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Culture and exploration on in vitro explant and instigating nacre gem development of freshwater pearl mussel mantle epithelial cell *Sinohyriopsis cumingii*

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Abstract

The foundation of natural pearl formation by mussels is calcium carbonate in the form of aragonite crystals, secreted essentially by the epithelial cells of mantle tissue as nacre. The *in vitro* explant culture of nacre secreting pallial mantle explants of freshwater pearl mussel was a vital step in the approach to the establishment of quality of pearl mussel species, by screening pearl mussel species that are able to form pearls with high efficiency. Moreover, the results of this research provide knowledge for the future *in vitro* colored pearl production. The aims of this research were to culture freshwater pearl mussel mantle epithelial cells and to investigate the capacity of their nacre-secretion when they were induced by some specific factors such as Ca^{2+}, FGF-2 and EGF. In this research, mantle epithelial cells were cultured in four different kinds of medium (DMEM/F12, L15-M199, IMDM, TCM) and temperature [(4, 24°C and room temperature (28°C)] to select the suitable environment for pearl mussel mantle epithelial cell culture for at least one month. After that, old medium was changed by fresh medium supplemented with three inducers (Ca^{2+}, FGF, EGF). Nacre secretion of these cells was evaluated via the nacre formation in culture medium. The results showed that the mantle epithelial cells may be cultured *in vitro* and secrete nacre in DMEM/F12 medium supplemented with 10% FBS, but the efficiency of secretion was independent with different inducers investigated. DMEM/F12 medium is the best for growing of mantle epithelial cells while IMDM medium is suitable for heamocyte –like cells.

Keywords: DMEM/F12, Ca^{2+}, EGF, FGF, Freshwater mussel, mantle epithelial cells, nacre secretion.

INTRODUCTION

In Vietnam and other countries (that is, Japan, China, India, etc.), the culture of mussels to produce pearl is carried out by combining natural conditions with core and tissue implanting (Janaki, 2003). This method brings a good result, increasing the pearl yield, reducing time, thus the profit is higher. However, it also has some difficulties and risks such as the effect of natural conditions on mussels, that is, mussel pathologies and a decrease on the ratio of good pearl production. Besides, the effect of core and tissue implanting is still low as well as limited pearl quantity because of long time for a pearl in natural condition. The research on the selection of mussel species with both high capacity of producing nacre and high quality of nacre was never performed neither in our country nor around the world. This may be due to the limit and complication of methods used to evaluate and screening.

In vitro mussel culture and the induction of nacre secretion may open doors to new screening methods of mussel quality and *in vitro* pearl production. In theory, the culture of nacre secretion cells and *in vitro* pearl production are promising and may overcome many disadvantages of traditional methods. The first manuscript on the successful culture of nacre secretion cells from mussels was published by Machii et al. (1989).

In 1961, the results from Wada showed that in pearl production, *Pinctada fucata*, pearl was formed from nacre crystals (Wada, 1961). After that Machii cultured mussel

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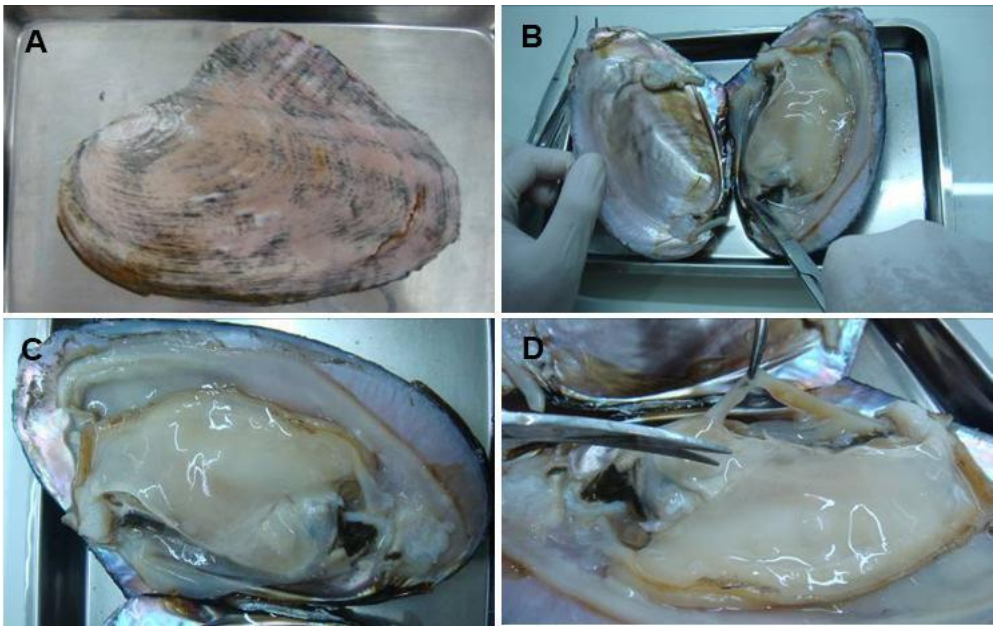

Figure 1. Mussel dissection method. Mussels were (A) processed with clear cover; (B) cut out cover-holding muscle; (C) ready to obtain tissues; (D) isolated epithelium.

mantle epithelial *P. fucata* by serum drop in Petri dish. In his culture, there are a lot of epithelial cells as well as myocytes appearing. And organic matters were accumulated in culture as well as nacre crystals (Machii and Wada, 1989). In 1994, Samata performed mantle epithelial cell culture and nacre crystals were formed after 15-30 day. Nacre crystals composed calcium and sulpher detected by SEM (scanning electron microscope) and EDS (*Energy Dispersive X-ray* Spectroscopy) analysis (Samta et al., 1994). Until now, nacre crystals were produced successfully from mussel mantle epithelial cell culture (Suzuki and Mori, 1991; Awaji and Suzuki, 1998; Dharmaraj and Suja, 2001; Shi et al., 2002; Barik et al., 2004; Li et al., 2005; Sugishita et al., 2005). However, efforts in *in vitro* creating pearls failed. So that some researchers used this method to study nacre production process (Pereira-Mouries, 2003; Tsukamoto et al., 2004; Zhang et al., 2004; Chen et al., 2005; Suja and Dharmaraj, 2005; Gong et al., 2008a, b).

In nature, nacre is secreted by mussel epithelial cells as a body's reaction for strange agents such as bacteria. Nacre is produced by the mixture of calcium carbonate and some proteins (the major protein is conchiolin) which are produced by mantle epithelial cells. However, under *in vitro* conditions, nacre may be induced when mussels contact with some stress causing agents, included in the culture medium. *In vitro* nacre was formed from matrix protein that mantle epithelial cells expressed (Mayumi et al., 2006) and calcium carbonate as component of culture medium.

Thus, the objectives of this research were to culture *in vitro* nacre secretion cells using different culture mediums (DMEM/F12, L15-M199, IMDM, TCM199) and temperature [4 24°C and room temperature (28°C)] and to examine cell capacity of nacre secretion induced by specific stress factors (Ca^{2+}, FGF, EGF).

MATERIALS AND METHODS

Mussel sterilization

Fresh mussels *Sinohyriopsis cumingii* in the size range of 35 × 20 – 55 × 38 mm in length × breadth were suppled from pear farms in Dongnai province (Vietnam). They were cultured for at least one week at laboratory in autoclaved tap water. After that, they were carefully washed by soap and pure water, rubbed with metal brush until the black outer cover was removed. Mussels were placed in an UVA chamber for 20 min, the mussel mouth was then open and sterilized with ethanol 70% for 60 s.

Isolation and sterilization mussel epithelium

Sterilized mussels were dissected to obtain mantle epithelium (Figure 1), which was sterilized by two different methods. Method A (Gong et al., 2008): tissue samples were soaked in antibiotic solution A (fungizone 2000 µg, penicillin 100.000 IU, streptomycine 100.000 µg, kanamycin 500.000 µg in 10 ml distilled water, sterilized by filter) four times, 15 s for each time. Then, samples were washed again with distilled water four times, 15 s per time. This last procedure was repeated three times. Method B (Edward and Frank, 1979): tissue samples were washed in pure water for six times, then soaked in ethanol 10% for 15 s. Afterwards, samples were firstly washed in distilled water four times; secondly steeped in antibiotic solution B (fungizone 5.000 µg, penicillin 200.000 IU, streptomycin 200.000 µg, kanamycin 100.000 µg, gentamycine

Table 1. The average bacteria quantity per area from 10 random areas.

Bacteria quantity per area (40X)	0 h	12 h	24 h
Method A	714.82	921.37	949.57
Method B	160.50	278.05	371.21

124.000 µg in 10 ml distilled water, sterilized by filter) for 10 min; and, finally, were washed in sterilized distilled water for six times.

The effect of decontamination by these two methods was evaluated through: the quantity of bacteria in the same area under microscope which was selected randomly, each well was counted for ten times; the degree of increasing or decreasing bacteria quantity after 0, 12 and 24 h.

Studying culture medium

The 1-2 mm^2 tissue fragments were cultured in 3.5 cm disk (Nunc, Roskilde, Denmark). All disks were divided in four treatments: each treatment consisted of three culturing disk with four different medium: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), Leibovitz: TCM 199 (L15-M199), Iscove's Modified Dulbecco's Medium (IMDM), Tissue culture medium 199 (TCM) (all bought from Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (Sigma-Aldrich, St Louis, MO); all disks were cultured in the same condition 24°C, 5% CO_2. The culture medium was evaluated by four criterions: the time at which spreading cells appear, the percentage of tissue fragments appeared spreading cells, the proliferation of cells in culture times after 12, 24 and 120 h.

Studying culture temperature

Cells cultured in IMDM 10% FBS in 3.5 cm dish (Nunc, Roskilde, Denmark) were divided in three treatments, three disks for each treatment, cultured in three different temperatures: 4, 24°C and room temperature (28°C). Evaluating temperature condition was based on: the time at which cells appearing, the quantity of cells according to time (cell proliferation rate).

Flow cytometry method

In different culture medium, isolated cells were analyzed in size and drop degree of cells. Cells were obtained, centrifuged and re-suspended at the concentration of 10^6cells/ml in Facsflow solution. They were analyzed in FASCalibur (BD Bioscience) machine using CellQuest Pro software with 10.000 events analyzed in two channels FSC and SSC.

Inducing nacre secretion and evaluating nacre secretion

All tissue fragments cultured in the best conditions of experimental studying will be used to culturing mantle tissue cells on 3.5 cm dish (Nunc, Roskilde, Denmark). Each dish contained 15 tissue fragments from mantle of one mussel which has size from 1-2 mm^2. After cells from tissue fragments spread and adhered surround their fragment, culture medium was replaced by nacre secretion inducing medium. Nacre secretion of *in vitro* culturing epithelial cells was evaluated by the appearance of nacre crystal under microscope. Treatment 1: Observing the appearance of nacre crystal after 7, 15, 21, 30, 37, 45, 52 and 60 culture days. Medium was changed per four days. Treatment 2: Evaluating the nacre production when adding some agents: 90% of nacre is $CaCO_3$, while the concentration of Ca^{2+} in medium is so low (according to D'MEM/F12), we supplemented Ca^{2+} into medium like the protocol of differentiation mesenchymal stem cells to osteoblasts, with fixed amounts is 100 mM. Treatment 3: Studying the ability of nacre formation when supplementing two growth factor bFGF (basic-fibroblast growth factor) (10 ng/ml) and EGF (epidermal growth factor) (20 ng/ml) (all bought from Sigma-Aldrich, St Louis, MO). These two factors stimulate the development of mantle epithelium. Thus, they may stimulate nacre secretion.

The quantity of nacre crystal from culture disk were obtained from these disks by the techniques of filtering all cells and medium through 75 µm filter (BD Bioscience) to receive crystals on the surface of filter. Then, the number of crystals of each disk was counted directly under microscope in grid Petri dish (Nunc, Roskilde, Denmark).

RESULTS

Decontamination effect

In method A, the bacteria quantity increased in studying time but it is insignificant. After 48 h, culture samples did not be opaque. However, the number of bacteria was still high in these samples. The reason could be the soaking time in antibiotic was not adequate to destroy bacteria effectively. And in method B, the number of bacteria increased a little. The bacteria quantity in medium was not much. Culture samples were not opaque after 48 h.

In Table 1, the increasing degree of bacteria in Method B was faster than Method A. However, method B was better than method A because the number of bacteria in method B was lower significantly.

Development and proliferation effects in different medium

DMEM/F12 medium

After 12 h, cells began to spread from tissue fragments, approximately one fragment per disk did not appear cells (3.33%). Compare with the research of Barik et al. (2004), cells only spread after 24 culture h. Thus, this research's result had spreading cells sooner. In the first stage, cells proliferated slowly (5.24%). The reason was the effect of many factors: (1) Cells might not adapt to new medium, (2) Contamination factors: even thought tissues were processed to reject contamination, it is impossible to remove all bacteria. This is one of the most difficulties in culturing mollusc cells. The proliferation of bacteria is much times faster than cells, thus it is required to supplement antibiotic to restrict and destroy bacteria. Supplementing antibiotics also limits the proliferation speed of cells. (3) Changing medium much times causes stress for cells: bacteria increasing quickly in culture medium both spend much nutrition factors, and change the physical and chemical properties of medium in the harmful direction for cells pH decreasing, a lot of hazardous wastes of bacteria. To solve this problem, it is

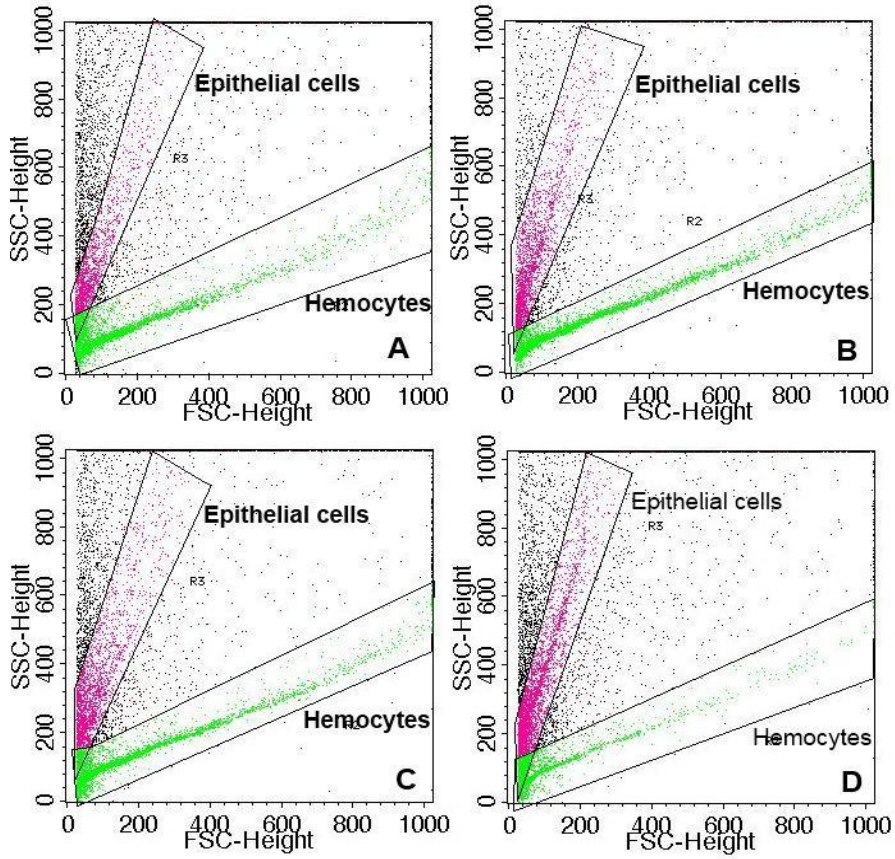

Figure 2. Results of analysis of primary culture cell population according to size and cell complexity (A) TCM-199, (B) L15-M199, (C) IMDM, (D) DMEM/F12.

needed to carry out the strategy of changing medium every 12 h. This strategy helps solve two problems: removing bacteria in medium and supply adequately nutrition factors for cells. However, this strategy also caused cells losing in suspension. Reaching to 120 h, the number of cells increased 147.85% compared to the time of 12 h (Figure 4). This proliferation results proved that cells were stable and they began to increase in number. It is showed that after decontamination time, bacteria were nearly completely removed, because the number of bacteria was low, antibiotics in medium were enough to restraint the proliferation of bacteria; furthermore, cells began to adapt to new medium.

Culture medium had two advantageous populations that were R2 and R3. R2 were the cells like heamocyte (cells have large size, low drop degree in cytoplasm), R3 were epithelial populations (cells have small size, high drop degree in cytoplasm) (Figure 3). Therefore, when culturing tissue fragments in DMEM/F12, the ratio of epithelial cells (42.18%) is more advantageous than hemocyte (20.23%) in the cells population. Outside the R2, R3 area, cells have average size and drop degree between two specific populations. Indeed, two R2 populations could be detected under microscope: one kind has no nucleus, not adhering and one kind has nucleus and adhering. Based on the diagram (Figure 2.), R2 population had irregular size and non-concentrated cytoplasm, this is hemocyte cells. R3 population had regular size and concentrated cytoplasm, this is epithelial cells. This result is similar to other research all over the world, they also reported that there is the appearance of these two kinds of cells when culturing tissue fragments from molluse with high ratio.

L15-M199 medium

After 12 culture hours, all tissue fragments appeared spreading cells (Figure 3). Compare with the research of culturing mantle tissue cells of Gong et al. 2008, cells like hemocyte appeared after 4 days (96 h). In this medium, there are two populations similar to DMEM/F12 medium, but the ratio of each type of cells was different: the percentage of epithelial cells and hemocyte population were 23.72 and 58.14% respectively.

IMDM medium

After 12 h, spreading cells appeared from tissue fragments

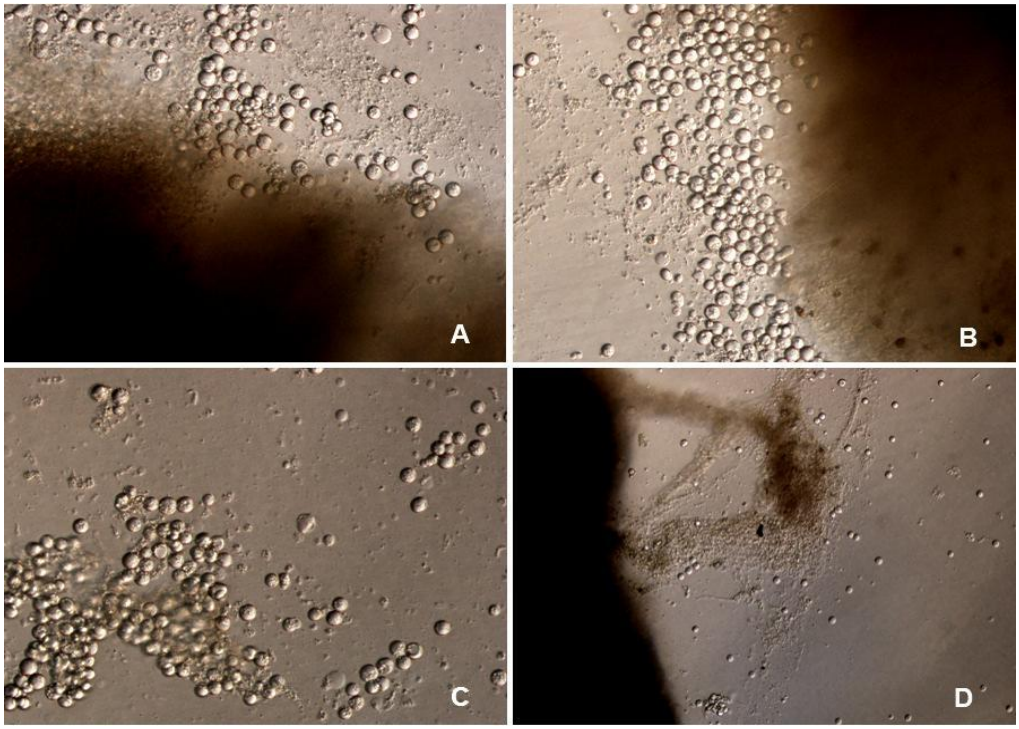

Figure 3. Cells developed after 120 culture h (A) DMEM/F12; (B) L15-M199; (C) IMDM; (D) TCM.

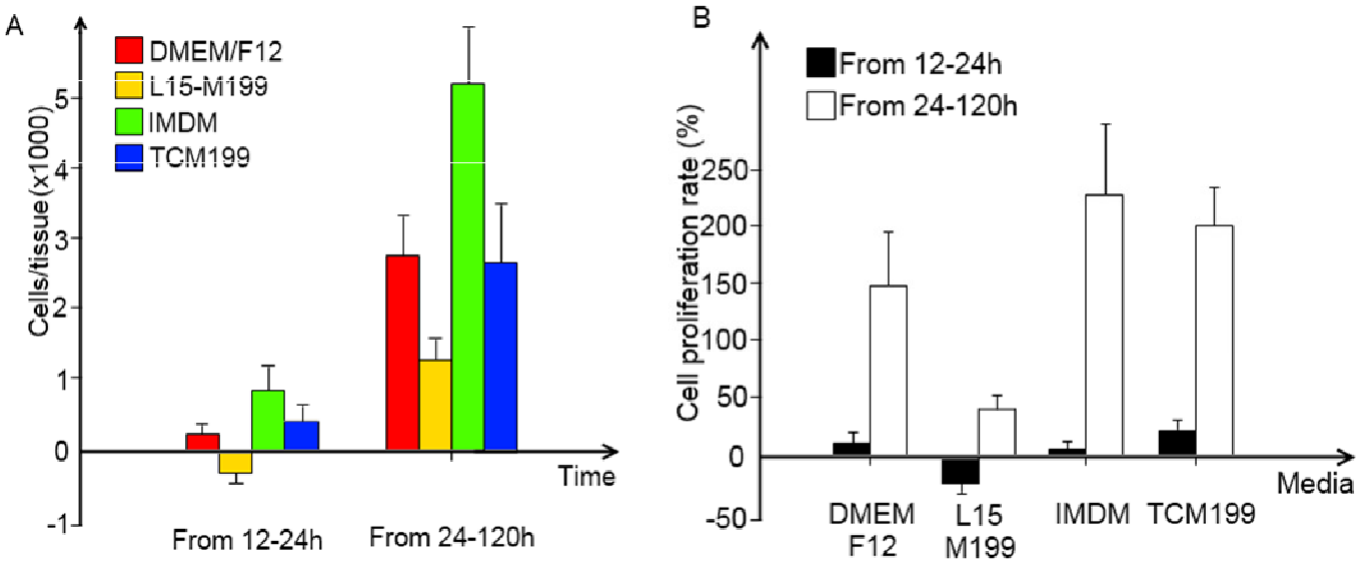

Figure 4. (A) Proliferation of cells according to time and (B) Cell proliferation rate in four media.

with the ratio of 97.66%. In the first stage (12-24 h), cells increased in number slowly, about 4.41%. In the next stage, cells adapted to new medium and proliferated strongly. Similar to two above medium, IMDM medium also have two advantageous cells population: hemocyte (42.12%) and epithelial cells (25.40%).

TCM medium

In the stage of 0-12 h of culture, cells appeared surrounding tissue fragments. In the stage of 12-24 h, TCM medium had the highest rate of proliferation cells among four kinds of medium (Figure 4). There were two

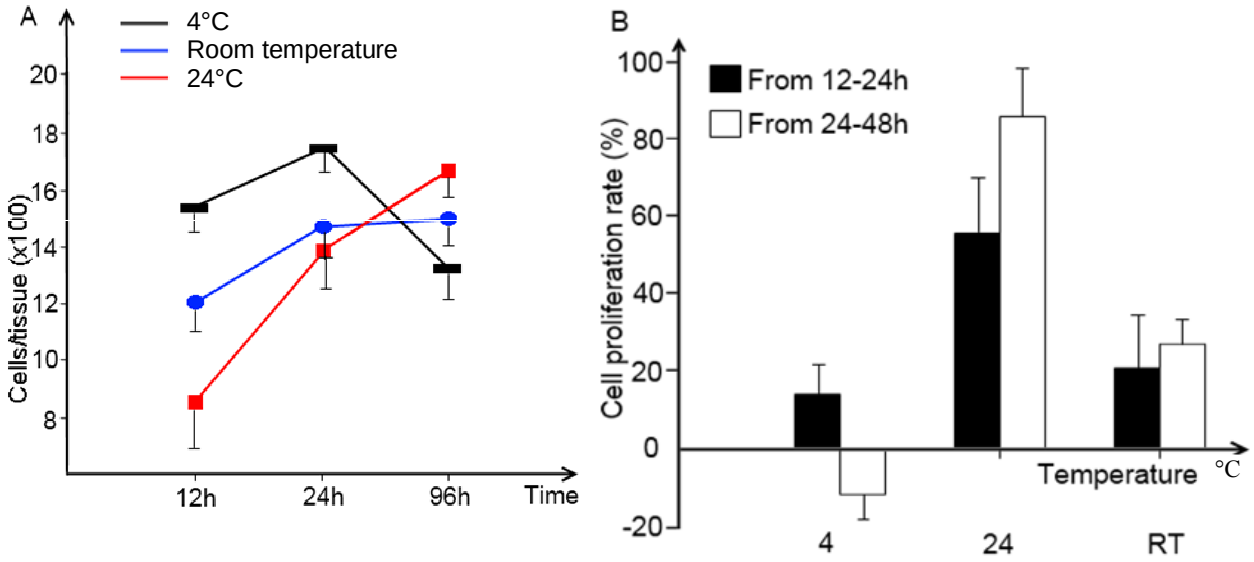

Figure 5. (A) Cell number per tissue fragment and (B) Cell proliferation rate according to time.

advantageous cells population that were hemocyte and epithelial cells. The ratio of epithelial cells is low (20.18%), the ratio of cells like hemocyte is high (43.73%).

Proliferation effect of cells at different temperature

At 4°C: The appearance of spreading cells was not different from other temperatures. In the first stage, the number of cells was increasing but not rapidly. It is proved that this temperature was not advantageous for cells proliferation. In the stage of 24-96 h, the cells number decreased. The reasons could be: (1) temperature was not favourable: metabolic process was slow when temperature decreased. Thus, the speed of biochemical reaction inside the cells will be slow much more than the cells cultured in suitable temperature. At 4°C, many enzymes were passive or inactive. However, cells did not stop all metabolic reactions, some cells adapted to low temperature by themselves, others could be died, the number of cells decreased. (2) Changing medium many times loses cells in suspension. Therefore, the selecting process might happen, cells generations could be stable after time.

At 24°C: During culturing time, the number of cells increased stably. In nature, mussels in Vietnam usually grow and develop well at temperature from 17 to 24°C, in *in vitro* culture cells had suitable conditions to proliferate.

Room temperature (28°C): The appearance time of spreading cells was 0-12 h. In this side, there was no difference to other temperatures. Because room temperature always changed from 20-28°C, on the favourable temperature cells could proliferate well, on the contrary, cells could be inhibited and decreased in number. In the first stage, cells developed well. In the next stage, cells increased slowly. There were some dead tissue fragments. The reason could be unstable temperature, biochemical processes were influenced. Moreover, cells might secrete inducing factors to adapt to cells concentration and surrounding environmental. When changing temperature, cells have to change function in the direction of adapting new medium in priority. Thus, proliferation speed will be slower.

Based on diagram 5A (Figure 5A), cells at 24°C had proliferation level better than other methods. When processing the data of each tissue fragments using StatGraphics software gave *P-value* > 0.05 in both three culture time. Thus, the results were not different significantly. The reason could be the un-identical size between tissue fragments, changing medium much times caused losing cells unequally. All these reason made an error on counting process. To improve the errors of counting average cells per tissue fragment, we evaluated the proliferation based on comparing the percentage of average proliferation cells at other times to 12 h. The results showed that there were differences significantly between proliferation rates at three temperature value. And 24°C is preferable for culturing mussel epithelial cells (Figure 5B).

Nacre secretion induction

Culturing in different inducing medium, after 30 days, all samples appeared nacre. The crystals have rectangular, cube shape with smooth angles and reflection of light

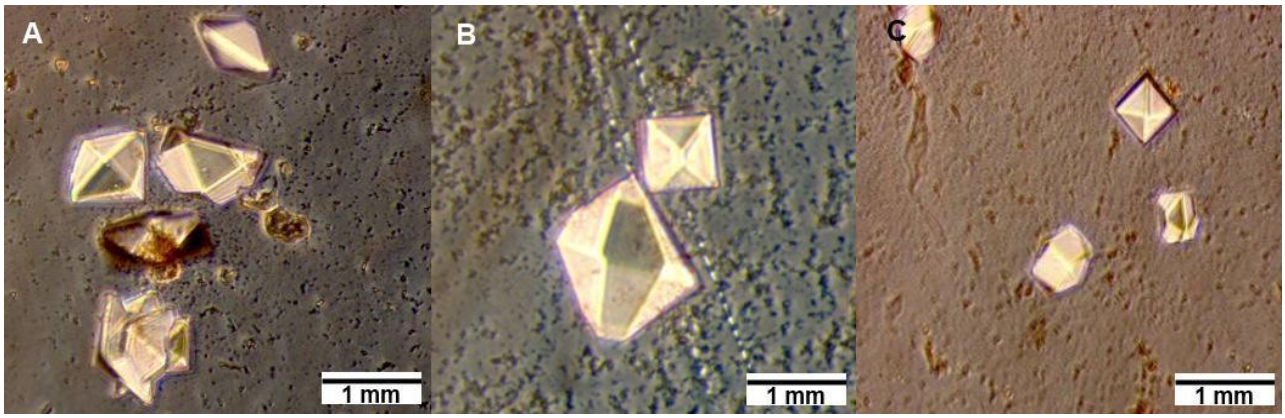

Figure 6. The crystals appeared in inducing medium at 200X magnification (A, D), 100X (B, E) and 50X (C, F) respectively of non-supplied inducing factors medium, Ca^{2+} supplementing medium, EGF and FGF supplementing medium after 45 days.

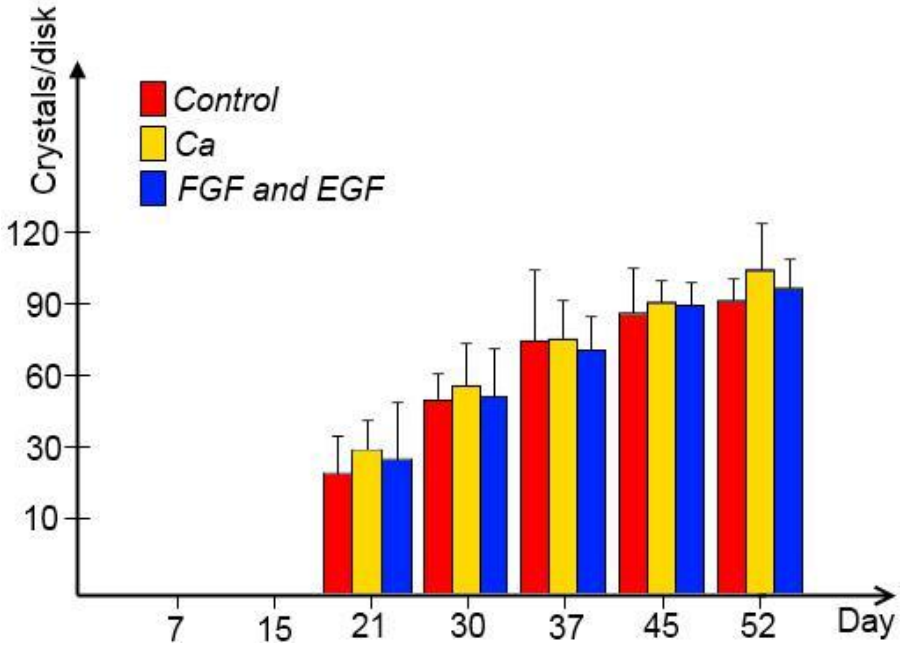

Figure 7. Increasing of crystals according to time in 3 treatments (control, supplementing Ca^{2+}, supplementing EGF and FGF).

(Figure 6). This result is similar to the result of Panha and Phansuwan (1996), and Domart-Coulon et al., (2001).

In the studying of L15-M199 medium, it contains Calcium salt solution, thus the crystals appearing in the culture disk were aragonite. This is the result of biological mineralization changing calcium salt to aragonite crystals contained $CaCO_3$ through epithelial mantle cells. This is proved that epithelial cells supply frame for nacre crystals, while control samples (only medium) did not have these frames, thus nacre crystals did not appear. However, the crystals appearing on this research should be evaluated by other profound methods such as X-ray absorbent, micro laser analysis, observing by scanning electromagnetic microscope, etc. to determine the characteristics and qualify the nacre crystals.

Supplying some inducing factors such as Ca^{2+}, FGF or EGF almost do not have nacre production effect (Figure 7). In the treatment of culturing cells in normal condition, cells still produced nacre at 30 days. At that time, treatment 2 and 3 appeared nacre crystals in culture disks. The number of nacre crystals increased in time at all three treatment; however proliferation degree was insignificant after 45 days. At each time, the number of nacre crystals per disk is equal, with 35.5±1.7, 75.2±5.5,

80.1±6.9, 100.8±3.4 and 110.3±8.2/disk for 30, 37, 45, 52 and 60 days, respectively.

DISCUSSION

Medium is the most important condition to help the animal cells proliferation. So that, from the first medium established for maintaining the tissue animal outside body by Sydney Ringer on 19th century to now, there are many kinds media were created. DMEM/F12, IMDM, TCM-199 and L-15 are the popular media in tissue and cell animal culture. Different media are differences in components, especially vitamins, amino acids, mineral salts… These differences effect to osmotic pressure, charge and nutrition which are suitable for some kinds of cells from different species or different organs in same body. So that, this research aimed at identifying which medium is suitable for mantle epithelial cells from freshwater pear mussels. Besides culture medium, temperature also is important factor effecting to success of cell culture. In this research, we evaluated the cell growth at 24°C based on the freshwater temperature. Moreover, we investigated the cell growth at 4°C and room temperature (28°C).

The results showed that all medium used in this research permitted mantle cell growth. At the first studying time (12 h), the spreading cells in four medium all appeared. The number of tissue fragments having spreading cells was different insignificantly. Based on this result, it might not be conclude that what is the best medium. It might be criticized that nacre producing cells of mussel are strong proliferation cells adapted to many kinds of medium. All four kinds of above medium might be suitable for culturing nacre producing cells of mussel. Calculating the average amount showed that cells in IMDM medium have highest speed of proliferation. However, this difference was not meaningful in statistics at reliability of 95%.

The comparison based on percentage of cells in time, the result showed that in the stage of 12-24 h, TCM medium had the highest percentage of proliferation cells. In the next stage, IMDM medium gave the highest rate of proliferation cells.

About cells composition, all three kinds of medium appeared both two kinds of cells (epithelial and hemocyte), in which hemocytes took advantage in IMDM and TCM medium, meanwhile epithelial cells took advantage in L15-M199 and DMEM/F12. Because of the culture condition, DMEM/F12 have to use bicarbonate buffer, we recommend that it should be used L15-M199 in later research because this medium uses HEPES buffer (independent on CO_2/bicarbonate buffer).

Because we want to produce *in vitro* pearls; we investigated some inducers which effect to nacre secretion. Ca^{2+} is main component of nacre. So it is used as an inducer for stimulating and enhancing nacre secretion in this research. Besides EGF and FGF are growth factors which usually stimulate epithelial cells and fibroblast. We hoped that they induced mantle epithelial cell growth. However, this result showed that Ca^{2+}, EGF and FGF were not nacre production inducing factor. Epithelial cells have this ability. Thus, we think that mechanical impacts on tissue and cells are inducing factors to produce nacre. Culturing time was longer, the concentration of cells was more dense, cells get older, and that could be the reason why nacre appear with lower speed in time.

Conclusions

From the results, treatments, primary culture method of epithelial cells are suggested as below: decontamination methods follows Edward and Frank (2004), culture temperature is 24°C and medium is L15-M199. Using this method, cells will appear after 12 h, but cells population is not identical about proliferation speed. Nacre crystals increase in time (for 60 days) and nacre secretion does not depend on supplementing some agents like EGF, FGF and Ca^{2+} at studying concentration.

The appearance of nacre crystals when culturing mussel mantle tissue cells is an important premise for searching and evaluating the pearl production of mussel to construct evaluating and screening techniques of good pearl producing mussel as well as directing to obtain *in vitro* nacre crystals for cosmetic application, pharmaceuticals and further application like producing pearl *in vitro.*

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