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Effect of media additives on biomineralization in primary cultures of mantle cells of *Paphia malabarica*

Lambert Niyoyitungiye[1, 3*], Marc Ndayisenga[2, 4], Simon Buhungu[1], Prudence Bararunyeretse[1]
[1] Department of Biology, Faculty of Sciences, University of Burundi, Bujumbura, Burundi
[2] Department of Biochemistry, Applied Pedagogy Institute, University of Burundi, Bujumbura, Burundi
[3] Department of Life Science and Bioinformatics, Assam University, Silchar, Assam, India
[4] Departement of Zoology, Goa University, Taleigao Plateau, Goa, India

Abstract
Media additives are believed to influence biomineralization *in vitro* mediated by mantle cells of *P. malabarica*. $CaCl_2$ and $MgCl_2$ were tested as media additives in the present study using 2 x L-15 solution to establish primary cultures for evaluation of their effect on biomineralization. Primary culture inoculated with media composition without additives was also designed to serve as control experiment. Results were collected through an inverted microscope-Olympus (Model no. CKX41). Our results demonstrate that both $CaCl_2$ and $MgCl_2$ additives influence biomineralization by increasing the rate of crystal deposition and the number of crystals in mantle cells primary cultures of *Paphia malabarica*.

Keywords: media additives, mantle cells, *Paphia malabarica*, Biomineralization

Introduction
Cell culture from marine bivalves is an important technique to investigate the mechanism of shell formation. Biomineralization *in vitro* has been studied by many researchers using mantle cells primary cultures from various species of shelled molluscs [1, 2, 3, 4, 5, 6]. Together, these studies provide important insights into some important aspects of cell and developmental biology. Mantle cells are believed to play a huge role in shell biomineralization by secreting a variety of matrix proteins that control crystal nucleation, growth and morphology [6]. In their natural living conditions, bivalves accumulate different chemicals in their tissues by filtering large volumes of water [4]. They uptake Calcium and bicarbonate ions from their environment and metabolize them to form Calcium carbonate found in the shell [1]. These ions are transported across the mantle epithelium into an Extrapallial Fluid (EPF), a solution in which the deposition of $CaCO_3$ crystals occurs [7]. In the process, protein secreted by the mantle surrounds the individual crystals and becomes the cement which binds them together as a shell [8]. The study such as that conducted by Wilbur and Jodrey [9] (1955) suggests that a part of shell carbonate has its origin in the carbon dioxide of metabolic processes, and its alternative source would be bicarbonate of seawater. L-15 medium is commonly used along with various additives to create an appropriate artificial environment for mantle cell growth and proliferation to study the *in vitro* biomineralization. Nevertheless, there is very little scientific understanding of biomineralization effect of media additives in mantle cells primary culture. The present study is a preliminary effort to evaluate the effect of Calcium chloride ($CaCl_2$) and Magnesium chloride ($MgCl_2$) on biomineralization in primary culture of mantle cells of *Paphia malabarica*.

Materials and Methods
Preparation of culture media
2 x L-15 medium was used in the present study as shown in the table 1. This was prepared by dissolving 21 g of L-15 into 1000 ml of distilled water. 100 ml of the solution was kept into a labeled sterile bottle (A) to be further used for control experiment. The remaining solution was kept into a beaker to serve for media additives preparation.

Preparation of media composition with $CaCl_2$
$CaCl_2$ solutions were prepared by dissolving 0.021 g and 0.014 g into two beakers, B and C respectively, each containing 100 ml of 2 x L-15 to make up a final concentration of 1.9 mM and 1.3 mM. pH of the medium was adjusted to 7.5.

Preparation of media composition with $MgCl_2$
$MgCl_2$ solutions were prepared by dissolving 0.14 g and 0.094 g into two beakers, D and E respectively, each containing 100 ml of 2 x L-15 to make up a final concentration of 14.7 mM and 9.9 mM. pH of the medium was adjusted to 7.5.

Media sterilization
Solutions into beakers A, B, C, D, and E were sterilized separately into a laminar air flow by filtration with 0.22 µm Millipore filter unit. After filtration, media were kept into sterilized bottle that were labeled to indicate the higher and lower concentrations. Media were finally placed into a refrigerator set at 16°C temperature prior to cell culture activity.

Table 1: Preparation of L-15 medium with additives tested

Sr.no	Medium	Formulations	Additive conc.
1	A	2 x L-15 (100 ml)	
2	B	2 x L-15 (100ml) + $CaCl_2$ (0.021 g)	1.9 mM
3	C	2 x L-15 (100ml) + $CaCl_2$ (0.014 g)	1.3 mM
4	D	2 x L-15 (100ml) + $MgCl_2$ (0.14 g)	14.7 mM
5	E	2 x L-15 (100ml) + $MgCl_2$ (0.094 g)	9.9 mM

Sr.no: Serial number, Conc.: Concentration

Establishment of mantle cells primary culture

Bivalves *P. malabarica* were obtained from Panjim local fish market (Goa state, India) and were maintained in seawater with antibiotics for 24 hours to reduce contamination. All procedures were carried out under a laminar flow hood. Bivalves were first sprayed with 70 % alcohol and kept in Petri dish under a laminar flow hood in order to dry. They were then opened in separate Petri dish with a scalpel. The mantle membrane was cut-off and scrapped-off the shell with the help of forceps and transferred to Petri dish containing L-15 medium. 10 mg/ml streptomycin, 2 µg/mL Nystatin and 10,000 IU/mL Penicillin were mixed and the solution were added to the basal medium for initial washing of tissue pieces after dissection and antibiotic antimycotic solution of Hi-Media was used as antibiotic drops at the time of final inoculation of the culture to prevent bacterial contamination.

The same rinsing procedure was repeated twice maintaining each time for 15 minutes in basal medium with antibiotics. Tissue pieces were now kept on sterile slide and the tissues were cut into pieces of dimensions of 2 mm^2 by using a sharp sterile surgical blade.

These pieces were then rinsed again in basal medium with antibiotics. For mechanical dissociation, tissue pieces were transferred to sterile 15 mL centrifuge tube with basal medium containing antibiotics and were triturated with ten strokes in and out of 10 mL pipette. Suspension was then filtered through appropriate nylon filter to sieve separated cells. Filtrate obtained from this process was subjected to centrifugation at 500g for 3 min to pellet out cells and were given three washes with inoculating medium. Pelleted cells were resuspended in inoculating medium and viable cell density count was performed. In brief, a sample of media containing the cells in suspension was added to the trypan blue dye.

The number of viable cells excluding the dye per square on a 1 mm^3 was counted with the help of inverted microscope and haemocytometer. The average of four counts was taken to denote the cell density at that given time. Cell count was adjusted to approximately 1 x 10^6 cells/mLBivalves *P. malabarica* were obtained from Panjim local fish market (Goa state, India) and were maintained in seawater with antibiotics for 24 hours to reduce contamination. All procedures were carried out under a laminar flow hood. Bivalves were first sprayed with 70 % alcohol and kept in Petri dish under a laminar flow hood in order to dry. They were then opened in separate Petri dish with a scalpel. The mantle membrane was cut-off and scrapped-off the shell with the help of forceps and transferred to Petri dish containing L-15 medium. 10 mg/ml streptomycin, 2 µg/mL Nystatin and 10,000 IU/mL Penicillin were mixed and the solution were added to the basal medium for initial washing of tissue pieces after dissection and antibiotic antimycotic solution of Hi-Media was used as antibiotic drops at the time of final inoculation of the culture to prevent bacterial contamination. The same rinsing procedure was repeated twice maintaining each time for 15 minutes in basal medium with antibiotics. Tissue pieces were now kept on sterile slide and the tissues were cut into pieces of dimensions of 2 mm^2 by using a sharp sterile surgical blade. These pieces were then rinsed again in basal medium with antibiotics. For mechanical dissociation, tissue pieces were transferred to sterile 15 mL centrifuge tube with basal medium containing antibiotics and were triturated with ten strokes in and out of 10 mL pipette. Suspension was then filtered through appropriate nylon filter to sieve separated cells. Filtrate obtained from this process was subjected to centrifugation at 500g for 3 min to pellet out cells and were given three washes with inoculating medium.

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- **Media suitability for biomineralization:** As shown in the table 2, suitability of media composition for biomineralization activity of primary free cell cultures of mantle of *p. malabarica* was split into three sets with their subsets as follows:
- **Control set:** Cultures set up and inoculated with 2 x L-15 medium without additive served as control sets and these were performed in triplicates. 2 mL of cell suspension in inoculating medium at 1 x 10^6 cells/mL was dispensed in UV activated Petri dishes. Petri dishes were then covered and sealed with parafilm, labeled and transferred to incubator set at 25^{o}C temperature.

- **Experimental set 1:** Cultures set up and inoculated with 2 x L-15 medium containing $CaCl_2$ (0.021 g, 0.014 g) served as experimental set 1 with two subsets to account for two different concentrations of $CaCl_2$ (1.9Mm, 1.3 mM). Each subset was performed in triplicates. 2 mL of cell suspension in inoculating medium at 1 x 10^6 cells/mL was dispensed in UV activated Petri dishes (diameter 20 mm). Petri dishes were then covered and sealed with parafilm, labeled and transferred to incubator set at 25°C temperature.
- **Experimental set 2:** Cultures set up and inoculated with 2 x L-15 medium containing $MgCl_2$ (0.14 g, 0.094 g) served as experimental set 1 with two subsets to account for two different concentrations of $MgCl_2$ (14.7 mM, 9.9mM). Each subset was performed in triplicates. 2 mL of cell suspension in inoculating medium at 1 x 10^6 Cells/mL was dispensed in UV activated Petri dishes.

  Petri dishes were then covered and sealed with parafilm, labeled and transferred to incubator set at 25°C temperature. Culturing of cells in many Petri dishes allowed us to select only those having no traces of contamination.

Detection of crystals

Cultured mantle cells were monitored everyday with an inverted microscope-Olympus. Photographs were taken and crystals counted to make comparison between the three types of experiments (i.e. control set, experimental set 1, and experimental set 2) by analyzing the differences in terms of the rate of crystals deposition and the number of crystals recorded. These allowed us to determine the effect of additive used on mantle cells-mediated biomineralization.

Table 2: Experimental design for primary free cell culture inoculation

Sr.no	Medium	Experiment	Additive conc.	Cell density	Vol/dish	No. of dishes
1	A	Control set	-	$1x10^6$ cells/mL	2 mL	3
2	B	Exp. set 1, subset 1	1.9 mM $CaCl_2$	$1x10^6$ cells/mL	2 mL	3
3	C	Exp. set 1, subset 2	1.3 mM $CaCl_2$	$1x10^6$cells/mL	2 mL	3
4	D	Exp. set 2, subset 1	14.7 mM $MgCl_2$	$1x10^6$ cells/mL	2 mL	3
5	E	Exp. set 2, subset 2	9.9 mM $MgCl_2$	$1x10^6$ cells/mL	2 mL	3

Results

In order to evaluate biomineralization effect of media additives, various concentrations of $CaCl_2$ and $MgCl_2$ were prepared using 2 x L-15 solution to serve for primary mantle cells culture. Besides this experimental design, a control setup was also planned for comparison. Results were obtained through microscopic observation and these were systematically recorded and analyzed after 10, 20, and 30 days of culturing. Two main factors such as the time (in days) after which crystal deposition was detected and the crystal numbers were took into consideration for our results analysis.

Mantle cells-mediated crystal deposition

The results of the correlational analysis are shown in the table 3. No crystal grew after 1 day either in primary cultures with additives or in control experiment. This result indicates that biomineralization does not occur spontaneously in cultured mantle cells. Small particles were observed attached to the cover surfaces of dishes in an attempt of forming a monolayer (Figure 3 A_1, Figure 4 B_1 and C_1, Figure 5 D_1 and E_1). These particles were assumed to be mantle cells. Crystals were recorded between 10-15 days both in control group and experimental sets 1 and 2. They appeared in the areas where mantle cell aggregates occurred indicating that mantle cells are directly involved in biomineralization. After 10 days, crystals were recorded only in primary cultures with additives whereas in control group they appeared after 25 days. Taken together, these results suggest that the rate of crystal deposition in cultured mantle cells is more favored when culture medium is supplemented with $CaCl_2$ or $MgCl_2$ additives.

Crystal growth and evolution in numbers

As it can be seen in the table 3, more crystals were recorded in experimental sets 1 and 2 than in control group. The number of crystals increased over time. The higher the concentration of additive, the better was the number of crystals. On day 25, crystals reached a final growth phase with somehow specific morphology showing a characteristic of biomineralization product (Figure 3 A_3, Figure 4 B_3 and C_3, Figure 5 D_3 and E_3

Table 3: Number of crystals recorded for each type of experiment per time unit.

Sr. No	Experiment type	No. of crystals recorded			Magnification
		Day 1	day 10	Day 25	
1	Control set	0	0	4	x 40
2	Exp. set 1, subset 1	0	4	7	x 40
3	Exp. set 1, subset 2	0	2	5	x 40
4	Exp. set 2, subset 1	0	2	6	x 40
5	Exp. set 2, subset 2	0	1	5	x 40

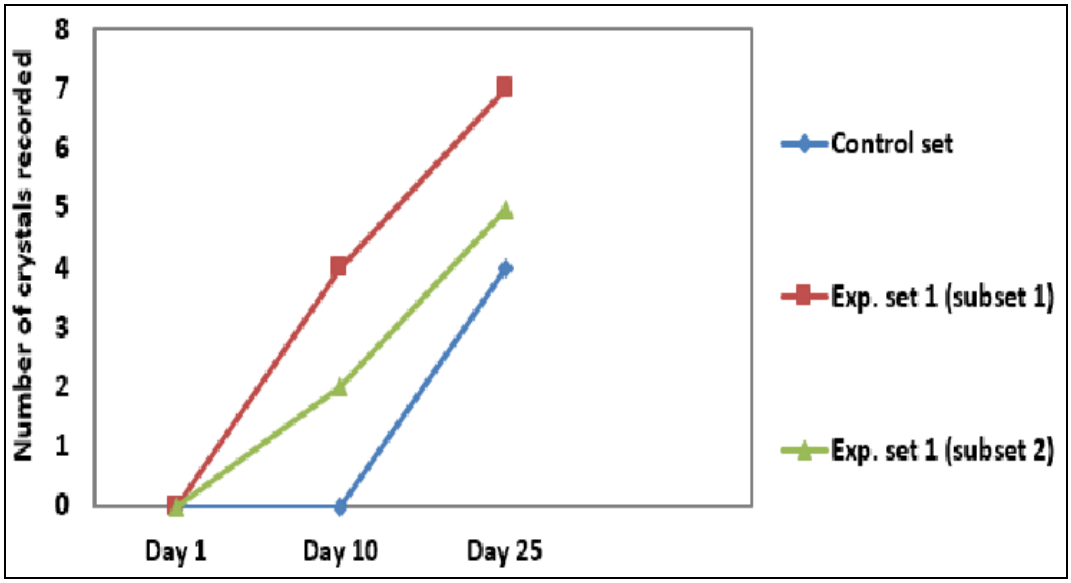

Fig 1: Number of crystals over time: comparison between control and experimental set 1

The figure 1 illustrates the evolution in numbers of crystals in control group and experimental set 1 (Exp. set 1). Subset 1 and 2 represent cultures set up with media composition containing 1.9 mM $CaCl_2$ and 1.3 mM $CaCl_2$ respectively. After 25 days, crystal numbers increased with $CaCl_2$ concentration used. Primary culture set up without additive (control set) shows less numbers of crystals.

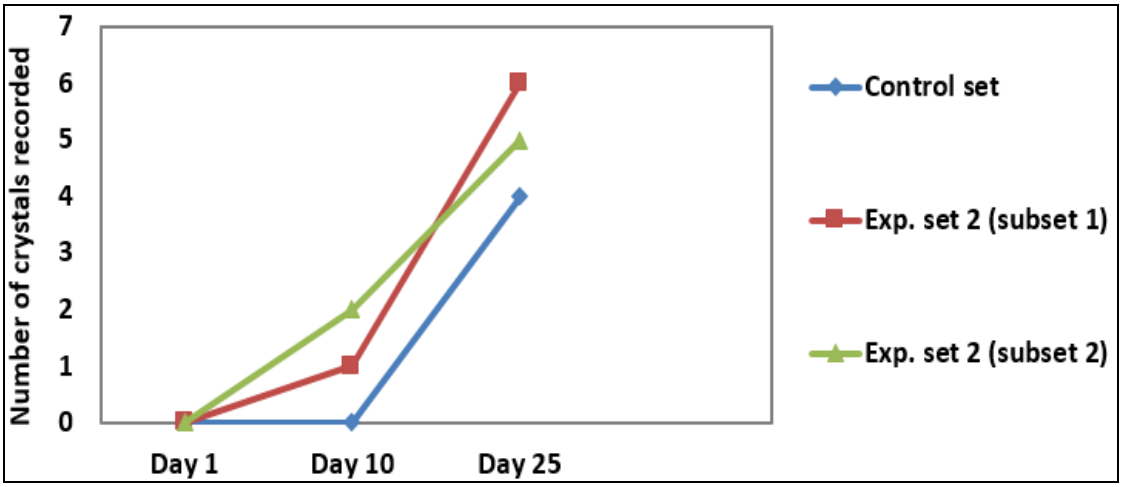

Fig 2: Number of crystals over Time: comparison between control and experimental set 2

The figure 2 illustrates the evolution in numbers of crystals recorded in control set and experimental set 2 (Exp. set 2). Subset 1 and 2 represent cultures set up with media composition containing 1.9mM $MgCl_2$ and 1.3mM $MgCl_2$ respectively. After 25 days, the number of crystals increased with $MgCl_2$ concentration used. Primary culture set up without additive (control set) shows less numbers of crystals.

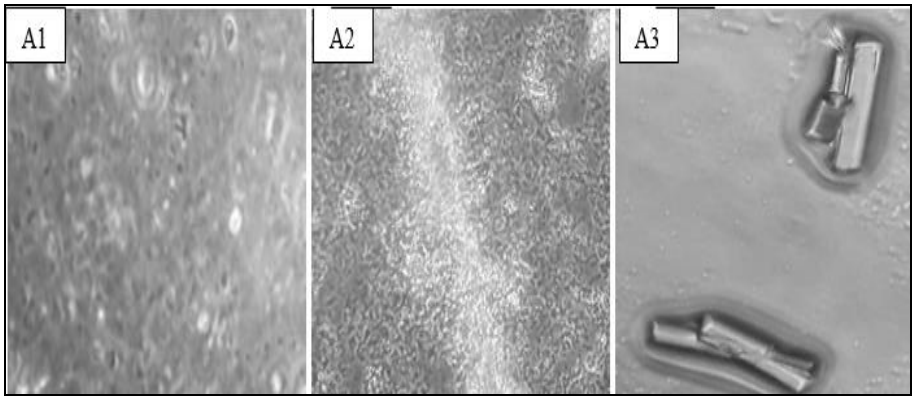

Fig 3: Control A1, A2, and A3, primary cultures on day 1, 15, and 25 respectively. No crystal appeared after 1 day and 10 days (A1, A2). Prismatic-Shaped crystals appeared after 25 days.

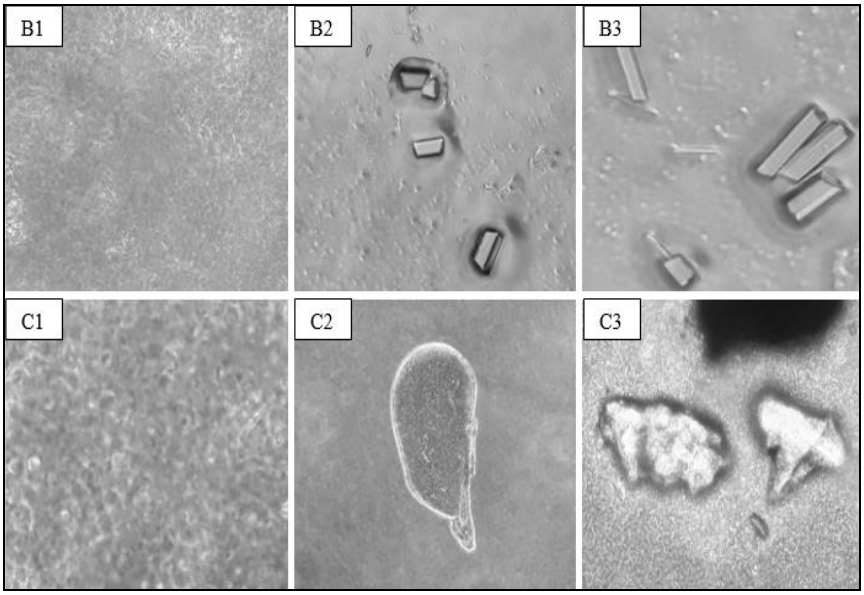

Fig 4: Experimental set 1 B_1, B_2, B_3, primary cultures set up with 2 x L-15 supplemented with $CaCl_2$ in a concentration of 1.9 mM on day 1, 10, and 25 respectively. C_1, C_2, C_3, primary cultures set up with 1.5 x L-15 supplemented with $CaCl_2$ in a concentration of 1.3 mM on day 1, 10, and 25 respectively. No crystal grew on day 1(B1, C1). Growing prismatic-shaped crystals surrounded by mantle cells were recorded in primary culture set up and inoculated with 1.9 mM $CaCl_2$ (B2). These crystals were piled up after 25 days (B3). A crystal with large mantle cells aggregate adhered on the surface in an attempt of forming crystal appeared after 10 days in primary culture set up and inoculated with 1.3 mM $CaCl_2$ (C2). Shaped crystals were observed after 25 days (C3).

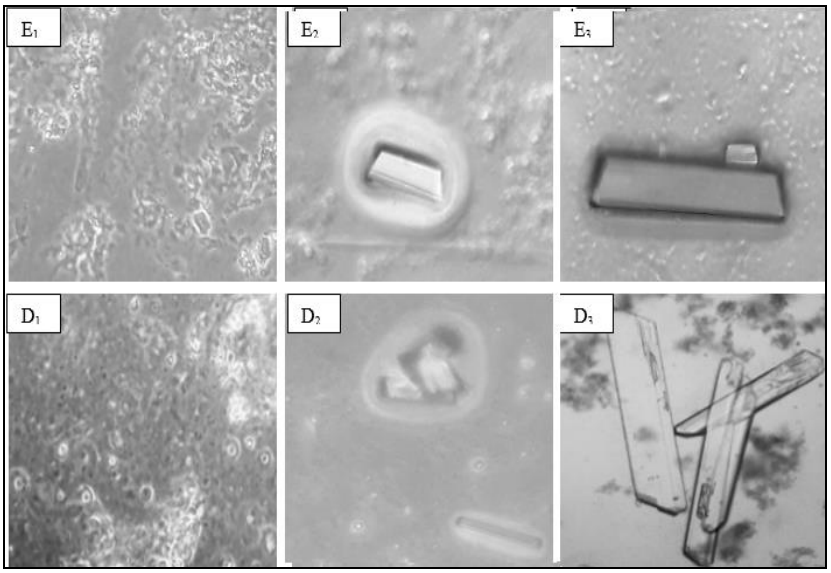

Fig 5: Experimental set 2. E1, E2, E3, Primary Cultures set up and inoculated with media composition containing 14.7 mM $MgCl_2$ on day 1, 10, 25 respectively. D_1, D_2, D_3, primary cultures set up and inoculated with media composition containing 9.9 ml $MgCl_2$ on day 1, 10, and 25 respectively. No crystal grew on day 1. After 10 days, small prismatic crystals appeared (E_2, D_2). On day 25, these were merged up together (E3, D3).

Discussions

The present study was designed to determine the effect of media additives on biomineralization in primary cultures of mantle cells of *P. malabarica*. $CaCl_2$ (1.9mM and 1.3mM) and $MgCl_2$ (14.7mM and 9.9mM) were tested as additives. Crystal formation in primary culture results from the normal metabolic activity of mantle cells [10, 11]. This observation was also in agreement with our results showing the presence of crystals both in control group and primary cultures inoculated with additives as a result of physiological activity of mantle cells. Furthermore, it is noteworthy that when our additives were added to culture media, they increased crystallization rate and the number of crystals. Interestingly, the more additive concentration, the better was the rate of crystal deposition and crystal numbers in cultured mantle cells. In general, it seems possible that $CaCl_2$ and $MgCl_2$ in an appropriate concentration are well-suited for biomineralization *in vitro*. A possible explanation for this might be that the additives used interacted with mantle cells to increase the secretion of soluble substances responsible for biomineralization. However, this result has not previously been described but it has been demonstrated by others that mantle cells have the ability to secrete matrix proteins that are involved in crystal nucleation, growth and remodeling [12]. This combination of findings provides an opportunity to develop our understanding of some important aspects of cell and developmental biology. Further studies on the current topic are therefore recommended to

characterize various crystals induced by mantle cells under the influence of $CaCl_2$ or $MgCl_2$ *in vitro* experiments.

Conclusion

The present study was designed to determine the effect of media additives on biomineralization in primary cultures of mantle cells of *P. malabarica*. Our findings support that the *in vitro* biomineralization is more favored when $CaCl_2$ or $MgCl_2$ is added to 2xL-15 medium. Thus, the results of this investigation show that these additives influence biomineralization processes by increasing both crystallization rate and crystals.

Declaration of Conflicting Interests

The authors declare that they have no potential conflict of interest to report with respect to the research, authorship and publication of this article.

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