PRIMARY INSECT CELL CULTURE FROM TOTAL EMBRYO AND EMBRYONIC BRAIN TISSUE OF PERIPLANETA AMERICANA: A PRELIMINARY STUDY

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Abstract: The aim of this preliminary study was to establish a primary insect cell culture from total embryos and embryonic brain tissues of Periplaneta americana, collected from Izmir, Turkey. Cells were cultured at 29ºC in Grace's insect medium for one month. In the embryonic brain tissue culture, single cells and cell clumps containing spherical and ovoid as well as dividing cells were observed. Single bipolar neurons were detected after 4 days in culture. Network systems comprised of bipolar neurons were observed on the 5th day of incubation. In addition, presumably glia cells were observed in the embryonic brain culture. In the total embryo culture, the cell population exhibited variable morphologies, including spherical, spindle-like, polygonal and giant cells after nearly 20 days; the culture covered almost half of the Petri dish area within 30 days. This preliminary study associated with Periplaneta americana primary cell culture is the first of its kind in Turkey. These results should contribute to the development of new insect cell lines that are indigenous to Turkey.

Key words: Insect cell culture; Periplaneta americana; embryo; brain tissue

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INTRODUCTION

Cultured insect cells obtained from different tissues are used in the fields of biology, genetics and medicine (Smagghe et al., 2009). Insect cell culture is a useful tool for cell-virus interaction studies (Gundersen-Rindal and Dougherty, 2000; Mudiganti et al., 2006; Schütz and Sarnow, 2006; Lennan et al., 2007), virus propagation (Li and Bonning, 2007; Lynn, 2007), development of new pesticides (Smagghe et al., 2009; Beckmann and Haack, 2003) and the elucidation of signal transduction pathways (Lemaitre and Hoffmann, 2007). Additionally, insect cell culture is successfully utilized in the production of recombinant proteins, which can be vaccine candidates (Iwabuchi, 2000; van Oers and Lynn, 2010).

Currently, there are more than 500 insect cell lines available. However, primary insect cell cultures established from different and new species are still necessary in order to uncover interactions among molecules in different cells and to find more effective cell lines producing recombinant humanized proteins in eukaryotic systems. Thus, various cells derived from ovaries, embryos, hemocytes, imaginal discs, fat bodies, midguts, neonate larvae, cuticles, the nervous, endocrine and muscular systems are cultured (Lynn, 2001).

In particular, cells from the nervous system are cultured because the cell lines associated with this tissue are rare or nonexistent (Lynn, 2001). It is suggested that cells isolated from the nervous system are significant for studies related to ionic channels, receptors of the nerve membrane, neurotransmitters, hormones, venoms, toxins, and cultured neural cells give important information about the behavior of small neuronal networks and the development of nerve and glial elements (Hicks et al., 1981; Muller et al., 1992; Boehm and Huck, 1997; Jensen et al., 1997; Brosenitsch et al., 1998; Angevin et al., 2000). To date, neural cells derived from a wide variety of insects, including cockroach, locust, honeybee, houseflies, crickets grasshopper, moths and fruit fly have been
cultured (Oh et al., 2007; Beadle, 2006). In addition to the nervous system, another important cell source is the insect embryo, which is preferred to other tissues since it has undifferentiated cell groups (Lynn, 1996).

There is a need for indigenous insect cell lines that can replace the use of biohazardous chemical pesticides, and produce lifesaving pharmaceuticals and vaccines (Sudeep et al., 2005). However, studies involving primary insect cell cultures obtained from insects that live in Turkey are nonexistent. The aim of this study was to establish a primary insect cell culture from total embryos and embryonic brain tissues of Periplaneta americana collected from Izmir, Turkey. In comparison to other previous studies associated with cell cultures from Periplaneta americana, we established a cell culture from the brain tissue of Periplaneta americana using Grace's insect medium, and obtained similar results to those of previous studies. In addition to brain tissue culture, we maintained the first cell culture from the total embryo of Periplaneta americana for one month, which contained different types of cells. These cells from the total embryo could be suitable for continuous cell lines. This study could be useful for future studies requiring the establishment of new and continuous insect cell lines, the development of new insecticides against insects that live in Turkey and the development of new insect cell lines that can be used in the production of recombinant proteins.

MATERIALS AND METHODS
Dissection of embryos

Oothecae were collected from Periplaneta americana adults grown in the insect laboratory of the Department of Zoology at Ege University. They were incubated at 29°C for 16–21 days, after which they were cut longitudinally with sharp scissors under stereomicroscope (Carl Zeiss) and rinsed with absolute ethanol (Sigma-Aldrich) for 10 min for sterilization. Finally, they were rinsed with sterilized water and dried at room temperature (Cochran, 1999; Bell et al., 2007).

Cell culture

Cell cultures from embryonic brain tissues were prepared from the brains of 16–21-day-old embryos of Periplaneta americana using the technique of Angevin et al. (2000) with some modifications from Schneider (1969). Heads of embryos (n:20) were cut with a microscalpel and collected in Rinaldini’s salt solution (RSS, 800 mg NaCl, 20 mg KCl, 5 mg NaH₂PO₄·H₂O, 100 mg NaHCO₃, 100 mg glucose, in 100 mL distilled water) containing penicillin/streptomycin (100 units/mL, AppliChem) and gentamycin (50 mg/mL, Sigma-Aldrich). Similarly, for total embryonic cell cultures, total embryos (n:20) were cut into 8 pieces with a microscalpel and collected in RSS. After this step, both cell cultures were prepared using the same procedures. Both embryonic head tissues and total embryo tissues were separately transferred to RSS containing pen/strep, gentamicin and 2% trypsin (Sigma-Aldrich), and then they were incubated at room temperature for 60 min and centrifuged at 500 g for 3 min. Thereafter, 10% FBS (fetal bovine serum, Sigma-Aldrich) prepared in Grace’s insect medium was added to the pellet for trypsin inhibition after the supernatant was removed. Finally, cells obtained from both the heads of embryos and total embryos were separately transferred to Petri dishes (Becton Dickinson, 35x10 mm) for cultivation with 200 µL Grace’s insect medium (pH: 6.7, Sigma-Aldrich) containing 10% FBS, pen/strep (100 units/mL), gentamycin (50 mg/mL) and sodium bicarbonate (0.35 g/L, Sigma-Aldrich). All solutions were sterilized through 0.22-µm pore-size filters (Sartorius Stedim, Minisart) prior to use. After the 1st day of cultivation, 1 mL of Grace’s insect medium was added to the pellet for trypsin inhibition after the supernatant was removed. Finally, cells obtained from both the heads of embryos and total embryos were separately transferred to Petri dishes (Becton Dickinson, 35x10 mm) for cultivation with 200 µL Grace’s insect medium (pH: 6.7, Sigma-Aldrich) containing 10% FBS, pen/strep (100 units/mL), gentamycin (50 mg/mL) and sodium bicarbonate (0.35 g/L, Sigma-Aldrich). All solutions were sterilized through 0.22-µm pore-size filters (Sartorius Stedim, Minisart) prior to use. After the 1st day of cultivation, 1 mL of Grace’s insect medium was added to the pellet. Cells were incubated at 29°C in an incubator (Binder, CB 53) and 500 µL of culture medium was added to the Petri dishes per week. The Petri dishes were routinely examined with an inverted microscope (Olympus, CK 40). When the Petri dishes reached 3 mL medium volume, all media were discarded except 500 µL, and 500 µL of fresh medium were added. This cycle was repeated for one month. Cell diameters were measured using Olympus LC Micro software from the cells in three different areas.
RESULTS

Embryonic brain tissue

Single cells (Fig. 1A) and cell clumps (Fig. 1B-C) were examined in culture a few days after dissociation of the nerve tissue. They were approximately 5-15 μm in diameter. Among them, there were both spherical and ovoid cells. Dividing cells in telophase of mitosis were rarely seen in culture (Fig. 1D). The common feature of these cells was that they did not have axons and dendrite-like structures yet. These cells were not observed after one week of incubation.

In addition to these cells, single surviving cells, which differentiated from bipolar neurons, were seen after the 4th day of culture. Most of these cells had long axons and a spherical and ovoid cell body. A few of them had long axons and dendritic-like processes (Fig. 2). All of them adhered to the bottom of the Petri dishes and survived for nearly 15-20 days.

Cell bodies produced numerous outgrowths called neurites in order to form a complex network, and some neurons established connections with other neurons (Fig. 3). After the 5th day of cultivation, networks of neurons were observed. In culture, certain networks remained stable during 30 days.

There were some cells that were not identified in culture. It seems they migrated from the explant and associated with each other, like neurons (Fig. 4). Also, they survived during 30 days in culture.

Total embryo

Embryonic cell growth rate during the primary culture was slow and cells adhering to the bottom of the Petri dishes were observed after nearly 20 days. The culture covered almost half of the Petri dish area within 30th day of incubation. The culture consisted of heterogeneous cells, which were spherical, elongated and irregular (Fig. 5). Diameters of cells were between 10-50 μm in culture. Neurons found in the embryonic brain tissue culture were not observed in the culture established from total embryos.
DISCUSSION

Brain neurons derived from embryonic cockroaches (*Periplaneta americana*) can differentiate up to three months of culture (Angevin et al., 2000). A few days after adhesion, neurons begin to produce undifferentiated neurites. Later, neurites break the symmetry and one of neurites elongates to establish connections with neighboring neurons. Thus, strong network systems are formed by neurites in culture (Yamamoto et al., 2012).

Angevin et al. (2000) suggested that the number of neurons in culture after dissociation remains unchanged up to 30-40 days. The cause of this stability is that neither cell death nor dividing cells are present during culturing. However, increasing numbers of viable cells in culture depend on living brain tissues, called explants, from which new neurons migrate. Neuroblasts of embryonic cockroaches generate neurons and glia *in vivo*, which do not survive *in vitro*. Contrary to the neuroblasts of cockroaches, it was demonstrated that neuroblasts obtained from *Drosophila* could be cultured *in vitro* (Wu et al., 1983).

One of the objectives of the present preliminary study was to establish a primary culture from the embryonic brain tissues of *Periplaneta americana*. Cells derived from embryonic brain tissues were cultured in Grace's insect medium (pH: 6.7) containing 10% FBS, and these cells survived for one month. Among these cells, some were single bipolar neurons, which have long axons and dendritic-like processes, and others were neurites and formed network systems. In addition to neurons, there were migrated cells from explants, which were not identified in culture.

The type of medium for growth and proliferation of cells is a significant factor because no medium is specific for every type of cell. Mixtures of Schneider's insect culture medium, Eagle's basal medium, Leibovitz's L-15 medium and Yunker's modified Grace medium have been generally used in studies associated with primary culture of embryonic cockroach neurons (Hicks et al., 1981; Beadle et al., 1982; Angevin et al., 2000). However, Grace's insect medium with serum was used in our study and the results of our study were similar to those of previous studies that used a combination of four media containing Schneider's insect culture medium, Eagle's basal medium, Leibovitz's L-15 medium and Yunker's modified Grace medium (Hicks et al., 1981; Beadle et al., 1982; Angevin et al., 2000). The results of our study demonstrated that the use of Grace's insect medium could be sufficient for establishing cell culture from brain tissue of *Periplaneta americana*.

In this study, single bipolar neurons and network systems comprised of neurites were observed in culture. Among them, single bipolar neurons survived until the 20th day; network systems survived for 30 days as well. Similarly, it was demonstrated that neuronal network systems could be maintained for at least 2 months and bipolar neurons survived for 3 weeks (Hicks et al., 1981; Howes et al., 1991). Moreover, in a previous study, it was observed that neuronal network systems remained stable for more than one month (Angevin et al., 2000).

In addition to nervous system cells, we also observed that cells that migrated away from the brain tissue (explants), had a spindle-like and flattened morphology (Fig. 4). These cells are presumed to be glial cells. Such morphological features were described in glial cell culture from cockroach (Keen et al., 1994). These cells survived in culture for 30 days, while glial cells derived from embryonic brain of *Periplaneta americana* were maintained in primary culture conditions for up to 3 weeks.

Another objective of the present preliminary study was to establish a primary culture from total embryos of *Periplaneta americana*. The cell population of total embryos in culture exhibited variable morphologies, including spherical, spindle-like and polygonal morphologies. Cells were first observed after nearly 20 days and the culture covered almost half of the Petri dish area within 30 days. Similarly, it is stated that cell growth and the formation of confluent monolayers in primary culture is slow (Bello et al., 2001). It is suggested that primary cultures had cells of heterogeneous forms and sizes (Rey and Ferro Bello, 2000). The results obtained from total embryos
are valuable because the total embryo culture offered here had various cells with different morphologies, such as spherical, elongated and irregular cells.

Interestingly, nerve cells were not observed in the total embryonic cell culture. It is known from the literature that establishing primary neural cell cultures is more difficult than establishing cultures of other cell types (Smaghe et al., 2009). Neural cells are more sensitive and fragile compared to other cells. Repression of these cells by other types of cells could be one of the reasons for the absence of neural cells. Also, when compared to other cell types, the number of neural cells in the total embryo is low.

In conclusion, this preliminary study of primary cell cultures from cockroaches living in Turkey is the first of its kind and it should contribute to the development of new insect cell lines. This work could be used in future studies related to the establishment of new and continuous insect cell lines, the development of new insecticides and the production of recombinant proteins that can be produced in insect cell lines by baculovirus expression vector systems.

REFERENCES


