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Short communication

# The cellular death pattern of primary haemocytes isolated from the black tiger shrimp (*Penaeus monodon*)

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ABSTRACT

A key to successfully generate the penaeid shrimp cell line is to find out how primary cells died. The most suitable period to culture *Penaeus monodon* haemocytes was in the first 48 h of culture because cells had normal morphology, high percent of viable cells (65.29 ± 5.43%), low percent of early (11.75 ± 1.30%) and late apoptotic cells (15.47 ± 11.71%) determined by Annexin V and TUNEL including constant IAP (0.06 ± 0.01–0.07 ± 0.01) and caspase-3 expression (0.30 ± 0.06–0.39 ± 0.10) by real-time PCR throughout the experiment. Moreover, adding 50 and 250 μM of the cell permeable pan caspase inhibitor Z-VAD–FMK produced some melanised cells since the 48$^{th}$ hour, while percent of viable cells was decreased since the 24$^{th}$ hour with no difference in percent of early and late apoptotic cells compared to control at each time point. No difference of IAP and caspase-3 expression level in both Z-VAD–FMK groups was found compared to control and vehicle groups at each time point, excluding caspase-3 in 250 μM Z-VAD–FMK at the 24$^{th}$ hour was higher than control and vehicle. Supplementing sodium fluoride (NaF) induced cell membrane damage and cellular shrinkage of primary haemocytes within 2 h. Even percent of viable cells was reduced down to zero and percent of late apoptotic cells was increased by 2 h of incubation in 25 and 50 mM NaF, IAP and caspase-3 in all NaF groups was not different from control. These results indicate that a number of primary haemocytes derived in this study die through the apoptotic process.

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## 1. Introduction

To succeed in the establishment of permanent cell lines is one of the key factors for increasing the knowledge of developmental biology, molecular biology and biotechnology in animals [1]. In crustacean, no continuous cell line has been established even many approaches e.g. improving the culture conditions, transformation and cellular fusion have been made [2]. If the map of cell death mechanism of each type of primary cells could be drawn clearly, it would further help in finding the way to generate continuous cell lines in the shrimp. In this study, haemocyte, which plays an important role in immunological system and disease prevention [3], was selected to be the model for investigation of cell death mechanism.

Apoptosis is an active form of cell death identified by cellular shrinkage, nuclear and cytoplasm condensation, chromatin fragmentation, cell membrane blebbing without no loss of cellular integrity and phagocytosis of dying cell. While, necrosis is a passive process in association with cellular and organelle swelling, plasma membrane rupture and leakage of cellular contents into the extracellular compartment. Different kinds of factors may stimulate either apoptotic or necrotic cell death depending on the type of cells and severity of injury. Moreover, the death of apoptotic cells requires the sufficient intracellular energy level and the redox state compatible with the caspase activation in order to complete the cell suicidal programme. Therefore, the depletion of ATP or severe oxidative stress possibly drives the apoptotic cell death to necrosis [4,5].

In the black tiger shrimp, some well-known key factors regulating the mechanism of apoptotic cell death e.g. caspase-3 and inhibition of apoptosis protein (IAP) are characterised [6,7], and morphological changes have been examined by some staining markers e.g. propidium iodide (PI), Hoechst, 4′,6-diamidino-2-

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phenylindole (DAPI), Annexin V and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labelling (TUNEL) assay, including the analysis of DNA fragmentation, and the investigation of caspase-3 expression, level and activity [6–12]. However, the determination of cellular death mechanisms with primary cell cultures has not been reported in the penaeid shrimp before. Some experiments dealing with cell death mechanisms were conducted in the isolated haemocytes incubated with nitrite for up to 3 h and lipopolysaccharide (LPS) for up to 2 h before they were then taken to investigate the apoptotic cell ratio by flow cytometry in *P. monodon* [10,13].

In this present study, our first experiment was aimed to figure out the most suitable culture period of primary haemocytes isolated from *P. monodon* by examining the cellular death characteristics of primary haemocytes cultures at different time points before other chemical substances e.g. *N*-Benzyloxycarbonyl-Val-Ala-Asp-[O-methyl]-fluoromethyl ketone (Z-VAD–FMK), a cell-permeant pan caspase inhibitor that irreversibly suppresses the function of caspase proteases and subsequently in the inhibition of apoptotic induction in many mammalian cell types both *in vitro* and *in vivo* [14–16], in association with being known as a chemical-induced necrotic cell death via the kinases of the receptor interacting proteins [17–20], which is called necroptosis [21], was used to test its effect with primary haemocytes in the second experiment. Whereas, fluoride, another chemical substance abundant on the surface of earth that can be found as small amounts in water, air, plants, and animals [22], was selected to determine its effect with primary haemocytes in the third experiment. Fluoride is also well-known as the element producing a variety of cellular and metabolic changes e.g. glycolysis inhibition, changes in membrane receptors and apoptotic induction. Supplement of NaF as the main source of fluoride to mammalian cells can induce apoptosis both *in vitro* and *in vivo* via the reactive oxygen species (ROS)-dependent, caspase-mediated and JNK signalling pathways [4,22–24]. Morphological changes and analysis of Annexin V, a well-known early apoptotic detection marker [25], and TUNEL, the outstanding detection assay of late apoptosis [9,26], including the investigation of the expression level of IAP and caspase-3 genes by real-time PCR technique, were undertaken to indicate the cellular death pattern of primary haemocytes in this study.

## 2. Materials and methods

### 2.1. Chemicals

Most of chemical substances and reagents used in this study were purchased from Sigma-Aldrich.

### 2.2. Animals

Both males and females of juvenile black tiger shrimp at the age of 4–6 months weighing between 15 and 20 g were purchased from Shrimp Genetic Improvement Center, Surat Thani, Thailand, they were kept at Shrimp Biotechnology Business Unit, Pathum Thani, Thailand, before use.

### 2.3. Preparation of haemocyte suspension

Haemolymph was withdrawn into a syringe half filled with 10% sodium acetate and then centrifuged at 3500 rpm for 5 min at 4 °C. The cell pellet was roughly washed with 10% sodium acetate twice before being washed once with culture medium [2× Libovitz L-15, 5% (v/v) lactalbumin solution, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% fungisone (v/v) and 10% each of FCS and shrimp meat extract]. Then, culture medium was used to resuspend the pellet and cells were seeded at the density of 0.8–1 x $10^5$ cells/well of 96-well plate. Trypan blue staining was the first method used to determine cell viability of primary haemocytes [27]. If the positive result of trypan blue staining of primary haemocytes was less than 5%, cells isolated from that shrimp would be further used in the experiments.

### 2.4. Experimental designs

There were three experiments conducted in this study. The first one was to find out the most suitable culture period of primary haemocytes by determining the death characteristics of primary haemocytes cultured in the medium at 24, 48, 72 and 96 h of culture. The second one was aimed to test the effect of Z-VAD–FMK at the dosage of 50 and 250 μM on primary haemocyte cultures whether it can prolong primary haemocytes in the culture or not, while the last experiment was to examine the effect of NaF at the concentrations of 5, 25 and 50 mM on primary haemocytes. In the second experiment, no addition of Z-VAD–FMK in the culture medium was used as the control group, while supplement of 0.2% DMSO in the medium was represented as the vehicle control. In case of the third experiment, no NaF supplemented in the culture medium was served as the control group. Culture medium was changed half daily and cells were roughly determined for the apoptotic and necrotic characteristics by using the morphological changes. The analysis of apoptotic cell death was performed using Annexin V and TUNEL assays, together with examining the level of IAP and caspase-3 genes by real-time PCR technique.

### 2.5. Detection of cellular morphological changes by Annexin V assay

Samples obtained from primary haemocytes seeding at the density of 0.8–1 x $10^5$ cells/well of 96-well plate in the culture medium for 24, 48, 72 and 96 h, together with samples collected from primary haemocytes treated with different dosages of Z-VAD–FMK for 24 and 48 h including those added with different concentrations of NaF for 10 min, 2 and 24 h were analysed with Annexin V assay modified from the manufacturer's instructions (catalog no. ab66108, abcam). Briefly, cells were washed twice with cold PBS (3×) and then followed by Annexin-binding buffer (1×) once. The combination of 50 μl Annexin-binding buffer (1×) and 2.5 μl Annexin V conjugate was added and incubated with cells at room temperature for 30 min in the dark before 1 μl of 100 μg/ml PI working solution was applied to incubate with cells at room temperature in the dark for 5 min. Then, samples were washed 3 times with Annexin-binding buffer (1×). Finally, cells were observed under an Olympus inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan). The excitation of 494 nm and the emission of 518 nm were undertaken for detecting Annexin V intensity, and the excitation of 535 nm and the emission of 617 nm for PI. Apoptotic cells showed Annexin V staining of the cellular membrane, while the late stage of apoptosis or necrosis showed both membrane staining by Annexin V and strong nuclear staining from the red PI, or only PI positive staining. The percent of viable cells was calculated by this equation, {[(Numbers of total cells - early apoptotic cells – late apoptosis/necrosis)/numbers of total cells] x 100, the percent of early apoptosis was calculated by this equation, {[(Numbers of Annexin V-positive cells - merge cells)/numbers of total cells] x 100} and the percent of late apoptosis/necrosis was calculated by this equation, {[(Numbers of PI-positive cells)/numbers of total cells] x 100}. In general, 2–4 representative fields of at least 100 cells were captured and scored. These pictures were taken using a magnification of 640×.

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