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A rational distribution pipetting method for the separation and culture of primary cortical

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Abstract: *On the basis of the existing methods of primary culture of neuronal cells, we optimized the extraction steps and extraction practices, and used a reasonable gradient mechanical discrete method to obtain in vitro neuronal cell models with high purity and good activity. In this study, Sprague-Dawley (SD) rats within 24 h of birth were used as experimental subjects. Brain tissues were obtained by the guillotine method and the cerebral cortex was isolated. The tissue digestion process was performed using a two-enzyme combined digestion system of papain and deoxyribonuclease I (DNase I), which was terminated by adding 1 mL of fetal bovine serum after digestion at 37°C for 20-30 min. Subsequently, single-cell suspensions were prepared by gradient mechanical disaggregation, and the cells were finally inoculated into polylysine-coated six-well plates for subsequent experiments. At 4 h, 24 h, 48 h and 7 d after inoculation, the cells were observed under the microscope and photographed. On the 7th day, the neuronal cells were identified by immunofluorescence. The cortical neuronal cells extracted by this method showed a typical morphology, with a full cytosol, clear boundaries, abundant dendritic and axonal branches, and formed a complex neural network; the purity of the neurons identified by MAP2 immunofluorescence technique reached more than 95%. Compared with the traditional primary cortical neuron extraction method, this method utilizes multiple details such as dual-enzyme co-digestion system and gradient mechanical discretization method to explore and improve, and successfully establishes a stable result, simple and feasible primary cortical neuron culture method, which is a more ideal model for the study of neuroscience field.*

Keywords: *Neuronal cells; Primary culture; Cerebral cortex; Stepwise blowing method; Papain; SD rat*

Primary neuronal culture technology plays an irreplaceable role in the exploration of neurological disease mechanisms. Studies have shown that primary cultured neuronal cells are close to the human body's own neurons, which can simulate neuronal ischemia-reperfusion injury after stroke, diabetic encephalopathy, and other neurological disease models [1]. To explore the neurological injurious diseases [2]. The experimental system of using primary neuronal cells for in vitro culture has significant advantages, the model can not only maintain the inherent physiological properties of neuronal cells, but also effectively reduce the influence of external interfering factors through the standardized operation process. This fine control of experimental conditions provides an ideal research model for in-depth investigation of the morphological changes of neurons and their intrinsic signaling mechanisms, which helps to reveal the key molecular regulatory networks of neurological-related diseases [3, 4]. Foreign literature shows that primary neurons can also be used to study the pathogenesis of neuroleptic viruses for assessing viral infection profiles and cellular immune responses to viral infection [5]. At present, for the isolation and extraction of neuronal cells still face many technical challenges. From the current status of domestic and international research, there are significant methodological differences in this field, mainly in the selection of digestive enzymes, the optimization of the culture system, and operational techniques. It brings challenges and opportunities for the research in this field [6].

1. Materials

1.1 Animals

9 adult SD rats of SPF grade, provided by Guangdong Viton Lihua Laboratory Animal Co., Ltd, with the ratio of male to female of 2:1, were successfully bred to obtain the pups after being raised in a

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combined cage. Four newborn SD rats of either sex were selected within 24 hours of birth. All experimental operations were performed in strict compliance with the "Regulations on the Administration of Laboratory Animals in China" and relevant ethical standards.

1.2 Main reagents

10×polylysine-contained goat serum, Tritonx-100, papain were purchased from Solarbio Company, Beijing, China; DMEMF12, DMEM high glucose, Neurobasal-A, Glutamine solution, B27 additive were purchased from Gibco Company, U.S.A. DNase I was purchased from MCE Company, U.S.A.; fetal calf serum was purchased from Suzhou Saiye Co. MAP2 and FITC-labeled goat anti-rabbit IgG were purchased from Wuhan Sanying Co.

2. Method

2.1 Pre-treatment of Petri dishes:

Dilute 10× poly-lysine into 1× with sterile double-distilled water one night in advance, encapsulate the six-well plate, and plant cell crawlers in the wells used for identification at the same time. 37°C incubator overnight, and then wash with PBS three times, each time for 5 min before use.

2.2 Configuration of digestion solution:

Configure 6 ml of papain (2 g/L) with DMEMF12 and add 300 μl of DNase I enzyme (5 g/L), and filter the solution through a 0.22 um filter to remove bacteria. It should be noted that the papain should be used as it is.

2.3 Neuronal seeding solution

DMEMF12 with 1% double antibody and 10% FBS.

2.4 Configuration of neuronal culture medium

Neurobasal-A with 1% B27, 0.5% glutamine, 0.5% double antibody

2.5 Primary cell isolation and culture

Four SD rat pups within 24 h after birth were selected as experimental subjects in this study. First, the pups were sterilized in 75% ethanol solution for 30 min. subsequently, the sterilized pups were transferred to an ultra-clean bench and dissected on a sterile ice box. Brain tissues were quickly removed and placed in pre-cooled DMEM high sugar medium and washed thoroughly twice. The hippocampus, cerebellum and telencephalon tissues were carefully isolated using microscopic instruments, and only the cerebral cortex was preserved. Subsequently, the vascular membrane tissue on the surface of the cortex was finely removed to ensure maximum cleanliness. Finally, the processed cortical tissue was transferred to a new sterile 10 mm Petri dish and cut into 1 mm × 1 mm × 1 mm pieces using surgical scissors for subsequent experiments. Add the pre-configured digestive solution, and digest the tissue in the incubator at 37°C for 20-30 min with shaking every 5 min. At the end of the digestion, add 1 ml of fetal bovine serum to terminate the digestion. Place the digested petri dish on ice to cool down, elevate one side of the petri dish, and allow the whole system to rest for 3 min, removing as much liquid as possible from the upper layer, while retaining the lower layer of tissue clumps. Add 2 ml of neuronal seed plate solution and 50 μl of DNase I enzyme to the petri dish, and gently blow the sample with a 5 mL pasteurized tube, and repeat the operation for 10 times. The blowing process should be gentle and slow. The sample was then left for 2 min to allow the cells to fully settle, at which time the free single cells were mainly distributed in the supernatant. This is the desired cell suspension, and the upper layer of the suspension was transferred to a pre-cooled 15 ml centrifuge tube. This step is known as the gradient mechanical disaggregation method, and it was repeated a total of three times. The cell counter was counted and repeated three times and the average value was taken. Six-well plates were grown at 1×106 per well. Placed in a 37°C incubator (CO_2 concentration of 5%, relative humidity of 95%), after 4-6 h DMEM was gently rinsed once to further remove the unattached cells and cell debris. The culture medium was replaced with neuronal culture medium. Half of the culture medium was replaced every 3

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d thereafter. Figure 1 Anatomical process of SD rats.

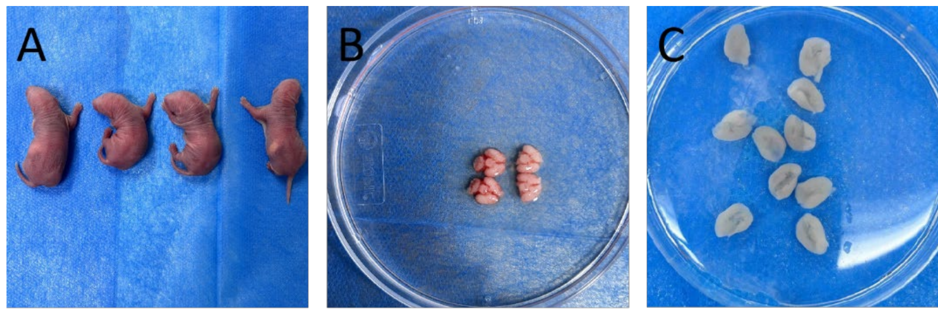

Figure 1: Lysis process of SD rats A: 24h newborn rats B: brain tissue stripped C: cerebral cortex with vascular membrane removed.

2.6 Immunofluorescence identification:

Neuronal cells were inoculated with 1×10^6 cells per well in a six-well plate containing cell crawlers, and after the cells had grown to about 7 d, they were washed three times with PBS for 5 min each time, fixed with a mixture of 4% paraformaldehyde and 20% sucrose for 20 min, and washed with PBS three times with PBS for 5 min each time, and then treated with 0.5% Tritons-100 permeabilized membrane at room temperature for 15 min. The membrane was washed 3 times with PBS, each time for 5 min. 10% goat serum was blocked at room temperature for 1 h. Goat serum was discarded, and 800 ul of neuron-specific antibody MAP2 (1:200) was added to each well and incubated overnight at 4°C. MAP2 was recovered and washed with PBS. MAP2 was recovered and washed 3 times with PBS for 5 min each time. 800 ul of FITC-labeled goat anti-rabbit IgG (1:200) was added to each well and incubated at room temperature for 1 h. The wells were washed 3 times with PBS for 5 min each time. coverslips were removed and sealed by Dapi, and the staining was observed and photographed under fluorescence microscope.

3. Results

3.1 Morphological observation on the growth of cortical neurons

4 h after inoculation with 1×106 cells/mL cell suspension, some of the cells were adherent to the wall, and the halo of the cytosol and nucleus was obvious, see Fig. 2A. 24 h after inoculation, the neuronal synapses were further grown and lengthened, and the neurons showed typical bipolar neuron morphology, see Fig. 2B. 48 h after inoculation, the neurons had a smooth surface, strong refractive index, and the synapses had grown obviously, and many synapses connected with each other to form a thin and sparse structure, see Fig. 2B. After 48 h of inoculation, the surface of the neurons was smooth and refractive, and the protrusions were obviously growing, and many of them were interconnected to form a sparse network, as shown in Figure 2C. 7 d after inoculation, the cytosol of the neurons began to aggregate, and the protrusions were thickened and grew, and the protrusions formed a more dense network, as shown in Figure 2D.

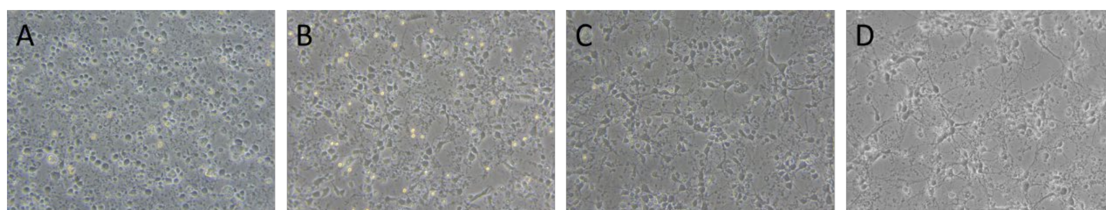

Figure 2: Morphology of primary cultured cortical cells at different periods under inverted microscope (20×10) A: cultured neuron cells for 4 h B: cultured neuron cells for 24 h C: cultured neuron cells for 48 h D: cultured neuron cells for 7 days

3.2 Immunofluorescence results of primary cortical neurons

in order to further determine the purity of primary cortical neurons, cells grown for 7 d were selected for MAP2 immunofluorescence identification, Figure 3 B, which was observed microscopically and

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morphologically showed that the neurons presented typical multipolar morphology features with contrasting nucleoplasmic staining, and the results of fluorescence labeling experiments showed that the dual-channel excitation The results of fluorescent labeling experiments showed that the cytoplasmic region showed bright blue-green bicolor fluorescent labeling under dual-channel excitation. Quantitative analysis showed that the purity of this cell population was more than 95%, which was in line with the quality control standard of neural cell culture in vitro. It is worth noting that 4% paraformaldehyde fixative alone, Figure 3 A often leads to disruption of neuronal connections, disappearance of protrusion breaks and fragmentation.

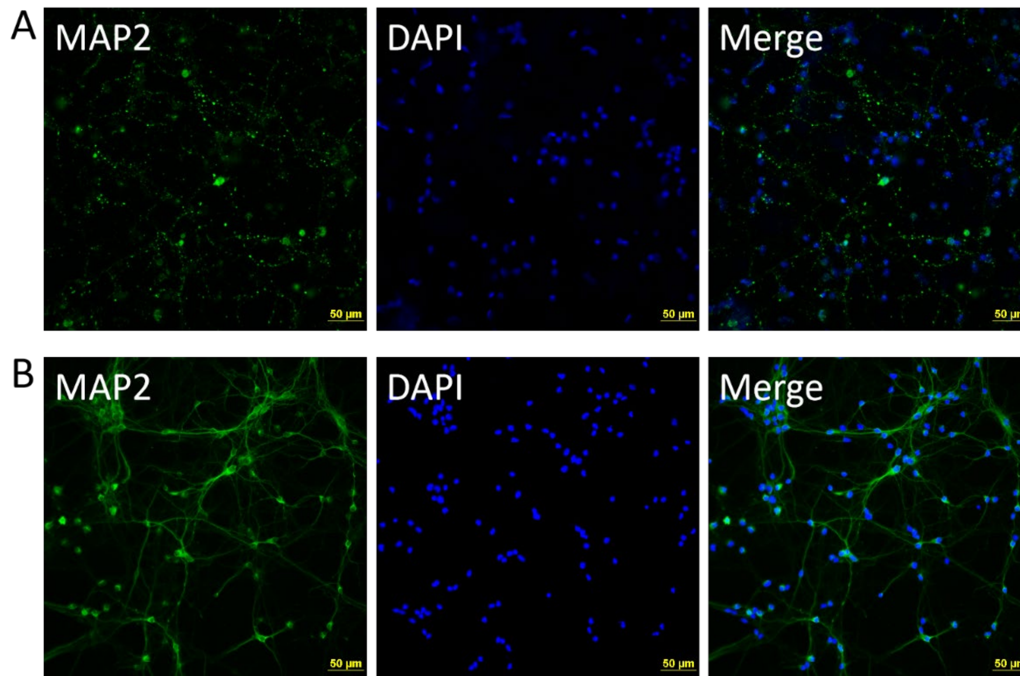

Figure 3: Identification of rat cortical neurons by MAP-2 immunofluorescence staining (40×10) A: paraformaldehyde fixation B: paraformaldehyde fixation containing 20% sucrose

4. Discussion

Primary neuronal cell culture is of great significance, and in vitro cellular models have become invaluable tools for studying the complex pathogenesis of neurodegeneration, identifying potential therapeutic targets, and assisting in drug discovery [7]. In addition, the co-growth of different cell types, such as neurons and astrocytes, used to mimic brain interactions and microenvironments promises to enhance our understanding and accelerate the development of effective therapeutic approaches [8]. Although primary neuronal cell culture provides a good in vitro model for the study of neurodegeneration in the field of neurology, there are many limitations of primary culture techniques, and primary neuronal cells are extremely demanding in terms of culture environment, extraction techniques, tissue sampling, and medium selection, so it is never easy to obtain neuronal cells with high purity and in good condition [6].

There are two main sources of animals for primary culture of cortical neuronal cells. One is newborn SD rat pups and the other is 15-18 d fetal rats. Although fetal rats can also obtain neuronal cells with high activity and good purity, the gestational age of fetal rats is difficult to grasp, and the operation process can easily cause the death of pregnant rats, so the overall consideration of this experiment is to choose the newborn 24 h pups. It is worth noting that when the sampling time of the pups exceeded 24 h, most of the neuronal cells would die after 48 h, although they could be attached to the wall. Therefore, it is important to strictly control the sampling time [9]. In the separation and digestion of tissues, except for the digestion step, the rest of the whole process needs to be operated on ice, that is, from the execution of the pups should be lowered to 0 ° C as soon as possible, and the solution used should be ice bath in advance, so as to greatly improve the survival rate of neurons. However, at 0°C neuronal cells still undergo this great degree of metabolism, so we need DMEM-high sugar or DMEMF12 plus horse serum to soak brain tissue during dissection to supply brain metabolism and inhibit neuronal apoptosis. Whether the stripping of the vascular membrane on the cortical surface is clean or not is an important factor

affecting the purity and quality of primary cell culture, and it is necessary to maximize the stripping of the meninges and blood vessels on the cortical surface. Failure to completely remove the vascular membrane during the experimental process will adversely affect neuronal isolation and culture. Standardized vascular membrane removal operation is a key prerequisite to ensure the quality of neuronal culture in vitro. For the selection of digestive enzymes, we found that trypsin digestion is very difficult to control the speed, although trypsin plays an important role in the digestion process, but in the experimental process, if the trypsin digestion rate is not controlled properly, it may lead to the rapid disintegration of the cell structure, which may lead to the release of a large amount of DNA. At the same time, the released DNA will interact with proteins to form flocculent complexes, which tend to adhere to the surface of tissues, preventing the internal tissues from being fully digested, thus affecting the results of the experiment. Papain has a mild action and high cell survival after digestion[10, 11]. For the concentration of papain, 2 g/L and digestion for 20-30 min are usually recommended. papain needs special attention that it must be ready to use. In addition, we will also add an appropriate amount of DNase I enzyme, DNase I enzyme digestion of DNA produced by ruptured cells, to avoid DNA and protein entanglement to form flocs wrapped in the surface of the tissue block, which will hinder further digestion. It is not recommended to use centrifuge tubes for digestion, but 6 cm or 10 cm petri dishes can be used, so that the digestive solution can completely cover the bottom of the petri dish, and the tissue mass can be evenly distributed in it, avoiding uneven digestion. Digestion should be carried out in an incubator with slight shaking every 5 min. The most critical step of the experiment is blowing, which should be done slowly and gently is the key to cell viability. In this study, the cell separation operation was carried out by the gradient mechanical dispersion method, and the specific steps were as follows: after every 10 mechanical dispersion operations, the mixed system was left for 2 min to make the natural stratification. At this time, the dissociated single cells were suspended in the upper layer of the liquid surface, while the incompletely dissociated cell aggregates settled at the bottom of the tube. The single cell suspension in the supernatant was collected using pre-cooled centrifuge tubes, and a low temperature environment was maintained throughout the operation. The cell clusters remaining at the bottom of the tube were supplemented with 1.5 ml of fresh medium and an appropriate amount of DNase I (50 μL), and the above dissociation-resting process was repeated to collect the free cells in the supernatant again, and the gradient separation process was repeated three times. Undissociated tissue blocks (suggesting incomplete endothelial stripping) that remained after three gradient separations should be discarded. The advantages of this gradient dissociation strategy are twofold: first, the staged operation effectively avoids the damage to cellular activity caused by excessive mechanical force in a single pass; second, the gradual dissociation significantly improves the single-cell acquisition rate. In particular, it is recommended to add appropriate amount of DNase I (50 μL) before each disaggregation, which can significantly improve the efficiency of single-cell isolation. Experimental studies have shown that the differentiation process of neurons is significantly correlated with the initial cell density. In terms of vessel selection, too large culture space (e.g., more than 6-well plate size) would lead to a gradient difference in the density of cell distribution between the central region and the edge, which in turn would cause a desynchronization of the cell maturation rate; on the contrary, too small culture units (e.g., the 96-well plate system) would lead to excessive aggregation of cells due to the spatial limitation. Based on the above spatial effect and density-dependent interaction mechanism, there is a significant negative correlation between the time of neuronal terminal differentiation and the initial population density due to the contact inhibition phenomenon. In this experimental system, we chose to use a 6-well plate to cultivate neuronal cells, and maintained an inoculum of 1×10^6 cells per well [12]. In the in vitro culture system of neuronal cells, the choice of medium is mainly divided into two categories: serum-added and serum-free. It has been shown that the use of serum-free medium is more favorable for neuronal culture in vitro, which is mainly due to the fact that serum components may promote the excessive proliferation of non-target cells, such as glial cells, which in turn reduces the purity and yield of target neurons. In traditional primary neuronal culture systems, cytarabine is often used as a cellular mitotic inhibitor [13-15], however, this substance may have adverse effects on long-term neuronal development [16]. Notably, culture experiments on primary neurons from rat cortex confirmed that a highly pure and active neuronal population could still be obtained without the addition of cytarabine. Among the existing medium systems, Neurobasal serum-free culture gene is considered as the gold standard for primary neuron culture because of its excellent culture effect [17]. Primary neuronal cells in the process of immunofluorescence identification we found that the choice of fixative is also critical, simple 4% paraformaldehyde fixation often leads to cell signaling breaks and disruption of network connectivity. It affects the observation and purity identification . Therefore, we added 20% sucrose to the 4% paraformaldehyde solution, and the cells were able to establish nerve fiber networks and form effective synaptic connections.

In conclusion, in order to extract primary cortical neuronal cells with high purity and good activity, we need to pay attention to many details. In this experiment, on the basis of the original method, we

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selected mice, separated and digested the enzyme, and utilized the mild gradient mechanical disaggregation method, and obtained cortical neuronal cells with high viability, good purity, good state, clean background and low mortality rate to the greatest extent by using the morphology of the cells, and the identification of immunofluorescence.

References

[1] NOAH G, K M R, J L P, ERKIN S. A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation [J]. Journal of neuroinflammation, 2020, 17(1): 155.
[2] GOSHI N, MORGAN R K, LEIN P J, SEKER E. A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation [J]. J Neuroinflammation, 2020, 17(1): 155.
[3] VANESSA D P, ANDRE B, ORESTES F, HELENA B. Effect of lithium at therapeutic and subtherapeutic doses in GSK3beta autonomous pathways at primary hippocampal neurons cell culture [J]. ABSTRACTS OF PAPERS OF THE AMERICAN CHEMICAL SOCIETY, 2019, 257.
[4] HOSSAIN M M, RICHARDSON J R. Nerve Growth Factor Protects Against Pyrethroid-Induced Endoplasmic Reticulum (ER) Stress in Primary Hippocampal Neurons [J]. Toxicol Sci, 2020, 174(1): 147-158.
[5] NAZNEEN F, BAI F. Isolate and Culture Mouse Primary Neurons for West Nile Virus Infection [J]. Methods Mol Biol, 2023, 2585: 23-31.
[6] M M M, P B A, A C E, et al. Primary culture of mouse embryonic spinal cord neurons: cell composition and suitability for axonal regeneration studies [J]. The International journal of neuroscience, 2019, 129(8): 762-769.
[7] GHIASVAND K, AMIRFAZLI M, MOGHIMI P, et al. The role of neuron-like cell lines and primary neuron cell models in unraveling the complexity of neurodegenerative diseases: a comprehensive review [J]. Molecular biology reports, 2024, 51(1): 1024.
[8] KIM H, LE B, GOSHI N, et al. Primary cortical cell tri-culture to study effects of amyloid-β on microglia function and neuroinflammatory response [J]. Journal of Alzheimer's disease : JAD, 2024, 102(3): 13872877241291142.
[9] SANGO K, TAKAKU S, NIIMI N, YAKO H. Isolation and Culture of Adult Rat Dorsal Root Ganglion Neurons for the In Vitro Analysis of Peripheral Nerve Degeneration and Regeneration [J]. Methods in molecular biology (Clifton, NJ), 2024, 2831: 301-313.
[10] LAURA F, D S S. Culture of Rodent Cortical, Hippocampal, and Striatal Neurons [J]. Methods in molecular biology (Clifton, NJ), 2018, 1727: 39-47.
[11] SUI-YI X, YONG-MIN W, ZHONG J, et al. A Modified Technique for Culturing Primary Fetal Rat Cortical Neurons [J]. Journal of Biomedicine and Biotechnology, 2012, 2012(X): 803930- 803930.
[12] XIAOKUN S, LINZHI D, HANG Z, et al. Neuritin Attenuates Neuronal Apoptosis Mediated by Endoplasmic Reticulum Stress In Vitro [J]. Neurochemical research, 2018, 43(7): 1383-1391.
[13] ORIGINAL/AU/AU-AFF. HMGB-1/RAGE signaling inhibition by dioscin attenuates hippocampal neuron damage induced by oxygen-glucose deprivation/reperfusion [J]. Experimental and therapeutic medicine, 2020, 20(6): 231-231.
[14] LAURA T, TERESA S, LISA M, et al. Deletion of calcineurin from GFAP-expressing astrocytes impairs excitability of cerebellar and hippocampal neurons through astroglial Na+ /K+ ATPase [J]. Glia, 2020, 68(3): 543-560.
[15] ABHINAV S, DIANA K, XIN H, et al. Improved Method for Efficient Generation of Functional Neurons from Murine Neural Progenitor Cells [J]. Cells, 2021, 10(8): 1894-1894.
[16] FAN X, ZHANG J, ZHANG X, et al. Acute and chronic morphine treatments and morphine withdrawal differentially regulate GRK2 and GRK5 gene expression in rat brain [J]. Neuropharmacology, 2002, 43(5): 809-816.
[17] NEDA V, MANSOOREH H, ABOLHASSAN A, LEILA D. Comparison of Rat Primary Midbrain Neurons Cultured in DMEM/F12 and Neurobasal Mediums [J]. Basic and clinical neuroscience, 2021, 12(2): 205-212.