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Effect of depuration of animals and use of antimicrobial agents on proliferation of cells and microbial contamination in *in-vitro* mantle explant culture of the abalone *Haliotis varia* Linnaeus

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ABSTRACT

The role played by depuration of test animals and surface sterilisation of tissues has been greatly felt in tissue cultures of molluscs so as to control contamination and to ensure viable cultures. The effect of depuration and the optimum dose of antimicrobial agents for sterilisation of mantle tissue in abalone mantle tissue culture and their effects in cell yield have not been reported with reference to cell proliferation and its survival. Abalone cell culture is being given importance in order to develop a technology for *in-vitro* pearl production, as the cultured pearls are highly valuable in terms of high quality. Separate studies were conducted to evaluate the effect of depuration and to standardise the optimum dose of antimicrobial agents. Mantle tissue from depurated and non-depurated abalones (*Haliotis varia*) was used simultaneously to study the effect of depuration in cell yield. During the study, contamination was found to be more in depurated tissues than in non-depurated ones. Role of antibiotics such as streptomycin and penicillin on cell proliferation and contamination was studied by washing tissues using solutions containing three different doses of antibiotics, prior to initiation of culture. In the first experiment, washing of tissue in seawater containing 1000 µg ml^{-1} of streptomycin and 2000 IU ml^{-1} of penicillin yielded more cell proliferation with less contamination. Fungizone was incorporated along with streptomycin and penicillin to minimise the fungal contamination in the second experiment. The dosage of 200 µg ml^{-1} of fungizone with above said antibiotic dosage yielded better cell proliferation and less contamination than other doses.

Keywords: Abalone, Cell proliferation, Depuration, Explant culture, *In-vitro* pearl

Introduction

Microbial contamination is one of the major constraints in cell cultures and hence elimination of such microbes needed a careful study. More reports were available on decontamination of microbes in bivalve cell culture systems whereas limited works have been reported in gastropods. Decontamination of microbes was carried out by appropriate use of antibiotics. Hansen (1979) reported a method of decontamination of snail embryos. Stephens and Hetrick (1979) gave a detailed account on decontamination process during primary cultures by depurating animals and using disinfectants and antibiotics. They also emphasised the need for cleaning animal shell surface and aquarium. Machii *et al.* (1985a) and Machii and Wada (1989) cleaned abalone larvae and veliger larvae of *Pinctada fucata* and *Pinctada margaritifera* respectively, by rinsing overnight in running sterile seawater and by depurating the test animals for several days in UV treated and filter sterilised (0.45 µm Millipore filter), running seawater prior to initiating tissue culture.

Ellis and Bishop (1989) kept the oyster, *Crassostrea virginica* and the clam, *Mercenaria mercenaria* in running seawater for one week. They used 1 to 5% Clorox solution for surface sterilisation of oyster and clam prior to culture. Chen *et al.* (1989) used L15 medium and 1% Clorox solution for washing the gill tissue of hard clam, *Meretrix lusoria.* Chen and Wen (1999) surface sterilised the mantle tissue of oyster, *Crassostrea gigas* by wiping with 70% alcohol. Dharmaraj and Suja (1998; 2001) depurated the pearl oyster; *P. fucata* for three days in UV sterilised running seawater when tissue culture experiments were organised.

Abalones are economically important marine gastropods known for their rainbow coloured gem quality pearls and delicious meat. Mantle tissue of the abalone *Haliotis varia,* was used for the present study. Success of cell culture lies on the extent of care taken on animal and tissue preparation. The present study aims on the effect of depuration of abalone and the effect of antimicrobial agents on cell proliferation and in preventing contamination.

Materials and methods

Haliotis varia in the size range of 35 mm x 20 mm to 55 mm x 38 mm (length x width) were collected from break water stones at Tuticorin Harbour basin (long.78°09' 70" to 78°11' 90" E; lat. 08° 47' 20" N) and transported to laboratory within two hours in a basin with seawater.

Depuration of animals

The outer shell surface of ten healthy abalones was scrubbed with brush to remove epifauna and micro encrustations. Out of this a set of five abalones were placed in a 75 l capacity tank for depuration. Continuous flow of UV treated seawater was maintained in the tank for three days. The depuration tank was cleaned everyday with disinfectants to avoid microbes. A second set of the remaining five abalones were used for the experiment without depuration, as control.

The depurated and non-depurated abalones of the same age group were used for the tissue culture experiment. The external surface of the shell was wiped with 70% ethyl alcohol soaked cotton and taken aseptically to the clean room for further processing. The mantle strip from depurated and non-depurated abalones was removed aseptically and kept separately. The mantle strip excised from each abalone yielded 5 to 8 pieces of explants and hence 3-5 abalones were scarified. The strips were cut in to small pieces of 1 mm^2 explants and inoculated in sterile tissue culture (T25) flasks. The process of removal of mantle strips, washing and preparation of explants and their inoculation, incubation and observation of cell proliferation were done as per the methods described by Suja and Dharmaraj (2005).

The difference in cell proliferation from explant tissues of both depurated and non-depurated abalones was studied. The suspended cells proliferated from explants were collected after eight days and fresh medium was added to the same flask without disturbing the explants. The cells were again collected after eight days for counting. The process of cell collection was continued till the explant ceased proliferation of further cells. The entire cells were scraped out from the flask mechanically before the final counting. The total number of cells proliferated in each week both from depurated and non-depurated abalones were estimated using haemocytometer and the effect of depuration was assessed. Viability of cells were assessed using trypan blue (Suja and Dharmaraj, 2005).

Standardisation of antibiotics and fungicide in washing solution

The non-depurated animals were taken up for this study to assess the optimum dose of antimicrobial agents for controlling contamination. The preparation of tissue was followed as described earlier (Suja and Dharmaraj, 2005). After this initial processing, the strips were sorted out to three sets for the two sets of experiments.

Streptomycin and penicillin in washing solution

In this experiment three sets of mantle strips were washed in three dosages of antibiotic solutions of streptomycin and penicillin separately twice for five minutes each as shown in (Table 1). The antibiotic treated strips were again washed thoroughly twice in sterile seawater to get rid of any adherent antibiotic remnants. The processed strips were taken to laminar flow hood by keeping in sterile seawater. Each mantle strip treated in different concentration of antibiotics were cut separately into smaller pieces of explants of 1mm^2 size with a sterile knife and inoculated separately in sterile T25 flasks. Three explants were placed in each flask using a sterile needle. Five replicates were kept for each treatment. Cell proliferation was assessed at eight days interval up to thirty-two days by taking out the entire medium with suspended cells and quantified the number of cells using haemocytometer. At the time of final counting the entire cells were scraped out from the flask and counted.

Streptomycin, penicillin and Fungizone in washing solution

In this experiment, three sets of mantle strips were washed separately in three dosages of antibiotic solutions of streptomycin, penicillin and fungizone (ketoconazole) as shown in Table 1. Processing of tissue, inoculation, incubation and cell counting were done as described earlier.

Table 1. Antibiotic concentrations used in washing solutions

Solution No.	Antibiotic concentrations		
	Streptomycin (µg ml^{-1})	Penicillin (IU ml^{-1})	Fungizone (µg ml^{-1})
1	250	500	-
2	1000	2000	-
3	4000	8000	-
4	250	500	50
5	1000	2000	200
6	4000	8000	800

Statistical analysis

The difference in cell proliferation in depurated and non-depurated animals was analysed statistically Analysis of Variance (ANOVA) using SPSS software.

Results and discussion

There was no regularity in cell proliferation at weekly intervals among the replicates from the explants of depurated animals. The highest mean cell proliferation was recorded in the first week and the intensity of cell

proliferation decreased towards fifth week. A slight increase in cells was noticed in sixth week. The overall cell proliferation from depurated explants was 24.96% (Table 2). Bacterial contamination was noticed in the beginning in one of the replicates. Fungal contamination noticed in one replicate from fourth week and in two replicates from fifth week.

In non-depurated explants, irregular pattern of cell proliferation was noticed among the replicates during six weeks period. The number of cells proliferated from non-depurated explants was more when compared to depurated explants. The total percentage of cell proliferation was 75.04 (Table 2).

Almost an identical result was obtained in live and dead cells of depurated and non-depurated explants (Table 3). Significant difference was observed in this experiment using ANOVA when considering the depuration treatment factor alone ($p=0.001$). The difference in cell proliferation during different periods was insignificant ($p=0.617$).

In spite of various measures followed by different workers, contamination is a major constraint in cell cultures. In the present study, the abalones were depurated in UV sterilised running seawater for three days and their shell surface was wiped with 70% ethyl alcohol. The dissected tissues, after washing in sterile natural seawater, were treated twice in 10 ml antibiotic solution containing 1000 µg ml^{-1} streptomycin and 2000 IU ml^{-1} penicillin for 5 min each. The survival rate of cells from depurated and non-depurated explants was found almost identical (Table 3). Ellis and Bishop (1989) avoided long depuration procedure in the laboratory as suggested by Stephens and Hetrick (1979) and disinfected the tissues in 1 to 5% Clorox solution for 1 min prior to culture. In the present study even though there was no definite pattern of cell proliferation at weekly intervals, the total cells proliferated from depurated explants were less than that from the non-depurated explants (Table 2). The difference in results between depurated and non-depurated treatments was significant statistically. Depuration of abalones for three days in UV sterilised running seawater was found to reduce the efficiency of explants in the proliferation of cells.

Table 2. Cell proliferation from depurated and non-depurated explants.

Treatment	Replicates	Weekly cell proliferation (x 10^4 cells ml^{-1})						Total (x 10^4 cells ml^{-1})	Cell proliferation (%)
		1	2	3	4	5	6		
Depurated	1	6.25	7.75	4.625	5.875	5.125	8.625	38.25	24.96
	2	-*	-*	-*	-*	-*	-*	-*	
	3	7.75	4.4	3.75	8.375	-*	-*	24.275	
	4	10.125	5.875	9.125	4.0	-*	-*	29.125	
	5	7.75	9.0	5.0	-*	-*	-*	21.75	
	Total	31.875	27.025	22.5	18.25	5.125	8.625	113.4	
	Average (%)**	6.375 (28.1)	5.405 (23.8)	4.5 (19.8)	3.65 (16.1)	1.025 (4.5)	1.725 (7.6)	22.68	
Non-depurated	1	11.875	11.215	9.125	3.7	-*	-*	35.965	75.04
	2	13.625	10.125	15.25	6.25	4.875	7.625	61.75	
	3	16.25	15.5	12.375	5.875	7.125	7.75	64.875	
	4	10.375	9.875	12.75	12.75	21.125	62.5	129.375	
	5	9.875	7.0	10.875	5.625	7.375	8.25	49.0	
	Total	62.0	53.715	60.375	34.25	40.5	90.125	340.965	
	Average (%)**	12.4 (18.2)	10.743 (15.8)	12.075 (17.7)	6.85 (10.1)	8.1 (11.9)	18.025 (26.4)	68.193	

-* Contaminated
** Weekly percentage of cell liberation from the mean of five replicates.

Table 3. Live and dead cells from depurated and non-depurated explants

Treatment	Cells	Mean of five replicates at weekly intervals (x10^4 cells ml^{-1})						Total (x10^4 cells ml^{-1})	Percentage of cells
		1	2	3	4	5	6		
Depurated	Live	31.875	27.025	22.5	18.25	5.125	8.625	113.4	92.0
	Dead	2.25	1.125	3.5	1.75	0.5	0.625	9.75	8.0
Non-depurated	Live	62.0	53.715	60.375	34.25	40.5	90.125	340.965	92.6
	Dead	4.2	4.7	6.4	4.2	6.6	1.2	27.3	7.4

The chances of contamination in explant cultures of depurated and non-depurated abalones were equal or less in the explants of non-depurated animals. The presence of contamination in the later stages of culture, that is, twenty days after the initiation might be mostly from the air borne fungal contaminants during the medium renewal process, as observed by Ellis and Bishop (1989). The cause of bacterial contamination earlier in the culture period of depurated abalones might be due to the injury to the exposed mantle tissue during collection of animal and the development of secondary infection during the course of depuration.

Effect of using antimicrobials in washing solution

Streptomycin and penicillin: The data on cell proliferation from the explants treated with three different concentrations of streptomycin and penicillin are presented in Table 4. With solution 1, cell proliferation observed was 23.7%. Here fungal contamination occurred in replicates 3 and 5 on 16th day and on 32nd day in replicate 4. Highest percentage of 42.8 was recorded in solution 2. Gradual decrease in cell proliferation was observed from 16th day to 32nd day from the explants treated in solution 3 (Table 4) with 33.5% cell proliferation. No contamination was recorded with solution 2 and solution 3. The difference in cell proliferation among the three dosages of antibiotics was not significant (p=0.060). The least significant difference (LSD) values showed that treatments between 1 (streptomycin and penicillin concentrations 250 µg ml^{-1} and 500 IU ml^{-1}) and 2 (streptomycin and penicillin solutions 1000 µg ml^{-1} and 2000 IU ml^{-1}) were significant (p= 0.020).

Streptomycin, penicillin and fungizone: The mean values of cell proliferation from explants washed with the three concentrations of antibiotics/fungicide are given in Table 5. With solution 4, the total percentage of cell proliferation was 25.8. Unlike the experiment with the bactericidal antibiotics alone, in this experiment, the cell proliferation showed steady increase from 8th day to 32nd day. In the third replicate, contamination occurred since 16th day. From the explants washed in solution 5, the mean values of cell proliferation were higher than the other two concentrations. There was steady increase in cell proliferation from 8th to 32nd day with a high (41.3%) percentage of cell proliferation. In the higher concentration of solution 6, the percentage of cell proliferation was 32.8. Frequency of contamination was more in solution 4. A single occurrence of contamination was recorded each in solution 5 and 6 (Table 5). There was no significant difference in cell proliferation between the treatments of three dosages.

Extensive work has been done on control of microorganisms in *in vitro* tissue culture (Machii and Wada, 1989; Chen and Wen, 1999). The dosage of antibiotics used by Chen and Wen (1999) in

Table 4. Number of cells liberated in cultures where tissues washed in different concentrations of streptomycin and penicillin

Concentration of antibiotics	Replications	No. of cells proliferated in days (x10^4 cells ml^{-1})				Total (x10^4 cells ml^{-1})	Cell proliferation (%)
		8	16	24	32		
Streptomycin (250 µg ml^{-1}) Penicillin (500 IUml^{-1})	1	4.625	2.125	2.25	2.125	11.125	
	2	2.125	2.75	3.125	1.375	9.375	
	3	2.375	-*	-*	-*	2.375	
	4	1.375	3.875	3.5	-*	8.75	23.7
	5	1.375	-*	-*	-*	1.375	
	Total	11.875	8.75	8.875	3.5	33	
	Mean	2.37	1.75	1.77	0.7	6.6	
Streptomycin (1000 µg ml^{-1}) Penicillin (2000 IU ml^{-1})	1	1.25	3.25	3.5	1.125	9.125	
	2	3.625	3.25	3.625	1.125	11.625	
	3	4.0	4.375	1.375	1.0	10.75	
	4	3.0	9.375	2.875	1.125	16.375	42.8
	5	3.0	4.625	3.125	0.875	11.625	
	Total	14.875	24.875	14.5	5.25	59.5	
	Mean	2.98	4.98	2.9	1.05	11.9	
Streptomycin (4000 µg ml^{-1}) Penicillin (8000 IU ml^{-1})	1	1.625	2.75	1.875	1.125	7.375	
	2	2.375	3.0	2.625	1.375	9.375	
	3	2.875	3.875	2.75	0.875	10.375	
	4	3.0	3.5	3.25	1.625	11.375	33.5
	5	1.375	3.625	2.25	0.875	8.125	
	Total	11.25	16.75	12.75	5.875	46.625	
	Mean	2.25	3.35	2.55	1.18	9.3	

* Contaminated

Table 5. Number of cells liberated in cultures with tissues washed in different concentrations of streptomycin, penicillin and fungizone

Concentration of antibiotics	Replications	No. of cells proliferated in days ($x10^4$ cells ml^{-1})				Total ($x10^4$ cells ml^{-1})	Cell proliferation (%)
		8	16	24	32		
Streptomycin (250 µg ml^{-1}) Penicillin (500 IU ml^{-1}) Fungizone (50 µg ml^{-1})	1	11.4	11.0	13.9	10.4	46.7	25.8
	2	6.0	7.9	8.9	7.1	29.9	
	3	5.4	-*	-*	-*	5.4	
	4	5.0	8.4	6.3	5.3	25.0	
	5	4.8	9.6	3.6	4.1	22.1	
	Total	32.6	36.9	41.3	42.2	129.1	
	Mean	6.52	7.38	8.26	8.44	25.8	
Streptomycin (1000 µg ml^{-1}) Penicillin (2000 IU ml^{-1}) Fungizone (200 µg ml^{-1})	1	10.7	11.4	10.7	19.6	52.4	41.3
	2	10.4	8.6	11.8	21.0	51.8	
	3	11.8	7.5	11.8	-*	31.1	
	4	3.4	8.6	12.3	11.3	35.6	
	5	5.7	6.4	11.9	11.6	35.6	
	Total	42.0	42.5	58.5	63.5	206.5	
	Mean	8.4	8.5	11.7	12.7	41.3	
Streptomycin (4000 µg ml^{-1}) Penicillin (8000 IU ml^{-1}) Fungizone (800 µg ml^{-1})	1	4.6	5.7	8.2	8.2	26.7	32.8
	2	8.9	7.1	18.6	8.6	43.2	
	3	8.9	12.1	7.1	14.6	42.9	
	4	11.6	7.2	5.7	8.4	32.9	
	5	8.1	6.8	3.7	-*	18.6	
	Total	42.1	38.9	43.3	39.8	164.3	
	Mean	8.42	7.78	8.66	7.96	32.8	

* Contaminated

Crassostrea gigas was practically low when compared to the dosages used in the present study on the abalone *H. varia.* Perlman (1976) suggested that amphotericin B should be used if fungal or yeast contamination is likely to occur, but not on a continuous basis. The present study indicated that the solution 2 (1000 µg ml^{-1} streptomycin and 2000 IU ml^{-1} penicillin) and 5 (1000 µg ml^{-1} streptomycin, 2000 IU ml^{-1} penicillin and 200 µg ml^{-1} fungizone), promoted high cell proliferation with less contamination. Single occurrence of contamination in the fifth and sixth concentrations recalled the statement of McGarrity (1976) that contamination may originate from any source in the environment (Table 5). More contamination at lower concentrations in both experiments (Table 4 and 5) might be due to insufficient dose of antimicrobial concentration. Nagai *et al*. (1998) maintained the primary culture of hemocytes from Japanese black abalone *Nordotis discus* for one month when the tissue was disinfected with 70% ethanol and for one week or less in spite of the animal's healthy appearance when no antibiotic was used for washing the tissues. He opined that it might be possible that the abalones used in his experiment had already been infected with an unknown pathogen in spite of their healthy appearance.

In the present study, the mean value of cell proliferation in the treatment with different doses of streptomycin and penicillin was less than in the experiment with the incorporation of fungizone in all combinations (Table 4 and 5). It was also seen that the intensity of cell proliferation showed no regular pattern at weekly intervals among different antibiotic concentrations. It might be probable that cells might need acclimatisation initially to the new environment *in vitro* and hence there was less cell numbers. Such a disparity was already indicated by Li *et al.* (1966). Stephens and Hetrick (1979) treated tissues with 10% ethanol solution in filtered bay water and washed in a mixture of antibiotic solution every 30 min for 2 h and final washing in 10 ml filtered bay water for 3 times. They emphasised that ethanol is effective in destroying protozoa contaminants peculiar to the oyster. Chen and Wen (1999) surface sterilised mantle tissue of the oyster, *C. gigas* with 70% ethyl alcohol and without any antibiotics. In the present study, the mantle tissue of *H. varia* was dipped in 35% ethyl alcohol for 15 sec before washing of the tissues in 10 ml of the three concentrations of antibiotic/antifungal solutions. The second concentration of antibiotic solution in both experiments was found effective for treating the mantle tissue of *H. varia* as there was less contamination and more cell liberation. In cell proliferation no significant difference was noticed statistically between the three concentrations. However, significant difference was noticed in between concentrations 1 and 2 ($p= 0.020$). The results of the present study indicates that the long process

of depuration procedure in the laboratory is not necessary for *H. varia* as non-depurated abalones proliferated more cells with less contamination. Washing the tissues in sterile seawater incorporated with antimicrobial agents at 1000 µg ml^{-1} streptomycin, 2000 IU ml^{-1} penicillin and 200 µg ml^{-1} fungizone promoted high cell proliferation with less contamination.

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