Observation of Pig Chromaffin Cells in Primary Culture

Chidtraporn Lamom[1], Kyle Lopin[2] and Prattana Lopin[1]*

[1] Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, 65000, Thailand
[2] Department of Physics, Faculty of Science, Naresuan University, Phitsanulok, 65000, Thailand
*Corresponding author. E-mail: prattanas@nu.ac.th

ABSTRACT

Adrenal chromaffin cells play an important role in animal physiology as a part of the sympathetic nervous system. They also have clinical applications in pain therapy, chronic fatigue, and hypertension. Due to their crucial function, chromaffin cells from several different kinds of animals have been used in research. Interestingly, not many people employ pig chromaffin cell for experiments even though pigs show many similarities to human in many physiological aspects. The goal of this study is to observe pig chromaffin cells in primary culture. We developed a primary culture of pig chromaffin cells by isolating cells from the adrenal gland of a pig. Chromaffin cells were identified by neutral red staining. We then observed the morphology, cell size, and cell density at different time points. We found that pig chromaffin cells could be isolated in primary culture, were viable and have a rounded morphology. In culture their size is approximately 15 μm in diameter. This procedure isolated chromaffin cells that could survive for up to 3 days, in sufficient numbers to be useful as an experimental model.

Keywords: pig, chromaffin cell, primary culture

INTRODUCTION

Chromaffin cells reside in the medulla of the adrenal gland. They are innervated by the sympathetic fiber and release the hormones epinephrine and norepinephrine in response to sympathetic activation (Aunis, 1998). Chromaffin cells are considered neuroendocrine cells, as they display both neural and endocrine properties. Epinephrine and norepinephrine released from chromaffin cells adjust the activities of organ systems; for example, they affect vascular tone, and cardiac contractility, resulting in proper body response to difference sympathetic stimulation (Chrousos and Gold, 1992; Habib et al., 2001). In addition to catecholamines, chromaffin cells also secrete peptide transmitters such as enkephalines and neuropeptide Y which have a role in pain relief and vasoconstriction, respectively (Crivellato et al., 2008).

Due to their critical functions, adrenal chromaffin cells of animals, including mouse, rat, cow, cat, and pig, have been used in research to reveal their basic biological mechanisms and to test for clinical applications. Interestingly, pigs have a similar genome (Humphray et al., 2007; Thomas et al., 2003), chromosome structure (Meyers et al., 2005; Murphy et al., 2005), organ structure and function (Tumbleson and Schook, 1996) to humans, more so than any other animal. Studying cells or tissues collected from pig can be a way to obtain biological materials for scientific study that is very similar to human physiology since there is a limitation in using human samples in terms

of availability and human ethics. However, not many laboratories use pig chromaffin cells for research and not much information on how to obtain a primary culture is available. Our goal in this work is to isolate pig chromaffin cells in primary culture, observe their physiology and access their potential as a model system for human physiology.

METHODOLOGY

Chromaffin cells isolation and primary culture

Pig adrenal glands were obtained from a local slaughterhouse and the Naresuan University Animal Care and Use Committee (NUACUC) approved the animal protocol. The glands were cut from the internal organs, put in a clean beaker, covered with foil, and transported on ice to the laboratory within 30 minutes. At the laboratory, the surrounding fat was removed from the adrenal glands. The glands were then perfused with 3 to 4 ml of warm Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, and 5 mM HEPES, adjust pH to 7.4 with NaOH) twice through the vein orifice to rinse off blood inside the glands and incubated at 37°C for 10 minutes. Next, the glands were injected with fresh collagenase solution (1 mg/ml, Worthington) and incubated at 37°C for 20 minutes. This step was repeated twice. After that, three sides of the adrenal glands were cut and the adrenal cortex tissue was separated from the adrenal medulla tissue using forceps to pull the upper and lower parts of the gland apart. The adrenal medulla tissue was collected and minced with a sterile scalpel blade. Minced medulla was digested with fresh collagenase solution and incubated at 37°C for 20 minutes. During incubation, the tissue was carefully triturated every 5 to 10 minutes to separate clumps of tissue using a sterile Pasteur pipette. The digested medulla tissue was then filtered through a 70-μm cell strainer into two 15 ml falcon tube to separate the chromaffin cells. The tubes were filled with 10 ml of warm Locke's solution and centrifuged at 1,000 rpm for 5 minutes. After the first centrifugation, the supernatant was discarded and the cell pellet was resuspended with 10 ml warm Locke's solution and centrifuged again at 1,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended with warm 5 ml DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/ml each). Isolated cells were then plated onto 6 mm diameter culture dish and cultured in the same supplemented DMEM at 37°C in 5% CO_2.

Chromaffin cell identification and morphological studies

Cell viability was determined by the trypan blue dye exclusion assay. Isolated chromaffin cells were incubated with 0.2% trypan blue for 2 minutes. Living and dead cells were then counted using a hemocytometer.

Chromaffin cells were identified by neutral red staining. Cells were incubated with 1 μM neutral red for 5 minutes and then washed with Locke's solution. Fluorescence images were acquired on an Olympus BX-50. Neutral red was excited at 543 nm and the light emitted with a wavelength between 600 and 700 nm was collected.

RESULTS AND DISCUSSION

Morphology and cell identity of pig chromaffin cells

We isolated adrenal chromaffin cells from pig and cultured them for 5 days. To determine chromaffin cell identity, we stained the cells with an acidic dye, neutral

red. This dye has been used as a standard staining method for chromaffin cell identification because the cells contain many acidic granules (Moreno et al., 2010; Role and Perlman, 1980). We found that more than 90% of cells in our culture were stained with neutral red as shown in Figure 1.

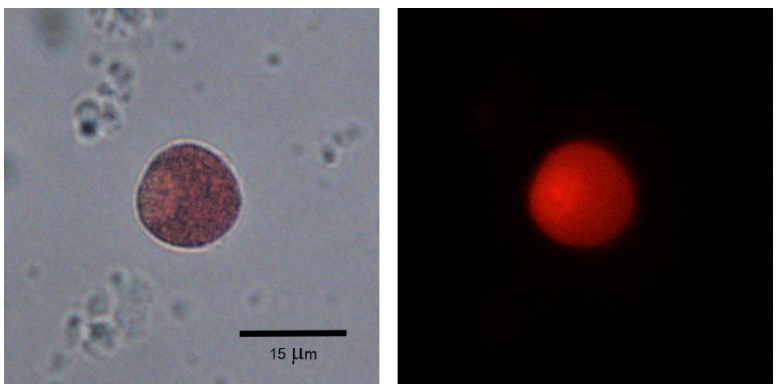

Figure 1 Chromaffin cell identification using neutral red staining: (Left) bright field and (Right) fluorescent images.

Our isolation protocol yielded approximately 90% cell viability tested with trypan blue exclusion assay. After cell plating, we found single chromaffin cells with some cell clusters. Live and healthy chromaffin cells showed a round structure with a clear plasma membrane (Figure 2, left). In contrast, the unhealthy chromaffin cells displayed blebbing or an unclear membrane (Figure 2, right). Pig chromaffin cells observed in our study showed similarity in their morphology to chromaffin cells obtained from the primary culture of other animals (Fenwick et al., 1978; Samasilp et al., 2014).

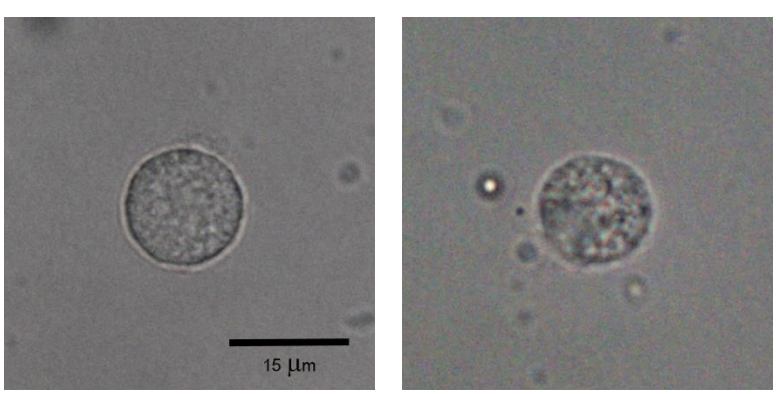

Figure 2 (Left) healthy and (Right) unhealthy pig chromaffin cells.

There were also other cell types in our primary culture. They were likely red blood cells and fibroblasts. These cells can be distinguished from chromaffin cells by their morphology and size. Red blood cells were much smaller than chromaffin cells and most of them did not attach to the culture dish (Figure 3, white arrow). We found a small number of fibroblasts, which had a spindle shape and attached to the culture dish (not shown in figure).

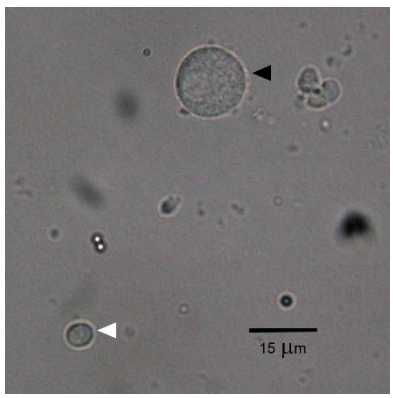

Figure 3 (Red arrow) pig chromaffin cells and (Black arrow) red blood cell.

Morphology of pig chromaffin cells during primary culture

We next observed the changes of the chromaffin cells over time. Figure 4 shows pictures of pig chromaffin cells from our primary culture on day 0, 1, 2, 3, and 4. There were healthy-looking cells in the culture dishes on day 0 (on the day of the cell isolation) until day 3 (Figure 4). After day 3, there was an increase in the number of unhealthy-looking and death cells; therefore, we stopped observing the cells after day 4.

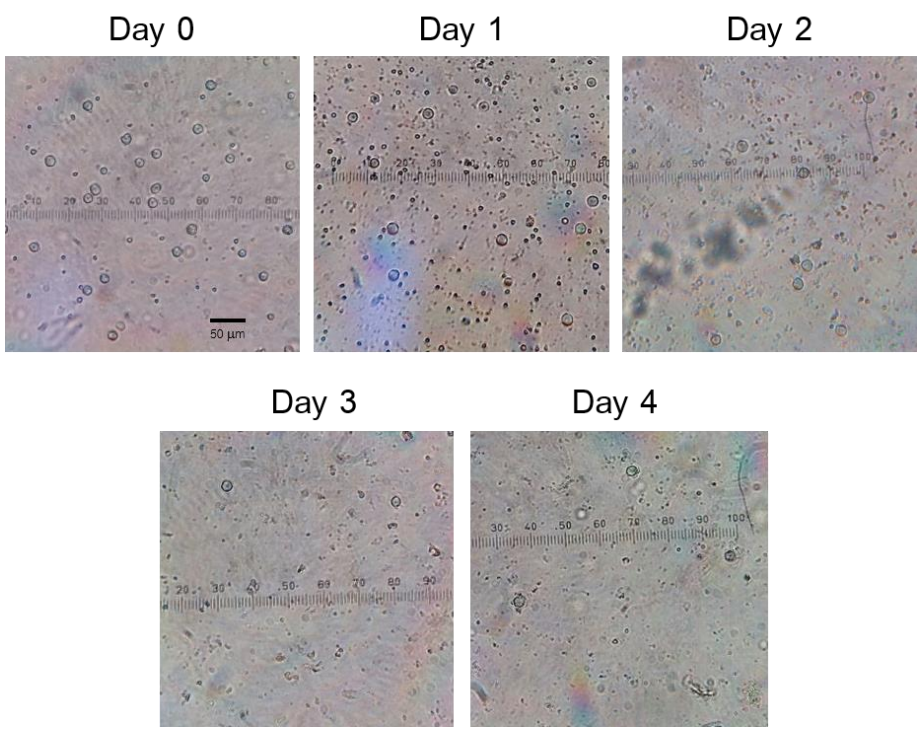

Figure 4 Pig chromaffin cells on Day 0, 1, 2, 3, and 4 of primary culture.

According to this observation, we recommend using pig chromaffin cells that are cultured during day 1 to day 3 for experiments.

Cell size and cell density of pig chromaffin cell primary culture

We measured the size of the chromaffin cell under 32x magnification objective. The average diameter of the pig chromaffin cells was 14.29 ± 0.05 μm (mean ± SE, n = 28 cells). The cell size is in the same range as chromaffin cells obtained from other animal (Fulop et al., 2008; O'Connor et al., 2007; Samasilp et al., 2014). We also counted the number of live and healthy chromaffin cells obtained from our primary culture. Table 1 shows the density of chromaffin cell on day 0, 1, 2, 3, and 4 of culture. We found that the number of healthy chromaffin cell was highest on the first day of isolation and then the cell density decreased over time in the culture (Table 1).

Table 1 Cell density of pig chromaffin cells in primary culture

Day	Average cell density (cell/dish)	SE	Preparation
0	2.16×10^4	7143	2
1	1.86×10^4	3887	4
2	1.66×10^4	3356	4
3	1.21×10^4	2657	4
4	7.86×10^3	728	4

Based on our observation, we suggest that our cell isolation and primary culture protocol is appropriate for single cell studies such as patch clamping. If one needs to use chromaffin cells for biochemical assay, we recommend to further purify the culture to add Percoll or Renografin density gradient step into the protocol to remove other cell types and to get a higher yield of chromaffin cells (O'Connor et al., 2007; Wilson, 1987).

CONCLUSION

In this study, we observed the morphology, cell size, and cell density of pig chromaffin cells for four days in a primary culture. Many healthy-looking chromaffin cells were present on the first three days of the culture and decreased over time. We observed that isolating pig chromaffin cells obtained from a slaughter house can yield a sufficient number of healthy cells that can be used for physiological studies.

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