Isolation, culture, and characterization of duck primary neurons

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ABSTRACT The duck is a representative and good model for studying the development and physiological mechanisms of the nervous system (**NS**) in waterfowl. Neurons are the basic structural and functional units of NS, but there is no detailed method for cultured duck neurons in vitro. An efficient and simple method for duck neuron culture is reported in this study. First, the sfigpecific markers (NSE and GFAP, respectively) were used to explore the timing of the development of neurons and astrocytes during the duck embryonic stage (E5−E18). The cytomorphology of tissues and cells was tracked with the microscope at different time points. The brain tissues from 10-day-old duck embryos were determined as the optimal sampling embryo age for neuron culture. Then, the brain tissue isolation method (papain digestion) and cell suspension inoculation density (7×10^5 cells/mL) were identified as the culture protocol to obtain target cells with high viability and high density. The purity of the cultured neurons was more than 95%. This experiment provides a supplement for the study of in vitro culture of waterfowl neurons and lays a good foundation for various subsequent studies.

Key words: duck, neuron, embryo, primary culture

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INTRODUCTION

The brain is a relatively complex organ. Different kinds of neurons, glial cells, and their fibers in the brain form a complicated network, dominating the normal operation of the body. The neuron is a helpful model in vitro for the research of neurogenesis, synapse formation, and neural circuit formation (Chen et al., 2013; Patel et al., 2019). Great emphasis has been placed on avian neurological diseases nowadays, but the lack of suitable infection models in vitro limits the in-depth study of related pathological mechanisms. Since primary neurons are directly isolated from the brain and cultured with the primary characteristics of the tissue, they can better reflect the real state of the interaction between the brain and pathogenic microorganisms (Giordano and Costa, 2011). What's more, compared to utilizing intact brain tissue for experiments, culturing primary neurons in vitro may ensure the controllability of conditions and relatively good operability during experiments, reducing the influence of cells unwanted and other nonessential factors (Roppongi et al., 2017). Therefore, establishing a method for culturing primary avian neurons is beneficial to further studying the avian nervous system (**NS**) disease in vitro.

The culture of neurons is particularly challenging and different from other types of somatic cells since mature neurons don't undergo cell division in vitro. Compared with mammals, the embryonic development of poultry is not strongly dependent on maternal support, which is conducive to scientific research. The methods of dissociating primary neurons from mice have been well studied in the past, but the contamination of glial cells is often a hindrance to culturing primary neurons in vitro (Beaudoin et al., 2012; Lu et al., 2016). At present, the most widely studied and used avian biological model is the chicken embryo (Heidemann et al., 2003; Kuang et al., 2015; Kumar and Mallick, 2016). Although the isolation methods of primary neurons from the chicken embryo are supposed to have important reference values for the application of duck embryos, there are many differences in the developmental characteristics of terrestores (represented by the chicken) and waterfowl (represented by the duck). For example, the embryonic period of chicken is about 20 d, and that of duck is about 28 d.

Both neurons and glial cells are derived from primary neuroepithelial cells (**NE**), and the timing of their occurrence is significantly different during embryonic development (Merkle and Alvarez-Buylla, 2006). Exploring the order of occurrence and maturation of neurons and glial

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cells is a prerequisite for determining the time to isolate primary neurons and reducing cell contamination. An optimal tissue digestion approach and moderate cell density may also be the key factors for getting pure primary neurons successfully. Here, we investigated the effects of different factors on primary duck brain neurons and tried to establish an effective model for the study of the avian NS in vitro based on the observation of the brain developing order at the embryonic period.

MATERIALS AND METHODS

Duck Eggs

Cherry valley duck eggs were purchased from Weishan Lake Farm (China). After being sterilized with 75% ethanol, eggs were incubated at 37°C, 75% humidity in an egg incubator (Keyu, China). Duck embryonic brain tissues isolated from embryonic d 5 (E5), E7, E10, E12, E18, and E21 ducklings were fixed in 4% paraformaldehyde for morphology analysis. In this study, 30 duck eggs were used for histological studies during duck embryo development, 50 eggs were used for the experiments in which duck neurons were isolated.

Reagents

DMEM/F12 (Gibco, Carlsbad, CA), Neurobasal medium (Gibco, Carlsbad, CA), Fetal bovine serum (Gibco, Carlsbad, CA), B-27 supplement (Gibco, Carlsbad, CA), Papain (Solarbio, China), Poly-D-lysine hydrobromide (Sigma-Aldrich, St. Louis, MO), Trypsin (Gibco, Carlsbad, CA), Penicillin (Gibco, Carlsbad, CA) were prepared for the cell isolation and culture. Mouse Monoclonal NSE Antibody (Proteintech, Rosemont, IL), Mouse Anti-Glial fibrillary acidic protein (**GFAP**) Antibody (Proteintech, Rosemont, IL), Alexa Fluor 488-AffiniPure goat anti-mouse IgG (H+L) (Abcam, UK), DAPI (Abcam, UK) were purchased for the cell identification.

Preparation for the Neuron Culture

Neurons are generally not easy to attach to the hydrophobic surface of glass or plastic. Therefore, changing the surface conditions of cell culture plates and cell slides with Poly-D-lysine is beneficial to the growth of neurons.

a) Six-well plates and 18 mm circular coverslips were used in the present study. The coverslips were left in 2 M HCl for at least 2 days before use to remove acid-soluble impurities that may be harmful to cell growth.
b) The coverslips were rinsed thoroughly with distilled water (dH_2O) several times, sterilized at 121°C for an hour in an autoclave, and then dried in an oven.
c) Fifty μg/mL poly-D-lysine was added to 6-well plates and Petri dishes containing coverslips and placed in a CO_2 constant-temperature incubator for 30 min.
d) Complete removal of poly-D-lysine by using sterilized PBS washing. The treated 6-well plates were dried naturally in a sterile environment, but the coverslips should be pretreated with the medium in a CO_2 incubator to prevent drying.

HE Staining and Immunofluorescence

Duckling brain tissues were fixed in 4% paraformaldehyde overnight and then processed for paraffin embedding and a conventional paraffin section with a thickness of 9 μm. The sections were then stained with hematoxylin and counter-stained with eosin (**HE**). Staining results were observed under a light microscope (Olympus; Tokyo, Japan).

The duckling brain paraffin sections were deparaffinized and dehydrated in xylene and ethanol respectively. They were then blocked with a 3% H_2O_2 solution, and heated in boiling sodium citrate buffer for 5 min. The Anti-NSE antibody and anti-GFAP antibody were used as primary antibodies. The next day, after being washed with PBS thoroughly, the samples were incubated with the corresponding secondary antibody. Images were captured using a fluorescence microscope (Olympus DP73, Japan).

The primary neurons fixed with 4% paraformaldehyde were soaked in PBS and washed mildly 3 times. Cells were sequentially permeabilized with 1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin (**BSA**) for 30 min. They were then incubated with primary antibody (NSE antibody or anti-GFAP antibody) in a humified chamber at 4°C overnight. Alexa Fluor 488-AffiniPure goat anti-mouse IgG (H+L) and DAPI were used the next day. Images were captured using a laser scanning confocal microscope (LSCM; Carl Zeiss, Oberkochen, Germany).

Embryo Dissection and Isolation of Neurons

Based on the result of HE and immunofluorescence, the fertilized eggs at E10 were selected to isolate the neurons. First of all, mark the outline of the air sac and sterilize the surface of the duck eggs with 75% ethanol thoroughly. Keep the position of the air sac facing upward. After breaking eggs along the outline of the air sac, duck embryos were transferred carefully to a clean glass petri dish filled with cold DMEM/F12 (Figure 1). Then, separate the brain and body, and carefully remove the brain. Since the meninges are rich in fibroblasts, they must be completely stripped to prevent contamination by other unwanted cells. The intact brains were collected in a clean petri dish and subjected to chopping into pieces by small sterilized scissors. All the steps mentioned must be operated upon the ice. The highly active single-cell suspension is a crucial factor in the culture process, and the cell activity largely depends on its digestion mode. Therefore, we have tested the effects of 3 digestion methods.

Scheme 1: 2 mg/mL papain for 30 to 40 min.
Scheme 2: 0.25% trypsin for 10 min.
Scheme 3: mixture of 0.25% trypsin and 0.2 mg/mL collagenase for 15 min.

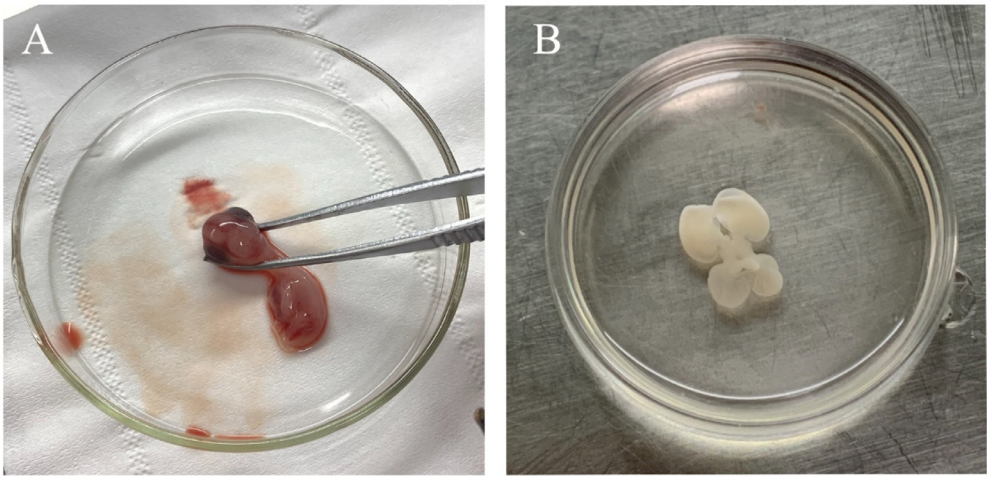

Figure 1. The isolation of duck embryo brain. (A) The body of a duck at embryonic day 10; (B) brain tissue after dissection of the meninges.

The Petri dishes filled with chopped brain tissues and enzymes were incubated in a 37°C CO_2 incubation for better digestion and shaken gently every 5 min. After digestion, DMEM/F12 containing 10% FBS was added slowly to terminate the digestion. The mixture was filtered through a 200 mesh stainless steel filter into a 15-mL centrifuge tube, and this process was repeated twice to remove most of the cellular debris. The digested tissue was centrifuged at 1,500 r/min for 10 min and resuspended in a fresh DMEM/F12 medium containing 10% FBS, 1% penicillin and streptomycin, and 1% B-27 supplement. The density of neurons is also one of the important factors to determine the growth state of neurons. Thus, the resuspended neurons were cultured in different concentrations (1×10^5 to 1×10^7 cells per milliliter). The sufficient cell suspension was transferred to 6-well plates and 12-well plates containing poly-D-lysine overlay. After incubation for 4 h in vitro (**HIV**), the DMEM/F12 medium was replaced with a fresh serum-free neurobasal medium containing 1% B-27 supplement, penicillin-streptomycin, and glutamine. Neurons were continued to be cultured in 37°C incubation until 9 d in vitro (**DIV**) and then collected to detect the purity by immunofluorescence. When the neuron purity reaches about 95%, follow-up experiments can be performed.

RESULTS

Morphological Changes of the Duck Brain During Embryo Development

The morphological analysis of duckling embryo mesencephalon and cerebrum at E5, E7, E10, E12, and E21 was shown in Figures 2 and 3. At E5 and E7, neurons were widely distributed in the brain, whereas the connections between cells were not tight, and the overall structure of brain tissue was loose. With the increase of embryo age, it was found that the number and thickness of layers in the mesencephalon at duck E10 became obvious and were relatively well developed. Early neurons migrated rapidly from E10, forming a multilayered initial structure. During the later period of brain development, glial cells began to develop and distributed between layers of neurons along with neurites. The precise distribution of glial cells, neurons, and neurites constituted the sophisticated structure of duck's mesencephalon. At E21, neurons tended to mature and had larger cell bodies. The enlarged intercellular space was filled with more neurites between cells, and the mesencephalon parenchyma structure was fully developed. The cerebrum showed similar trends to the mesencephalon (Figure 3).

Expression of NSE and GFAP During Duck Embryo Brain Development

To investigate the isolation time of primary neurons separated from duck embryos, it is necessary to study the structure and constitution of duck embryo brain tissues at different embryonic days. Immunohistochemical was used to identify the occurrence and distribution of neurons and glial cells. Mature neurons expressing large amounts of NSE could be seen at both E10 and E12 (Figures 4B and 4C). An important consideration in the isolation of primary cultures is the purity of the cell. NSE, is a kind of brain-specific glycolytic enzyme, the expression level of which gradually increases with the development and maturation of brain neurons (Marangos et al., 1980). However, as an important marker of astrocyte maturation, from E5 to E15, the GFAP could be detected from E12 under the fluorescence microscope (Figures 4G and 4H), suggesting that gliogenesis began during the embryonic age of 10 d to 12 d in the duckling brain. These results complied with the finding that neurons develop earlier than glial cells in avians (Powell et al., 2014). Therefore, duck embryos of E10 can be selected for isolation and extraction of primary neurons.

Primary Ducking Brain Neurons Culture

Making highly active single-cell suspension is a crucial step in the culture process of neurons. Enzymatic digestion is usually used to isolate tissue, so we incubate brain

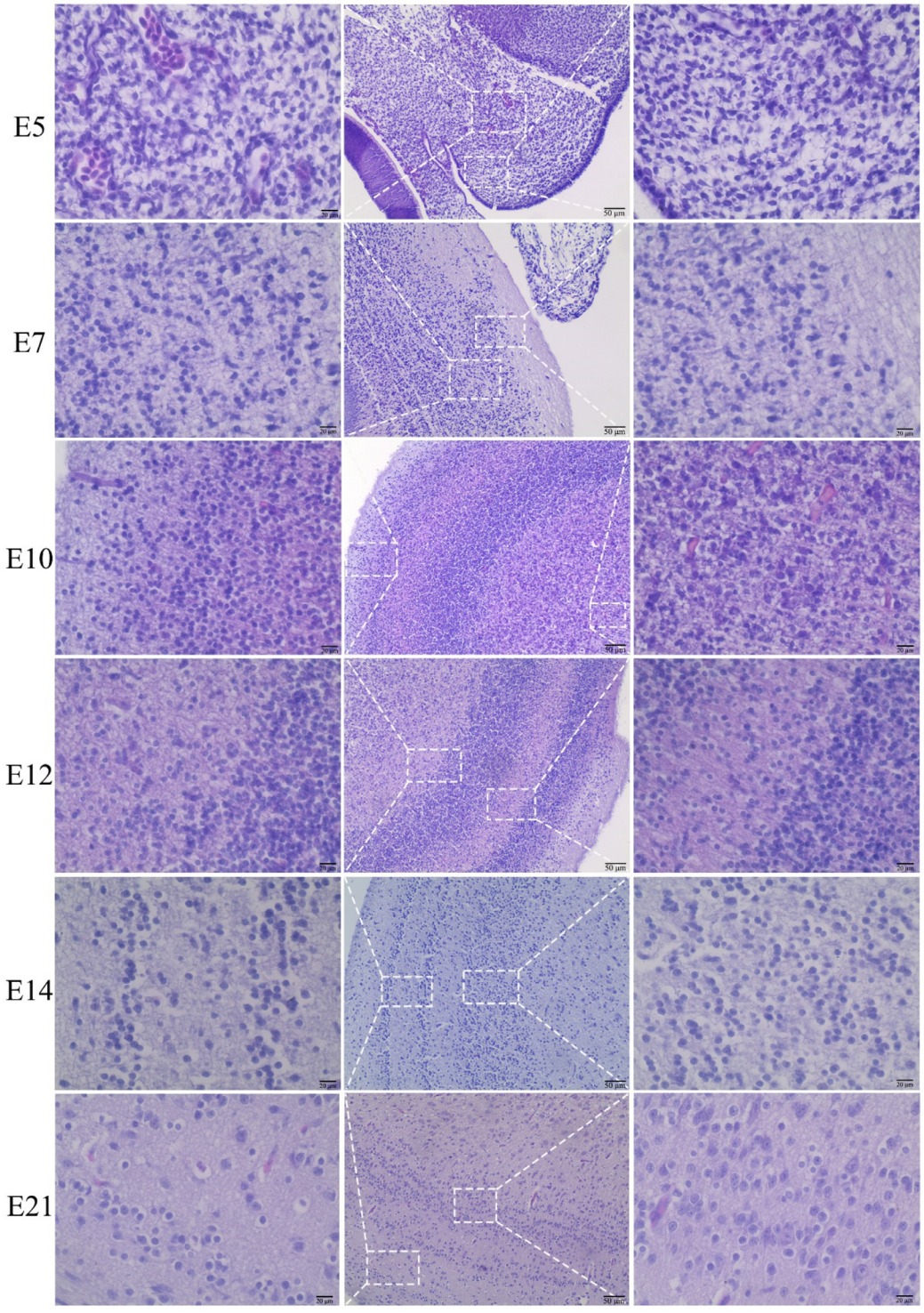

Figure 2. Morphological results of the cerebrum during duck embryonic development (HE). E5, E7, E10, E12, E14, and E21 mean the ages of duckling embryos are 5, 7, 10, 12,14, and 21 days, respectively.

tissue through different digestive enzymes to obtain single-cell suspension with high activity. Different enzymes have different digestive abilities and action sites, so it is essential to screen digestive enzymes according to the characteristics of tissues. Trypsin mainly breaks down the protein components of tissue interstitium and has a strong digestive effect. When digesting cells, if the time is too long, the cell membrane proteins may be further digested and the cells are damaged. Collagenases can specifically digest collagen fibers in the intercellular matrix, and their side effect is alleviated. Papain is a proteolytic enzyme isolated from Carieapapaya that can decompress extracellular matrix. In this experiment, we showed that the three protocols of trypsin, trypsin-collagenase, and papain were able to isolate neurons. Papain was the enzyme with the highest activity of neurons in the above three schemes, and the activity of neurons was not strong and the growth was slow after trypsin digestion. The digestion ability of pancreatic enzymes is strong, and their digestion time has to be controlled to

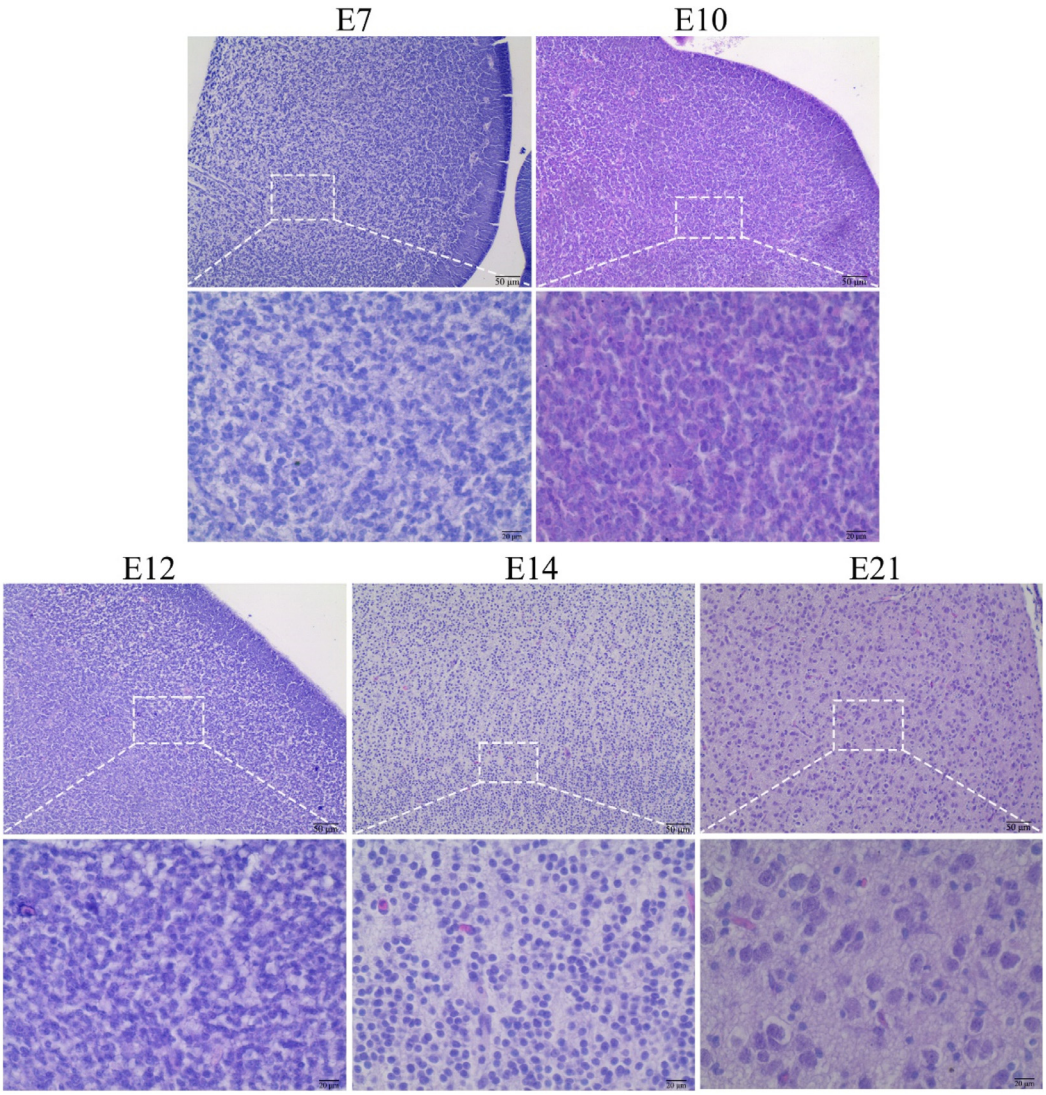

Figure 3. Morphological results of the cerebrum during duck embryonic development (HE). E7, E10, E12, E14, and E21 mean the ages of duckling embryos are 7, 10, 12,14, and 21 days, respectively.

avoid over-digestion (Figure 5A). After inoculation of the single cell suspension digested with trypsin, the cells gathered into spherical growth (Figure 5B) and gradually shed and died with the extension of culture time (Figure 5C). In addition, papain generally has a mild effect without excessive digestion, and its digestion time can be up to 30 min, which is conducive to experimental operation. Based on the comparison of experimental operation simplicity and neuronal viability, Papain's protocol is recommended. Thus, in this study, we chose the digestion method of papain enzyme to observe the in vitro growth of neuron growth.

Unlike other cells, neurons are a type of highly differentiated terminal cells, hence the number of cells available for experiments is much more limited. To evaluate the contribution of cell density to the growth of neurons, we next compared the neuronal growth after inoculation of low to high-density ($1 \times 10^5 \sim 1 \times 10^7$ cells per milliliters) cell suspensions into the culture plates. In low-density cases (1×10^5 and 7×10^5 cells per milliliters), almost no multicellular aggregations and far fewer neurite fasciculations were exhibited even on the sixth day after seeding (Figures 6A and 6B). However, compared with the 7×10^5 to 1×10^5 case, the cells were evenly plated, the intercellular connections were frequent, and the neuron cell body had a stronger refractive index. In the case of 1.5×10^6 cells per milliliters, multicellular aggregations could be observed and developed neurites to form complicated nets. Excessive neurites may join together to become neurite fasciculations (Figure 6C). In the first 2 d, neurons with high density (1×10^7 cells per milliliters, Figure 6D) began to migrate together to form multicellular aggregations (also known as soma aggregations).

Immunofluorescence Characterization of Primary Neurons

Nine DIV neurons were immunostained with anti-NSE antibody and anti-GFAP antibody respectively. Results showed that NSE-positive cells accounted for about 95.3% ± 1% with scarce anti-GFAP-positive cells (Figure 7). The purity of primary neurons was about 95%, which can be used as experimental cell models in vitro.

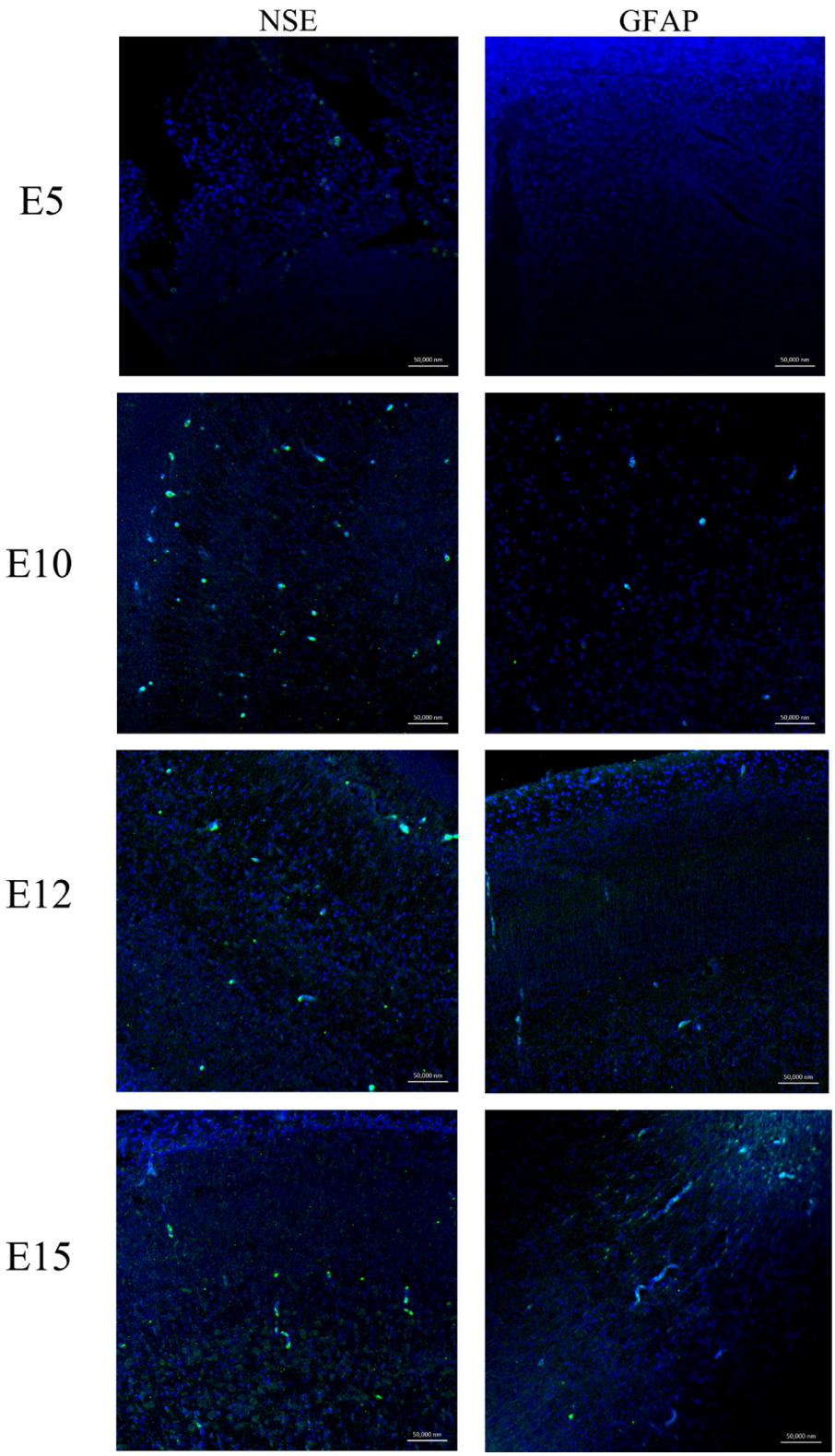

Figure 4. Expression of NSE and GFAP during duck embryo brain development. E5, E10, E12, and E15 mean the ages of duckling embryos are 5, 10, 12, and 15 days, respectively.

Morphological Changes of Neurons During Culture in Vitro

Most neuron cells adhered to the wall 4 h after inoculation, which were spherical. And a few cells had small protrusions, which were tadpole-like (Figures 8A and 8B). After 6 h, obvious neurites were seen. At 24 h, the neuronal cells were completely adherent, whose halo was obvious, and showed a multipolar morphology (Figures 8C and 8D). With the extension of the culture time, the neurites thickened and elongated, and gradually differentiated into dendrites and axons, and cell protrusions are intertwined into a loose mesh structure. After 3 d, the cells were larger and more diverse, mostly

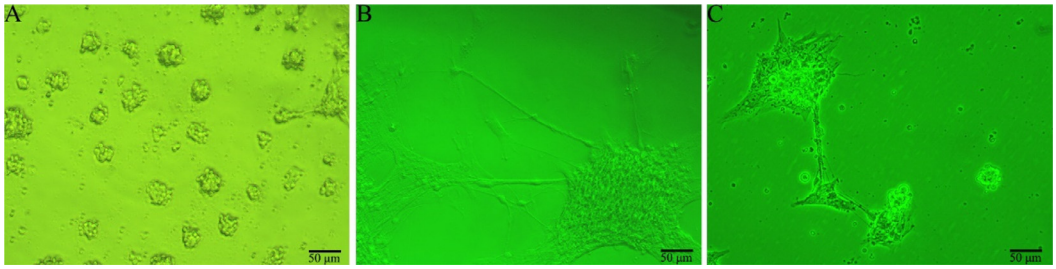

Figure 5. Effects of enzymes on neuron growth. (A) Cells were damaged after over digestion; (B) neurons grew in aggregation; (C) neurons aggregated, gradually fell off and died.

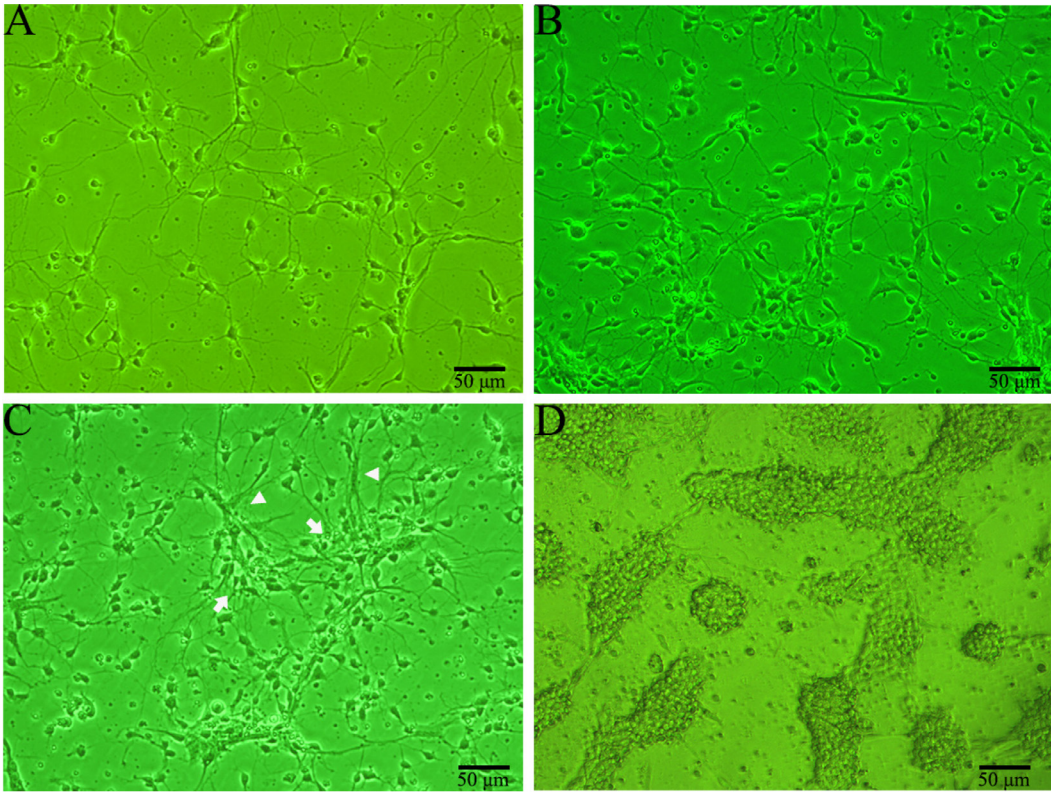

Figure 6. Effects of inoculation density on neuron growth. (A) Inoculated 1×10^5 cells per milliliters cell suspensions at 4 DIV. (B) Inoculated 7×10^5 cells per milliliters cell suspensions at 4 DIV. (C) Inoculated 7×10^5 cells per milliliters cell suspensions at 4 DIV, soma aggregations (arrows) and excessive neurite fasciculations (arrowheads) could be observed. (D) Inoculated 1×10^7 cells per milliliters cell suspensions at 2 DIV, neurons began to clump together in clusters. DIV, days in vitro.

in the shape of spindle and triangle (Figures 8E and 8F). After culturing for 7 d, the neuron cell body further increased and the process lengthened, forming a complex and dense neuron network (Figures 8G and 8H). Combined with the results of Figure 7, it can be preliminarily inferred that duck neurons began to mature and stabilize from the 9th day, which could be used as an experimental model for further experiments.

DISCUSSION

Nerve cell culture is an important technology in the field of neurobiomedical research, including cell function, neurodevelopment, degenerative diseases, neurotoxicology, etc., and the nerve cell has become an important experimental material and model tool in neuroscience research. Various neurological diseases are directly related to avian neurons, but the studies on duck neurons are few. Therefore, we explored a high-purity and high-vitality culture method of duck neurons in this study.

Classically, neurons and glial cells appear at a sequential time during embryonic development. After neurogenesis, NE gradually transforms into radial glial cells, which can form early neurons directly through asymmetric division or intermediate progenitor cells (**IPCs**) division. Gliogenesis generally occurs in the late embryonic development until the eggs hatch. Some late radial glial cells can divide to generate astrocytes or indirectly form oligodendrocytes and astrocytes by generating different subtypes of IPCs (Kriegstein and Alvarez-Buylla, 2009; Yang et al., 2022). Briefly, the differentiation process of neural stem cells into neurons and glial cells has a distinct time-dependent differentiation pattern (Qian et al., 2000), and the fate of embryonic progenitor cells is regulated by certain developmental regulatory signals

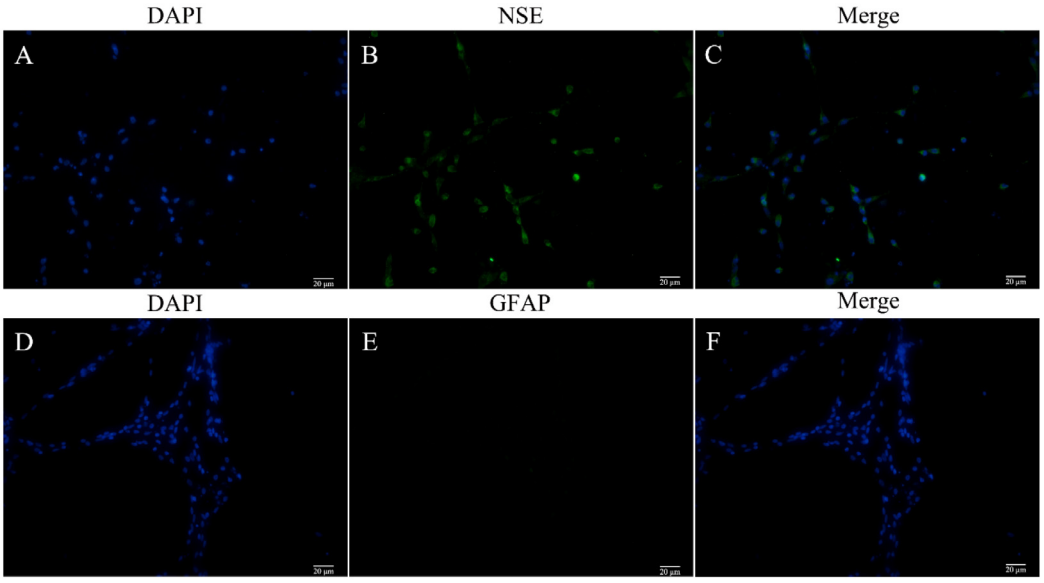

Figure 7. Neurons (9 d after seeding) characterization by immunofluorescence.

in vivo (Morrow et al., 2001; Dinh Duong et al., 2019). In our study, we found that duck neurons were initially widely distributed and loosely connected in the early embryonic phase. As duckling brain tissue matured, neurons released synapses and connected to form a complicated network. At the same time, glial cells began to develop and filled between neurons, providing nutrients and supporting the continued development of neurons. In later stages of development, the overall structure of the brain tissue became compact. Therefore, we speculate that the timing difference between neurogenesis and gliogenesis is favorable for the formation of neural networks.

Currently, many viral and neuronal studies are performed on cell lines. Meanwhile, the drawback of cell lines is that they might have many important physiological differences from the cell types that they originate from. Hence, the use of primary cultures is highly desirable because they are not tumor-derived and hence are more likely to duplicate the characteristics of neurons in vivo. Methods of isolating primary neurons from mice have been well studied in the past (Chen et al., 2011; Jones et al., 2012). Due to the essential differences in brain tissue structure between birds and mammals (Kloos et al., 2019; Stacho et al., 2020), their primary neuron culture methods may also differ significantly. Culturing avian primary neurons in vitro can better reflect the dynamic order of avian brains infected with viruses, so it is required that avian primary neurons are used as infection models to further study the relevant pathogenic mechanisms of avian neurological diseases such as Newcastle Disease Virus (Wang et al., 2019), Marek's disease virus (Gimeno et al., 2001), Tembusu virus (Sun et al., 2014), and Japanese Encephalitis Virus (Di et al., 2020).

Based on the developmental order of neurons and glial cells in the duckling embryo brain, we first successfully cultured the neurons reducing the contamination of glial cells from the source by selecting the appropriate embryonic age for ducklings. There are certain differences in the timing of neurogenesis and gliogenesis in duck embryos and chicks (Lever et al., 2014). The majority of mature neurons but scarce glial cells existed at E10 in duck embryos, suggesting that duck embryos at E10 are favorable materials to isolate and extract primary neurons. Serum-containing culture can promote cell recovery, adaptation to the environment, and stable growth, but serum stimulates glial cell division, which may have adverse effects on subsequent experiments. The neurobasal medium is a serum-free neuronal cell basal medium that facilitates the survival and growth of neuronal cells. In this experiment, a serum-containing culture medium was used to inoculate cells, while a serum-free culture medium was used to maintain cell survival and growth. Combining the advantages of the above is more beneficial to the purity and viability of cultured neurons. In addition, primary neurons may exhibit different activities and morphology when various enzymes and cell densities are included in the experiment. Many protocols recommend 0.25% trypsin for its good digestive efficiency, but it is not easy to control (Xu et al., 2012). In this study, we explored that mechanical digestion combined with 2 mg/mL papain could reduce cell damage and improve digestive efficiency. Besides, the culture density of neurons has a great influence on their growth rate (Kaneko and Sankai, 2014). When cultured at an ultra-low density, neurons tend to die because they have less chance to connect to each other, making it hard to get paracrine trophic support from adjacent neurons and glia (Kaech and Banker, 2006). However, when the cell density is high, with the extension of the culture time, excessive neurite bundle contraction and somatic cell aggregation can be observed (Wang et al., 2010).

In conclusion, we presented a simplified and optimized approach for the preparation of primary neurons isolated from duckling embryo brains without glial cell contamination. 10-days-old duck embryos and the combination of mechanical digestion and 2 mg/mL papain

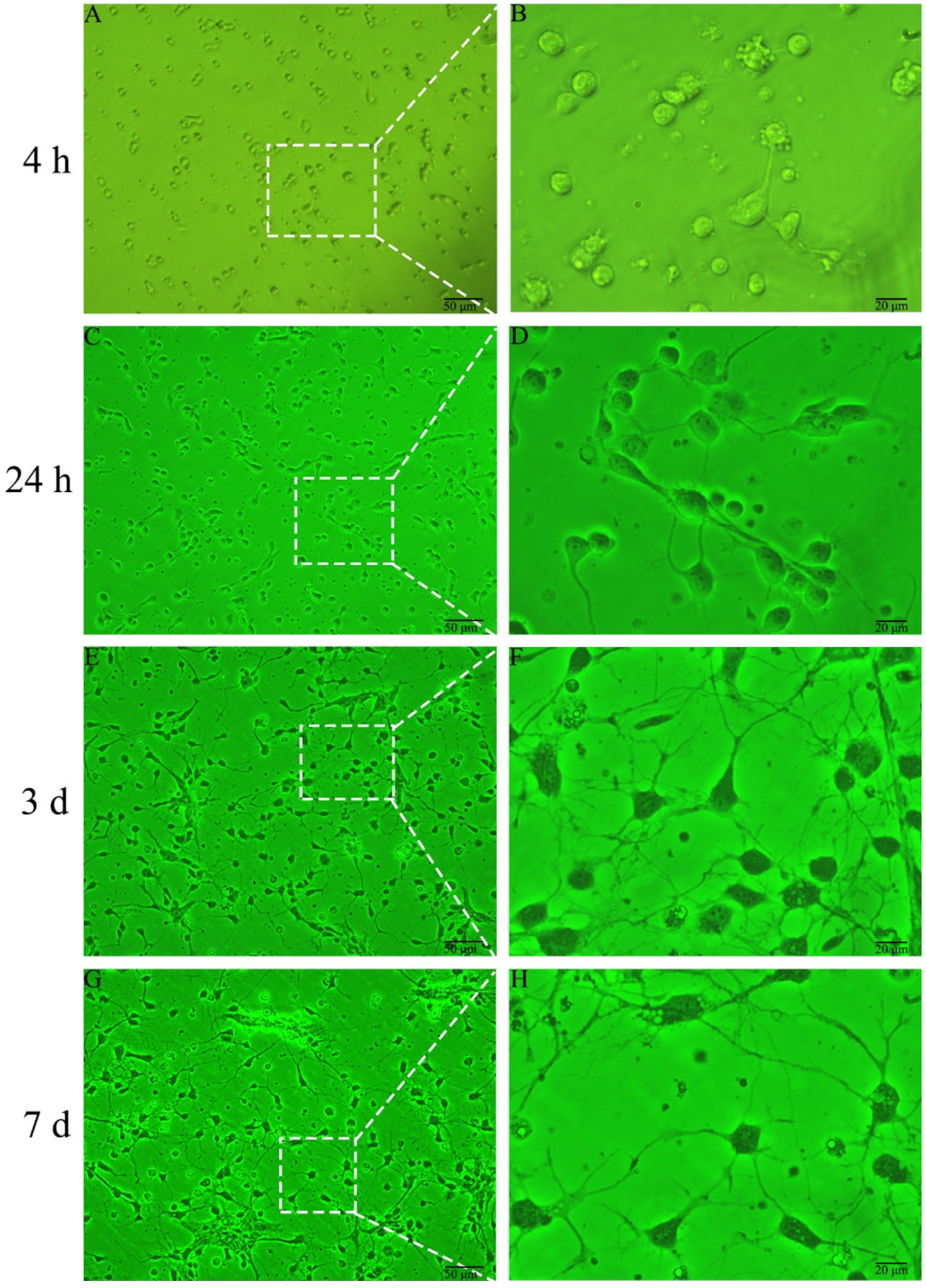

Figure 8. Morphological changes of neurons during culture in vitro.

are preferable to the isolation of primary neurons. Primary neurons may remain better state when cultured at a medium density (approximately 7×10^5 cells per milliliter of cell suspension). This method can provide a useful experimental basis for various applications and research on pure neurons of ducks.

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DISCLOSURES

The authors who have taken part in this study declared that they have no competing interests.

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