

## CHARACTERISTICS OF VARIOUS CELL TYPES IN PRIMARY INSECT CELL CULTURE FROM LARVAL OVARIES OF SILKWORM (*BOMBYX MORI* L.)

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### ABSTRACT

As insect cell lines can be used in baculovirus expression systems for producing recombinant proteins, there is a need to establish new cell lines. Insect cell lines are thought to be useful in production of humanized recombinant proteins. Hence, we aimed to establish a culture from larval ovaries of *Bombyx mori* to investigate the cell characteristics and growth properties. Two sets of primary cell cultures were established from ovaries of fifth instar larvae. Characterization of cell line was carried out by comparing the PCR products of cell line and silkworms from *COI* (*cytochrome c oxidase subunit I*) specific primers. In the first set of cultures, the culture population was found to be heterogeneous and cell sizes were variable. Fibroblastic, epithelial-like, spindle-like, and round cells were observed after 7<sup>th</sup> day. Interestingly, contractions were observed in muscle-like cells at the end of first month, and stayed stable until the fourth month. At the beginning of the fourth month, because cell death increased significantly, cell concentrations decreased. In the second set of cultures, it was determined that doubling time was about 72 h with high viability and confluency of cells were higher in early passages. PCR which was conducted for characterization of cells resulted in a 710 bp product in each cell line and silkworms. In conclusion, growth rate detected in this study was observed to be compatible with literature. Also, it was thought that if primary culture is supported with growth stimulators, growth rate can be more accelerated and this primary culture can be used more efficiently in recombinant protein production studies.

**Keywords:** Primary cell culture, *Bombyx mori*, characterization, ovary, cell morphology, growth analysis.

### INTRODUCTION

In recent decades, science and technology focus on human needs, especially adequate nutrition and medical treatment, because of the rapid increase in human population. To address the medical needs, research and pharmaceutical industries are exploring ways to use natural compounds or synthetic biological systems to produce a wide range of pharmaceuticals. Various manufacturing methods are used for this purpose and one of the most preferable is recombination, which allows generation of humanized products (Shi and Jarvis, 2007). Studies in recent years have greatly increased in the direction of producing large quantities of humanized proteins in other organisms (Kost *et al.*, 2005). While prokaryotic cells are preferred for their high growth rates in production of recombinant human proteins, some protein functionality problems arise because they cannot perform post-translational modifications like eukaryotic cells (Kamionka, 2011). Therefore, insect cell cultures are expected to provide solutions to this problem (Smaghe *et al.*, 2009).

Use of insect cell culture offers many benefits in a variety of scientific fields such as immunology (Taniai *et al.* 2006), developmental biology (Liu *et al.*, 2007; Akiduki and Imanishi, 2007) and, biopesticide (Woo *et al.*, 2007) and biopharmaceutical researches (Orihara *et*

*al.*, 2008; Pan *et al.*, 2010). Since the 1950s, more than 320 lines from 65 species have been established from a range of tissues (Lynn and Harrison, 2016). While some of these lines are suspended, others have adherent characteristics. Since *Bombyx mori* is a somewhat larger insect than the others and its genome is completely sequenced, it has become an organism frequently used in the cell culture studies in recent years. A few cell lines from ovaries and embryos have been established (Khurad *et al.*, 2006, 2009, 2013; Matindoost *et al.*, 2008; Zhang *et al.*, 2014) but only a few can support replication of BmNPV. Agathos (1993) reported in his review that more efficient and more widely applicable cell lines were needed for baculovirus replication and foreign gene expression. Although many years passed since the establishment of BmN cell line, there is a necessity to introduce a new cell line from silkworm.

New cell lines are generally established in order to find fast growing and suitable cell lines for production of recombinant proteins. Moreover, it should be noted that the behavior and growth rates of the cell lines must be well known. Many studies have been conducted in order to determine which organs and cell types are the best candidates for recombinant protein production (van Oers and Lynn, 2010). Initiation of primary cultures is laborious and difficult, and patience is required. These difficulties are as follows: (1) In the first days of

culturing, a heterogeneous cell population is obtained from the tissue and these cells have slow growth rates, (2) homogenous cell lines with high grow rates are obtained after so many passing processes (Lynn, 1996).

To characterize the new cell lines, PCR-targeted mitochondrial *COI* gene fragments (710 bp) are generally used. The *COI* gene is a major target gene of the DNA Barcoding Project (Kress *et al.*, 2005). DNA barcoding is a method which is used to identify species of organisms based on matching to short genetic markers. Therefore, specific PCR results are highly applicable to phylogenetic analysis of the Bombycidae species.

In this study, we aimed to establish a primary ovary culture from silkworms and to examine its behavior in the culture, growth rate and ability for passaging. In order to characterize our cell culture, we carried out a *COI*-PCR analysis and compared the specific products from cell culture and insects.

## MATERIALS AND METHODS

**Primary culture:** The silkworm eggs were provided by Bursa Kozabirlik Company, Turkey. The eggs were reared in the laboratory at  $25 \pm 1$  °C and  $75 \pm 5$  % relative humidity on fresh mulberry leaves. Two sets of primary cell cultures were established to investigate cell morphologies, cell behaviors, and growth rates. The first culture was established to investigate cell morphologies and cell behaviors; the other was established to examine ability of culture to be passaged and to determine the culture growth rate. Firstly, the surfaces of 5<sup>th</sup> instar larva silkworms were sterilized by 70 % ethanol for 10 min. Then ovaries were dissected from the insects under a stereo-microscope (Carl Zeiss) and pooled into cell dissociation solution (C1544, Sigma Aldrich). Each primary culture was established using 8-10 5<sup>th</sup> instar larvae ovaries. All the pooled ovaries were cut into small pieces and then centrifuged at 2000 rpm for 10 min. The supernatant was removed and tissue fragments were suspended with 1 ml of Grace insect medium (G8142, Sigma Aldrich) supplemented with pen/strep (10000 units penicillin and 10 mg streptomycin/mL, P4333, Sigma Aldrich), gentamycin (50 mg/mL, G1397, Sigma Aldrich), amphotericin B (A2942, Sigma Aldrich) and 10

% fetal bovine serum (FBS; F9665, Sigma Aldrich) that was inactivated at 56 °C for 30 min. Finally, suspended cells were transferred into 35x10mm culture flasks (Becton Dickinson) and were incubated at 26 °C in a cell culture incubator (Binder, CB 53). 500 µL of culture medium was added to the petri dishes each week. When the petri dishes reached 3 mL medium volume, all media was discarded except 500 µL, and then 500 µL of fresh medium was added. This cycle was repeated for 4 months. All solutions were sterilized through 0.22-µm pore-size filters (Sartorius Stedim, Minisart) prior to use. Three replicates of each culture set were carried out in this experiment.

**Subculture:** For the second set of cultures, when the cells fully covered the bottom of the flask, 1 mL of 0.25 % trypsin-EDTA solution (T4049, Sigma Aldrich) was applied to the culture and detachment of cells was carried out after incubation for 30 min. Detached cells were centrifuged at 2000 rpm for 10 min and the supernatant was removed. The resulting cell pellet was suspended with 1 mL insect medium and half of the culture suspension was transferred to a new flask containing fresh culture medium.

**Cell morphology:** The cultured cells were observed using an inverted microscope (Olympus, CK 40). Images were taken with Olympus LC Micro software. Cell sizes were calculated according to a calibrated magnification factor. Average cell dimensions were determined from measurements of 10 cells.

**Growth rate analysis:** The growth rate of the cell culture was measured according to the cell density analysis protocol (Murhammer, 2007). Cells were seeded into 35x10 mm culture flasks at a density of approximately  $2 \times 10^5$  cells/mL in 2-mL medium. The cell suspension was sampled from culture flasks every day. The cell density was analyzed by hemocytometer (Thoma, Isolab) using trypan blue at every 24 h. The growth curve was constructed according to the average cell density and the doubling time was calculated by the equation shown below (Mitsubishi, 2002). Experiments were performed in triplicate and repeated three times.

$$DoublingTime = \frac{duration * \log(2)}{\log(FinalConcentration) - \log(InitialConcentration)}$$

**Characterization:** Cells were collected when the growth curve reached a plateau level. Detachments of cells were performed by pipetting and then detached cells were centrifuged at 4 °C for 10 min at 300 g. From a pellet of approximately  $1 \times 10^5$  cells, genomic DNA was extracted as described by Iwanaga *et al.*, (2000) and used for PCR. The following primer pair consistently amplified a 710-bp fragment of *COI* (*cytochrome c oxidase subunit I*)

across the broadest array of silkworm and cell line (Folmer *et al.*, 1994):

LCO1490: 5'-gggtcaacaatcataaagattggg-3'  
 HC02198: 5'-taaactcagggtgaccaaataca-3'

The 25 µL amplification reaction included 2.5 µL 10× buffer, 2 µL dNTP (2.5 mM each), 10 picomole of each primer, 1.5 U Taq polymerase, 30 ng of genomic DNA and rest milli Q water. The PCR amplification

reaction was performed using the following calculated-control protocol: 3 min initial denaturation step at 94 °C, followed by 35 cycle of 1 min at 94 °C, (denaturation), 1 min at 42°C, (annealing), 2 min at 72 °C, (extension) and a final extension of 7 min at 72 °C. The reaction product was electrophoresed on 2 % agarose gel at 100 V for 40 min. Then, the agarose plate was stained in 0.75 µg/mL ethidium bromide and visualized under UV imaging system.

## RESULTS

After the primary culture was established from the ovaries, cells were examined every day. For the first set of cultures which were used for cell morphology determination, attachment of explants began after 24 h of incubation. In the first week of primary culture incubation, cell growth was slow. It was observed that adhesion of some tissue explants continued for three weeks. Among these explants, there were different kinds of explants producing the different types of cells. The population of the primary cell culture was heterogeneous and different cell sizes were identified. Different cell types including fibroblast-like, epithelial-like, spindle-like and round were observable after the 7<sup>th</sup> day of the culture. Fibroblastic (or fibroblast-like) cells were bipolar or multipolar and had elongated shapes. Epithelial-like cells were polygonal in shape with more regular dimensions. Spindle-like cells were characterized as a combination of epithelial-like and fibroblast-like cells. They had both elongated shapes like fibroblastic and shapes with more regular dimensions like epithelial cells. Also, there were round cells that were spherical in shape and usually grew in suspension without attaching to any surface. Most of the culture population was determined as adherent and very few of them were suspended.

Briefly, the morphology of these cell types are as follows: The epithelial cells were irregular in shape, and were in varied sizes ( $20 \pm 5$  µm in width) and attached strongly to the culture flask (Figure 1). The fibroblast-like cells were predominantly ellipsoidal with two long extensions on opposite sides (Figure 2). They attached strongly to the surfaces of the flask, and were in varied sizes ( $100 \pm 30$  µm in length and  $10 \pm 2$  µm in width). The spindle-like shaped cells strongly attached to the surfaces of the flask like the fibroblastic cells and were also varied size ( $40 \pm 20$  µm in length and  $10 \pm 2$

µm) (Figure 1). The round cells were the smallest cell type which attached to the surfaces of the flask and had an average diameter of  $15 \pm 2$  µm (Figure 1). In the first month of the culture, the spindle-like (40 %) and epithelial-like (30 %) cells were observed as the most common cell types, followed by the fibroblast-like cells (20 %) and round cells (10 %). After the first month, the amount of fibroblastic cells within the population became equal to the epithelial-like cell population.

First contacts between adherent cells were observed on the 10<sup>th</sup> day and stayed stable for 3 to 5 months. Interestingly, it was observed that fibroblastic and spindle-like cells started to construct dense porous structures after the 2<sup>nd</sup> week and these structures were observed in high amounts until the end of 3<sup>rd</sup> month (Figure 2C). Fortunately, at the end of first month, some contracting cell groups like smooth muscle cells were observed. Their contractions were observed everyday and it was noted that they continued to contract until the end of the 4<sup>th</sup> month (Figure 3). In the first days, contraction of cells was very frequent and this frequency continued until the end of the 3<sup>rd</sup> month. At the beginning of the 4<sup>th</sup> month, significant cell deaths started and cell concentration decreased dramatically. These sets of cultures were finished at the end of the 4<sup>th</sup> month because of the high levels of cell death.

The second set of cultures was established to determine growth rate and doubling time of the cells.  $2 \times 10^5$  cells/mL in 2 mL medium were seeded into culture flasks. The cell densities in five areas of each flask were determined at 24 h intervals for 7 days of incubation. At the end of 7<sup>th</sup> day, cell population had increased more than 2-fold (Figure 4). As a result of this growth rate analysis, the doubling time of cells was estimated to be approximately 3 days (72 hours). After the 1<sup>st</sup> month of primer culture, the cells approached confluence and they were sub-cultured. In the fourth passage, no explants were seen in the culture flasks. Individual cells were attached in different areas of the flasks. Because there were no explants in the culture, confluency of cells was observed lower than in early passages.

For characterization of cell line, *cytochrome c oxidase subunit I (COI)* primers were used and resulting product from cell line was compared with product from insect larva (Figure 5). A 710 bp product was found in each cell line and insects.

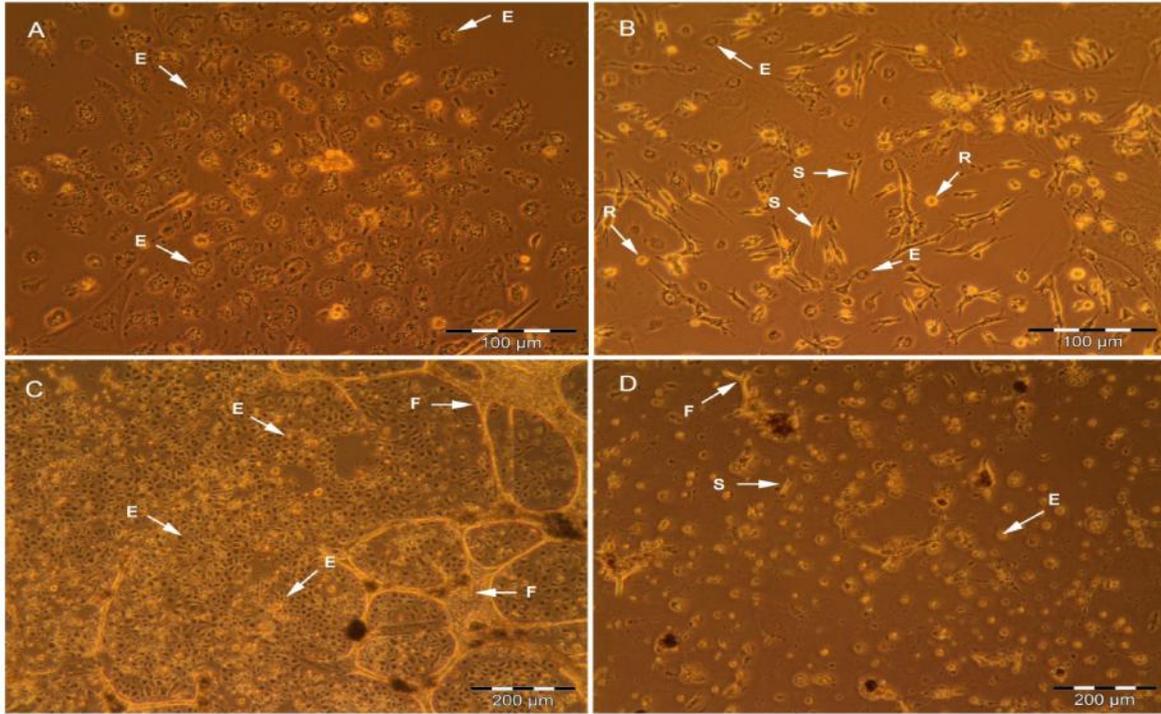


Figure 1. Epithelial-like and spindle-like cell population in culture. Confluent monolayers of cell culture from ovaries of *Bombyx mori* showing the predominantly epithelial-like and spindle-like cell population in 9 days (A), 23 days (B), 54 days (C) and 96 days (D) after being cultured. E: Epithelial-like, S: Spindle-like, R: Round cells, F: Fibroblastic.

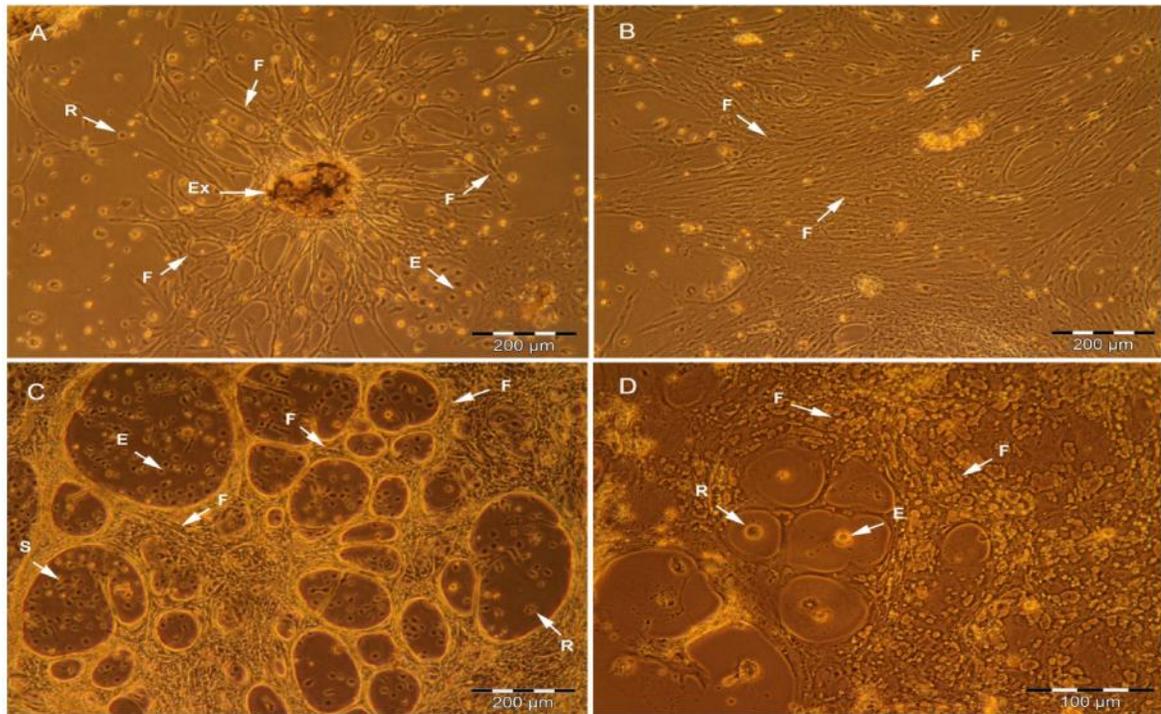


Figure 2. Fibroblastic-like cell population in culture. Confluent monolayers of cell culture from ovaries of *Bombyx mori* showing the predominantly fibroblastic-like cell population in 9 days (A), 23 days (B), 54 days (C) and 96 days (D) after being cultured. E: Epithelial-like, S: Spindle-like, R: Round cells, F: Fibroblastic, Ex: Explant.

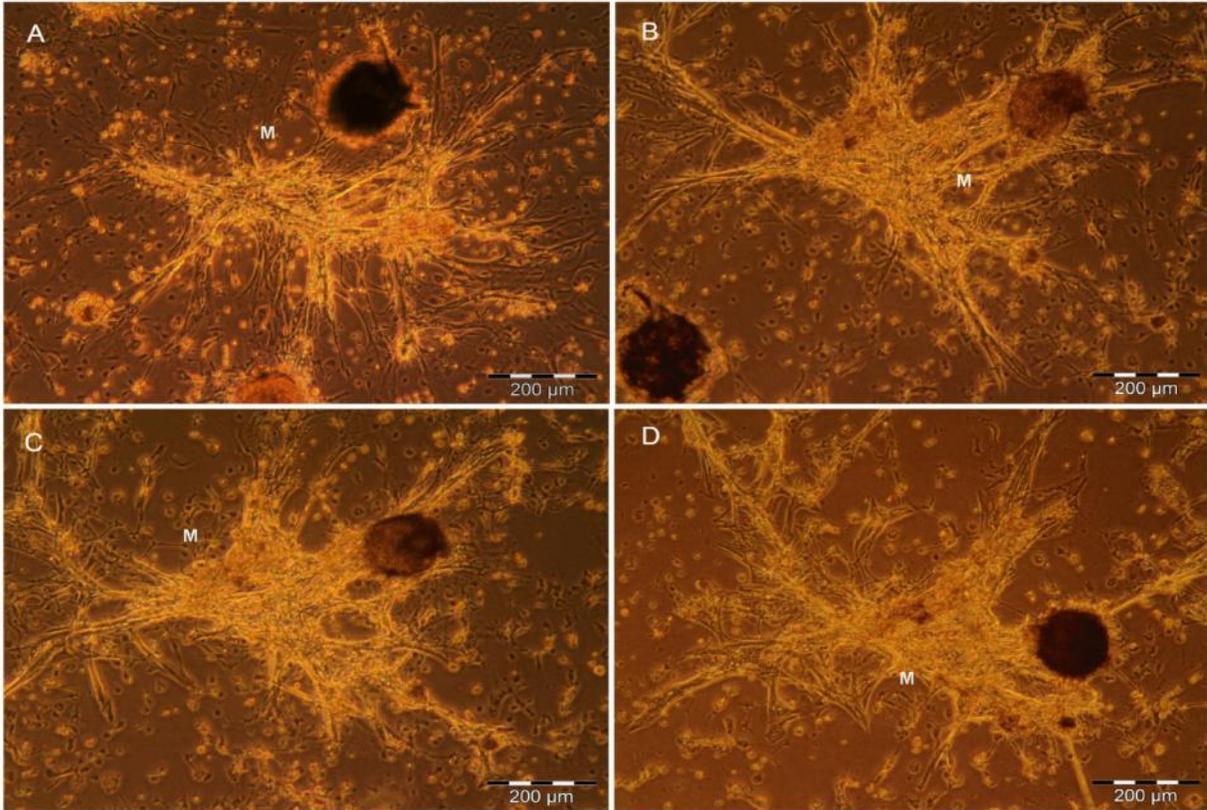


Figure 3. Muscle-like cell population from 34 days (A), 49 days (B), 62 days (C) and 96 days (D) after being cultured. M: Muscle cell explant.

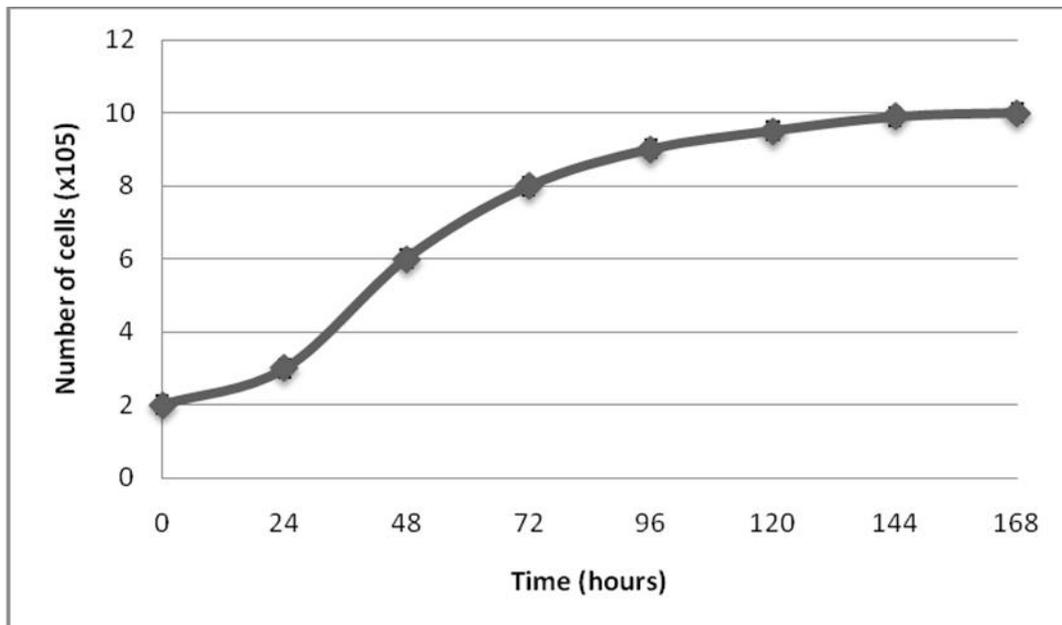
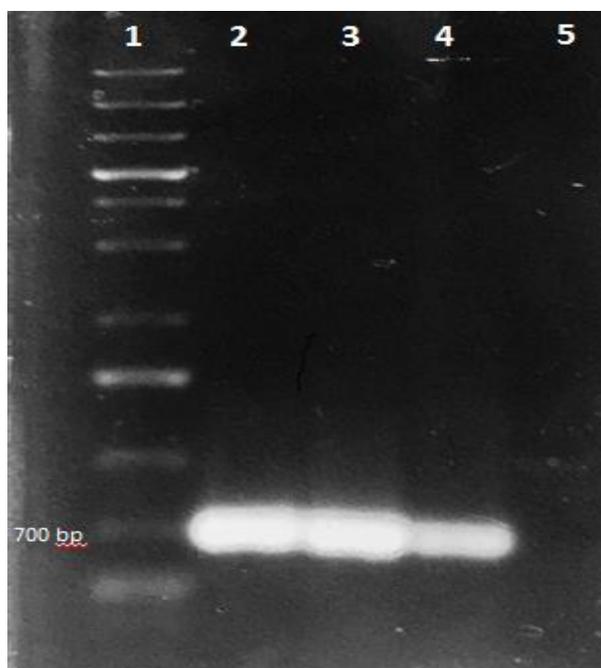


Figure 4. The growth curve of ovary cells. The cell densities in five areas of each flask were determined at 24 h intervals for 7 days incubation. Results are expressed as mean ± SEM of three determinations.



**Figure 5.** Gel electrophoresis of *COI*-PCR products of cell line and silkworm. 1<sup>st</sup> lane: DNA Ladder, 2<sup>nd</sup> and 3<sup>rd</sup> lanes: 710 bp products from cell line, 4<sup>th</sup> lane: 710 bp product from insects, 5<sup>th</sup> lane: Negative control without DNA.

## DISCUSSION

In recent years, many insect cell lines have been established from different insect species. Cell cultures originating from larval ovary (Pant *et al.*, 2002; Khurad *et al.*, 2006; 2009; Pan *et al.*, 2010; Zhang *et al.*, 2014), pupal ovary (Li *et al.*, 2012), pupa (Wu and Wang, 2006; Wu *et al.*, 2012; Yeh *et al.*, 2007), larval fat body (Zhang *et al.*, 2006; Iwanaga *et al.*, 2009; Liu *et al.*, 2015), embryo (Sudeep *et al.*, 2002a; Pan *et al.*, 2007; Matindoost *et al.*, 2008; Soya *et al.*, 2015), neonate larva (Zhang *et al.*, 2012; Ding *et al.*, 2013), larval hemocyte (Sudeep *et al.*, 2002b), testes (Goodman *et al.*, 2001; Can *et al.*, 2017), midgut (Garcia *et al.*, 2001; Li *et al.*, 2015) and neural tissues (Beadle *et al.*, 2006; Soya *et al.*, 2015) were established by different researchers. One of the most investigated species is *Bombyx mori* since it has become a model organism for lepidopteran studies. However, these cell lines originating from different species or tissues have displayed different growth rates and characteristics.

In this study, we aimed to examine cell morphologies and growth characteristics of *Bombyx mori* ovary cells in *in vitro* conditions. In order to do that, we generated two sets of cultures. First set of cultures was used for cell morphology observations and the second one was established to determine the growth rates of the cells. In the first set of primary cultures, cells started to migrate from the explanted ovaries 24 h after the cultures were initiated. Pan *et al.* (2010) noticed in the first days

that only fibroblastic cells extended from the adherent tissue masses (Pan *et al.*, 2010). Also Iwanaga *et al.* (2009) observed that their culture had mainly two types of cells which were round and spindle-like. Khurad *et al.* (2006) discussed in their study that epithelial-like and round refractive cells made up their *Bombyx mori* ovary line. In an ovary cell line studied by Zhang *et al.* (2014), two cell lines showing different morphological characteristics were found. The BmN-SWU1 cell line mainly appeared as elliptical and spindle-like cells while the BmN-SWU2 contained a large number of long needle-shaped cells in addition to round cells. Similar to these studies, we mainly observed four different cell types in our culture, including epithelial, fibroblast, spindle-like and round cells. Among these cell types, epithelial-like cells were observed most abundant. In the first set of cultures, cells were viable until the end of the 3<sup>rd</sup> month. In the beginning of the 4<sup>th</sup> month, significant levels of cell death began and cell concentration decreased dramatically. Also, many vacuole-like structures were observed in these cells. This set of culture was finished at the end of 4<sup>th</sup> month because of the high level of cell death.

In the second set of cultures, the cell amounts were calculated at every 24 h for 7 days and a growth curve was generated (Figure 4). The cell doubling time of our culture was determined to be 3 days (~72 hours). The growth rate of our culture was found similar when it was compared to other cell lines. In the silkworm ovary cell line of Zhang *et al.* (2014), doubling times of BmN-

SWU1 and BmNSWU2 cells were approximately 60 h and 96 h, respectively (Zhang *et al.*, 2014). In addition, Pan *et al.* (2010) found that the doubling time of their ovary cell line was 57.7 hours (Pan *et al.*, 2010). Interestingly, Khurad *et al.* (2013) reported in their primary culture that, cell proliferation from the explants was very slow and it took almost one year to cover the whole area of the culture flask. In that study, the first subculture was made about 356 days after the primary culture was established. The cells were passaged once in 10 days until passage 15. After 15<sup>th</sup> passage, the cells actively proliferated and were passaged at 5 day intervals. In our study, after passage 4, low cell proliferation was observed. It was thought that this condition can be caused by lack of explants in culture flasks.

It was concluded that if this primary culture is supported with growth stimulators, growth rate can be more accelerated and the culture can be used in several types of research fields such as production of recombinant proteins.

**Conflict of Interest:** The authors of this research and article declare that, there is no any conflict of interest regarding the publication and dissemination of knowledge.

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