$CaCO_3$ crystallization in primary culture of mantle epithelial cells of freshwater pearl mussel

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The foundation of natural pearl formation by molluscs 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 *Lamellidens marginalis* was attempted and vital steps to accomplish this included depuration of pearl mussels with different physical and chemical agents to eradicate various commensal flora and fauna, removal of pallial mantle ribbon, aseptic preparation of explants from the ribbon and transfer of those explants into tissue-culture petri dishes. Special synthetic tissue-culture media enriched with additives, viz. inactivated calf foetal serum and antibiotics were poured into plates with explants. The culture plates were incubated in a carbon dioxide incubator at 5% CO_2 and at 30°C. After 12 h epithelial-like cells began to migrate out and they formed a complete cell sheet surrounding the explant within 7–10 days. Most importantly, cells in the culture indicated functional viability, as subsequently after 38–40 days of culture typical aragonitic crystals of $CaCO_3$ could be observed throughout the culture plates.

PEARLS have been admired and adored as a symbol of beauty, power and love. Natural pearls are formed when an irritant like a grain of sand is swept into the pearl mollusc and is lodged within it where it gets coated by a microthin layer of nacre, a silvery substance that is about 90% $CaCO_3$. The mantle epithelial cells are directly responsible for pearl formation by the deposition of aragonitic $CaCO_3$ crystals and the secretion of an organic protein matrix, conchiolin, to hold them. However, the cellular mechanism signalling nacre secretion is yet to be fully elucidated. Primary culture of outer epithelial cells of the mantle tissue is important to study the pearl-formation process and most reports in this aspect have been concentrated on marine pearl oyster. The present investigation is on tropical freshwater pearl-producing mussel, *Lamellidens marginalis*. Research is being conducted by us to establish a sustainable and functional cell line of mantle epithelial cells of *L. marginalis*, as a preliminary investigation to carry out further applied research on tissue-cultured pearl.

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L. marginalis is predominantly used for pearl production in freshwaters in the Indian context as it is abundantly distributed throughout the freshwater habitats of the country. Pearls are produced either by cavity-insertion method, where a shell bead (< 6.0 mm diameter) is placed into the umbonal cavity between the outer mantle layer and inner shell surface[1], or by grafting technique which is a delicate surgical intervention conducted by skilled personnel[2]. In case of the former, the mantle epithelial cells produce nacreous coatings around the bead to give rise to a shell-attached pearl. During grafting, a live tissue fragment or graft (approximately 3 mm^2) prepared from the pallial zone of the mantle of a donor pearl mussel is implanted into the gonad or mantle tissue of the recipient pearl mussel. A small bead, often referred to as the nucleus (approximate diameter, 3–6 mm) and made generally from the shell of freshwater mussel is also inserted to be in contact with the graft. After the operation, the outer epithelial cells of the mantle graft become squamous, migrate and proliferate to encapsulate the nucleus. They eventually form a follicle called the pearl sac around the bead and start secreting pearl or nacreous layers on the latter[3]. Hence, the quality of pearl is directly related to the differences in function of the epithelial cells. Detailed physiological studies on function of epithelial cells, however, have not been conducted adequately, mainly due to unavailability of a proper experimental model. However, one of the techniques that will serve to partly reveal the function of mantle epithelial cells is primary culture of epithelial cells. While studies have been carried out mainly with regard to marine pearl oyster tissue culture, the observations made in the present investigation are from experiments with *L. marginalis*. The effect of depuration and antibiotics on cell proliferation from mantle tissue of *Haliotis varia*[4] and *Pinctada fucata*[5,6] and effect of tissue extracts on abalone explant culture[7] are some of the preliminary studies made on cell culture of marine mussels in the Indian context.

L. marginalis (> 8.0 cm in shell length and > 35.0 g in wet weight) was collected from ponds situated inside the campus of the Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga and brought to laboratory within 2–3 h. The encrusted and superficial mud, flora and fauna, if any, were removed by scraping with the help of a hard brush and a sharp knife. The mussels were kept in clean freshwater without feeding for 48–72 h with daily water exchange, to reduce any further external risk of contamination before initiating the cell culture experiment[8]. The mussels were then kept immersed in CaO (7.5 mg l^{-1}) solution for 3 days to eradicate leeches[9] followed by repeated washings in clean, aged tap water for 24 h. Later, the mussels were subjected to 1% (v/v) sodium hypochlorite immersion treatment for 24 h for effective surface disinfection[8]. The mussels were then washed thoroughly in clean, aged tap water several times for 2 days to remove residues of the chemicals used in preceding

steps, which might interfere during the cell culture operation. Finally, the mussels were given an immersion treatment with chloramphenicol (100 mg l^{-1}) for 24 h.

All the surgical instruments and glassware were sterilized. Before operating, the pre-depurated mussels were washed repeatedly with sterile tap water followed by thorough wiping with sterile cotton swabs soaked in 70% alcohol. The animals were then exposed to UV light for 30 min for further sterilization. The mussels were opened by cutting both the adductor muscles situated at their anterior and posterior ends. Then the pallial ribbon along the pallial line (a strip of around 1 cm width along the ventral side of the animal) was cut and removed from the shell. The ribbon was transferred immediately into a petri dish with pre-cooled, sterile, isotonic salt solution (NaCl 8.26 g, KCl 0.36 g, $MgSO_4$ 1.60 g, $MgCl_2$ 0.73 g, $CaCl_2$ 0.37 g, $NaHCO_3$ 0.10 g, NaH_2PO_4 0.01 g, glucose 0.10 g, distilled water 1000 ml) on ice.

The pallial mantle ribbon is slippery to handle due to the presence of slime and mucus, which were removed by keeping the ribbon in a sterile petri dish on ice and gently scraping (once or twice) with a sterile, absorbent cotton pad soaked in pre-cooled, sterile isotonic salt solution. The mantle ribbon was then transferred to another petri dish on ice and initially trimmed (from all four sides) to a size of 5–6 cm length and 4 mm width. The mantle strip was then washed 6–8 times with pre-cooled sterile balanced salt solution (Gibco BRL) mixed with antibiotics, amphotericin B (Sigma) (2.8 mg l^{-1}) and kanamycin (200 mg l^{-1}). After the final washing, explants (2 mm^2) were cut from the mantle strip and kept ready inside a sterile petri dish on ice for immediate use.

For primary culture the earlier prepared explants were transferred under laminar air flow cabinet into tissue-culture petri dishes (1–2 explants per dish; Tarsons, 60 mm diameter, radiation-sterilized) and kept undisturbed for 5–7 min to facilitate proper attachment of the explant with the treated surface of the petri dish. Then 3–5 ml filter-sterilized (0.22 µm) tissue-culture medium, DMEM; Dulbecco's Modified Eagle Medium, Gibco BRL; pH adjusted to 7.4 was aseptically dispensed into each dish with explants. The medium used was enriched with inactivated (incubated at 56°C for 30 min), filter-sterilized (0.22 µm) foetal bovine serum (10%; Gibco BRL), and supplemented with antibiotics, viz. penicillin (200 µg ml^{-1}, Sigma) and streptomycin (200 µg ml^{-1}, Sigma). The tissue-culture plates were then incubated in a CO_2 incubator ('open type' culture; LSL Secfroid) at 5% CO_2 and at 28–32°C. The culture dishes were regularly observed for any cellular development under an invert phase contrast microscope (Lieca) and the medium was exchanged at every 3–4 days interval. Desirable control culture plates without explants were also maintained at the same experimental conditions.

The tissue sterilization step is critical for the success of any cell-culture study and the tissue decontamination procedures adopted in this study were adequate for successful culture of the mantle of the outer epithelial cells of freshwater pearl mussel. The reduction of mucus from the mantle tissue was appreciable, as the residual mucus did not cause any kind of observable interference during tissue handling and tissue culture.

During the primary cell-culture experiment, spherical epithelial-like cells began to migrate out (Figure 1 *a*) and they formed a cell sheet surrounding the explant within

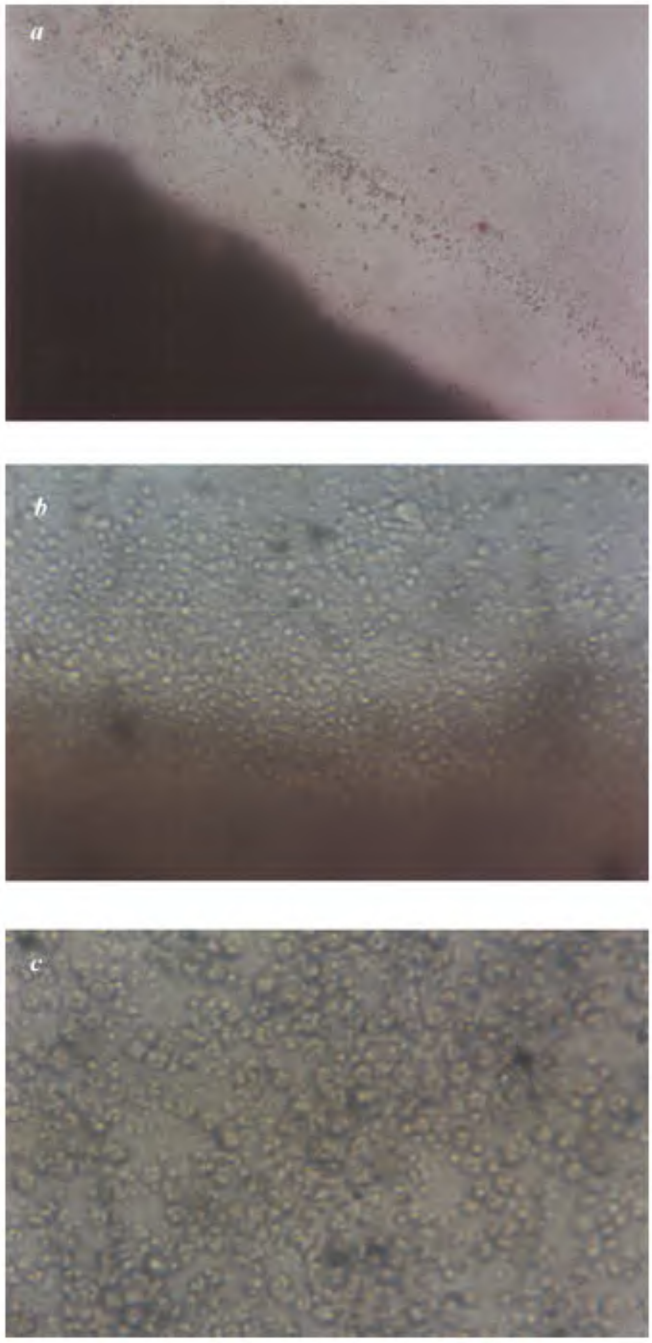

Figure 1. ***a***, Initial stage of migration of cells from explant margin on day 3 (40 ×). ***b***, Epithelial cell migration from explant and initiation of monolayer on day 7 (200 ×). ***c***, Complete monolayer of epithelial cells on day 15 (400 ×).

24 h of the culture incubation (Figure 1 *b*). During the following 7–10 days, surface-adhered cells emerging out from the explant margin occupied most of the surface of culture plates forming a complete monolayer (Figure 1 *c*) of epithelial cells. The culture was maintained for more than 70 days without any contamination, with periodic addition of fresh media. Most importantly, cells in the culture indicated functional viability as subsequently after 38–40 days of culture, typical distinct aragonitic crystals of $CaCO_3$ could be observed, mostly singly, throughout the culture plates (Figures 2 *a* and *b*). Gradually, as the culture progressed, the crystals started aggregating together (Figure 2 *c*) after 65 days. Further, crystals were also observed on the explant surface itself (Figure 2 *d*).

Since mussels are mucoid filter feeders, tissues like mantle are in direct contact with the surrounding environment, which results in the accumulation of considerable number of microorganisms. Hence a critical step in conducting primary cell cultures from these tissues involves the elimination of contaminating microbes. More extensive use of a battery of antibiotics during tissue sterilization is recommended for the cell culture of marine bivalves[10]. Although the methods adopted during this study cannot prevent microbial contamination completely, it is not often to lose cultures because of contamination. During this study the antibiotics used were kept to a minimum, though many reports[11,12] reveal extensive use of a variety of antibiotics at the same time. Mulcahy[13] recommended reduced use of antimicrobials, as they are toxic to the cells. Another hindrance generally encountered during mantle tissue processing is the mucus-like substance, which causes problems during tissue handling and interference during culture. Though Awaji and Suzuki[14] treated the mantle tissue (approximate thickness 1.0 mm) of marine oyster with an enzyme mixture (collagenage, DNAase I and hyaluronidase) to solve the problem, it was however avoided here in view of the very thin (0.3–0.5 mm) and soft nature of the pallial mantle tissue and to avoid the risk of epithelial tissue dissociation from the explant. The procedure adopted effectively reduced the thickness of the slime surrounding the pallial mantle tissue in this study.

Primary culture of the outer epithelial cells of pearl oyster mantle has been conducted mostly by tissue explant culture[15]. As mentioned earlier the morphological features of the migrating epithelial cells resemble those described for regenerating outer epithelial cells at *in vivo* wound sites[3,16]. Janaki Ram *et al.*[9] maintained the primary culture of mantle epithelial cells of *L. marginalis* for 42 days. Panha and Phansuwan[17] reported deposition of pearl substance as crystals within the first month of experiment. The production of aragonite crystals in *in vitro* primary cell culture from hard coral was also reported by Domart-Coulon *et al.*[18]. Panha and Phansuwan[17] observed the aggregation of crystals in freshwater pearl mussel, *Chamberlainia hainesiania*. Because, the medium (DMEM) used for the primary cell culture contained

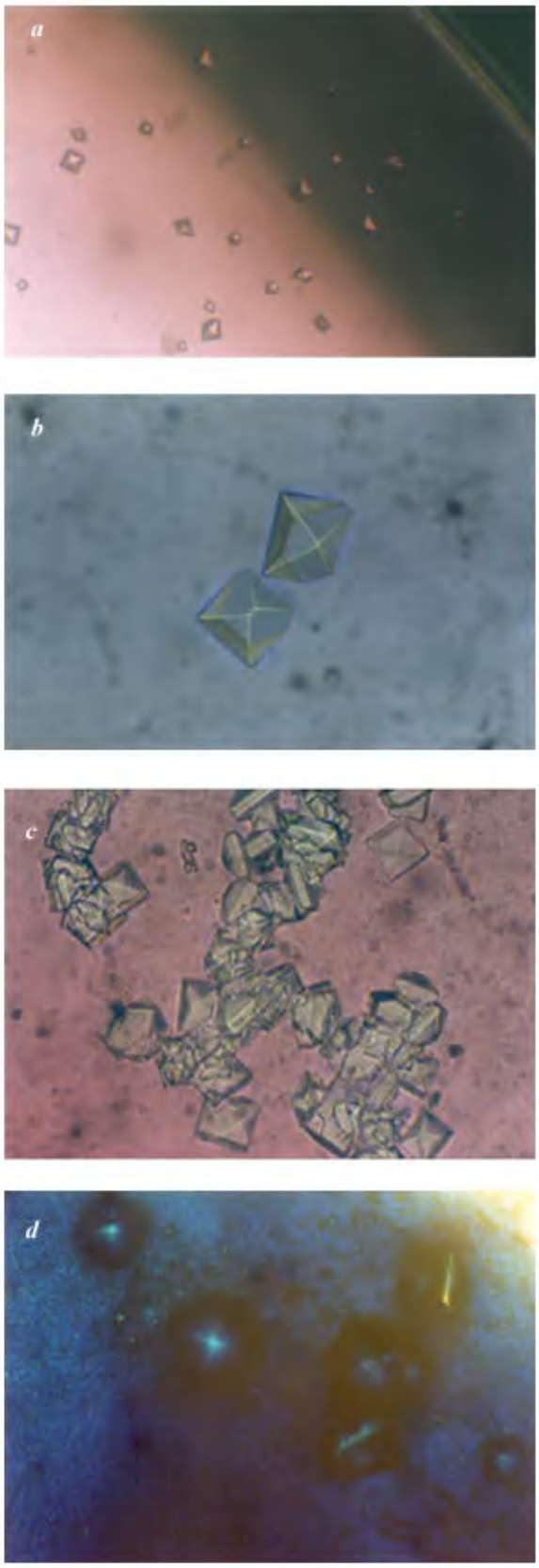

Figure 2. ***a***, Appearance of individual crystals in the plate on day 40 (40 ×). ***b***, Aragonite crystals at different orientations in the plate (200 ×). ***c***, Aggregation of crystals after day 75 (100 ×). ***d***, Crystals on explant (100 ×; after staining the culture plate with methylene blue[22]).

calcium salts in solution, the crystals formed in the culture dish were envisaged to be aragonite crystals, as during the natural process, the dissolved calcium salts present in ambient water get converted into aragonite crystals of $CaCO_3$ of pearl through the biomineralization process essentially mediated by mantle epithelial cells. However, the crystals formed during mantle explant culture of *Pinctade fucata* are considered as *in vitro* pearls[15]. The aragonitic crystal structures (prismatic, rhomboidal) and characters (translucency, fluorescence, etc.) observed during this study conform with those observed in previous studies[15,17]. However, further studies like X-ray absorption, laser microanalysis, scanning electron microscopy, etc. are essential for complete characterization and confirmation of these crystals[19]. During the primary culture we have not come across the occurrence (or if occurred might have escaped our detection) of organic matrix (trefoil bodies), as observed by Machii and Wada[15] during explant culture of marine pearl oyster, *P. fucata*.

In this experiment, the absence of $CaCO_3$ crystals in control experiments and occurrence of some crystals within the cultured explant indicate that this aragonite crystal polymorph is biogenic and not an inorganic precipitation. The occurrence of aragonite crystals of $CaCO_3$ – the building block of pearl nacre skeleton – indicates that the epithelial cells in *in vitro* cell culture retained properties (cell migration, aragonite crystal biomineralization, etc.) of intact epithelial cells *in vivo*. The results demonstrate that *in vitro* crystallization of aragonite in primary mantle explant epithelial cell culture is possible, and it provides an innovative approach to investigate pearl formation at the cellular level.

In India, the base technology has been developed and well standardized for commercial culture of pearls in fresh and marine waters. But to compete globally, a suitable technology has to be developed by which gem-quality pearls could be obtained in a relatively quicker time and this could only be achieved by the application of modern tools like cell culture. Though the outcome of this study merits encouragement, it will be premature to predict the success of tissue-cultured pearl production in India. In fact, the blue print for developing cell-cultured pearl has been conceptualized[20], whereby instead of implanting mantle graft tissue, one has to inject cell suspension of the functional mantle epithelial cells along with the nucleus. This seems unrealistic and impractical unless a pure and functional cell line of mantle epithelial cells is established. If this would be a success, there are some other prospects associated with it, viz. (i) the quality of pearl can be modified as desired using cell lines from different species with excellent secretory function in epithelium tissue (because the quality of pearl is a direct function of the nature of epithelial cell secretion); (ii) the nacre coating over the nucleus would be uniform, unlike the conventional method (cell suspension would be uniformly attached around the nucleus), i.e. avoidance of uncertainty of improper contact of graft and nucleus, and (iii) minimizing sacrifice of donor mussels for preparation of mantle grafts contributing indirectly to mussel conservation.

Recently, a gene nacrein, which is expressed in the pearl oyster mantle and codes for a shell matrix protein, has been cloned[21]. The use of such specific markers for the culture of epithelial cells should be considered for cell identification. The development of the cell line would provide an invaluable tool for both basic (role of epithelial cells in aragonite biomineralization, etc.) and applied research (tissue-cultured pearl, *in vitro* pearl, etc.) involving this commercially important mussel.

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