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Analysis of isolation of cerebral cortical neurons in rats by different methods

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Abstract: The aim of this study was to find a way to efficiently separate neuronal cells from the cerebral cortex of adult rats, providing a reference method for rapid acquisition of neuronal cells from the adult rat brain. Fifteen SD rats were randomly divided into three groups, with five SD rats in each group. Then, neuron cells were isolated from the adult rat cerebral cortex by the grinding method, the trypsin method, and the collagenase II method, respectively. The expression of anti-NeuN in the neurons of each group was analyzed by flow cytometry. The acquisition rates and morphology of neurons of each group were observed by immunofluorescence staining. The grinding or collagenase II method is more suitable for rapid acquisition of neuronal cells from an adult rat's cerebral cortex. The number of neuron cells obtained by the trypsin method were very few, so it is not convenient for later experiments.

Introduction

The study of specific types of cells in the central nervous system, especially neurons, is becoming more and more common. Different cell populations have specific functions for the development of the central nervous system (Hoye *et al.*, 2018; Kopec *et al.*, 2018; Lioy *et al.*, 2011; Sarlus and Heneka, 2017; Yu *et al.*, 2018). The separation and culture of neuronal cells from the central nervous system are essential for the study of neuropharmacology, neurodegenerative diseases, and nerve regeneration mechanisms. Common methods for obtaining single-cell suspensions of neurons are the grinding method and the collagenase and trypsin methods (Him *et al.*, 2017; Katzenell *et al.*, 2017; Leong *et al.*, 2013; Yu *et al.*, 2016; Zhang and Hu, 2013), and the grinding method are applied to extract and separate astrocytes to study the effect of ADP-ribosylation on the aging rat brain (Manoochehr and Azadeh, 2019) and the neural stem cells were extracted and separated to study the Gene expression profile of Sox1, Sox2, p53, Bax and Nestin in adult mouse brain tissues (Wang *et al.*, 2019), but it is still not clear which method can quickly and efficiently obtain neuronal cells from the adult rat brain. Although embryonic neurons are a simple and rapid source of primary neuronal cells, their developmental stages are not suitable for studying many neurodegenerative diseases that occur in later life. Some studies have shown that embryonic neurons differ from adult neurons in the areas of pharmacology, electrophysiology, development, and pathology (Brewer *et al.*, 2006; Parihar and Brewer, 2007). In this study, three common methods, including the grinding, trypsin, and collagenase methods, were used to isolate neurons from the cerebral cortex of adult rats to compare the effects of these three methods on obtaining neurons, to find a best way to isolate neurons from adult rats, and to provide a reference method for the rapid acquisition of neurons from an adult rat brain.

Materials and Methods

Animals

The animals used in this experiment were SPF-level SD male rats, 8-10-weeks-old and weighing between 250 grams and 280 grams. They were purchased at the Qinglong Mountain Animal Breeding Farm in Jiangning District, Nanjing City, China (license number: SCXK (Shanghai) 2018-0004). A total of fifteen rats were randomly divided into three groups, and there were five rats in each group.

Main reagents and instruments

Trypsin (27252-018), collagenase II (QN0512), and DMEM (12800-017) were purchased from GIBCO. Fetal bovine serum (04-001-1ACS) was purchased from Biological

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Industries. Polylysine (P0296) was purchased from Sigma. DAPI (ab104139), anti-NeuN (ab177467), and Goat anti-rabbit IgG H&L (FITC, ab6717) were purchased from Abcam. BD Pharm Lyse lysing solution (No. 555899) was purchased from Becton Dickinson. The stationary liquid 0.01 M PBS solution) contained 3% formaldehyde and 20% sucrose. BD FACS Celesta Flow cytometry was obtained from BD Biosciences-US, and Cytation5 was obtained from BioTek.

Treatment of 6-well plates with polylysine

To disinfect the slides, a 12 mm cover glass was placed in an iron box covered with aluminum foil and sterilized in an oven at 225°C for six hours. An 0.1 mg/ml working solution diluted with ultrapure water and a filter was prepared. Then, 1 ml of polylysine was added to each well of a 6-well plate , and the slides in 6-well plate were gently shaken at 4°C to fully soak. The refrigerator was left on overnight, and the slides were washed twice with HBSS on the next day (Yang *et al*., 2017).

Preparation of rat brain cell suspension by the grinding method

Five SD rats were anesthetized with a 10% chloral hydrate solution and sacrificed by cervical dislocation. After the rats were disinfected with 75% ethanol, the brain skin was removed, the skull was cut off, and the brain was removed into a cell culture dish that contained an HBSS buffer solution (270–305 mOsm/kg) for cleaning. Meninges and blood vessels were removed from the cerebral cortex by using forceps under a microscope. An 0.1 g organization was placed on a 48 mm sterile stainless-steel filter net, under which was a 35 mm cell culture dish. The tissue was grinded by using a glass abrasive bar, and 10 ml of DMEM medium containing 10% fetal bovine serum was added while grinding. The cell suspension was transferred from the culture dish to a 15 ml centrifuge tube, and it was filtered twice with a 48 μm sterile stainless-steel filter during the transferring. The cell suspension was gently mixed and placed on ice for five minutes to discard tissue debris, and cell clusters were deposited on the centrifuge tube. The upper 8 ml of cell suspension was transferred to another 15 ml centrifuge tube for later use.

Preparation of rat brain cell suspension by the trypsin method

Five SD rats were anesthetized with a 10% chloral hydrate solution and then sacrificed by cervical dislocation. After the rats were disinfected with 75% ethanol, the brain skin was removed, the skull was cut off, and the brain was removed into a cell culture dish containing an HBSS buffer solution (270–305 mOsm/kg). A total of 0.1 g of tissue was placed in a 35 mm cell culture dish, and the tissue was cut as small as possible with a surgical knife. Then, 1 ml of an 0.125% trypsin solution was digested in an incubator at 37°C for 15 minutes. The cell culture dish was shaken gently every 2–3 minutes to completely digest the tissues. The digestion reaction was terminated by adding 2 ml of pre-warmed DMEM medium containing 10% fetal calf serum. The liquid in the culture dish was transferred to a 15 ml centrifuge tube, filtered twice through 48 μm sterile stainless-steel mesh, and supplemented with a 10 ml DMEM medium containing 10% fetal calf serum. After gently mixing with the cell suspension, it was placed on ice for five minutes to discard tissue debris, and cell clusters were deposited on the centrifuge tube. The upper 8 ml of cell suspension was transferred to another 15 ml centrifuge tube for later use.

Preparation of rat brain cell suspension by the collagenase II method

Five SD rats were anesthetized with a 10% chloral hydrate solution and then sacrificed by cervical dislocation. After the rats were disinfected with 75% ethanol, the brain skin was removed, the skull was cut off, and the brain was removed into a cell culture dish containing an HBSS buffer solution (270–305 mOsm/kg). A total of 0.1 g of tissue was placed in a 35 mm cell culture dish, and the tissue was cut as small as possible with a surgical knife. Then, 1 ml of 300U collagenase II was added, and it was digested in an incubator at a temperature of 37°C for 15 minutes. The cell culture dish was shaken every 2–3 minutes to completely digest the tissues. The digestion reaction was terminated by adding 2 ml of pre-warmed DMEM medium containing 10% fetal calf serum. The liquid in the culture dish was transferred to a 15 ml centrifuge tube, filtered twice through 48 μm sterile stainless-steel mesh, and supplemented with 10 ml of DMEM medium containing 10% fetal calf serum. After gently mixing with the cell suspension, it was placed on ice for five minutes to discard tissue debris, and cell clusters were deposited on the centrifuge tube. The upper 8 ml of cell suspension was transferred to another 15 ml centrifuge tube for later use.

Immunofluorescence

The 6-well plate (containing cover glass) was cleaned twice with HBSS buffer, which was treated with polylysine one day before using. Three replicates of 150 μl cell suspension solution (prepared in the previous steps) were given to each rat, and 2 ml of DMEM medium containing 10% fetal bovine serum was added. The 6-well plate was shaken so that the cells could be dispersed. The 6-well plate was placed in a CO_2 incubator at 37°C for two hours so the cells could adhere to the wall. After two hours, the culture medium was removed, and 2 ml of 0.01 M PBS buffer was added to each well for cleaning. When adding PBS buffer, the operation was gentle so as to avoid washing the cells off the cover glass. A total of 1 ml of stationary liquid was added to each hole and fixed for 10 minutes. After washing three times with a PBS buffer, an 0.5% Triton-X-100 solution was added and treated for five minutes to improve the permeability of cells and to facilitate the entry of antibodies. After washing with a PBS buffer three times, 1 ml of a 5% BSA solution was added and blocked for two hours. After three washings with a PBS buffer, a 100 μl anti-NeuN (Rabbit, 1:300) solution was added to each slide and incubated for one hour. After washing with a PBS buffer three times, a 100 μl Goat anti-Rabbit IgG H&L (FITC, 1:1000) solution was added to each slide and incubated for one hour. Finally, a 100 μl DAPI (1:100) staining solution was added to each slide and reacted for five minutes. After cleaning with a PBS buffer, a Cytation five-cell imaging system was used to take photos.

Flow cytometry

The supernatant was discarded from the 1 ml of cell suspension, as prepared in the previous steps (1000 r/min, centrifuged for 10 minutes). A total of 1 ml of red cell cleavage solution was added to each 5 ml flow-cytometry cell tube. After the addition of RBC lysate buffer, it was immediately swirled slightly to blend the cells. It had reacted on ice for 15 minutes and gently mixed for twice. After the RBC lysate was added, it was slightly swirled to mix the cells. It again reacted on ice for 15 minutes and gently mixed for twice. It centrifuged at 450 g for 10 minutes, and the supernatant was carefully discarded. A total of 1 ml HBSS was added to the vortex and mix, and it was centrifuged at 450 g for 10 minutes. The supernatant was then carefully discarded. With a 1 ml HBSS solution, cells were resuspended and counted. A total of 2×10^6 cells were added to each flow tube and fixed with 1 ml of an 80% methanol solution for five minutes. The membrane was then ruptured with 1 ml of 0.01 M PBST solution for 20 minutes. A total of 2 ml of supernatant was removed, and 1 μl of anti-NeuN (1:100) was added—it was then incubated at room temperature for 30 minutes while being protected from light. The cells were washed once with an HBSS buffer, they were centrifuged at a speed of 400 g for five minutes, and they were then resuspended with 500 μl of pre-cooled HBSS. A total of 1 μl of Goat anti-Rabbit IgG H&L (FITC) (1:500) was added to each tube, mixed, and incubated for 20 minutes at room temperature in the dark. HBSS was used to wash the cells once, and they were centrifuged at a speed of 400 g for 5 minutes, and they were resuspended with a 400 μl pre-cooled HBSS solution. This was immediately detected by flow cytometry.

Statistical analysis

GraphPad Prism 5.0 software was used for data collation and analysis. The measurement data were normally distributed and expressed by mean ± standard deviation. A single factor design variance analysis was used to compare variance between multiple groups. Post hoc comparison between groups used Tukey's multiple comparisons test. A two-sided p value of 0.05 was considered statistically significant.

Results

Cell suspension and cell adherence

Cell suspension was obtained by three methods and observated under optical microscope, and it found that there were some larger cell clusters beside cells by the grinding method (Fig. 1a) and the collagenase II method (Fig. 1b), but was less by the trypsin method (Fig. 1c). After adhering the cells for two hours, the results showed that cells obtained by the grinding method and the collagenase II method had more cells adhering to the cover glass (Figs. 1d and 1e). However, the number of adherent cells treated with trypsin was less (Fig. 1f), so they may have needed a longer time to adhere to the cover glass.

Immunofluorescence

A vertebrate neuron-specific nuclear protein called NeuN (neuronal nuclei) is an excellent marker for neurons in primary cultures and in retinoic acid-stimulated P19 cells. NeuN is a neuron-specific, DNA-binding nuclear protein in vertebrates. In mice, NeuN is observed in most neuronal cell types throughout the nervous system, including the cerebellum, cerebral cortex, hippocampus, thalamus, and spinal cord, as well as the dorsal root ganglia, sympathetic chain ganglia, and enteric ganglia of the peripheral nervous system. In order to identify living nerve cells, NeuN was used to label nerve cells, and DAPI was used to label the nuclei of living cells. Immunofluorescence results showed that there were more living nerve cells obtained by the grinding method and the collagenase II method (Fig. 2). Cytation 5 software was used to analyze the number of NeuN$^+$ cells. The results showed that the acquisition rate of NeuN$^+$ cells by the grinding method was 42.7% ± 1.2% (Fig. 3a), and it was 47.5% ± 3.3% by the collagenase II method (Fig. 3b), and it was 61.6% ± 2.1% by the trypsin method (Fig. 3c). The acquisition rate of the trypsin method was higher than that of the grinding method and the collagenase II method ($p < 0.001$) (Fig. 3d). Under a 10-fold objective lens, the average number of NeuN$^+$ cells in each field of vision was 68% ± 12% by the grinding method, and 60% ± 9.8% by the collagenase II method, and only 30% ± 2% by the trypsin method. Thus, the grinding method and the collagenase II method was higher than the trypsin method ($p < 0.001$) (Fig. 3e).

Flow cytometry

The results of flow cytometry showed that NeuN$^+$ cell acquisition rate was the least by the grinding method, which was 43.0% ± 1.6% (Fig. 4a). The collagenase II method was 52.9% ± 4.2% (Fig. 4b), and the NeuN$^+$ cell acquisition rate was the highest by trypsin method, which was 64.8% ± 2.7% ($p < 0.001$) (Figs. 4c and 4d). The results were consistent with the immunofluorescence.

The culture of adult rat neuronal cells is increasingly used in neuropharmacology and neurodegenerative diseases (Brewer *et al.*, 2006; Parihar and Brewer, 2007), and different isolation methods often lead to different results. In this study, the neuronal cells of a rat cerebral cortex were isolated by the grinding method, the collagenase method, and the trypsin method. After two hours, it was found that the number of single cells obtained by the grinding method was higher than that of the trypsin method ($p < 0.001$) and the collagenase method ($p < 0.001$) under the microscope, which was also confirmed by immunofluorescence staining. Besides, the state of cells adherent isolated by the grinding method was better than the enzymatic method under optical microscopy. Studies have shown that neurons, astrocytes, and microglia can be simultaneously isolated from fresh brain tissue (Martin *et al.*, 2017; Smith *et al.*, 2014).Therefore, the grinding method can fully grind the tissue under the condition of guaranteeing cell viability, and it can remove large impurity debris through 48-micron stainless steel mesh filtration to obtain as many single cells as possible.

Trypsin is a serine protease extracted from the pancreas of pigs, cattle, and sheep. It acts as a digestive enzyme in mammals, fish, and some bacteria (Rajabi *et al.*, 2019), and it can cut off the ends of carboxyl, lysine, and arginine residues in the polypeptide chain. Trypsin also has strong specificity. In this study, the rate of rat cerebral cortical neuronal cells obtained by the trypsin method was higher than that of the grinding method ($p < 0.001$) and the

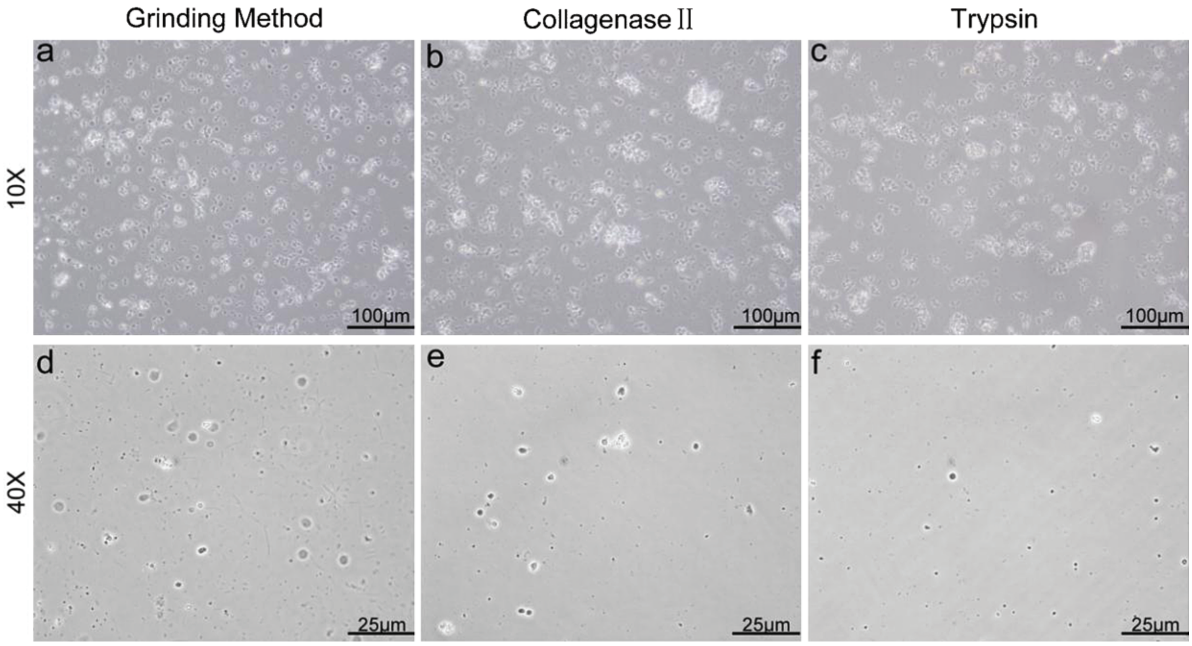

FIGURE 1. Cell suspension and cell adherence results.
Letters a,b, and c mean that cell suspensions (10×) were obtained by the grinding method, the collagenase II method, and the trypsin method, respectively. Letters d,e, and f mean that cells that were treated by the grinding method, the collagenase II method, and the trypsin method, respectively, were incubated for two hours in 37°C CO_2 incubator.

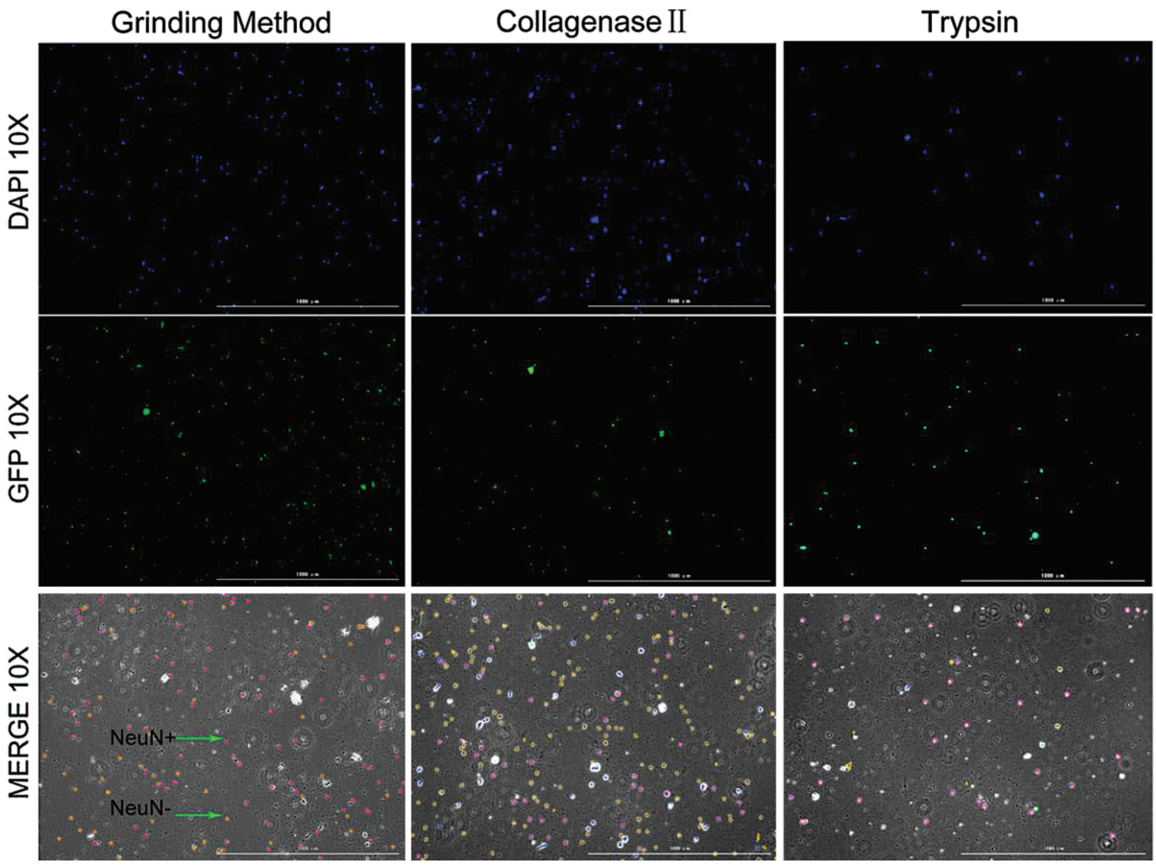

FIGURE 2. Immunofluorescence results the merge graph was overlapped by fluorescence and a bright field, and $NeuN^+$ cells and $NeuN^-$ cells were labelled by Cytation5 software.

collagenase method ($p < 0.001$) through flow cytometry and immunofluorescence staining, which may have been caused by the specificity of trypsin.

At the same time, studies have shown that trypsin does not damage neuronal cell surface receptors (Azari *et al.*, 2010), so this may also be the reason why most researchers

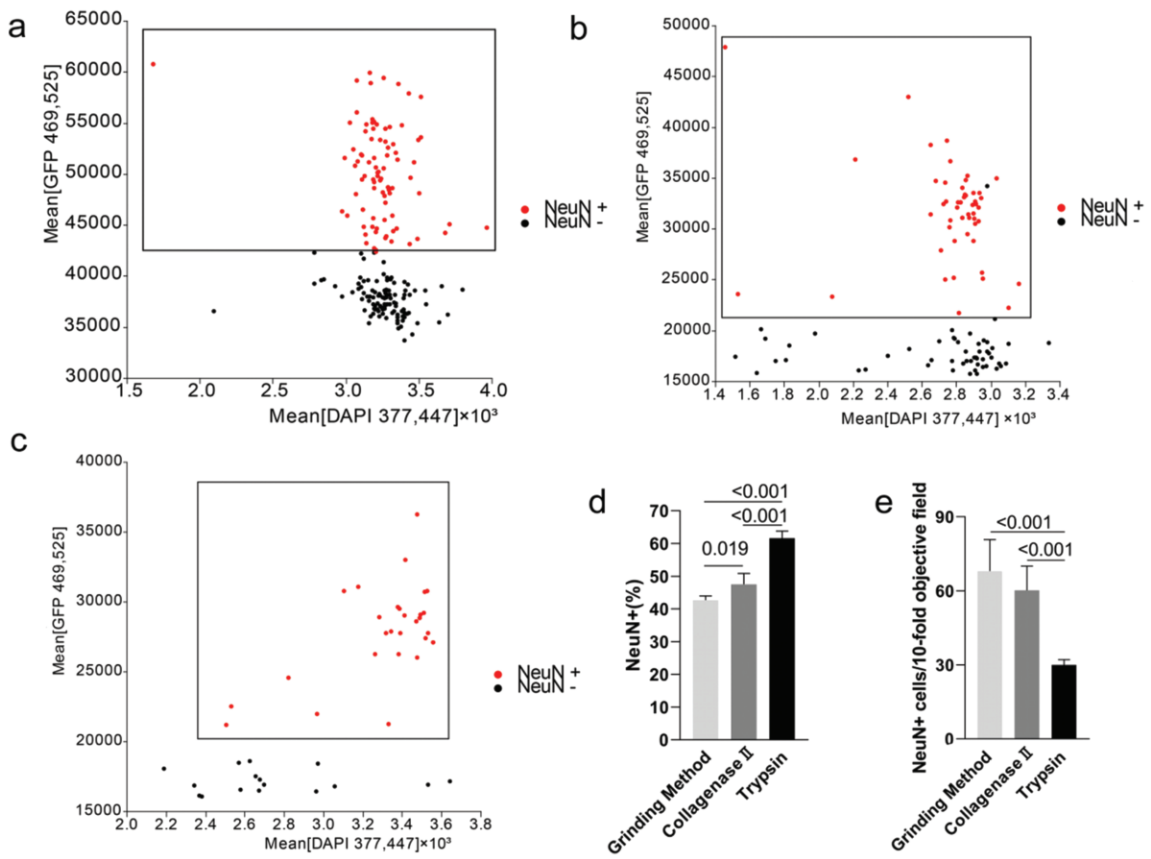

FIGURE 3. Neuronal cell acquisition rate after treatment of the rat cerebral cortex by three different methods.
(a, grinding method; b, collagenase II method; c, trypsin method; d, neuronal cell acquisition rate; e, the average number of NeuN$^+$ cells per field under a 10× microscope).

choose trypsin when culturing neuronal cells. However, in this study, we found that the number of neuronal cells obtained by the trypsin method was relatively small. For subsequent experiments requiring a large number of neuronal cells, the trypsin method may not be suitable for obtaining neurons from a rat cerebral cortex.

Collagenase is a product isolated and purified from clostridium histolytica. It is mainly used for tissue and cell separation because it can degrade natural collagen and reticular fibers and because it can hydrolyze connective tissue (Seifter *et al.*, 1959). In this study, the number of single cells obtained by the collagenase method was lower than that of the grinding method and was higher than that of the trypsin method. The acquisition rat of neuronal cells was higher than the grinding method and was less than trypsin. Some researchers choose the collagenase method when culturing the neuronal cells (Brun and Akbarali, 2018; Katzenell *et al.*, 2017; Munst *et al.*, 2018), and because collagenase can degrade collagen, reticular fibers hydrolyze connective tissue. Thus, the number of single cells obtained by the collagenase method was more than that of the trypsin method. The grinding method can obtain single cells to the greatest extent by controlling a certain degree of strength to ensure cell integrity, and the number of single cells obtained by the grinding method was more than that by the collagenase method and the trypsin method. At the same time, trypsin and collagenase were greatly affected by temperature, pH, and incubation time, while the mechanical method was less affected by this condition.

Therefore, we found that the grinding method can obtain more neuronal cells from the adult rat cerebral cortex to a greater extent than the collagenase method and the trypsin method so as to avoid unnecessary waste of rare sample resources.

Discussion

The culture of adult rat neuronal cells is increasingly used in neuropharmacology and neurodegenerative diseases (Brewer *et al.*, 2006; Parihar and Brewer, 2007), and different isolation methods often lead to different results. In this study, the neuronal cells of a rat cerebral cortex were isolated by the grinding method, the collagenase method, and the trypsin method. After two hours, it was found that the number of single cells obtained by the grinding method was higher than that of the trypsin method ($p < 0.001$) and the collagenase method ($p < 0.001$) under the microscope, which was also confirmed by immunofluorescence staining. Besides, the state of cells adherent isolated by the grinding method was better than the enzymatic method under optical microscopy. Studies have shown that neurons, astrocytes, and microglia can be simultaneously isolated from fresh brain tissue (Martin *et al.*, 2017; Smith *et al.*, 2014).Therefore, the grinding method can fully grind the tissue under the condition of guaranteeing cell viability, and it can remove large impurity debris through 48-micron stainless steel mesh filtration to obtain as many single cells as possible.

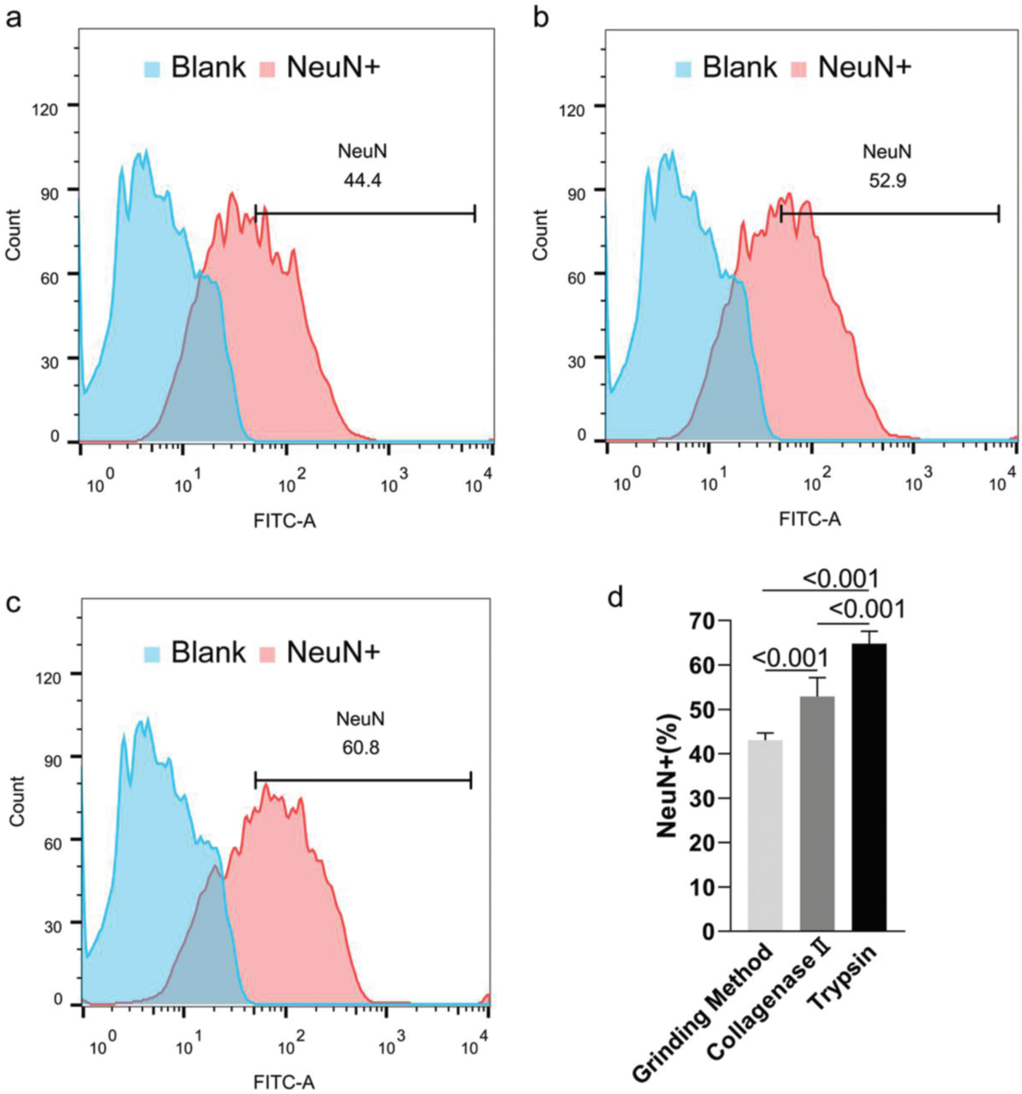

FIGURE 4. Achievement rate of $NeuN^+$ cells by three methods of flow cytometry. (a, grinding method; b, collagenase II method; c, trypsin method; d, $NeuN^+$ cell positive rate).

Trypsin is a serine protease extracted from the pancreas of pigs, cattle, and sheep. It acts as a digestive enzyme in mammals, fish, and some bacteria (Rajabi *et al.*, 2019), and it can cut off the ends of carboxyl, lysine, and arginine residues in the polypeptide chain. Trypsin also has strong specificity. In this study, the rate of rat cerebral cortical neuronal cells obtained by the trypsin method was higher than that of the grinding method ($p < 0.001$) and the collagenase method ($p < 0.001$) through flow cytometry and immunofluorescence staining, which may have been caused by the specificity of trypsin.

At the same time, studies have shown that trypsin does not damage neuronal cell surface receptors (Azari *et al.*, 2010), so this may also be the reason why most researchers choose trypsin when culturing neuronal cells. However, in this study, we found that the number of neuronal cells obtained by the trypsin method was relatively small. For subsequent experiments requiring a large number of neuronal cells, the trypsin method may not be suitable for obtaining neurons from a rat cerebral cortex.

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Therefore, we found that the grinding method can obtain more neuronal cells from the adult rat cerebral cortex to a greater extent than the collagenase method and the trypsin method so as to avoid unnecessary waste of rare sample resources.

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Statement of Ethics: Our studies was conducted ethically in accordance with the World Medical Association Declaration of Helsinki, and have been approved by the Ethics Committee of the Qinghai university affiliated hospital on animal research.

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