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Research Article

PRIMARY NEURONAL CULTURE IS THE BEST MODEL TO STUDY CYTOLOGICAL CHANGES DURING NEURONAL DEVELOPMENT AND DEGENERATION

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ABSTRACT
Study of neuronal development is very fascinating. Early embryologists studied the developmental anatomy of the brain and spinal cord in various model organisms like frog, chick, mouse etc. Due to advent of cell culture procedures, microscopy and imaging techniques it is quite possible to literally play with the cells in the laboratory. One can see the cytological alterations during cell growth and development *in vitro.* Neurons being terminally differentiated cells, never undergo cell division. Therefore, neurons are most vulnerable to ageing related degenerative alterations. Many reports have elucidated the consequences of neuronal aging and its mechanism including the neuropathological alterations like of aggregation of neurofibrillary tangles, accumulation of lipofuscin granules, deposition of β amyloid etc. in the histological sections of brain autopsy samples and using brain of experimental animals. Organotypic slice cultures maintain the three dimensional architecture of the tissue and are used to study electrophysiology, biochemical analyses etc. Dissociated neuronal culture model is very popular in the study of neuronal growth and development. In the present investigations, the hippocampal pyramidal neurons from E17 mouse embryos were grown on poly-L-lysine coated coverslips. The neurons exhibited typical neuronal morphology on the third day of incubation. The synapses and neuronal networking was seen on the fifth day. The degenerative alterations were also recorded. The degenerative alterations were nuclear condensation, nuclear fragmentation, cytoplasmic vacuolation, loss of axon hillock, swelling of the axon, loss of axon and dendrites. As these observations were visible at the individual neuronal level unlike histological sections, primary neuronal culture is the best model to study the neurodegenerative alterations.

Keywords: *Primary culture, Neuronal culture, Neuronal differentiation, Neurodegeneration*

INTRODUCTION
Primary cell culture is useful for the study of cell proliferation and cell death in dissociated cells. Neuronal culture attracted many scientists to investigate the developmental process, neurophysiology and neuropathology. Banker and Cowan (1977) for the first time developed an *in vitro* system to study the isolated hippocampal neurons from rat embryos. Ray *et al.* (1993) demonstrated that basic Fibroblast Growth Factor can induce survival and proliferation of embryonic hippocampal progenitor neurons *in vitro* in a dose-dependent manner. The concentration of bFGF from 50 pg to 1 ng/ml assisted the survival, whereas, the higher concentration ranging from 10-20 ng/ml stimulated the proliferation in serum free medium. The cells were differentiated morphologically into neurons. Haydar *et al.* (1999) studied the neurogenesis, apoptosis and neuronal migration in organotypic brain slice cultures of the E13-E14 mouse embryos. Organotypic brain slice culture is a well-established technique where 350-400µ thick slices of the brain are cultured. The three-dimensional organization of the neural tissue is well maintained in the slice culture (Gähwiler *et al.* 1997). Therefore, it is used to study the integrated brain physiology, including synaptogenesis, cell dysfunction, death, pathology development linked to aging and simulated pathogenic conditions (Duff *et al.* 2002). However, this culture system has its own limitations, where individual neuron cannot be visualized. Contrary to this, the dissociated neuronal culture provides an access to monitor the growth and degenerative changes. In the present study, hippocampal pyramidal

neurons from E17 mouse embryos were cultured on poly-L-lysine coated coverslips. Their growth and degeneration was studied at an interval of twenty four hours.

MATERIALS AND METHODS

1) Neurobasal medium (Invitrogen Cat. No. 2113-049)
2) 2.5% Trypsin (Sigma Aldrich Cat No. 49427C)
3) HBSS (Invitrogen Cat No. 14170-112)
4) Poly-L-lysine solution, 1mg/ml in 0.1M borate buffer pH 8.5 (prepared using Poly-L-lysine hydrobromide, Sigma-Aldrich Cat. No. P 6282)
5) B27 Supplement (Invitrogen Cat No. 17504044)
6) Trypsin inhibitor from Soyabean (Himedia TCL068)

I. Preparation of poly-L-lysine coated coverslips:

a. Cleaning of the coverslips: The glass coverslips were soaked in concentrated nitric acid for 18 hours. Thereafter, the coverslips were washed four times in glass distilled water. Each wash was of two hours duration. Then the coverslips were soaked in ethyl alcohol for 6 hours and finally air dried in desiccator at 80^{o}C. Each coverslip was wrapped individually in an aluminium foil and transferred to an oven maintained at 225^{o} C for 8 hours.

b. Coating the coverslips with Poly-L-lysine: Each coverslip was coated with 50 µl of filter sterilized Poly-L-lysine solution (1 mg/ml in 0.1M borate buffer at pH 8.5) in the laminar airflow. After spreading a drop of Poly-L-lysine solution uniformly on the surface, the coverslips were allowed to stand in laminar air flow for 24 hours. Thereafter, the poly-L-lysine coat was soaked in sterile water for two hours. Such two washes of sterile water were given. Finally the coverslips were soaked in HBSS up to 24 hrs.

c. Preparation of poly-L-lysine coated coverslips for seeding the neurons: One hour before seeding the neurons, HBSS surrounding the Poly-L-lysine coated coverslips was replaced by neurobasal medium supplemented with 2% B27 and incubated in CO_2 incubator at 5%CO_2 and 37^{o}C.

II. Primary culture of hippocampal pyramidal neurons:

a. Dissociation of neurons from embryonic hippocampus:

Hippocampal pyramidal neurons were isolated from E17 mouse embryos. All procedures were carried out with due permission of Institutional animal ethics committee. Under mild anaesthesia, E17 embryos (Embryonic day 17) were surgically removed from the pregnant mice. All surgical procedures and dissection of embryonic brain were carried out aseptically in laminar airflow using sterilized surgical instruments. The brain of each embryo was dissected by gently removing the skull and the meninges. The hippocampi were carefully separated under stereoscopic dissecting microscope in 60 mm cell culture plate (Eppendorf) containing chilled HBSS (Gibco Cat No. 14170-112). All separated hippocampi were collected in 15 ml conical centrifuge tube (Tarson) filled with 5 ml HBSS.

The isolated hippocampi were trypsinised for 8 min. in calcium-magnesium free HBSS containing 0.25% trypsin, incubated at 5% CO_2 and 37^{o}C (Galaxy 48R CO_2 incubator Eppendorf). 0.25% trypsin solution was decanted and the hippocampi were resuspended in HBSS and 100µL of Soybean trypsin inhibitor (Himedia TCL068) was added in it. Using a sterile fire polished Pasteur pipette the hippocampi were triturated to obtain isolated neuronal cells. The viable cell count was carried out using trypan blue dye exclusion method. Dissociated hippocampal neurons were seeded on poly-L-lysine coated 18 mm glass coverslips.

b. Seeding the hippocampal pyramidal neurons on poly-L-lysine coated coverslips: During seeding the hippocampal neurons, each coverslip from petri dish was gently removed without disturbing the poly-L-lysine coat**.** 50 µl of the cell suspension having the viable cell count 50,000 cells/ml was seeded at the centre of each coverslip. The seeded coverslips were allowed to stand for 10 min. in CO_2 incubator for adhesion of the cells to the coverslips.

After ensuring the cell attachment by examining under inverted phase contrast microscope, the seeded coverslips were transferred to 60 mm cell culture plates containing neurobasal medium supplemented

with 2% B27 and 1.25µl Gentamycin per 10ml. On every third day, 2/3rd of the culture medium was replaced by fresh culture medium. On the 5th day of the seeding, the neurons were fully grown with their axons and dendrites and developed the synapses.

c. Study of Neuronal growth and cytopathological alterations:

The neurons were observed after every twenty four hours under inverted phase contrast microscope (Lawrence and Mayo) and live cell imaging was carried out using Jenoptik camera.The neurodegenerative changes like nuclear alterations, cytoplasmic vacuolation, dendritic degeneration, axonal degeneration were studied.

RESULTS AND DISCUSSION

In the present investigations, the synapses were evident on the fifth day of seeding. The dissociated neurons appeared spherical in shape soon after the seeding (Figure 1 image 1). From the second day onwards the cells exhibited neuritic outgrowth (Figure 1 image 2). One of the outgrowths continued to elongate to form the axon (A in figure 1 image 2), whereas the others were comparatively smaller in length which were dendrites (D in figure 1 image 2). On the third day of incubation, the cells exhibited typical neuronal morphology (figure1 image 3). Dotti *et al.* (1988) also have examined the events of neuronal development during the first week of culture. Cerebral cortex, hippocampus, striatum, substantia nigra, dentate gyrus etc. are used for the primary culture of the neurons (Banker and Cowan 1977, WEIss *et al.* 1985, Stichel and Müller 1991 Brewer 1995).

In the present investigations, the neurodegenerative alterations were conspicuously revealed. 8% neurons exhibited nuclear condensation in which the nuclei appeared brighter. In 5% neurons nuclear fragmentation was evident (Figure 1 image 5 NF). In 7% neurons dendrites were degenerated (Figure 1 image10 DL). There was axonal precipitation in 5% neurons. (Figure 1 image 10AP). In 3% neurons, cytoplasmic vacuolation was observed (Figure 1 image 10 CV). 4% neurons exhibited loss of axon hillock (Figure 1 image 4 LA). 7% neurons exhibited axonal swelling (Figure 1 image 4 AS) and 5% neurons exhibited axonal precipitation (Figure 1 image 10 AP). Such cytological alterations can be revealed at individual neuronal level only in dissociated cell culture.

Guroff (1985) studied the PC12 cells as a model of neuronal differentiation. PC12 is a cell line derived from a neuroendocrine tumor of the rat adrenal medulla called as pheochromocytoma. Many of the cells produced short processes which were evident within 24 hours of plating. When these cells were subjected to the treatment of nerve growth factor (NGF), more processes were developed. SH-SY5Y is the human neuroblastoma cell line frequently used as an *in vitro* model for the study of dopaminergic neurons. Proliferating SH-SY5Y cells can be induced to differentiate into dopaminergic neurons by using 10 mM retinoic acid (RA). However, the cells derived from a tumor may not show the properties of a normal neuronal cell. According to Mao and Wang (2003), primary neural culture is an *in vitro* approach, attempting to reduce the complexity which is otherwise seen in *in vivo* system. It establishes a relatively purified model for studying the nervous system at a single cell level. Since the culture environment can be readily controlled and the cells derived from the neural tissue exhibits the biological properties similar to the original tissue, unlike the neural cells derived from the tumor, many *in vitro* neurodegenerative model systems have been used for the study of Alzheimer disease, Parkinson disease and to examine the effects of neurotoxins like amyloid-beta peptides, apolipoprotein E, 6-hydroxydopamine (6-OHDA) etc. (Kim 2007). Primary neuronal cultures and neuronal cell lines derived from rodents are widely used to study basic physiological properties of neurons, and represent a useful tool to study the potential neurotoxicity of chemicals (Giordano and Costa 2011).

However, *in vitro* model has some limitations also. The cell survival time is too low i.e. up to two weeks, which is far smaller than the *in vivo* life. The pattern of cell death in all the neurons is similar; like, vacuolation, loss of axon, nuclear fragmentation, etc., which may not be the situation in *in vivo* condition. Moreover, it is a monotypic culture where a single type of cells from one particular region of the brain such as hippocampus, are cultured. Therefore, the observations evidenced cannot be applied as a thumb

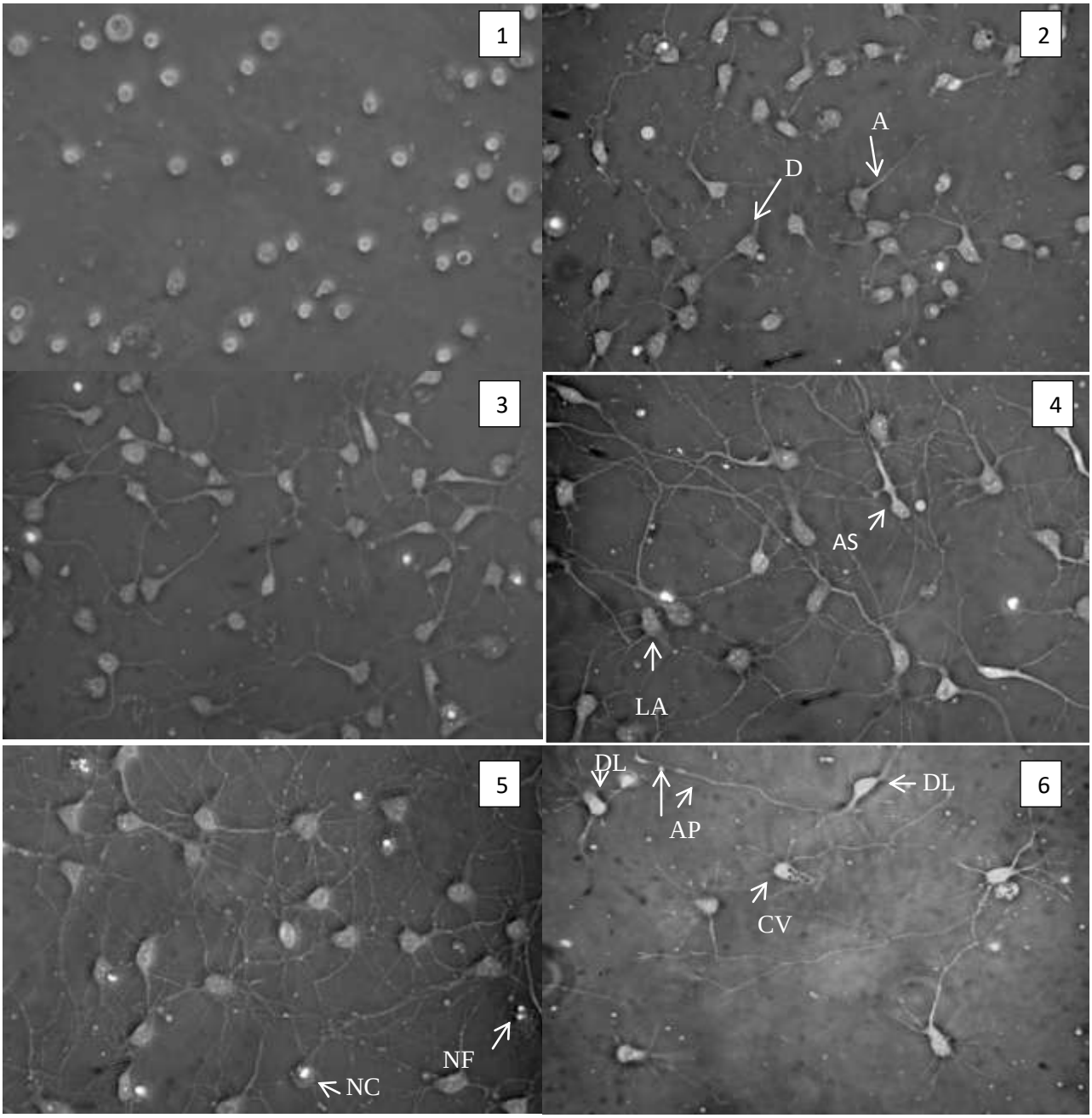

Figure 1: Inverted phase contrast microscopic images at 400X showing day-wise growth and development of hippocampal pyramidal neurons on Poly-L-Lysine coated coverslips incubated in Neurobasal medium+ 2% B27supplement at 37°C and 5% CO_2 in the CO_2 incubator. 1 to 10 indicates days of incubation. 1indicates stage immediately after seeding, 2 indicates second day of incubation and so on. Abbreviations: A: Axonal outgrowth, D: Dendritic outgrowth, AS: Axonal swelling, LA: loss of axon hillock, NF: Nuclear fragmentation, NC: Nuclear condensation CV: cytoplasmic vacuolation, DL: dendritic loss, AP: axonal precipitation

rule to the entire brain. In spite of these limitations the primary culture gives access to visualize the degenerative alterations by live cell imaging. Thus, dissociated neuronal culture is the best tool to study degenerative alterations.

CONCLUSION

Primary neuronal culture helps to examine the neuronal growth and development. Besides, it aids to study the neurodegenerative cytological alterations such as nuclear condensation, nuclear fragmentation, loss of axons, axonal swelling, loss of dendrites and synapses. It will help in the study of various therapeutic agents targeted to slow down the neurodegenerative alterations. It will also be an effective model to study the neurotoxic potential of various substances using primary neuronal culture.

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