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Original Article

A novel method for astrocytes isolation: compared with a classical method

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Abstract: Astrocytes have attracted much attention due to their preferable functions since they have been isolated in vitro. This study aimed to explain a novel method for astrocytes isolation and to illustrate the influence of novel method on astrocytes biological characteristics compared to the traditional method. Neonatal Wistar rats were sacrificed for the cerebral cortices collection. Both the classical shaking method and the novel method were used to isolate Astrocytes. As for the novel method, rat brain tissues were digested with trypsin instead of shaking or other complicate steps as described in classical methods. Furthermore, immunofluorescence was used to observe the isolated astrocytes with the two kinds of methods. Moreover, cell proliferation ability, cell migration ability and secretion ability were measured with CCK-8 assay, Transwell chamber and ELISA assay, respectively. Compared with the traditional isolation method, astrocytes isolated with the novel method performed high purity, with phenotype marker of glial fibrillary acidic protein (GFAP). Moreover, astrocytes isolated with the novel method retain similar abilities on proliferation, migration, and secretion abilities compared to that obtained using the classical method ($P>0.05$). Our study suggested that the novel method resulted in a high-yield, easy, and high pure preparation for astrocytes isolation.

Keywords: Astrocytes, novel method, classic method, isolation, trypsin, in vitro

Introduction

Glial cells are some nervous system associated cells, and accounts about 90% among these kinds of cells [1]. Astrocytes are some glial cells that are abundant and are widely distributed in cell subsets, which are very important in the immune system associated brain diseases, including ischemia, neurodegenerative diseases, inflammatory demyelinating diseases, and neoplastic diseases [2, 3]. It has been demonstrated that astrocytes played variety of important functions in many biological processes, such as the central nervous system development and pathology, the intake, inactivated, and supplement of neurotransmitters, antioxidant and repair of nutrients, and inhibitory excited neurons transition [4, 5]. Therefore, to investigate the biological structure and biological functions of astrocytes will be of great significance for immune system associated with brain diseases in clinical.

Previous studies have showed that isolation for astrocytes in vitro culture have been tried for many years, and several methods have been explored including isolation from CNS tissue by sedimentation field flow fraction and immune panning (Saura, 2003). So far, the most common used protocol for astrocytes isolated is reported by MacCarthy and De Vellis in 1980, which involves separation of astrocytes from primary mixed cultures of the newborn cerebral cortex on a rotary shaker and has been named as the classical method [6]. However, many drawbacks have been found about this classical method, 1) too much time cost, usually it costs 3 weeks to obtain rats before astrocytes isolation; 2) complicate steps, the frequent and prolong shaking step will be needed to remove the cells with low adhesion ability, which results in high economic cost and low cell quantity of astrocytes [7]. Thus, maintaining astrocytes in culture for a prolonged time will be difficult [8]. Hence, it is an urgent need to explore a new

method with easy and rapid steps for astrocytes isolation and to obtain pure astrocytes.

In recent years, many researchers have focused on investigating some more simple and high-yield isolation methods for microglial and astrocytes from CNS cells. For instance, Yoshihiro et al. expounded a novel simple and high-yield method for microglial cultures utilizing Aclar plastic film, which save the time cost and produced high yield and purity of microglial [9]. Josep and his colleagues invented a high-yield isolation method for murine microglia by mild trypsinization, which was convenient and economic [10]. Wang et al. isolated high purity astrocytes from the newborn 1 day SD rats by combining the mechanical and trypsin digestion methods [11]. In this present study, we described a novel protocol for astrocytes isolation from postnatal rats based on reviewing the literatures that associated with astrocytes isolation methods. We prepared astrocytes from neonatal rat brain and isolated astrocytes with a novel method using trypsin digestion only. Comprehensive experimental methods were used to compare the cell abilities including cell proliferation, cell migration, and secretion ability of the isolated astrocytes by the classical and novel methods. This study aimed to explore a new method for astrocytes purification, which is rapidly, efficiently, economically, and conveniently. Our novel approach for astrocytes isolation may be beneficial for the treatment of brain correlated disease in clinical.

Materials and methods

Cell preparation

All the experimental procedures were approved by the relevant local research animal ethics committee. The newborn Wistar rats (purchased from Animal Center, Southern Medical University) aging at 1-3 days were sacrificed for astrocytes collection. Briefly, cerebral hemispheres from neonatal rat brain were dissected out, and brain regions such as meninges, hippocampus, basal ganglion and olfactory bulb were carefully removed using microsurgical instruments under a microscope (Olympus, Japan). After that, the remaining cerebral cortical cells were seeded in DMEM-F12 (Dulbecco's modified Eagle medium, CellGro, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) at 37°C in humidified 5% CO_2 to adjust cell density at 60,000 cells/cm^2.

Classical method for astrocyte isolation

The collected cells were seeded in medium on 75 cm^2 flasks with medium changed for one time in every 3 days. After 3 weeks cultivation, cell cultures were rinsed gently with complete medium for 3 times to move the floating cells. Then 10 mL fresh medium was added into each flask for 2 h at the condition of 5% CO_2. Followed by removing flasks from chamber and tightening the caps completely, and securely fixing the flasks onto the surface of orbital shaker. The flasks were shaken for 15-18 h at 37°C (250 rpm, stroke diameter of 1.5 in) if cells were secured. After that, flasks were rinsed for 3 times to remove the suspended cells, and then 10 mL fresh medium was added into each flask. Finally, flasks were shaken vigorously by hand until the total oligodendrocytes had detached from the surface (observed using a light microscopy [12]), then cells were rinsed for 5 times with fresh medium supplemented with trypsin. Then astrocytes preparation was obtained.

Novel method for astrocyte isolation

Total collected cells were seeded in 75 cm^2 flasks. The medium was changed every 2-3 days and after for 2 weeks cultivation, each flask was subjected to gentle agitation for 2 min and then rinsed with fresh medium for 5 times to remove cells with low adhere ability. After that, the diluted trypsin (trypsin 0.25%: DMEM-F12=1:3, GIBCO, USA) was added into each flask, followed by putting them into a culture chamber for 40 min. Finally, suspended cells from all flasks were collected, and an enriched astrocytes preparation was obtained.

Immunofluorescence

Cells isolated with the classical method were cultivated for 3 weeks while cells isolated using the novel method were cultured for 2 weeks. Total cells isolated with two kinds of methods were seeded in 24-well plates to produce cell density of 1×10^5 cells/well. After 3 days of culture, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by immunostaining with primary antibody rabbit anti-rat GFAP (glial fibrillary acidic protein) (1:500, BD, USA) and secondary antibody goat anti-rabbit antibody. Then the cells were rinsed with PBS buffer for 3 times, and a nuclear counterstaining with DAPI (4',6'-diamidino-

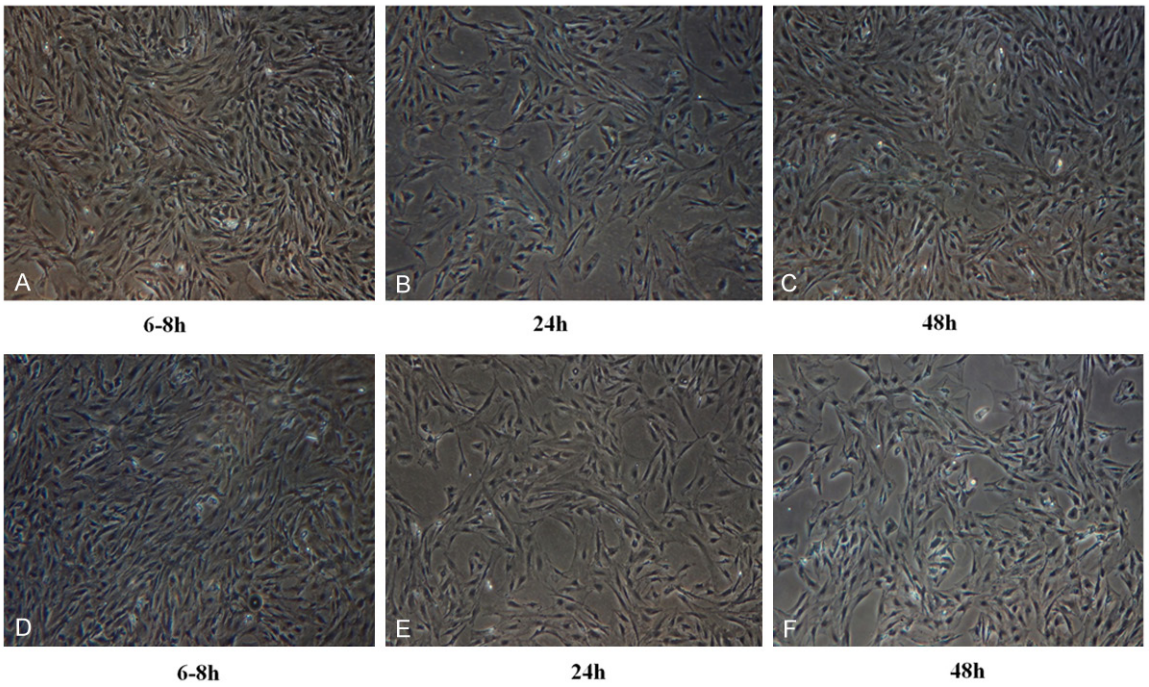

Figure 1. Astrocyte isolated from both new method and classical method. A-C: Astrocytes isolated with the novel method at different time point; D-F: Astrocytes isolated with the classical method at different time point.

2-phenylindole, Invitrogen, USA) was performed 5 min at RT. The ratio of stained cells/number of total cells was calculated for 3-5 fields to evaluate the percentage of positive cells.

Cell proliferation

The astrocytes isolated with the two kinds of methods were seeded in the 96-well plates at a density of 1×10^4 cells/well within fresh DMEM-F12 medium supplemented with 10% FBS. After 4 days of culture, cell growth of astrocytes was measured using Cell Counting Kit-8 (cck-8; Dojindo Molecular Technology, Gaithersburg, MD) each day according to manufacturer's protocols [13]. Briefly, 10 μL of CCK-8 was added into each well, and cells were incubated at 37°C for 2 h. Absorbance of cells in each well was detected using a multiwall spectrophotometer (Multiskan MK3, Thermo Scientific Company) at wavelength of 450 nm. The experiment was performed in 3 independent experiments.

Cell migration

The isolated astrocytes were re-suspended to obtain the suspensions and then were digested using 0.25% trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA in HBSS). The Transwell chamber was prepared to detect the migration ability of astrocytes isolated using the two kinds of methods. Astrocytes were rinsed for 3 times to remove the cell suspensions and then digested using trypsin as previously described. At the end of digestion, mixtures were centrifuged at 12,000 rpm at room temperature and re-suspended with PBS buffer (PH 7.4) for 2 times. Followed with re-suspended the harvested astrocytes in serum-free medium supplemented with bovine serum albumin (BSA) to adjust cell density of 5×10^5/mL. After that, 100 μL of cell suspension were added into the Transwell chamber, and then 600 μL of DMEM-F12 medium supplemented with 10% FBS was added into the 24-well plates. After 4-12 h of culture (time which was mainly depended on the migrate ability of astrocytes), the upper side filter was scraped with a cotton tip to eliminate cells without migration ability. Cells that migrated to the lower side of filter were calculated using a microscope. The experiments were performed in triplicate and ≥10 fields were counted in each experiment.

Enzyme-linked immunosorbent assay

In order to analyze the secretion capacity of isolated astrocytes with the two kinds of methods,

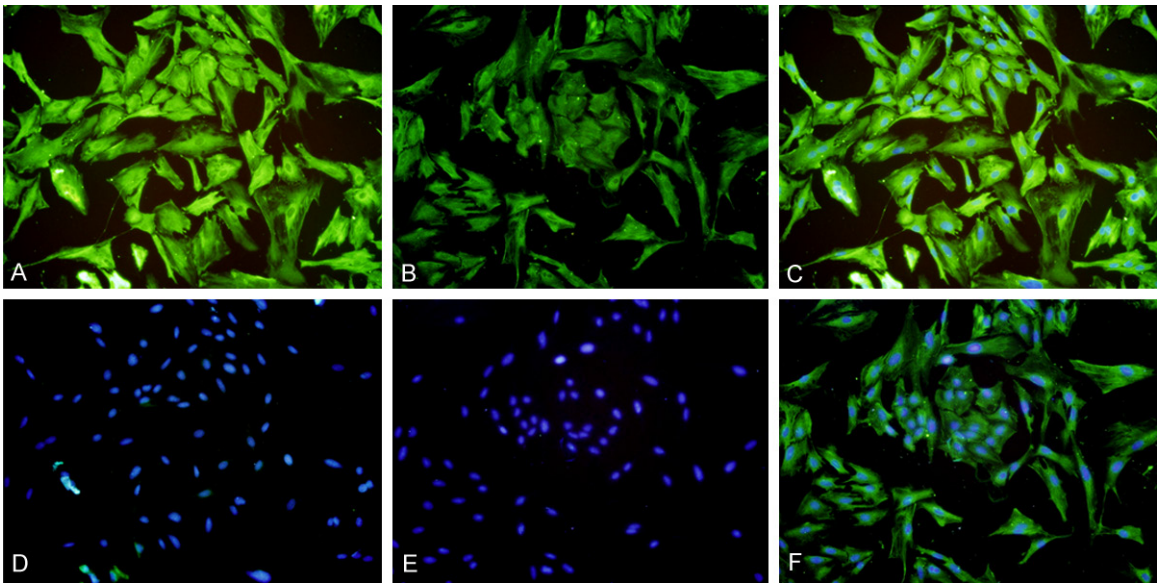

Figure 2. Immunocytofluorescence analysis of astrocytes isolated by the classical and novel methods. A-C: Astrocytes isolated by the novel method; D-F: Astrocytes isolated by the classical method.

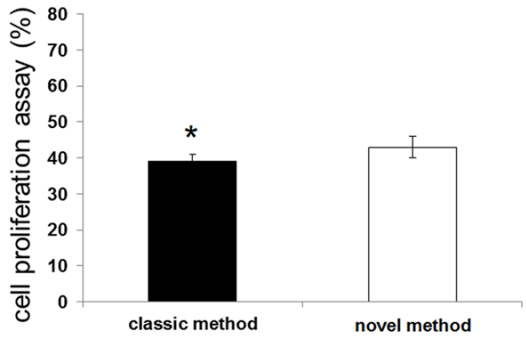

Figure 3. Cell proliferation assays of astrocytes isolated by the classical and novel methods. *: P>0.05, compared with the novel method.

cells were seeded in 6-well plates. Then 2 μg/mL of lipopolysaccharide (LPS) was mixed with cells to stimulate astrocytes. After 24 h of culture, supernatant of astrocytes was collected for the analysis of the amount of IL-1β using the enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction [14]. The experiments were conducted for three times independently.

Statistical analysis

All data were expressed as mean ± SEM (standard error of mean). Statistical analysis was completed using SPSS 16.0 (IL, CA, USA). Significance among groups was calculated using ANOVA (one-way analysis of variance). $P<0.05$ was considered as the statistically significant.

Results

Cell culture

Astrocytes isolated by the two kinds of methods were observed under the inverted phase contrast microscope (**Figure 1**). The photos showed that during the process of astrocytes purification, most of the cells have been adherent after 6-8 h, become oval, spindle shaped, and irregular shaped (**Figure 1A** and **1D**). After 24 h of cultivation, cells become are adherent, with an obvious halo, and mixed cultures of astrocytes have cluster shaped growth (**Figure 1B** and **1E**). At the time of 48 h for culture, cells have completely fused and astrocytes increased with more slender branches (**Figure 1C** and **1F**).

Immunocytofluorescence

Immunocytofluorescence was used to detect astrocytes under a fluorescence microscope (**Figure 2**). Astrocytes with GFAP-positive marker could present green florescence [15]. In this study, positive astrocytes in four visual fields (top, bottom, left, and right) were observed. The photos showed that about 95% astrocytes had

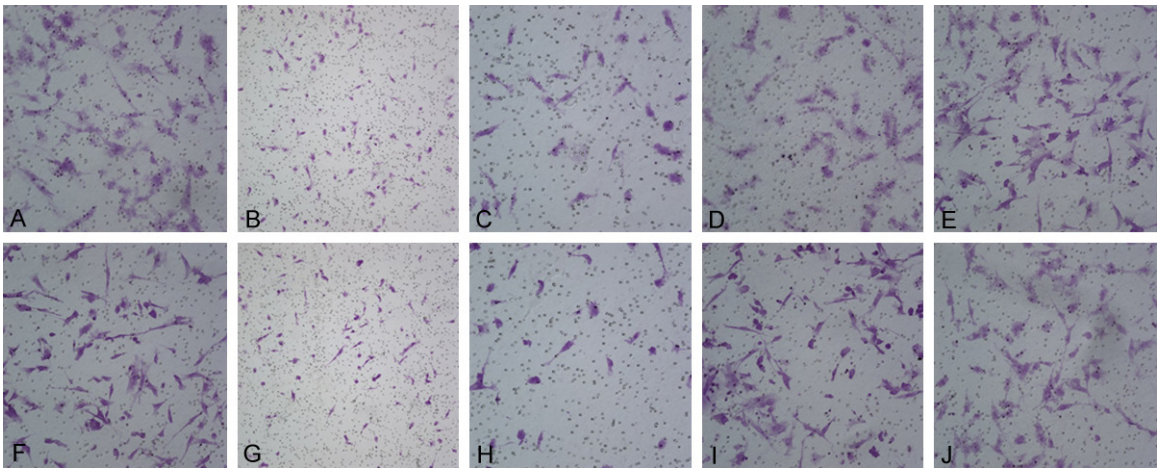

Figure 4. Changes of astrocytes migration level of acquired by Transwell assay for different old and new methods. A-E: The number of astrocytes going across the membrane with a new method; F-J: The number of astrocytes going across the membrane with the classical method. Count 5 field for observing cells under microscope with × 400.

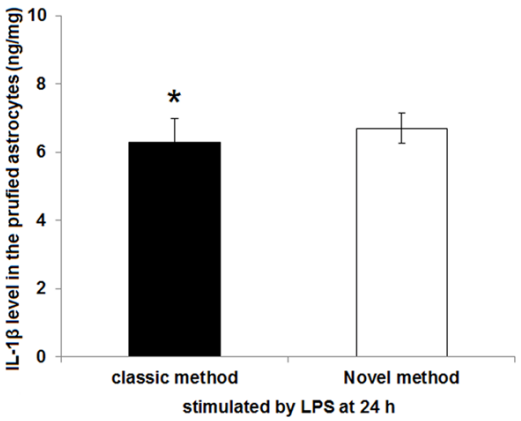

Figure 5. Enzyme-linked immunosorbent assay of astrocytes isolated with novel and classic methods. The level of IL-1β with the old method was detected as 6.31 ± 0.68 ng/mg, while IL-1β levels of the purified glial cells in the new approach was 6.72 ± 0.45 ng/mg. *: the difference of changes in their levels of IL-1β of astrocytes purified by the two methods was not statistically significant (P>0.05).

GFAP marker in the whole light microscope. Also, there was no difference of GFAP-marked astrocytes isolated by the classical and novel methods.

Cell proliferation

CCK-8 assay was used to detect the cell proliferation ability of astrocytes isolated by the two kinds of methods (**Figure 3**). The results showed that there was no significant difference of astrocytes proliferation that isolated with the classical and with the novel methods (P>0.05).

Cell migration

Transwell chamber was used to assess the cell migration ability of astrocytes isolated by two kinds of methods (**Figure 4**). The photos showed that there was no significant difference for migrated cell number of astrocytes isolated by two kinds of methods. Also, migrate cells from the two isolated methods performed no difference at different time points (P>0.05).

ELISA assay

ELISA assay was used to measure the proteins or cytokines secreted by astrocytes that isolated with the classical and novel method (**Figure 5**). Our results displayed that the level of IL-1β secreted by astrocytes isolated with the novel method was 6.31 ± 0.68 ng/mg, while IL-1β level of the purified astrocytes in novel method group was 6.72 ± 0.45 ng/mg. Also, there was no significant difference of the IL-1β level secreted by strocytes between the two groups (P>0.05).

Discussion

The glial cells were one of the two kinds of nerve tissue cells, and play pivotal roles in supporting neurons and promoting regeneration post-injuries [16]. Astrocytes were some most widespread and with largest number in nerve

tissues, and play crucial roles in immune associated brain diseases [17]. Although many studies have devoted to the exploration of astrocytes isolation, the inventive methods used for astrocytes were not so good which often result in high economic cost and low purity [6, 11]. In this study, we reviewed the previous studies that aimed on astrocytes isolation and described a novel method for astrocytes isolation with trypsin. Our data revealed that cell culture character of astrocytes isolated by our novel method was the same as that isolated by the classical method ($P>0.05$). Moreover, biological characters such as proliferation, cell migration, and secretion ability of astrocytes isolated by our novel method were similar as that isolated with the classical method ($P>0.05$).

Studies have demonstrated that astrocytes were characterized with huge volume and large nucleus, lots of cell number, star or ovoid during development [18, 19]. The astrocytes isolated with the two kinds of method performed adherent ability after cultured for about 6-9 h, and cells become oval, spindle shaped, and irregular shaped. Consequently, cells become are adherent, with an obvious halo, and mixed cultures of astrocytes have cluster shaped growth at time point of 24 h. Moreover, cells have completely fused and astrocytes increased with more slender branches, implying that our novel method could not change astrocytes biological characters.

In our study, immunofluorescence analysis showed that astrocytes isolated by the two kinds of methods had GFAP-marker. GFAP, a kind of intermediate filament protein, exists in cytoplasm of astrocytes, which is crucial for maintaining the steady of astrocytes structure and the growth and extension of synapse and is considered to be the characteristic symbol for astrocytes [20]. Several papers that devoted to the useful exploration of astrocytes isolation methods and other biological processes had all experimented the GFAP marker for cells [21, 22]. Therefore, our data suggested that there was no significant difference of astrocytes isolated by the classic and novel method. On the other hand, when we measured the biological characters for astrocytes isolated by classic and novel method, we observed that there was no significant difference of cell migration and proliferation between the two kinds of methods ($P>0.05$), indicating that novel method could not change the cell proliferation and migration ability during isolation.

Meanwhile, IL-1β is one of the strongest inflammatory mediators that secreted by the activated astrocytes, and can not only promote amyloid precursor protein expression but also accelerate entanglement of nerve fibers [23, 24]. Release of IL-1β contributes the cell adhesion, leukocyte migration, and reactive oxygen species (ROS) generation, which results in inducing cascade reactions of cytokines [25, 26]. Abundant IL-1β secretion would lead to inflammatory damage and malnutrition axons in nervous system associated diseases such as AD [27, 28]. Our data presented that the isolated astrocytes purified with the novel could secret 6.72 ± 0.45 ng/mg IL-1β, implying the cell viability for astrocytes. Besides, the IL-1β secreted by astrocytes isolated with classical method was 6.31 ± 0.68 ng/mg, and there was no significant difference of IL-1β yield secreted by astrocytes purified with two methods. Thus, we speculated that novel method could not change the cell viability and secretion ability of astrocytes.

In conclusion, the data presented that there were no significant differences between characteristics of astrocytes isolated by two methods such as cell biological character, migration, and proliferation ability, suggesting that novel isolation method would save the tedious operation steps for astrocytic isolation compared to the classic method. Our study suggested that the novel isolation method on astrocytes is high-yield, easy operation, and high pure preparation. This study may provide basis for the method exploration on astrocytes in vitro and may reduce the economic cost for clinical application.

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Disclosure of conflict of interest

None.

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