Research Paper: Comparison of Rat Primary Midbrain Neurons Cultured in DMEM/F12 and Neurobasal Mediums

Neda Valian¹, Mansooreh Heravi¹, Abolhassan Ahmadiani¹, Leila Dargahi¹*

¹. Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

* Corresponding Author:
Leila Dargahi, PhD.
Address: Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Tel: +98 (21) 22429768-9
E-mail: l.dargahi@sbmu.ac.ir

ABSTRACT

Introduction: Midbrain dopaminergic neurons are involved in various brain functions, including motor behavior, reinforcement, motivation, learning, and cognition. Primary dopaminergic neurons and also several lines of these cells are extensively used in cell culture studies. Primary dopaminergic neurons prepared from rodents have been cultured in both DMEM/F12 and neurobasal mediums in several studies. However, there is no document reporting the comparison of these two mediums. So in this study, we evaluated the neurons and astroglial cells in primary midbrain neurons from rat embryos cultured in DMEM/F12 and neurobasal mediums.

Methods: Primary mesencephalon cells were prepared from the E14.5 rat embryo. Then they were seeded in two different mediums (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 [DMEM/F12] and neurobasal). On day 3 and day 5, half of the medium was replaced with a fresh medium. On day 7, β3-tubulin-, GFAP (Glial fibrillary acidic protein)- and Tyrosine Hydroxylase TH-positive cells were characterized as neurons, astrocytes, and dopaminergic neurons, respectively, using immunohistochemistry. Furthermore, the morphology of the cells in both mediums was observed under light microscopy on days 1, 3, and 5.

Results: The cells cultured in both mediums were similar under light microscopy regarding the cell number, but in a neurobasal medium, the cells have aggregated and formed clustering structures. Although GFAP-immunoreactive cells were lower in neurobasal compared to DMEM/F12, the number of β3-tubulin- and TH-positive cells in both cultures was the same.

Conclusion: This study’s findings demonstrated that primary midbrain cells from the E14.5 rat embryo could grow in both DMEM/F12 and neurobasal mediums. Therefore, considering the high price of a neurobasal medium, it can be replaced with DMEM/F12 for culturing primary dopaminergic neurons.
1. Introduction

In vitro studies play an essential role in understanding the biological processes in a more isolated context (Giordano & Costa, 2011). It offers a controlled environment to test specific cellular and molecular hypotheses with a less experimental variation of in vivo models (Polikov et al., 2008). Neuronal cell lines derived from rodents and primary neuronal cultures are widely used to study the physiological properties of neurons and the potential neurotoxicity of chemicals (Giordano & Costa, 2011).

Midbrain dopaminergic neurons (mesostrial, mesolimbic, and mesocortical pathways) are involved in various brain functions, including motor behavior, reinforcement, motivation, learning, and cognition (Iversen & Iversen, 2007). Therefore, understanding the extra- and intracellular signaling events that increase the development or survival of these neurons may improve the potential of therapies for dopamine-related disorders like Parkinson Disease (PD) (Orme, Bhangal, & Fricker, 2013).

Several cell lines of dopaminergic neurons (mesostrial, mesolimbic, and mesocortical pathways) include human neuroblastoma (SH-SY5Y) (Deloncle et al., 2017; Wongprayoon & Govitrapong, 2017; Yeo et al., 2018), immortalized human dopaminergic precursor (Lund Human Mesencephalon; LUHMES) (Höllerhage et al., 2017; Zhang, Yin, & Zhang, 2014), and PC12 (Li, Wang, Lan, Yue, & Liu, 2011) cell lines are used in cell culture studies. However, these cell lines may have genetic instability due to high passage numbers. Besides, neurites may not represent true axons or dendrites, and then, cell-cell interactions will be missed (Harry et al., 1998). So, primary neurons prepared directly from fresh brain tissues can be a reliable model in which neurons acquire a neuronal phenotype and differentiation and ultimately die (Giordano & Costa, 2011). Primary dopaminergic neurons derived from the rodent embryonic central nervous system are among the most relevant models to challenge dopaminergic neurons against various stresses and neurotoxins for evaluating the neuroprotective compounds to prevent neuronal degeneration (Gaven, Marin, & Claeyisen, 2014).

Different culture mediums such as DMEM/F12 and neurobasal are used for culturing neuronal cell lines and primary cultures (Ciron, Lengacher, Dusonchet, Aeberscher, & Schneider, 2012; Munee, Alikunju, Szlachetka, & Haorah, 2011). Neurobasal medium is used extensively for primary hippocampal (Beaudoin et al., 2012; Henderson, Peng, Trojanowski, & Lee, 2018), cortical (Cui, Deng, Zhang, Yin, & Liu, 2018; Tan et al., 2017), cerebellar (Gustafsson, Katsioudi, Issazadeh-Navikas, & Huang, & Li, 2018; Li et al., 2017), immortalized human dopaminergic precursor (Lund Human Mesencephalon; LUHMES) (Höllerhage et al., 2017; Zhang, Yin, & Zhang, 2014), and PC12 (Li, Wang, Lan, Yue, & Liu, 2011) cell lines are used in cell culture studies.
Kornum, 2016), and striatal (Nguyen, Rymar, & Sadikot, 2016) neurons culturing. In the case of primary dopaminergic neurons, in vitro studies have used both neurobasal (Bayer Andersen, Leander Johansen, Hentzer, Smith, & Dietz 2016; Collo et al., 2013; Orme et al., 2013) and DMEM/F12 (Choi, Kim, & Xia, 2013; Choi, Kruse, Palmeter, & Xia, 2008; Collins et al., 2016) medium. Since there is no study documenting the difference of these mediums in culturing primary dopaminergic neurons, this study was designed to compare primary midbrain cells in DMEM/F12 and neurobasal cultures.

2. Methods

2.1. Animals

Adult female and male Wistar rats, weighing 220-250 g, from our breeding colony, were used in this study. The animals were maintained and handled in compliance with the institutional guidelines approved by the Ethics Committee for animal research of the Shahid Beheshti University of Medical Sciences. Male and female rats were housed in the cage for 12 hours, and then the males were removed. Fourteen days later, the female rats were shortly anesthetized using CO2, and the pregnant rats were characterized by touching the belly. Then, they were used for the preparation of embryonic mesencephalon cells from the E14.5 embryo.

2.2. Preparation of primary mesencephalon cells

Primary mesencephalon cells were prepared as reported previously (Choi et al., 2013). The pregnant females were dammed by CO2 inhalation, and after euthanizing by cervical dislocation, the embryos were taken out from the uterus and amniotic sac. The extracted embryos were washed in Ca2+- and Mg2+-free Hank’s Balanced Salt solution (HBSS; Sigma Aldrich, USA), from our breeding colony, were used in this study. The pregnant females were dammed by CO2 inhalation, and after euthanizing by cervical dislocation, the embryos were taken out from the uterus and amniotic sac. The extracted embryos were washed in Ca2+- and Mg2+-free Hank’s Balanced Salt solution (HBSS; Sigma Aldrich, USA), and Mg2+-free Hank’s Balanced Salt solution (HBSS; Sigma Aldrich, USA), the tissues were incubated with 0.05% trypsin solution (Gibco, USA) for 20 minutes at 37°C. By transferring pieces of tissue to HBSS, including 10% fetal bovine serum (FBS; Gibco, USA), the tissues were incubated with 0.05% trypsin solution (Gibco, USA) for 20 minutes at 37°C. By transferring pieces of tissue to HBSS, including 10% fetal bovine serum (FBS; Gibco, USA), trypsin was deactivated. The tissues were gently triturated in DMEM/F12 medium (Gibco, USA) with a fire-polished glass pipette 7 times to dissociate into single cells. Viable cells were counted using the hemocytometer method after diluting with trypan blue solution (1:10). The dissociated cells were plated in 0.01% poly-l-lysine (Sigma Aldrich, USA) coated plates at density of 4×105 cells/well in 6-well plates in DMEM/F12 medium (containing 10% FBS, 1% glutamine [Gibco, USA] and 1% penicillin/streptomycin [Gibco, USA]) (Choi et al., 2013) or neurobasal (containing 1% B27 [Gibco, USA], 1% glutamine and 1% penicillin/streptomycin) (Bayer Andersen et al., 2016). For cells cultured in DMEM/F12 medium, half of the medium was replaced with fresh medium on Day in Vitro (DIV) 3. Then, the cultures were replenished by replacing half of the medium with serum-free DMEM/F12 containing 1% B27 (Gibco, USA) on DIV5. In the case of cells cultured in neurobasal, on DIV3, half of the medium was also replaced by a fresh medium containing Arabinose C (AraC) (3 μM) to prevent glial cell proliferation. On DIV5, half of the medium was renewed by a fresh neurobasal medium. Seven days after culturing, i.e. the time required for dopaminergic neurons stabilization and maturation, immunocytochemistry assay was performed. On DIV1, DIV3, and DIV5 of culturing, the cells were evaluated morphologically under light microscopy.

2.3. Immunocytochemistry

Seven days after culturing, immunocytochemistry was performed to visualize neuronal and glial cells (Collins et al., 2016). Cells were washed twice in PBS and then fixed in 4% paraformaldehyde for 12 min at room temperature. After three washes with TPBS (0.05% Tween 20 in PBS), cell permeabilization was performed using 0.02% Triton x-100 in PBS for 15 min. The cells were incubated with 1% Bovine Serum Albumin (BSA; Merck, Germany) in TPBS for 1 h at room temperature to block nonspecific antibody binding-sites. Primary antibody incubation was done with anti-β3-tubulin antibody (1:1000; ab18207, Abcam, USA), anti-Tyrosine Hydroxylase (TH) antibody (1:500; ab112, Abcam, USA) and anti-Glial Fibrillary Acidic Protein (GFAP) antibody (1:1000; ab7260, Abcam, USA) overnight at 4°C. Secondary antibody (1:150; anti-rabbit IgG FITC conjugated, cell signaling, USA) was added for 1 h after three washing, followed by nuclear staining with DAPI (0.4 µg/mL in PBS) just before visualization. Immunoreactive cells were observed at ×10 and ×20 magnifications under an Olympus microscope. The immunostaining assay was repeated 3 times for DMEM/F12 and neurobasal mediums.

3. Results

3.1. Morphology of primary midbrain cells in DMEM/F12 and neurobasal

Morphology of primary midbrain cells 1, 3, and 5 days after culturing in DMEM/F12 and neurobasal mediums were evaluated using light microscopy at ×10 (Figure 1A) and ×20 (Figure 1B) magnifications. As shown in
Figure 1. Morphology of primary midbrain cells obtained from E14.5 of rat embryo 1, 3, and 5 days after culturing in DMEM/F12 and neurobasal mediums.

A: ×10; and B: ×20 (b) magnifications.

Figure 1, on day 1, after culturing, the cells were almost spherical with very short processes in both mediums. On DIV3 and DIV5, the formation and branching of axons and dendrites were clearly visible, and synaptic communications were formed completely in both cultures. Our morphological findings showed that although midbrain cells grew well in both cultures and reached the final morphology, in the neurobasal medium, the cells were clustered and formed sun-like structures. It seems that mesencephalon cells have better morphology in the DMEM/F12 medium.

3.2. Beta3-tubulin-positive cells in DMEM/F12 and neurobasal mediums

Seven days after culturing, β3-tubulin-positive cells were characterized as neurons in DMEM/F12 (Figure 2A-C) and neurobasal (Figure 2D-F) using immunocy-
tochemistry. Beta3-tubulin-positive cells (Figure 2A, D) and the nucleus of glial and neuronal cells (Figure 2B, E) were visualized using β3-tubulin antibody and DAPI, respectively. Beta3-tubulin-positive cells and DAPI-stained nucleus were merged to represent the cells better (Figure 2C, F). There was no difference between the number of immunostained neurons in DMEM/F12 and neurobasal cultures. However, the cells in the neurobasal medium were highly clustered.

3.3. GFAP-Positive neurons in DMEM/F12 and neurobasal mediums

Using immunocytochemistry, astrocytes were visualized using GFAP (astroglial cell marker) antibody in DMEM/F12 (Figure 3A-C) and neurobasal (Figure 3D-F) mediums. GFAP-positive cells, DAPI-stained cell’s nucleus, and merged the nucleus with GFAP-immunoreactive cells have been demonstrated in Figure 3. Since neurobasal is the appropriate medium for culturing neurons, not astroglial cells, as well as because of adding B-27 to neurobasal medium, the number of astrocytes in neurobasal was very low compared to DMEM/F12.

3.4. TH-positive cells 7 days after culturing in DMEM/F12 and neurobasal

Dopaminergic neurons were characterized by immunostaining against TH antibody, 7 days following culturing in DMEM/F12 (Figure 4A-C) and neurobasal (Figure 4D-F). Similar to β3-tubulin-positive cells, despite clustering appearance in neurobasal, the number of TH+ neurons was not different in the two cultures.

4. Discussion

This study’s findings indicated that cells’ morphology was better in DMEM/F12 than that in neurobasal, but there was no difference in dopaminergic neuron number. It is suggested that primary dopaminergic neurons can grow and survive in both cultures. Consistent with our results, several in vitro studies reported survival of primary mesencephalon neurons in both cultures (Bayer Andersen et al., 2016; Choi et al., 2008; Collins et al., 2016; Collo et al., 2013). Neurobasal media is a neuron-specific culture and suppresses glial cells proliferation to less than 0.5% of the nearly pure neuronal population, as demonstrated by immunocytochemistry for GFAP. It has been shown that glial cell growth is suppressed in a neurobasal medium. Moreover, the neurobasal medium enhances the neuronal gene expression and neuronal survival due to lower osmolarity, presence of glutamine and cysteine, and lower toxic ferrous sulfate in comparison to DMEM/F12. (Brewer, 1995; Brewer, Torricelli, Evege, & Price, 2008; Brewer, Torricelli, Evege, & Price, 2008; Brewer, Torricelli, Evege, & Price, 2008; Brewer, Torricelli, Evege, & Price, 2008; Brewer, Torricelli, Evege, & Price, 2008; Brewer, Torricelli, Evege, & Price, 2008.)
Besides, B27 (an essential component of neurobasal medium) contains free radical scavenging enzymes such as catalase, superoxide dismutase, and glutathione (Nguyen et al., 2016), which provide a suitable environment for neuronal cell survival. Furthermore, using AraC in neurobasal culturing inhibits astroglial proliferation as well.

**Figure 3.** GFAP-immunoreactive cells in primary mesencephalon cells 7 days after culturing
A-C: GFAP-positive cells are characterized as astrocytes in DMEM/F12; and D-F: Neurobasal at ×10 magnification.
The immunostaining assay was repeated 3 times for each medium.

**Figure 4.** Dopaminergic neurons in primary midbrain culture on DIV7
A-C: Using immunocytochemistry, TH-positive neurons were visualized in DMEM/F12; and D-F: Neurobasal (at ×20 magnification).
The immunostaining assay was repeated 3 times for each medium.
well. So as expected, the astrocytes number was higher in DMEM/F12 compared to the neurobasal medium.

Neurotrophic factors released by astrocytes, especially Glial-Derived Neurotrophic Factor (GDNF), are critical for dopaminergic neuron survival (Kramer & Liss, 2015). In serum-supplemented DMEM/F12 media, the glial cell proliferation continued, and therefore we observed a lot of astrocytes in DMEM/F12 medium, which provided necessary neurotrophic factors. In our study, besides FBS exposure, the cells cultured in DMEM/F12 have been exposed to B27 on DIV5, which enriched the medium to survive neurons. We observed the dopaminergic neurons in DMEM/F12 similar to neurobasal and parallel with glial cells.

In general, the finding of this study indicated that primary midbrain cells can be cultured and survived in DMEM/F12 and neurobasal, with no significant differences in dopaminergic neuron number. However, DMEM/F12 was better regarding the morphology of the cells and lower cost compared to neurobasal medium as well.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors’ contributions

Methodology, bench work, writing - original draft: Neda Valian; Bench work: Mansooreh Heravi; Supervision, funding acquisition: Abolhassan Ahmadiani; Conceptualization, supervision, writing- review & editing: Leila Dargah.

Conflict of interest

The authors declared no conflict of interest.

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