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Culturing Adult Rat Hippocampal Neurons with Long-Interval Changing Media

Shohreh Majd*[1], Asadollah Zarifkar[1], Karim Rastegar[1] and Mohammad Ali Takhshid [2]

[1]*Dept. of Physiology and* [2]*Dept. of Biochemistry, Shiraz University of Medical Sciences, Shiraz, Iran*

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ABSTRACT

Background: Primary cultures of embryonic neurons have been used to introduce a model of neurons in physiological and pathological conditions. However, age-related cellular events limit this method as an optimal model in adult neurodegenerative diseases studies. Besides, short-interval changing media in previous cultures decreases the effectiveness of this model. As an example of this matter, we can refer to the study on some special neuronal secreted factors or the influence of some experimental materials on neurons. Meanwhile, short-interval changing media could remove the effects of some released factors from the environment. In this study, the method for isolation and culturing adult rat hippocampal neurons with long-intervals medium changing has been described. **Methods:** The hippocampal neurons of adult male rats were cultured. We used Neurobasal A/B27 culture medium, papain (2 mg/ml), trypsin 0.25% and collagenase (1 mg/ml) for neuronal isolation, OptiPrep density gradient for separation of neurons from other cell types and also debris and FGF2 (10 ng/ml) for increasing neuronal survival and regeneration. **Results:** The neuronal sprouting and viability were increased by using papain and mild triturating ($P<0.05$). Adult neuronal culturing and their regeneration were impossible without FGF2. It was shown that adding new fresh medium every 4 days and exchanging half of it every 8 days had no detrimental effect on neuronal viability. **Conclusion:** This investigation shows the possibility of culturing adult neuronal cells and their maintaining in long-interval media. It could be happened because of adult neurons rely significantly on the neighboring cells secreted factors for living and making synaptic connections. This model is very useful in physiological and pathological studies which need stable conditions of neuronal culture in a long period of time. *Iran. Biomed. J. 12 (2): 101-107, 2008*

Keywords: Adult neuron culture, Long-interval, Regeneration, Media changing, Hippocampal neurons

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INTRODUCTION

For many years, there was just a main successful method to cultivate primary neurons of the brain, which was embryonic culture. However, it was an easy and accessible method but some critical age-related neuronal parameters in physiological [1, 2] and neuro-degenerative states like Alzheimer and Parkinson were dismissed [3]. Besides, changing media in short-intervals were done before removing the detrimental factors produced by cellular metabolism [4, 5]. Such media changing decreases the effectiveness of this model in some investigations. For example the aim of some investigations is to study the neuronal secreted factors in a relatively long time point. In those cases, the media could not be changed in regular short-intervals. Also, in many other studies, the influence of some experimental materials on neuronal function in a desired time course is the main subject of investigation. Thus, washing out the media in short-intervals could interfere with the final results. Besides, short-term changing media could remove many secreted neuronal factors from environment [6].

Although most attention has focused just on keeping adult neurons alive for a period of time, there is growing recognition that in some investigations, it is needed to culture neurons for a rather long period with minimal media changing. It is helpful to study on the effects of some neuronal released factors and the effects of some experimental materials on them for a period of time. Besides, the presence of different factors and their maintenance

*Corresponding Author; Tel. (+98-711) 230 2026; Fax: (+98-711) 230 2026; E-mail: majdsh@sums.ac.ir

in neuronal spaces have a noticeable effect on neuronal condition, their health and their impressibility from other cells [7]. So, we have described the isolation and preparation of cultured rat hippocampal neurons and their maintenance without changing media in short-intervals. It has been shown that by using specific media and papain as a mild digestive agent, the cell viability increased. It was also demonstrated that adding a growth factor like FGF2 had a critical effect, not only on cell viability but also on their regeneration, and without it, the neurons would die in a short time. Changing half of the media every 8 days with adding new fresh media in the amount of half of the media in plating day in every 4 days did not make any decay in neuronal viability and the neurons stayed alive for more than 40 days.

MATERIALS AND METHODS

Rat hippocampal neuron culture. Hippocampal neuronal cultures were established from adult male Sprague-Dawly rats of 8-10 months old. The rats were anesthetized with ether and decapitated. The hippocampi were transferred into a 2-ml phosphate-buffered saline tube (Sigma, USA) supplemented with 2 ml Hibernate A (BrainBitLLC) and B27 (Invitrogen,USA) at 4°C. Hibernate A is a form of neurobasal media with common salts, glucose, pyruvate, 17 amino acids and nine vitamins that buffered for use with adult neurons at ambient CO_2. B27 is a serum-free medium supplement containing 20 ingredients that enables culture of neuron at high viability. Then, the slices of 0.5- mm thickness with fine scissor were cut perpendicular to the long axes of the hippocampi and transferred to a 5ml Hibernate A/B27 tube. After shaking at 150 rpm, at 30°C for 8 min, slices were transferred into another tube at 30°C containing papain (Worthington, USA; 12 mg/6 ml Hibernate A). Papain was prepared by dissolving 12 mg in 6 ml HibernateA and then it was sterilized. Slices were incubated for 30 min in a 30°C water bath with a platform rotating at 170 rpm and then were transferred into a 15-ml tube containing 2 ml Hibernate A/B27 at 30°C for 5 min in room temperature and triturated 10 × 3 times in 6 ml Hibernate A/B27.

Gradient isolation of cells. The cells were applied to the top of a gradient of OptiPrep 1.32 (Sigma, USA) and centrifuged at 800 × g for 5 min. Debris above 4 ml was discarded and fractions (fractions 2 and 3) containing neurons collected and diluted into 5 ml Hibernate A/B27. Then they were centrifuged at 200 × g for 2 min. This step was repeated one more time. The pellets then were resuspended in 3 ml main culture medium [Neurobasal A (Gibco,USA)/B27, 0.5mM glutamine, 10ng/ml FGF2 (Sigma, USA) and penicillin/ streptomycin (Sigma, USA)]. After mixing 20 µl of cell suspension with 20 µl of 0.4% trypan blue (Sigma, USA), live (dye-excluding) cells were counted in a hemacytometer.

Cell plating. We obtained 250,000-350,000 cells from each hippocampus (totally near 500,000-700,000 neurons from two hipocampi). We used 24-multi-well plates which were precoated with 100 µg/ml Poly-D-Lysine (Sigma, USA) for 3 hours and washed with sterile deionized water. The number of neurons that were plated was 250,000 cells in one milliliter in main culture medium with 0.5 ml in each well. The neurons then were incubated at 37°C, 5% CO_2. After 1 hour, the wells were rinsed 2 times with Hibernate A/B27 and they were filled with 0.5 ml of main culture medium in each well. Half of the medium was exchanged with fresh media every 8 days and 0.25 ml of fresh media was added to each well every 4 days.

Viability assay. Cell survival was assessed by morphological criteria and trypan blue exclusion, through counting the number of viable neurons in premarked microscope fields in three or four wells. At least six fields (1mm^2) were counted in each well. Cells were considered non-viable by the presence of cell body vacuolization and dendritic beading or the absence of trypan blue-exclusion.

Other proteases. Two other proteases were tested for neuronal damaging effect: 0.25% trypsin and 1 mg/ml collagenase (Sigma, USA) in Hibernate A/B27.

Statistical analysis. Differences between means were assessed by student's *t*-test and one-way ANOVA which using SPSS software. The data were presented as the means ± standard error. $P<0.05$ was taken as the criteria for statistical significance.

RESULTS

Long-term adult rat hippocampal neuron cultures were only possible when neurons were plated in a

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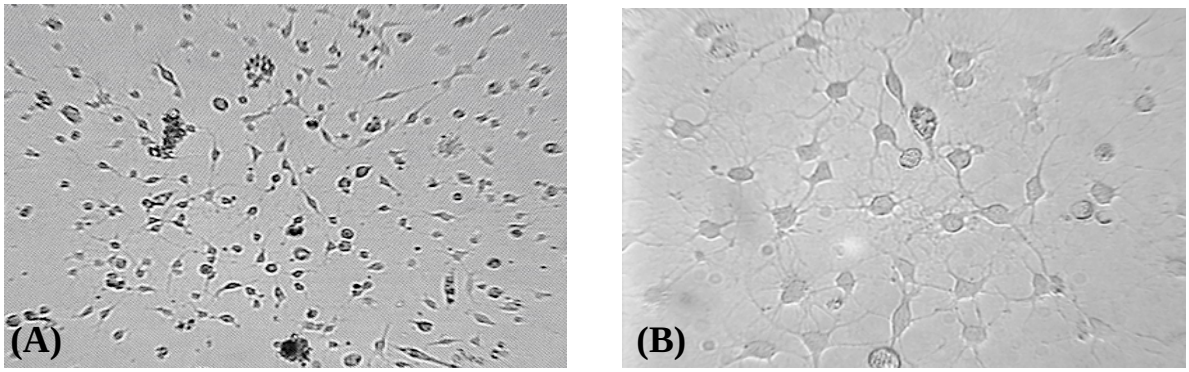

Fig. 1. Hippocampus neurons after **(A)** 10 days and **(B)** 14 days showed extensive regeneration of neuritis next. Pyramidal shapes with numerous neuritis which participated to an extensive neuronal network were noticeable in most of them. Magnification of A was 25 × objective and B was 40 × objective.

fine controlled condition. Under inverted microscopy, hippocampal neurons extended prominent neuritis within hours in Neurobasal A which contained critical factors for adult neuronal survival. Regeneration of neuritis and synaptic connections continued for the next days (Fig. 1) with pyramidal shapes and numerous neuritis in extensive neuronal networks.

Not only the number of live neurons decreased even after a few hours without using basic fibroblast growth factor (Fig. 2A) in comparison to those cultures which were done with FGF2 (Fig. 2B), we could not see any neuritis regeneration from the few alive ones.

The isolation procedure itself might be harmful to the adult neurons. Our culture system mostly contained neuronal cells as we collected just the most concentrated neuronal layers (Fig. 3).

Many studies have used trypsin and collagenase, but we showed that using a milder protease like papain had a less damaging effect. Neuronal survival increased by using papain (Fig. 4A) instead of trypsin (Fig. 4B) and collagenase (Fig. 4C). Besides, repeated and harsh triturating for 60 times had an irreversible damaging effect on neurons (Fig. 4D) in

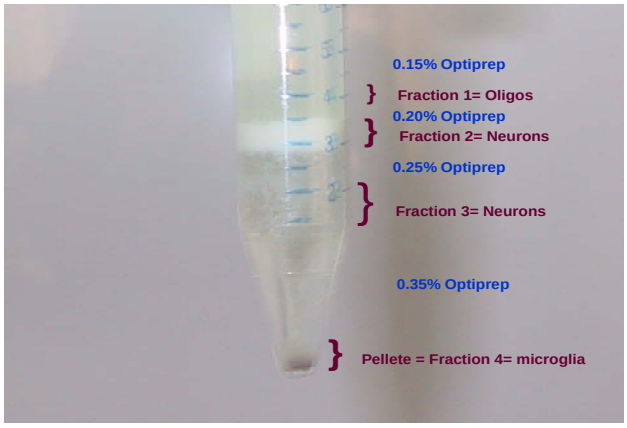

Fig. 3. OptiPrep density gradient (4 ml) of adult hippocampal neurons in a 15-ml centrifuge tube. Gradient fractions were identified for further analysis.

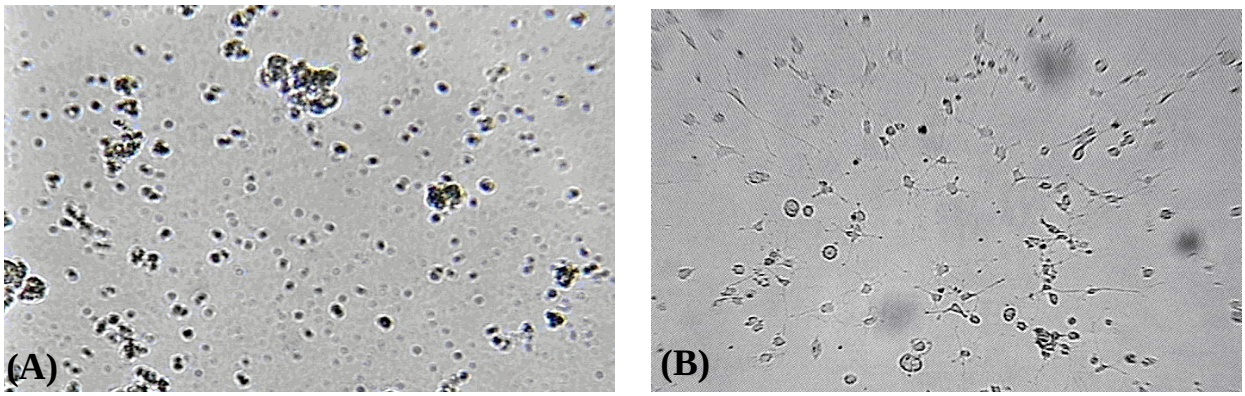

Fig. 2. Hippocampus neurons after **(B)** 10 days in culture in Neurobasal A/B27+10ng/ml FGF2 showed extensive regeneration of neuritis. **(A)** The number of live neurons in the absence of FGF2 obviously decreased and there was no processes regeneration. Magnification of A and B were 25 × objective.

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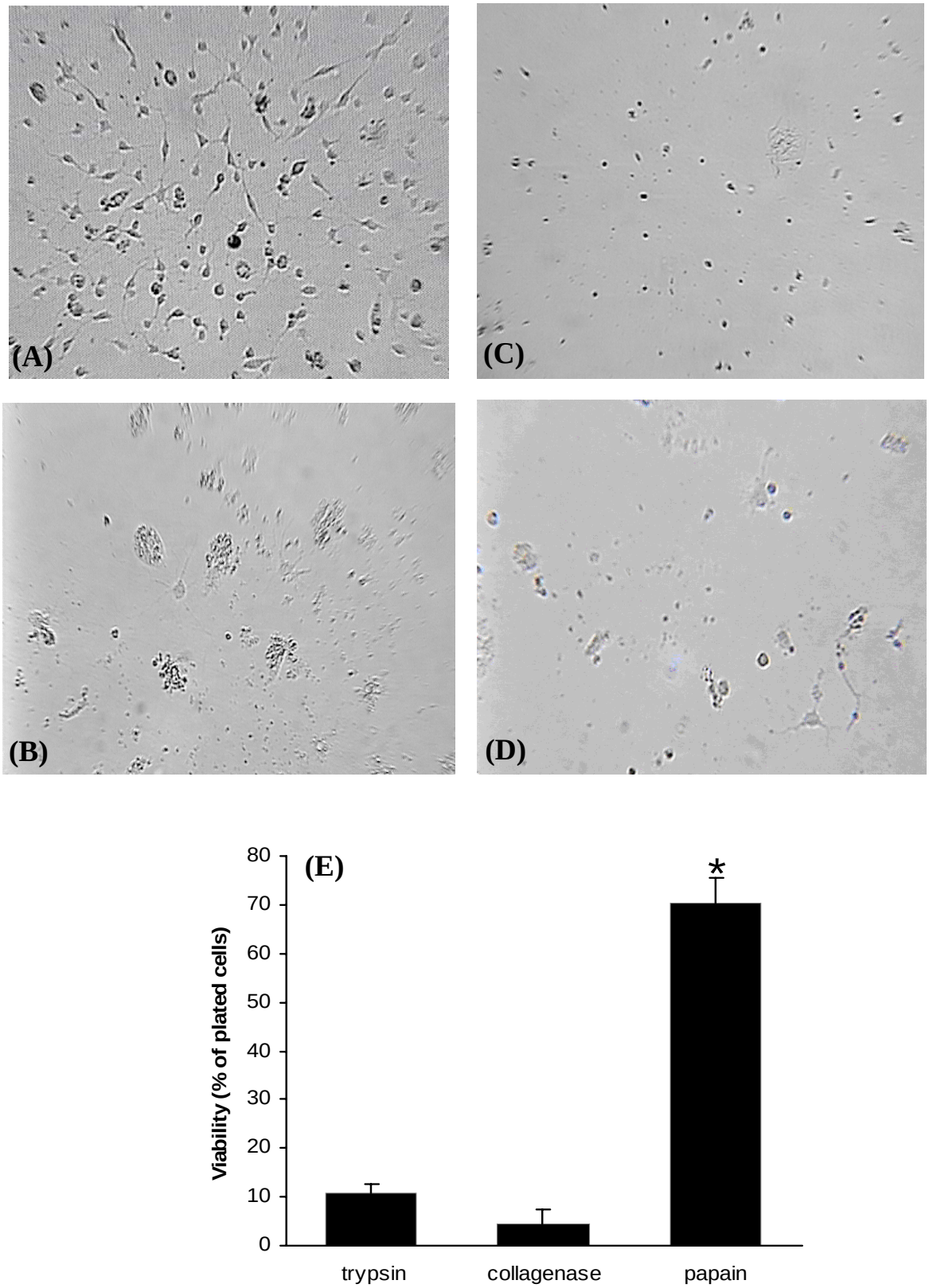

Fig. 4. By using papain, the number of live hippocampus neurons after **(A)** 10 days in culture were high with extensive neuritis regeneration. The number of live neurons by using trypsin **(B)** or collagenase **(C)** decreased. Repeated and harsh triturating for 60 times had a damaging effect on neurons **(D)**. Magnification of A and D were (10 × objective) and B and C were (25 × objective). Using papain resulted in a higher neuronal survival in comparison to trypsin and collagenase after 5 days **(E)**. Values are means ± S.E. ($P<0.01$).

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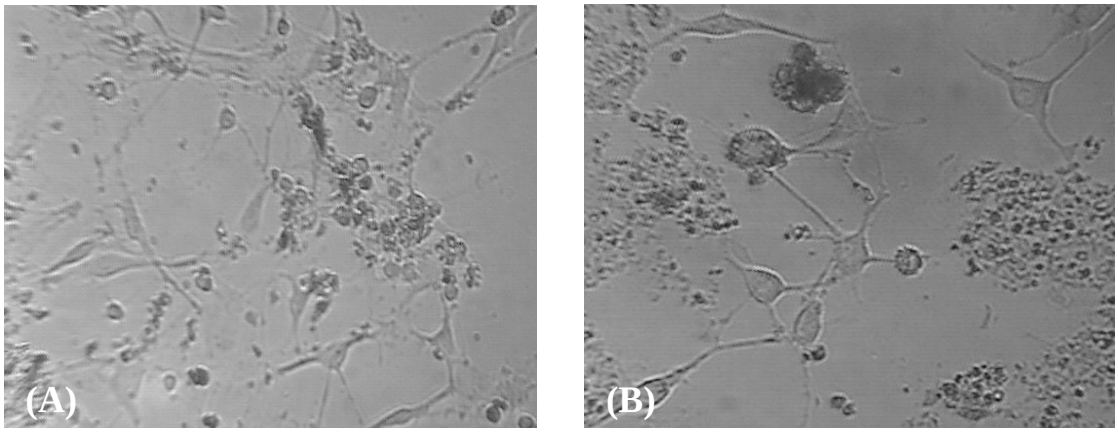

Fig. 5. Hippocampus neurons after (A) 40 days in culture with every **(A)** 8 and **(B)** 10 days half media changing. The fresh media (0.25 ml) were added every 4 days. The neurons were almost reasonable in number with extensive regeneration of neurits. Magnification of A and B were 40 × objectives.

comparison to mild triturating (Fig. 3A). It has been demonstrated that papain significantly increased neuronal viability in comparison to trypsin and collagenase (Fig. 4E).

To have a culture with the minimal change to its environmental and media condition which is appropriate for those experiments that need to be carried out on some neuronal released or experimentally added factors on neuronal cells, we changed half of the old media every 8 and 10 days instead of 4 day replacement which was used in previous cell culture studies and added 0.25 ml of fresh media. Using this method, we were able to culture adult neurons up to 40 days (Fig. 5). This method of media changing had no deteriorative effects on neuronal survival (Fig. 6)

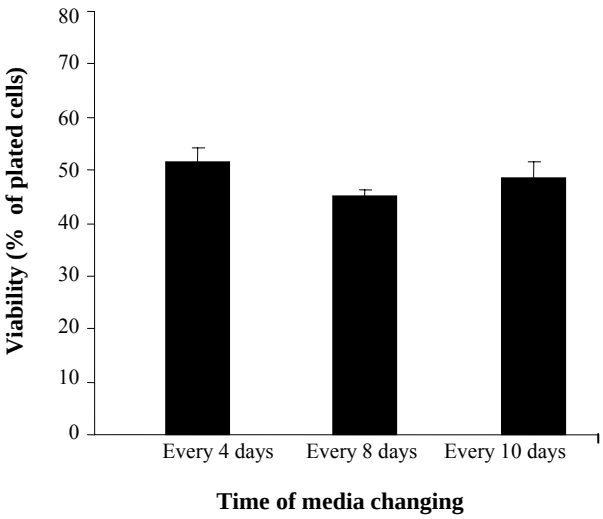

Fig. 6. Long-intervals media changing every 8 and 10 days had no destructive effect on neuronal viability in comparison to short-interval media changing after 5 weeks. The half volume of media in plating day was added to each well every 4 days.

DISCUSSIONS

The cultures of embryonic [8, 9] or postnatal neurons [10] were used in different studies. It is even performed in those diseases which happen merely in adulthood [11], because often the adult neurons failed to live or regenerate themselves in culture. Besides, it was a problem to keep medium unchanged for a rather long time to investigate on some neuronal released or experimentally added factors. We showed that in a controlled environment, with long-interval media changing, adult neuronal culturing is possible for a long time.

We demonstrated that adult neuronal tissues are sensitive to neuronal isolation methods and triturating as well. Trypsin [12, 13] and collagenase with less frequency [14] were previously reported as digestive materials according to the opinion that adult brain tissue is connected with many synapses and glial-neuronal adhesion as well as neuronal matrix adhesion. We showed that a mild digestive agent like papain is less harmful than stronger ones like trypsin and collagenase. It was demonstrated that there was a high neuronal viability with low triturating times when it was done very slowly and in a mild manner. The triturating procedure has a very critical role in neuronal surviving in our studies and it was in contrast to the previous study [15] that indicated adult neurons were resistant to isolation and triturating procedure.

We used an adult specific culture medium that promotes adult neuronal survival and their regeneration as well [1]. This is against some concepts which mentioned that adult neurons lose the ability to elongate and regenerate [16]. It seems that adult neuronal plasticity could be gained by

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removing debris and other inhibitory factors as it was supported by many evidences [17, 18]. As some studies have shown, presence of many essential factors like pyruvate or FGF2 is necessary for adult neuronal survival [18] as it had the same effect for fetal neurons [19]. An important point in our study is that besides the role of FGF2 in neuronal surviving, the sprouting rarely happens without it in the same time. It seems that trophic effects of FGF2 on neurons had not been limited to on survival and it affects neuronal regeneration as well.

A noticeable aspect of our study is that we showed it was possible to have a non-exchanged media around neurons for a rather long time. In most investigations in fetal and adult neuronal cultures [4, 5, 18-20], the media were exchanged every 3 or 4 days. However, it seems that dead factors released from debris or dead cells have inhibitory influences on cell survival, short-interval changing media decreases the effectiveness of this model. In some studies like those on special secreted factors or investigations on the influence of experimental materials on neurons for a period of time, short-interval changing media were a problem. Long term-interval media changing with providing the needed vital materials not only did not decrease neuronal viability, but also made our neurons to live more than 40 days. It has been demonstrated that some factors seem to be necessary for neuronal growth, their regeneration and helping neurons to find each other for making a neuronal network [21, 22]. The long living of differentiated process bearing neurons in our study may indicate that the maintenance of some critical neighboring factors in the media could overcome destructive effects of inhibitory factors on neuronal survival. As a conclusion, neuronal culturing is possible in studies which the assessment of neuronal secreted factors is favorable without washing them out from the media. Also, it could be used in those researches on the influence of some experimental materials on neurons during the time.

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