In vitro explant culture of mantle epithelium of freshwater pearl mussel

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Received 11 August 2003; revised 23 August 2004

The in vitro culture of nacre secreting pallial mantle explants of freshwater pearl producing mussel, Lamellidens marginalis (Lamarck) included depuration of pearl mussels with different physical and chemical agents to eradicate various commensals, 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 fetal serum and antibiotics were poured into plates with explants. The culture plates were incubated at 30°C in a CO₂ incubator at 5% CO₂. The cultures could be maintained for 42-45 days without any contamination. After 12 hr epithelial like cells began to migrate out and formed a complete cell sheet surrounding the explant within 12-15 days. The epithelial cells in the culture indicated functional viability as subsequently after 38-40 days of culture, typical aragonitic 'nacre' crystals of CaCO₃ could be observed throughout the culture plates.

Keywords: Explant culture, Epithelium, Mantle epithelium, Freshwater pearl mussel, Lamellidens marginalis

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 micro-thin layer of nacre, a silvery substance that is about 90% CaCO₃. The mantle tissue lying just beneath the outer shell of mollusc is directly responsible for shell formation by the deposition of CaCO₃ crystals and the secretion of an organic protein matrix to hold them.

The typical freshwater pond mussel Lamellidens marginalis (Lamarck) is the principal species used for pearl production in freshwaters in Indian context¹. Pearls are produced either by cavity insertion method where a shell bead of desired shape and size is placed into umbonal cavity in between the outer mantle layer and inner shell surface² or by grafting technique, which is a delicate surgical intervention³. In former the mantle epithelial cells produce nacreous coatings around the bead to give rise to a shell-attached pearl. In grafting, a small live mantle graft prepared from donor mussel along with a tiny nuclear bead is implanted into the gonad or mantle tissue of recipient pearl mussel. After the implantation, the outer epithelial cells of the mantle graft become squamous, migrate and proliferate to encapsulate the nucleus and subsequently form a follicle called pearl sac around the bead and start to secrete pearl or nacreous layers on the latter⁴. Hence, the quality of pearl is directly related to the quality of the epithelial cells.

Detailed physiological studies on function of epithelial cells, however, have not been conducted adequately, mainly due to lack of an ideal experimental model. However, one of the techniques that will help to unveil partly the function of mantle epithelial cells is primary culture of epithelial cells. While studies in India have been carried out mainly with regard to marine pearl oyster tissue culture, the present investigation is attempted on freshwater pearl mussel, L. marginalis and is the first report with respect to freshwater pearl culture.

Materials and Methods
The freshwater pearl mussels L. marginalis were collected from the aquaculture ponds situated inside the Institute's campus and brought to laboratory within 2-3 hr. The encrusted and superficial mud, flora and fauna, if any, were removed by scraping with hard brush and sharp knife. The animals were kept in clean freshwater without feeding for 72 hr with daily water exchange to reduce any further external risk of contamination before initiating the cell culture experiment⁵. The mussels were then kept immersed in CaO (7.5 mg l⁻¹) solution for 3 days to eradicate leeches etc., if any, followed by repeated washings in clean aged tap water and then were

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subjected to 1% (v/v) sodium hypochlorite immersion treatment for 24 hr for an effective surface disinfection\(^2\). This was followed by thorough washing of mussels in clean aged tap water for several times for 2 days for removal of residues of the chemicals used in preceding steps. Finally, the animals were given an immersion treatment with Chloramphenicol (100 mg l\(^{-1}\)) for 24 hr.

All the required surgical instruments and glassware were sterilised. Before operating, the 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 U.V. light for 30 min for further sterilisation. The mussels were opened by cutting both the adductor muscles situated at anterior and posterior ends of the animals. The pallial ribbon along the pallial line (a strip of 5-6 cm long and 4 mm wide) was cut and removed from the shell. The mantle strip was then transferred to another petri dish. Grafts were placed in two different manners in tissue culture media; LSL Secfroid) at 5% CO\(_2\) and at 28°-32°C. The culture dishes were regularly observed for any cellular development under an inverted phase contrast microscope (Lieca) and the medium was exchanged at every 3-4 day interval. Proper control sets without explants were maintained throughout the study.

**Results and Discussion**

Within 12-15 hr of the culture, epithelial cells began to migrate out and formed cell sheet surrounding the explant. During the next 7-10 days the surface adhered cells emerged out from explant margin and occupied most of the surface of culture plates forming a complete and confluent monolayer (Figs 1 and 2) of epithelial cells. The primary culture of the outer epithelial cells of pearl oyster mantle has been conducted earlier mostly by tissue explant culture\(^3\). In these studies, the culture could be maintained for 45 days without any contamination with periodic addition of fresh media. Cells in the culture showed 36% viability and more importantly they indicated functional viability as subsequently after 35-37 days of culture typical distinct aragonitic crystals of CaCO\(_3\) could be observed (Fig. 3); they were mostly singly throughout the culture plates. Interestingly, crystals were observed abundantly in all the plates where the outer surfaces of the grafts were attached to the culture plates. This indicates the specific secretory function of outer epithelial cells of mantle. However, crystals were also seen in very few numbers in some plates where inner epithelium was attached to plate, which could possibly be due to some outer epithelial tissue proliferation from the explant margin. This observation and particularly the procedure bears significance when compared to mantle explant culture of marine pearl oyster, where the inner layer of mantle epithelium could be removed by dispase digestion\(^7\). This procedure is not suitable
for freshwater pearl mussel due to the very thin and soft nature of mantle tissue when compared to that of marine oyster. However, strict morphological segregation could not be observed between the cells in the monolayers originated from explant grafts placed in two different manners in the petri dishes. Panha and Phansuwan reported secretion and 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. Because, the medium (DMEM) used for the primary cell culture contains calcium salt in solution, the crystals formed in the culture vessel are envisaged to be aragonite crystals, as in natural process the dissolved calcium salts present in ambient water get converted into aragonite crystals of CaCO₃ of pearl through biomineralisation process essentially mediated by mantle epithelial cells. The aragonitic crystal structure and characters (translucent, fluorescent, etc.) (Figs 4 and 5) observed in the present study match with those observed in earlier studies. However, further studies like X-ray absorption, laser microanalysis, etc., are essential for complete characterisation and confirmation of these crystals. In the present experiment, the absence of CaCO₃ crystallisation in control experiments indicates that this aragonite crystal polymorph is biogenic and not an inorganic precipitation. The occurrence of aragonite crystals of CaCO₃ — the building block of pearl nacre skeleton indicates that the epithelial cells in in vitro cell culture retained many properties of intact epithelial cells in vivo. The results demonstrate that in vitro crystallisation of aragonite in primary mantle explant epithelial cell culture is possible, and it can provide an innovative approach to investigate pearl formation at the cellular level.

Conclusion
The base technology has been developed and well standardised in India for commercial production of pearls in fresh- and marine water as well. But to compete globally a suitable technology has to be evolved by which gem quality pearls could be obtained in a relatively quicker time and this could only be achieved by application of modern tools like cell culture. In fact, the blueprint for developing cell cultured pearl has been conceptualised whereby in stead of implanting mantle graft tissue one has to inject cell suspension of the mantle epithelial cells.
Fi gs 4-5—A single crystal showing blue fluorescence typical of aragonite (x 200); A single crystal showing translucent (cells are visible beneath the crystal) nature (x 400)

along with the nucleus. This seems unrealistic and impractical unless a pure cell line of mantle epithelial cells is established. If this would be a success there are some other advantages associated with it, viz., (1) the quality of the pearls can be modified as desired, by using cell lines from different species with excellent secretory function in epithelial tissue (as the quality of pearl is a direct function of nature of epithelial cell secretion), (2) the nacre coating over the nucleus would be uniform unlike the conventional method (cell suspension would be uniformly coated) i.e., avoidance of uncertainty of improper contact of graft and nucleus, and (3) minimising sacrifice of donor mussels for preparation of mantle graft aiding indirectly for mussel conservation. Having mentioned this, one has to appreciate the fact that best gem quality pearls are and will always be Nature’s work of art.

Acknowledgement
This work has been carried out under the ‘Team of Excellence’ project on “Freshwater Pearl Culture” awarded by National Agricultural Technology Project of ICAR, New Delhi.

References
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