

## **Effects of different culture media on optimization of primary neuronal cell culture for in vitro models assay**

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### **Abstract:**

**BACKGROUND:** In vitro model studies are becoming increasingly popular for experimental research designs. They include isolation and expansion of cells of a particular tissue, such as the nervous tissue which contributes to understanding the underlying mechanisms in many pathologies. It enables the scrutinization of intracellular signaling pathways responsible for cell death. **OBJECTIVES:** In the literature, there are different methods for the isolation and culture of rat embryonic cortical neurons. However, this study developed a feasible, rapid and easily performable method. **METHODS:** Isolation of neurons was performed without using enzymatic digestion. Primary cortical cultures neurite outgrowth and neuron numbers per field of common mediums were compared for neuronal cells isolation and expansion. In this study, three different culture mediums were considered: Medium I: Neurobasal medium, B-27 and L-glutamine; Medium II: DMEM, FBS and L-glutamine; and Medium III: DMEM/F-12, FBS and L-glutamine. **RESULTS:** High survival rate and number of neurons was obtained with the current method. The best neuronal growth was achieved by Medium I, while Medium II and III had moderate effect on the neurite outgrowth. **CONCLUSIONS:** Enzyme-free treatment was introduced and Medium I was used as an alternative method for optimal neuron isolation and expansion. The neuronal cultures are similar to nervous tissue in physiological aspects. Hence, Medium I is more similar to the in vivo condition compared to Mediums II and III.

### **Introduction**

The cerebral cortex contributes to many central neural functions such as memory and motor functions (Briz et al., 2011). Cerebral cortex functions are affected by many diseases which generally triggers inflammatory procedures by degeneration of neural networks and synapses in the CNS (Kim and Magrane,

2011; Li et al., 2014). Primary cultures of cortical neurons provide an opportunity to unveil different cellular and molecular characteristics of neurons in vitro, such as synaptic transmission, neurite development, and neuritic transport (Harrill et al., 2013; Kim and Magrane, 2011). Till date, some neuronal cell lines, including the PC12 cells, are available for various assessments. However, primary neuronal

cultures give more precise and reliable results as a model of neuronal behavior in vitro (Harrill et al., 2011). Some researches utilized primary neuronal cultures as a suitable design model for investigating the effect of various chemicals on neuronal cells in vitro (Harrill et al., 2011, 2013); for example, some pharmacological or chemical products have been identified, such as K252a, Na<sub>3</sub>VO<sub>4</sub>, Bis-1, arsenic, and ethanol which impairs neurite outgrowth (Barclay et al., 2005; Harrill et al., 2013; Maekawa et al., 2013). The presence of high glucose in the medium decreases neurite outgrowth (Yan et al., 2012) while the use of bFGF, BDNF and simvastatin increases neurite outgrowth in neuronal cultures (Hasan et al., 2013; Li et al., 2010; Liu et al., 2014; Noshita et al., 2012; Wu et al., 2012).

The efficiency of primary cultures basically depends on some technical issues such as isolation of homogeneous cell populations from cortical tissue and preventing cellular loss (Kim and Magrane, 2011).

Isolation of embryonic and postnatal neurons seems to be an easy process to a certain extent because synapses do not form perfectly. However, mature adult neurons containing intracellular tight junctions are required to gently isolate and prevent irreversible fragmentation (Brewer and Torricelli, 2007). Neuronal cell culture in serum free medium provides a condition excluding hormonal, non hormonal, vascular and inflammatory effects. Dose-response prevents complication in cerebral cortex experiments. This culture medium must be enriched by B-27 supplement (serum-free) containing five beneficial antioxidants for neuronal survival (Brewer and Torricelli, 2007).

Medium such as Dulbecco's modified Eagle's medium (DMEM) or DMEM/F-12 supplemented by 10% fetal bovine serum (FBS) were used in some previous studies (Dhandapani et al., 2005; Koo et al., 2006), but due to lack of specificity for neuronal growth, it was not appropriate for neuronal cell culture

studies. DMEM does not contain sufficient nutrients for neuronal growth. DMEM/F-12 has many nutritional elements that make it an appropriate enriched medium for different types of cells cultures but is not a specific medium for neurons. It should be noted that FBS increases non neuronal cells growth, in spite of adding proliferation inhibitor such as Cytosine-β-D-arabinofuranoside (Ara-C).

This study developed a new and feasible method of isolation and culture of primary neuronal cells, used for in vitro model designed assessment. Also, three common mediums used previously, were investigated in this study by counting neurons per microscopic field as an indicator of cell survival and neurite outgrowth measurement as a prominent indicator of neuronal growth and development.

## **Materials and Methods**

**Animals:** Both male and female Wistar rats were used for this study, housed at a temperature of 22±2°C with approximately 55% humidity in a 12 h light/dark cycle and ad libitum access to food and water (Kim et al., 2012). The male rats were allowed to mate with female rats for a period of 3 days. The embryos of pregnant rats were collected by dissecting them in E16.5-18.5. According to previous studies, younger embryos are hard to dissect and older embryos have more contaminating glial cells (Kim and Magrane, 2011). Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and to the Iranian codes of practice for the use of laboratory animals.

**Neuronal cells isolation and culture procedure:** The isolation and culture of neurons was performed as previously described by Kim et al. with some modifications (Heber et

al., 2000; Kim and Magrane, 2011; Pacifici and Peruzzi, 2012; Xu et al., 2011). Twelve-well plates were coated by Poly L-Lysine (PLL, MW = 70,000-150,000 g/mol; 10 µg/ml, Sigma, St Louis, MO) and incubated for 2 h at 37°C. The plates were then rinsed with distilled water and kept until use.

Pregnant rats at gestational day 16.5- 18.5 were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation. Abdominal section was disinfected with 70% ethanol, the skin and abdominal muscles were incised and kept. Each uterine horn containing embryos was incised and then transferred to a dish containing cold Hank's Balanced Salt Solution (HBSS), calcium- and magnesium-free containing 1% penicillin/streptomycin. The area between two eyes of the embryos were cut under a stereo microscope and was used for dissection and isolation of neurons. The cranial part of the skull was removed using scissors and curved forceps. The brains were collected in a Petri dish containing cold HBSS. The olfactory bulb and cerebellum were removed to obtain the cortex. The cerebral hemispheres were separated and the midbrain was removed from each hemisphere and the hippocampus was also discarded. Finally, after removing the meninges, dissected cortices were transferred to a 15-ml conical tube, washed with cold HBSS three times, and centrifuged at 110 g for 5 min and ultimately supernatants were discarded. A volume of 1 ml Neurobasal medium (Gibco, Paisley, UK) was added to the tube. The cortical tissues were triturated gently using fired-polished Pasteur pipette 8-10 times and the particles were allowed to settled down. Finally, viable cell numbers were counted using 0.4% trypan blue staining and hemocytometer. Seeding density was regarded as 15,000 cells/cm<sup>2</sup> (Harrill et al., 2013).

**Culture mediums:** The culture Mediums were considered as follows: **Medium I:** Comprised of Neurobasal medium supplemented

with 2% B-27 (Gibco, Paisley, UK), 0.5 mM L-glutamine (Gibco, Paisley, UK), and 1% Penicillin/streptomycin.

**Medium II:** DMEM (Gibco, Paisley, UK) supplemented with 10% FBS (Gibco), 2 mM L-glutamine and 1% penicillin/streptomycin. Cytosine-β-D-arabinofuranoside (Ara-C, Sigma, St Louis, MO) was added 48 h after culturing.

**Medium III:** DMEM/F-12 (Gibco, Paisley, UK) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin. Ara-C added 48 h after culturing.

Glial proliferation inhibitor such as Ara-C was not added to Medium I because neurobasal medium inhibits glial proliferation.

**Assessment of neurite outgrowth:** The neurite outgrowth assessment of cells was done in DIV 4 as described previously (Harrill and Mundy, 2011; Nabiuni et al., 2012). Briefly, the experiments were done in triplicate, and three random fields of each well were recorded. Only cells that they had at least one neurite which was equal or longer than the cell body diameter were selected for measurement. Images were taken using an inverted microscope with a 10× objective. The length of the longest neurite was measured using the Image J 1.47 software (NIH, Bethesda, MD).

**Grown neuron numbers estimation:** The numbers of grown neuron was estimated by counting neurons on 3 random microscopic fields of 10× objectives. The counted cells had at least one neurite which was equal or longer than the cell body diameter (Harrill et al., 2011).

**Statistical analysis:** The significant differences between the three mediums were analyzed with one-way analysis of variance (ANOVA) followed by Tukey tests;  $p < 0.05$  was considered statistically significant.

## Results

The potency of three different culture me-

diums and their efficacies on primary cortical cell cultures of embryonic rat were investigated and compared (Fig. 1). After the expansion of neurons in three different mediums, neurite outgrowth and neuronal cell numbers between Mediums I, II, and III in DIV 4 were measured and compared

**Quantitative assessment of neurite outgrowth:** The length of the longest neurite of each neuron in the microscopic field was measured. Data were obtained for at least 50 neurites in each well. The average length of neurites in Mediums I, II, and III are shown in Table 1. An increase in total neurite length occurred in Medium I ( $105.43 \pm 4.24 \mu\text{m}$ ) compared to Medium II ( $63.99 \pm 3.28 \mu\text{m}$ ) and Medium III ( $66.52 \pm 3.45 \mu\text{m}$ ) and the difference was statistically significant ( $p < 0.001$ ).

**Grown neuron numbers estimation:** The number of neurons containing at least one neurite was equal or longer than the cell diameter, and they were counted in 3 fields of each well and averaged (Table 2). Neuronal cell numbers in Mediums I, II, and III were  $74.55 \pm 7.07$ ,  $31.88 \pm 3.18$ , and  $31.22 \pm 4.96$ , respectively. It is indicated that neuronal cell survival in Medium I was more than Mediums II and III and this difference was statistically significant ( $p < 0.001$ ).

In Mediums II and III, fewer neurons are present in the microscopic field and the number of neurites cannot be compared with the neurons observed in Medium I and this difference is statistically significant (Tables 1 and 2).

## Discussion

Primary cultures of neurons are appropriate models of neuronal maturation of normal brain in vitro. These cells in convenient environment produce axons and dendrites similar to the in vivo morphology of neurons (Harrill et al., 2013). It has been reported that changes in the morphology of primary neurons and neural cell lines after treatment with chemicals has

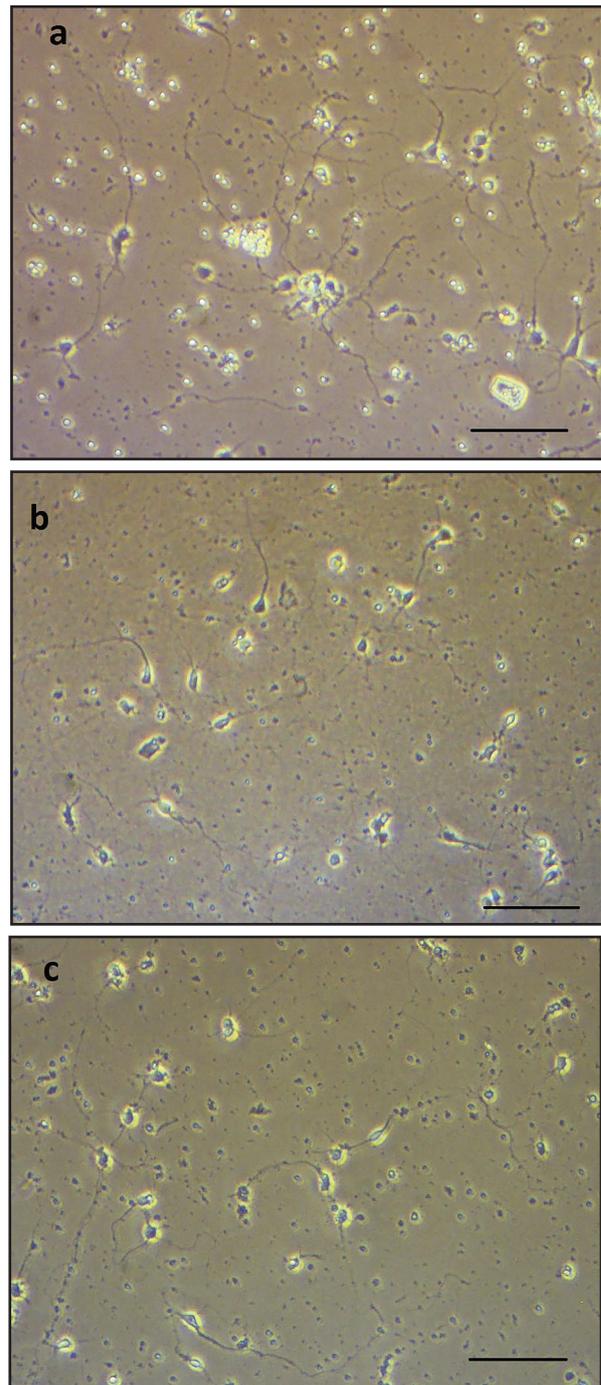


Figure 1. Primary cortical cultures of embryonic rat in (a) Medium I: Neurobasal medium supplemented by 2% B-27 and 0.5 mM glutamine, (b) Medium II: DMEM supplemented by 10% FBS and 2 mM glutamine, and (c) Medium III: DMEM/F-12 supplemented by 10% FBS and 2 mM glutamine in DIV 4 (scale bar = 100 microns).

been the subject of many studies in the area of developmental neurotoxicity (Harrill et al., 2013).

The growth and development of dendrites and axons also known as “neurite outgrowth”

Table 1. Effects of medium on neurite length. The length of the longest neurite was measured in DIV 4. Data was obtained from every cell with an identifiable neurite from three fields in each well (approximately 25 cells per field) and averaged. Data shown is the mean  $\pm$  SEM from three wells (n = 3), <sup>(a,b)</sup> different superscript letters are significantly different (p<0.001).

	Neurite length ( $\mu\text{m}$ ) per cell	
	mean $\pm$ SEM	
Medium I	105.4 $\pm$ 4.24 <sup>(a)</sup>	
Medium II	63.99 $\pm$ 3.28 <sup>(b)</sup>	
Medium III	66.52 $\pm$ 3.45 <sup>(b)</sup>	

Table 2. Effects of medium on neurons survival. The number of neurons was measured in DIV 4. Every cell with an identifiable neurite from three fields in each well was counted. Data shown is the mean  $\pm$  SEM from three wells (n=3), <sup>(a,b)</sup> different superscript letters are significantly different (p<0.001).

	Neurons per field	
	mean $\pm$ SEM	
Medium I	74.56 $\pm$ 7.07 <sup>(a)</sup>	
Medium II	31.89 $\pm$ 3.18 <sup>(b)</sup>	
Medium III	31.22 $\pm$ 4.96 <sup>(b)</sup>	

have been used in various neurotoxicity studies (Harrill and Mundy, 2011). Neurite outgrowth assessment is considered a more accurate factor than other cell health assays; hence, it has high sufficiency to be regarded as a subtle utility for distinguishing developmental neurotoxicants (Harrill et al., 2010). This study was based on live cells imaging using phase contrast microscopy followed by measuring neurite length as previously described by Harill et al. with slight modification (Harrill and Mundy, 2011). In this research only the longest neurite emerging from cell was assessed after images were taken with a digital camera. The advantage of this method is the availability of cultures for repeated imaging because the cells are not stained.

In this study, three different culture mediums were subjected to neuron isolation and expansion, and Medium I provided the best condition for neurons culture and isolation.

Neuronal cell cultures in Mediums I, II, and III at DIV 4 are shown in Figure 1.

High impact of neurite formation document-

ed in Medium I cultures (Fig. 1a) has been compared to other mediums (Fig. 1b, 1c) (Kim and Magrane, 2011). These formations have typical characteristics of normal neurons and can meet required criteria for having appropriate growth of neurons (Harrill et al., 2013). Also, no evidence of glial cell proliferation was found. Some tiny cells in this culture represent microglial cells which mainly showed dead modality in cell viability assessment by trypan blue exclusion assay.

The neurite outgrowth and neuron numbers in Medium I was 105.43 $\pm$ 4.24  $\mu\text{m}$  and 74.55 $\pm$ 7.07, respectively. This is somewhat consistent with the study of Harrill et al. (2011) who reported that the average length of neurite outgrowth was 129.8 $\pm$ 12.6  $\mu\text{m}$  and the number of neurons was 50.2 after 48 h of plating in a medium containing DMEM, 10% horse serum, sodium pyrovate, HEPES and Gluta-MAX. The difference in neurite outgrowth can be related to the seeding density of cells (6.66  $\times 10^4$  cells/cm<sup>2</sup>), which is about 4 times more than the density used in this study. Higher seeding density has an enhancing effect on neurite length (Jeerage et al., 2012; Radio et al., 2010). Also, this shows that Medium I is more reliable for the survival of neurons. In spite of the fewer seeding density of neurons, more neurons were present in comparison to the medium used in the study of Harrill et al.. In this regard, Wu et al. (2012) showed that using Neurobasal medium; same as Medium I; causes high growth of neurites (about 800  $\mu\text{m}/\text{cell}$ ) in DIV 5. This high difference can be attributed to high seeding density (10.9  $\times 10^4$  cells/cm<sup>2</sup>), which can induce high neurite outgrowth because of short distance between cells (Jeerage et al., 2012; Radio et al., 2010). The role of serum in medium is to enhance neuronal attachment and neurite outgrowth, which increases neuronal long-term survival (Yu and An, 2002).

In recent years, developmental neurotoxicity tests have been moved from rodent mod-

els to in vitro studies such as mammalian or non-mammalian species cell cultures for developing more credible tests. These types of investigations are useful as primitive tests for assessing neurotoxicity; however, these data cannot be extended in vivo (Ku wagata, 2012). For estimating the effects of different toxins on the nervous system, neuronal cultures similar to nervous tissue in physiological aspects were obtained. In this case, it seems that Medium I is more similar to the in vivo condition, compared to Mediums II and III. Primary neuronal cultures initially described by Choi et al. (1987) and Koh and Choi (1988) were used for glutamate neurotoxicity studies. The isolation of fetal neurons was carried out by enzymatic digestion. The used medium is not a special medium for neurons, they used Eagle's minimal essential medium (MEM) supplemented with 10% FBS and 10% horse serum. The percentage of neuronal cell survival and growth seems to be low compared to Medium I. The presence of glial cells non-intended can be considered in spite of using Ara-C or 5-fluoro-2'-deoxyuridine 24 or 48 h after culture.

It should be noted that  $5 \times 10^3$  cells/cm<sup>2</sup> is an appropriate seeding concentration (Kim and Magrane, 2011; Pacifici and Peruzzi, 2012). In the method used by Chen et al. the density of cells was too high ( $3 \times 10^5$  cells/cm<sup>2</sup>) (Chen et al., 2012; Zeron et al., 2002) and it resulted to clump formation.

Medium III included DMEM/F-12 which contains 50  $\mu$ M L-glutamic acid, which was indicated as a neurotoxic concentration by Xu et al. (2011), who stated that exposing rat cortical neuron cultures for 3 h in 50  $\mu$ M glutamate, reduces neuronal cell cultures viability by 53% (Xu et al., 2011). However, no difference was noted in neurite length or neuron numbers per field between DMEM/F-12 and DMEM which lacked the L-glutamic acid ingredient. Also, DMEM/F-12 was used by Zhang et al. (2000) previously for primary neuronal cultures and such toxicity was not indicated (Zhang et al.,

2000). Based on the neurobasal medium manufacturer's recommendation, 25  $\mu$ M of L-glutamic acid (Pinton et al., 2013) should be added for initial plating.

In conclusion, the described procedure can be considered as an optimized protocol for isolation and culture of rat cortical neurons for neurotoxicity experiments. There are many differences in the procedure of isolation and culture of primary cortical neurons in previous studies, but they have never been compared. Neuronal cell survival and neurite outgrowth (as important indicators of appropriate culture) were used in this research to show the efficacy of available methods and describe the optimal method for later in vitro excitotoxicity studies on primary neuronal cultures. This method is easy, can be done quickly and shows the high viability of neurons after the isolation procedure. Many neurons can survive and show normal growth of neurons in the mentioned medium. Interestingly within this experiment, no enzymatic dissociation such as trypsin or TripLE Express (a recombinant enzyme) (Pacifici and Peruzzi, 2012) was applied. Primary neuronal cultures are cost-effective, efficient and have the capability of neuronal development assessment of large number of chemicals (Harrill et al., 2013).

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## تأثیر محیط کشت‌های مختلف بر روی بهینه‌سازی کشت اولیه سلول‌های عصبی برای ارزیابی مدل‌های برون تنی

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### چکیده

**زمینه مطالعه:** مطالعه مدل‌های برون تنی برای طراحی تحقیقات تجربی رو به افزایش است. از جمله این نوع مطالعات جداسازی و کشت سلول‌ها از بافت‌های مختلف مانند بافت‌های عصبی می‌باشد، که در شناخت مکانیسم‌های اساسی آسیب‌ها می‌تواند بسیار کمک کننده باشد و ما را قادر می‌سازد تا مسیرهای سیگنالینگ درون سلولی مسئول مرگ سلولی را به دقت مورد بررسی قرار دهیم. **هدف:** اگر چه تا کنون، روش‌های مختلفی برای کشت نورون‌های قشری جنین رت توصیف شده است. با این حال، مطالعه موجود روش عملی و سریعی را معرفی می‌کند که می‌تواند در این نوع مطالعات مورد استفاده قرار گیرد. **روش کار:** در این مطالعه، از سه محیط کشت مختلف، به ترتیبی که توضیح داده می‌شود برای جداسازی و کشت نورون‌ها در کشت اولیه قشری بدون استفاده از هضم آنزیمی استفاده گردید: محیط‌های مورد استفاده عبارت بودند از: محیط I: محیط نوروبازال، B-۲۷ و ال-گلوتامین؛ محیط II: DMEM، FBS و ال-گلوتامین؛ و محیط III: FBS، ۱۲-DMEM/F، و ال-گلوتامین. **نتایج:** میزان زنده مانی نورونی در این مطالعه بسیار قابل توجه بود و بهترین رشد نورونی در محیط I مشاهده گردید، در حالیکه محیط‌های II و III اثر متوسطی بر روی رشد نوریت داشتند. **نتیجه‌گیری نهایی:** نتایج این مطالعه نشان می‌دهد محیط I بیشترین تشابه با شرایط برون تنی را در مقایسه با محیط‌های II و III ایجاد نموده و سلول‌های نورونی بیشتر قادر به حفظ خصوصیات فیزیولوژیک خود در این محیط می‌باشند.

**واژه‌های کلیدی:** نورون‌های قشری، جداسازی، کشت اولیه سلول، رت

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