell culture
· Cell viability
· Rat

**Abstract**

***Purpose:*** Glutamate is a major excitatory neurotransmitter in mammalian central nervous system. Excessive glutamate releasing overactivates its receptors and changes calcium homeostasis that in turn leads to a cascade of intracellular events causing neuronal degeneration. In current study, we used neural stem cells conditioned medium (NSCs-CM) to investigate its neuroprotective effects on glutamate-treated primary cortical neurons.

***Methods:*** Embryonic rat primary cortical cultures were exposed to different concentrations of glutamate for 1 hour and then they incubated with NSCs-CM. Subsequently, the amount of cell survival in different glutamate excitotoxic groups were measured after 24 h of incubation by trypan blue exclusion assay and MTT assay. Hoechst and propidium iodide were used for determining apoptotic and necrotic cell death pathways proportion and then the effect of NSCs-CM was investigated on this proportion.

***Results:*** NSCs conditioned medium increased viability rate of the primary cortical neurons after glutamate-induced excitotoxicity. Also we found that NSCs-CM provides its neuroprotective effects mainly by decreasing apoptotic cell death rate rather than necrotic cell death rate.

***Conclusion:*** The current study shows that adult neural stem cells could exert paracrine neuroprotective effects on cortical neurons following a glutamate neurotoxic insult.

## Introduction

Glutamate is a major excitatory neurotransmitter in mammalian central nervous system (CNS). Excessive glutamate releasing over-activates its receptors, promotes calcium overloading and initiates subsequent intracellular events that lead to neuronal degeneration.[1] Neuronal dysfunction and cell death are mediated by different mechanisms such as the production of reactive oxygen species, releasing neuronal zinc, poly (ADP-ribose) polymerase-1 activation, mitochondrial dysfunction, and overstimulation of enzymes such as calpains and other proteases, phospholipases, and endonucleases.[1-5] Oxidative glutamate toxicity is another mechanism in which cystine/glutamate transporters are inhibited by excessive glutamate concentration. Furthermore, the cystine uptake is inhibited which decreases intracellular glutathione levels and finally lead to neuronal oxidative stress.[6] Glutamate-induced excitotoxicity has been reported in both acute injuries such as ischemic stroke, and chronic brain disorders such as Alzheimer's and Parkinson's diseases.[2] Studies showed that glutamate receptors antagonists are not efficient to protect neurons against excitotoxicity and hence it is necessary to find new approaches to limiting glutamate-induced toxicity.[5]

Adult mammalian brain contains neural stem cells (NSCs) that give rise into new neurons and glial cells throughout life.[7-9] Two main areas of adult mammalian brain, the lateral subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in hippocampus accommodate NSCs. By virtue of their neuroprotective properties, neural stem and progenitor cells, with capacity to self-renew and generating different cell types of the CNS, have been extensively used for an array of nervous tissue disorders.

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The therapeutic effects of NSCs or NSCs conditioned medium (NSCs-CM) was shown *in vivo* on axon regeneration of corticospinal tract after spinal cord injury because of their protective effects on injured neurons,[10] also neuroprotective effects of NSCs and NSCs-CM was demonstrated *in vitro* on organotypic spinal cord cultures after glutamate induced-excitotoxicity.[11] This therapeutic effects is mainly due to the migration capabilities of NSCs to the injured areas,[11] and their anti-inflammatory and chaperone-like actions that inhibit neuronal devastation.[12,13] Remerging data suggest that inherent therapeutic effects of NSCs does not limit to only cell replacement, but they also exert paracrine effects on damaged cells and microenvironments.[14] NSCs and/or NSCs-CM has been showed to exert *in vitro* neuroprotective effect on neurons against degenerative procedures by releasing neurotrophic factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), glial-cell derived neurothrophic factor (GDNF), and nerve growth factor (NGF).[11,15] Primary cortical culture is a potent tool to study the effect of neurotoxic and neuroprotective agents on neuronal cell metabolism and survival.[16] Similarly, many efforts have been undertaken to exploit NSCs as a promising tool in neurotoxicity studies,[17] neurodegeneration modeling, drug discovery and gene or cell therapy.[18] So far, studies on the effects of NSCs have not completely elucidated the underlying mechanism with regard to neuronal cultures exposed by toxic agents. In current study to examine whether NSCs-CM could abolish glutamate-induced excitotoxicity or not, glutamate-treated primary cortical neurons were incubated with NSCs-CM and the levels of survival in different concentrations of glutamate-induced excitotoxicity were measured.

## Materials and Methods

### *Experimental animals*

Pregnant Wistar rat E 16.5-18.5 embryos as well as adult male Wistar rats (5-8 weeks old), were used for primary cortical neurons and adult NSCs isolation, respectively. Rats were housed in an animal facility maintained at 22 ± 2°C and 55 ± 5% relative humidity under a 12/12 h light/dark cycle and food and water were available *ad libitum*. Animal handling and experimental procedures were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and Iranian Ethical Guidelines for the Use of Laboratory Animals.

### *Isolation and culturing of adult NSCs*

Adult NSCs were isolated from male Wistar rats, as described previously by Azari et al.[19] In brief, subventricular zones of rats were collected, dissected and placed in cold Hank's Balanced Salt Solution (HBSS). The collected tissue was minced and enzymatically dissociated by using 0.05% trypsin/EDTA solution (Gibco, Paisley,UK) approximately for 5 min in 37°C water bath. Next, cells were resuspended in DMEM/F-12 (Gibco) containing 2% B-27 (Gibco, Grand Island, USA), 1% N-2 (Gibco, Grand Island, USA), 2 mM L-glutamine (Gibco, Paisley, UK), 20 ng/ml bFGF (Sigma-aldrich, St Louis, USA), 20 ng/ml EGF (Sigma-aldrich, St Louis, USA), and 1% penicillin/streptomycin (Sigma-aldrich, St Louis, USA). Cells were plated on a T25 flask cultured in 5% CO2 and 95% humidity at 37°C. Medium was replaced every 4 days to potentiate cell growth and neurosphere formation (Figure 1 a & b). Resulting neurospheres were passaged to expand the NSCs pool. In passage 2, when the neurospheres reached 50µm size the medium was replaced with fresh medium and was incubated for 72 h. overnight Then the medium containing neurospheres centrifuged at 400*g* for 5 min and the supernatants filtered through a 0.20-µm filter and stored at -80°C until needed.[20,21]

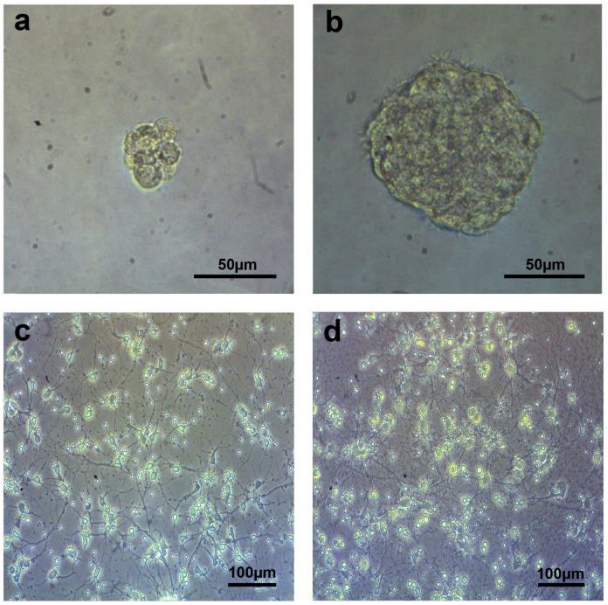


**Figure 1.** Representative bright-field images of primary adult NSCs culture 3 (a) and 9 (b) days post-seeding. The micro-spikes are distinctively evident at the periphery of neurosphere mass indicating viable neuroshperes. Panel c and d illustrate primary cortical cultures of embryonic rat 4 and 12 dyas after plating, respectively. The primary cortical neurons provided extent neurite outgrowth attributing to form integrated network of processes in culture within 4 (c) and 12 (d) after primary isolation.

### *Primary cultures of cortical neurons and glutamate exposure*

Cortical neurons were harvested from Wistar rats using method by Kim et al.[22] and Pacifici et al.[23] with some modifications. In brief, cerebral cortices of rat embryos were dissected. The cortical tissues were dissociated mechanically by trituration with Pasteur pipette. Then, the cells were resuspended in Neurobasal medium (Gibco, Paisley, UK) containing 2% B-27, 0.5 mM L-glutamine, and 1% penicillin/streptomycin and seeded at a density of $5 \times 10^4$ cells/cm.$^2$ Prior to seeding, 24- and 96-well plates were coated with Poly L-Lysine (PLL, MW = 70,000-150,000 g/mol; 10 µg/ml, Sigma, St

Louis, MO) at 37°C overnight. Isolated neurons cultured at humidified 37°C with 5% $CO_2$ incubator. Half of the culture medium was replaced every 3-4 days (Figure 1 c & d). The neuronal cultures were exposed to different concentrations of glutamate (10, 100 µM to 1, 10 and 100 mM) for 1 h to induce excitotoxicity *in vitro*. To evaluate neuroprotective effect of NSCs-CM on glutamate-induced toxicity, the wells were rinsed by HBSS and the medium containing primary neuronal culture medium and NSCs-CM (mixed at a 1:1 ratio) was added to each well containing cortical neurons pre-treated with glutamate amino acid. Some wells were replaced with fresh primary neuronal culture medium without any NSCs-CM to serve as control.

### *Immunocytochemistry*

Neurospheres were fixed with 4% paraformaldehyde, permeabilized with 0.05% Tween-20, and blocked in 0.01% PBST (PBS+ Triton-X 100) solution containing 10% horse serum for one hour at room temperature. Then, the neurospheres were incubated in primary antibodies (Nestin (Millipore, MAB353) and GFAP (DAKO, z0334)) diluted at appropriate concentrations in 0.01% PBST solution at 4 °C overnight. After washing with PBS, primary antibodies were detected appropriate secondary antibodies conjugated with Alexa-Flour-488- and -568 (Invitrogen). Hoechst was used in secondary antibody solution for nuclei staining. The immunostained neurospheres were observed under fluorescent microscope using appropriate filters (Figure 2).[24]

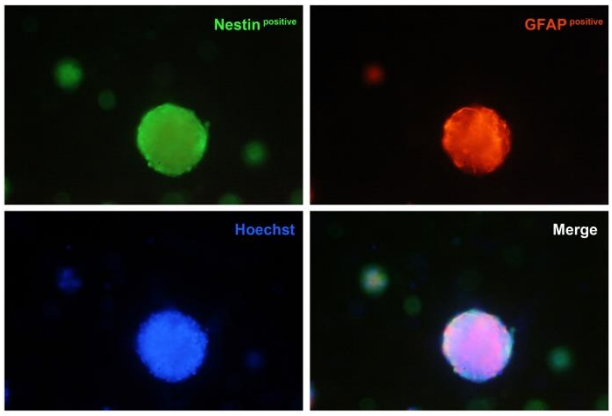


**Figure 2.** Immnuophenotype characterization of NSCs-associated neurospheres (a-d). As shown, undifferentiated isolated neurospheres were strongly positive for nestin and GFAP markers (a and b)(20 X Magnification).

### *Measurement of neuronal cell viability using Trypan blue Assay*

Trypan blue exclusion assay was used to determine cell viability as previously described.[25] In brief, 1.5% solution of trypan blue was applied for neuronal cultures at RT for 3 min, and rinsed with HBSS. To calculate the percent of viable cells, the number of blue colored cells as well as unstained cells were counted in 15 randomly selected fields at high magnification (40X). Finally, the viable cells ratio was calculated based on following formula: Viable cell ratio (%) = (non-stained cells number / total cells number) × 100.

### *Measurement of neuronal cell viability by MTT Assay*

To evaluate effect of glutamate on cell viability of primary cortical neurons, MTT assay was used 24 hours after one-hour exposure of primary cortical neurons to different doses of glutamate, as previously described by Selvatici et al.[26] In brief, Neurobasal medium containing 5 mg/ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide solution (MTT, Sigma, St Louis, MO) was prepared. A total volume of 10 µl of MTT solution was rinsed to each well of 96-well plates and incubated in 37°C for 4 h. At the end of incubation, medium was removed and 100 µl of acidic isopropanol were added to solubilize formazan crystals. Samples were read at wavelength of 550 nm with background subtraction at 630 nm. The absorbance data were expressed as percentages of control groups.

### *Measurement of percent viable, apoptotic, and necrotic neurons with Hoechst 33342 and Propidium Iodide (PI)*

The effect of NSCs-CM on necrotic and apoptotic cells ratio was determined with a combination of Hoechst 33342 and PI staining as described by Fricker et al.[27] Twenty four hours after one-hour glutamate exposure of primary cortical neurons, cells were stained with 1 µg/ml of Hoechst 33342 and 1 µg/ml PI for 15 min. Hoechst 33342 was used to determine apoptotic nuclear morphology while PI indicates dead cells. The cells with fragmented or condensed nuclei were regarded as apoptotic cells. After excluding Hoechst 33342 positive apoptotic cells, PI positive cells were considered necrotic cells. The number of apoptotic or necrotic cells in treatment groups were compared with control group.

### *Statistical analysis*

Statistical analysis was prepared using one-way analysis of variance (ANOVA) followed by Tukey's post-test, and two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The data represent means ± SEM and the level of significance was set at $P < 0.05$. All experiments were repeated three times in this study.

## Results

The importance of glutamate-induced excitotoxicity on cortical neurons has been shown extensively in many neurodegenerative diseases.[28] In this study, the neuroprotective effect of NSCs-CM against glutamate-induced excitotoxicity on primary cortical cultures of rat embryos was assessed.

### *Effects of NSCs-CM on mitochondrial function and viability of neuronal cells after glutamate exposure*

To determine the protective effect of NSCs derived CM on neuronal cells after exposure to glutamate, various concentrations of glutamate including 10 and 100 µM to 1, 10 and 100 mM was added to culture medium for 1 h. After exposure of primary cortical neuron to glutamate

neurotransmitter, culture medium is replaced by NSCs-CM or primary neuronal culture medium as control and they incubated for 24 h. Using MTT assay we could observe a trend of increased viability in NSCs-CM groups especially in groups treated with 1 and 10 mM glutamate but these differences were not statistically significant. Moreover, using trypan blue exclusion assay the same trend on increased viability in NSCs-CM groups were noticed and this increase was significantly higher in groups treated with 1 and 10 mM glutamate ($p<0.01$ and $p<0.001$ respectively). It is concluded that NSCs-CM could significantly nullify glutamate-induced excitotoxicity especially at 1 and 10 mM of glutamate concentration (Figure 3 a & b).

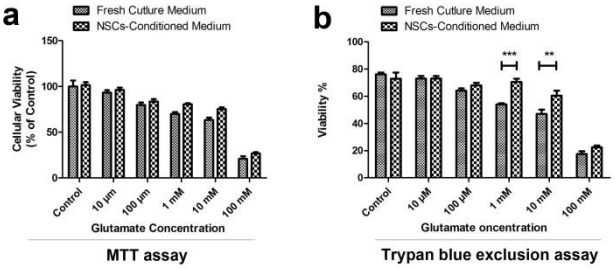


**Figure 3.** The positive neuroprotective effect of NSCs-CM on glutamate-induced excitotoxicity in primary rat cortical neurons culture analyzed by MTT (**a**) and trypan blue exclusion (**b**) assays. Panel a and b demonstrated neuroprotective effect on cell viability changes after glutamate treatment. Data are expressed as mean ± SEM. ***P* < 0.01 and ****P* < 0.001

### *NSCs-CM decreased apoptotic and necrotic cells ratio after glutamate toxicity*

Glutamate toxicity leads to cells death either via apoptotic or necrotic pathways. Hoechst 33342 usually used as an indicator of apoptotic cell death in which cell nuclei are condensed and fragmented (Figure 4 f). We used both Hoechst 33342 and PI staining together in order to distinguish the apoptotic and necrotic cell death as well as their proportions. We observed that increasing glutamate concentration resulted in cell death either by apoptotic or necrotic pathways and it was significantly higher at 1 mM, 10 mM, and 100 mM of glutamate concentrations as compared to the control (Figure 4 a). In addition, our analysis showed that up to 10 mM glutamate concentrations, the cell death pattern was mainly apoptotic but at 100 mM it dramatically reversed and the necrotic cell death become the main feature (Figure 4 b, * $p<0.05$, ***$p<0.001$). The addition of NSCs-CM rescued neuronal cells from apoptosis. This protective effect was significantly higher in 10 mM glutamate concentration as compared to primary neuronal culture medium (Figure 4 c, d). Interestingly, NSCs-CM could significantly decrease the amount of necrotic cell death at 100 mM concentration (Figure 4 e, *$p<0.05$). Hence, both of the apoptotic and necrotic cell changes were decreased by 24 h application of NSCs-CM post glutamate exposure.

## Discussion

Two main mechanisms including overactivation of glutamatergic receptors and glutamate/cystine anti-porter dysfunction have been described for glutamate-induced cell death in different CNS pathologies.[29] This study was aimed to assess the efficacy of NSCs-CM on neuronal survival after inducing neurotoxicity *in vitro* and to compare their protective effects on different concentrations of glutamate-induced acute excitotoxicity. We found that after exposing primary cortical cultures with different concentrations of glutamate, cellular viability decreases. Our results show apoptosis is the main death pathway in neuronal cultures, and necrotic pathway overtakes from apoptotic pathway in highest concentration of glutamate (Figure 4 b, $P < 0.01$). After adding NSCs-CM to glutamate-exposed cultures, we observed that cellular survival increased and both of the apoptotic and necrotic neurons rate decreased.

The neuroprotective effect of NSCs is highly related to its neurotrophic factors that increase neuronal cells survival in diseased nervous tissue. Interestingly, blocking neurotrphic signaling or omitting them from neuronal cells environment leads them to enter in apoptosis pathway.[30] Even with neutralization of growth factors, neuroprotective effects of stem cells diminishes to limited levels and this low levels of neuroprotectivity is related to other molecules that they secrete such as osteopontin, clusterin, cystatin-c, and tissue inhibitor of metalloproteinase 1.[31] Previously, neuroprotective effect of murine adipose derived stem cells conditioned medium on SH-SY5Y cell cultures survival has been shown after glutamate exposure.[32] Moreover, the neuroprotective effect of mesenchymal stem cells conditioned medium (MSCs-CM) has been shown on glutamate-induced excitotoxicity. This effect was related to cytokines and growth factors that MSCs secrete and generally modifies gene expression in neuronal cultures. They improve neuronal function, angiogenesis and immunity. Also, MSCs-CM treatment of cortical cultures 24 h prior NMDA (a glutamate ionotropic agonist) exposure reduced glutamate receptors expression followed by decreasing glutamate toxic effect.[33] Similarly, using human umbilical cord blood stem cells in glutamate-induced excitotoxicity of primary cortical neurons decreased apoptosis in millimolar concentrations.[28] Furthermore, it has been shown that NSCs was neuroprotective after inducing glutamate excitotoxicity in organotypic spinal cord cultures.[11] NSCs secrete neurotrophic factors such as GDNF and NGF constitutively which support and develop neurite outgrowth.[11] These factors diffuse in organotypic spinal cord culture and move to the damaged area that is located far apart from NSCs in the environment. NSCs neuroprotective effect has been demonstrated against glutamate-induced excitotoxicity both *in vivo* and *in vitro* NSCs-CM also shows therapeutic effects similar to the NSCs. The evidences indicate that the major neuroprotective effect of NSCs-CM is related to GDNF interaction with GDNFα receptors.[11] Interestingly, in our

study NSCs-CM could prevent cell death in all different toxic concentrations mediated by glutamate. The maximum protective effect of NSCs-CM on neuronal survival was recorded statistically significant at 10 mM concentration of glutamate either with trypan blue exclusion assay and Hoechst/PI staining (Figure 3 b & 4 c). Neuroprotective effects of NSCs-CM are highly related to its neurotrophins, which mainly trigger PI3-K/Akt signaling pathway for cell survival.[11,28,31] Activation of this pathway increases XIAP expression that limits caspase-3 activity and prevents creation of biological and morphological characteristic of apoptosis in glutamate-induced cells. Also the other anti-apoptotic effect of this pathway is inhibition of caspase-3 and -7 activity by increasing cellular levels of anti-apoptotic Bcl2 and decreasing Bax proapoptotic molecules.[28,31,34] ERK signaling is another pathway against neurotoxicity that begins by neurotrophins.[31]

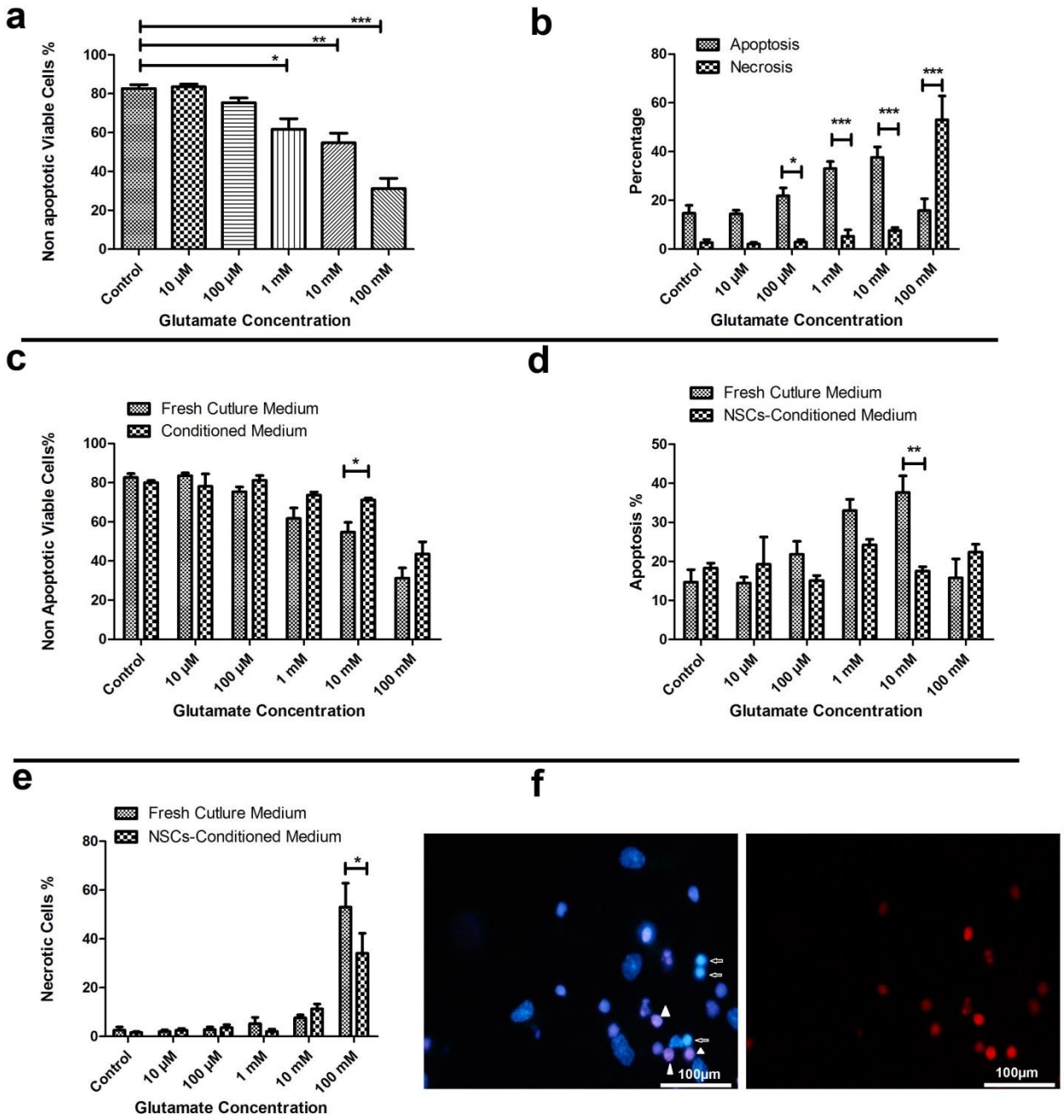


**Figure 4.** Representative effect of glutamate on apoptotic and necrotic nuclear changes in primary cortical neuronal cells were identified by Hoechst/PI staining after glutamate-induced excitotoxicity (a-f). Panel **a** represents the percentage of non-apoptotic viable neurons in primary rat cortical cultures. Part **b** compared the apoptotic and necrotic cells. C, d and e panels showed the neuroprotective effect of NSCs-derived CM on percentage of non-apoptotic viable, apoptotic and necrotic cell population. Nuclear morphology of primary cortical neurons stained by Hoechst 33342 and PI in DIV 13. Arrowheads showed condensed apoptotic nuclei whereas triangles indicated necrotic cells. Data expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001

As we used B27 supplement in neuronal cultures medium, we should consider its beneficial effect on neuronal survival as it contains antioxidant chemicals that could decrease oxidative glutamate toxicity and increase cellular viability.[28] Moreover, neuronal cultures sensitivity to glutamate-induced toxicity is associated to their age. Younger cultures can resist better against glutamate-induced neurotoxicity,[28] so we used cultures 12 days *in vitro* (DIV) for glutamate exposure, because mature neurons are more sensitive to induction of excitotoxicity.[35] Generally, Ionotropic receptors play major role in glutamate-induced neurotoxicity, and they can blocked by their antagonists.[28] Then, it is suggestible to investigate their probable enhancing effects on NSCs-CM neuroprotective effects in future studies.

## Conclusion

In conclusion, the importance of glutamate neurotoxicity studies arise from contribution in neurodegenerative diseases like Alzheimer's and Huntington's diseases or traumatic brain injuries such as ischemic stroke. Hence, it is necessary to find effective ways to deal with glutamate toxicity in acute or chronic manners. Stem cells, especially NSCs have potent neuroprotective effects against glutamate neurotoxicity. We observed that NSCs-CM has neuroprotective activity following glutamate excitotoxic damage on cortical neurons. This study showed protective effects of NSCs-CM *in vitro* neurotoxicity model that can be continued further *in vivo* studies. This neuroprotective effect of NSCs-CM is thought to be associated to soluble factors secreted by NSCs such as GDNF and NGF. This suggests that these conditioned mediums can be used in clinical trials to attenuate neuronal death through inhibiting excitotoxicity and oxidative stress during acute and chronic neurodegenerative diseases.

## Acknowledgments

We would like to acknowledge Ms Esfahani for her skillful technical helps and to appreciate Dr. Ghanbari and Dr. Aligholi for their devotional helps and guides.

## Ethical Issues

Not applicable.

## Conflict of Interest

The authors report no conflicts of interest.

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