中国科技论文在线 http://www.paper.edu.cn

ELSEVIER

Journal of Experimental Marine Biology and Ecology
267 (2002) 175–184

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

In vitro culture of embryonic cells from the shrimp, *Penaeus chinensis*

Ting-Jun Fan *, Xiao-Feng Wang

Department of Marine Biology, College of Marine Life Sciences, Ocean University of Qingdao, 5 Yushan Road, Qingdao 266003, PR China

Received 15 May 2001; received in revised form 8 August 2001; accepted 21 September 2001

Abstract

Studies were carried out in our laboratory to develop cell cultures from embryonic tissue of *Penaeus chinensis*. Good yields of dissociated, uncontaminated, viable cell suspensions were obtained by physical disruption of harvested embryonic tissues. Primary cultures in the form of proliferating foci of cells were readily initiated using MPS medium (osmolality: 2.4%) with 20% heat-inactivated fetal bovine serum. Insulin-like growth factor-II (IGF-II) and basic fibroblast growth factor (bFGF) at different concentrations were also added into the culture medium. Passage of primary cultures resulted in rapid proliferation and good adherence of lymphocytes in the presence of IGF-II at 80 ng/ml and bFGF at 20 ng/ml. Cells maintained in subculture for up to 10 passages still had good cellular morphology and division rates. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Embryonic cells; Culture; Growth factor; *Penaeus chinensis*

1. Introduction

It is widely recognized that infectious diseases are major limiting factors in the development of aquatic invertebrate culture. At present, at least 17 virus diseases of penaeid shrimp have been documented and more than 30 viruses overall identified in the Crustacea (Lu et al., 1991; Chou et al., 1995; Wang, 1995). Although many aspects of crustacean virus diseases have been investigated in the host animals, suitable in vitro tissue culture systems are necessary for the proliferation, isolation, purification and characterization of the infective agents and also for the development of diagnostic methods and the

* Corresponding author. Tel.: +86-532-2032-459; fax: +86-532-2032-276.
E-mail address: tjfan@ouqd.edu.cn (T.-J. Fan).

0022-0981/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0022-0981(01)00364-1

转载

中国科技论文在线 http://www.paper.edu.cn

production of material for immunological and vaccination studies (Chen and Kou, 1989; Lightner, 1992; Chou et al., 1995; Tapay et al., 1997; Chen and Wang, 1999).

An interest in invertebrate cell culture dates back to the early days of tissue culture development, but no permanent cell line derived from crustacean tissue has yet become available. Chen et al. (1986) reported the formation of confluent monolayers of cells from cultured ovarian tissue fragments from *Penaeus monodon*, with successful subcultivation for three passages before the cells finally degenerated. Primary monolayer cultures were similarly established from haematopoietic tissue (Chen et al., 1988) and lymphoid organ (Chen and Kou, 1989) using double strength L-15 medium and various serum, haemolymph and muscle tissue extract supplements. Itami et al. (1989) found medium 199 to be superior to L-15 for the maintenance of *P. japonicus* lymphoid organ primary cultures. Hu et al. (1990) also used serum -supplemented 199 for the in vitro culture of hepatopancreas tissue from *P. orientalis* and Rosenthal and Diamant (1990) successfully initiated primary cultures of haematopoietic tissue, hepatopancreas and ovary from *P. semisulcatus* with the same basal medium supplemented with serum and shrimp haemolymph. In a study designed to improve methods for producing primary cell cultures of penaeid shrimp, Luedeman and Lightner (1992) and Fraser and Hall (1999) were able to obtain cultures of ovarian cells from *P. stylirostris*, *P. vannamei* and *P. monodon* using modified Grace's Insect Medium or $2 \times$ L-15 media supplemented with hybridoma quality fetal bovine serum. Tong and Miao (1996) successfully initiated primary cultures of lymphoid tissues from *P. chinensis* with the self-designed serum–supplemented medium for penaeid shrimp (MPS), but failed to obtain successful subculture passages.

It is widely recognized that embryonic tissues, as undifferentiated and rapidly multiplying cells from developing life stages, tend to adapt to in vitro growth more readily than the specialized cells of mature shrimps. However, successful initiation of primary cell culture of embryonic tissue obtained from fertilized eggs has not yet been reported, with the exception of the freshwater prawn *Macrobrachium rosenbergii* (Frerichs, 1996), mainly because of the early problems with aquatic bacterial and fungal contamination. The present paper reports the establishment of cultures from embryonic cells of the shrimp, *P. chinensis*, and also the stimulating effect of insulin-like growth factor-II (IGF-II) and basic fibroblast growth factor (bFGF) on embryonic cell proliferation.

2. Materials and methods

2.1. Tissue preparations

Clusters of eggs were removed from ovigerous *P. chinensis* 8–10 days after fertilization and suspended for 1 h at room temperature in phosphate buffered saline (PBS)–antibiotic solution (penicillin 400 IU/ml; streptomycin 400 μg/ml). Eggs were then pelleted by low-speed centrifugation and resuspended in a few milliliters of 1:10 buffered iodophore (Buffodine: Evans Vanodine) with added malachite green (0.01 mg/ml). After holding for 20 min, the whole suspension was transferred to a mortar and pestle at room temperature and gently ground to break up the eggs and disperse the embryonic tissue in the disinfectant solution.

中国科技论文在线 http://www.paper.edu.cn

The disrupted tissue was recovered by centrifugation (1000*g*, 5 min), resuspended in PBS–antibiotic solution and passed through a stainless steel strainer to separate the cellular component from residual gelatinous debris. The strained cells were again harvested by centrifugation and finally resuspended in the required volume of fresh PBS–antibiotic solution for distribution into 25-cm^2 culture flasks.

2.2. Culture media

MPS medium specially designed for shrimp cell culture was used during this study (Tong and Miao, 1996). Sodium chloride was added to adjust its osmolality to 2.4%. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were routinely added to the medium. After filtration through a 0.22-μm filter, the medium supplemented with 20% heat-inactivated fetal bovine serum (FBS, Gibco) was used (Table 1).

2.3. Cell culture

Embryonic cells suspended in antibiotic FBS-containing MPS were distributed in 5 ml over the surface of 25-cm^2 culture flasks and incubated at 22 °C at about 10 thousands of cells per flask. Media were refreshed at 3-day intervals by discarding half of the old medium and adding half volume of fresh medium as used for primary culture initiation. The additional growth factors of IGF-II (insulin-like growth factor II, Sigma) and bFGF (basic fibroblast growth factor; Sigma) should also be included in those refreshing media for the cultures in which the effects of these factors were to be tested.

2.4. Growth factor

IGF-II at concentrations of 60, 80 or 100 ng/ml was added into the serum-supplemented MPS medium, along witb bFGF at concentrations of 10, 20 or 30 ng/ml, 3 days

Table 1
Medium and supplements for embryonic cells of *Penaeus chinensis*

Basal medium	Supplementation
MPS medium	Antibiotics
	Penicillin (100 IU/ml)
	Streptomycin (100 μg/ml)
	Salt
	12 g NaCl added to give an osmolality of approximately 2.4%
	$NaHCO_3$ (0.75 g/l)
	Sodium pyruvate (0.55 g/l)
	Serum
	Fetal bovine serum (15–20%)
	Chitosan (2.0 g/l)
	Nerval nodule extracts[a] (100 μl/flask)

[a] Extracts from the homogenate of nerval nodules isolated from fresh shrimps *P. chinensis*. Nerval nodules from 10 shrimps were extracted with 2 ml PBS solution, filtrated with 0.22 μm filter before use.

中国科技论文在线 http://www.paper.edu.cn

after culture initiation. Subculture passages were carried out by discarding the old medium, adding double volume of fresh medium as for primary culture, mechanically scraping and pipetting cells from the culture flasks, and redistributing the cells into two culture flasks.

3. Results

3.1. Effects on cell growth of different medium supplements

Results are reported as accumulated observations from numerous successive trials comparing the cell maintenance and growth properties of varied combinations of medium supplements.

Serum-supplemented MPS with pH adjusted to 7.0–7.2 appeared compatible with maintaining the viability of dissociated shrimp tissue. Adjusting the osmolality of MPS to 2.4% with sodium chloride was essential for maintaining the integrity of *P. chinensis* cells. Observations on cell culture performance with various concentrations of fetal bovine serum supplementation indicated that heat-treated serum at the 20% level gave the best results.

After initial seeding, at approximately 10 thousands of cells per flask, the general pattern of culture development was observed to be adherence of a proportion of tissue material to the vessel surface followed within a few days by the establishment of foci of proliferating cells (Fig. 1). The morphology of the cells was fibroblast-like. Slow colony extension continued during partial changes of medium at 3-day intervals, and confluent

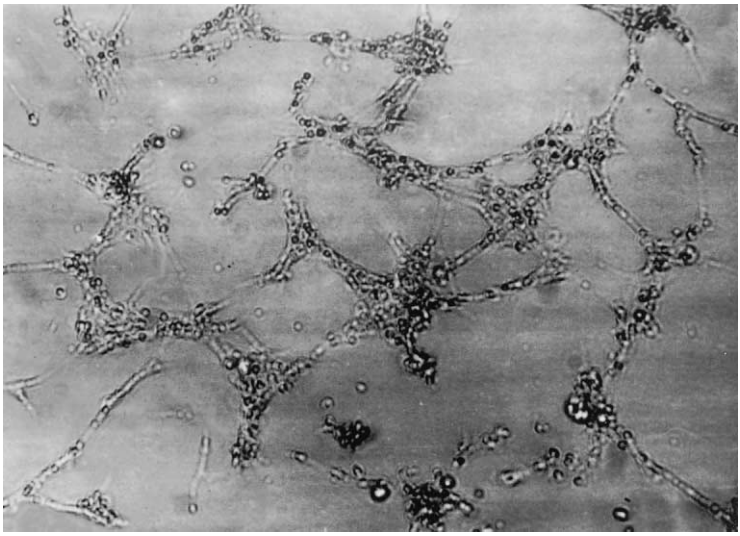


Fig. 1. Primary culture of embryonic cells from *P. chinensis* (250 ×). The embryonic cells formed a lot of colonies in the antibiotics and serum-supplemented MPS.

中国科技论文在线 http://www.paper.edu.cn

monolayers were obtained at day 6. The intact cell sheet could be maintained for at least 20 days and some of them survived for more than 2 months if the MPS medium was changed every 3 days. Cells in these monolayers seemed to be division arrested, although trypan blue dye exclusion tests indicated that the cell populations retained viability over at least four passages of subculture.

The MPS medium supplemented with antibiotics (Penicillin, 100 IU/ml; streptomycin, 100 μg/ml) and fetal bovine serum (15–20%) seemed much more favorable for cell survival and proliferation than in MPS alone. Attempts to induce cell attachment, growth and proliferation by addition of chitosan (2.0 g/l) were also successful in this study. At the presence of chitosan, almost all of the cells attached to flask surface at hour 6 after culture initiation. Contrastingly, most of the cells needed almost 12 h for successful attachment at absence of chitosan. Successive observation revealed that the number of cells was almost twice at the presence of chitosan than that of its absence at day 3 (Fig. 2).

Supplement of nerval nodule extracts was also tested in this study (Table 1). After 0.1 ml of nerval nodule extracts being added into the MPS medium of each culture flask, the number of adherent cells increased markedly, and cell growth was enhanced obviously, compared to that of their absence (data not shown).

3.2. The effect of growth factors

IGF-II and bFGF added simultaneously into the MPS medium starting 3 days after culture initiation had an enhancing effect on embryonic cell proliferation. This effect on

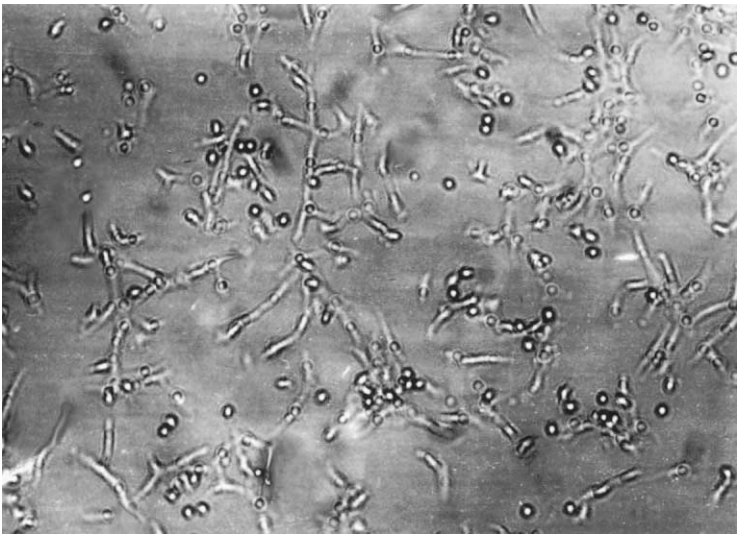


Fig. 2. Embryo cell attachment, growth and proliferation at the presence of chitosan (250 ×). The dispersed embryonic cells suspended in primary culture was well surface-attached, and they were in good growing and proliferating appearance. The concentration of chitosan added was 2.0 g/l.

中国科技论文在线 http://www.paper.edu.cn

Table 2
Stimulating effects of bFGF and IGF-II on embryonic cells under different combinations

Flask no.	bFGF (ng/ml)	IGF-II (ng/ml)	Cell growth and proliferation (cells/mm^2)
0[a]	0	0	30–50
1	10	60	50–70
2	20	60	90–120
3	30	60	70–90
4	10	80	70–90
5	20	80	100–120
6	30	80	70–90
7	10	100	60–80
8	20	100	90–120
9	30	100	90–120
10	20	0	70–90
11	0	80	60–80

[a] A control with no growth factor added (see Section 3.1).

cell proliferation differed with different combinations of concentrations (Table 2). The most effective combination observed was IGF-II at 80 ng/ml and bFGF at 20 ng/ml (Fig. 3). Under these conditions the embryonic cells grew and proliferated rapidly Many cells migrated from the embryonic tissue fragments and the cells formed confluent monolayers at day 3. These cells could be subcultured for at least 10 passages on

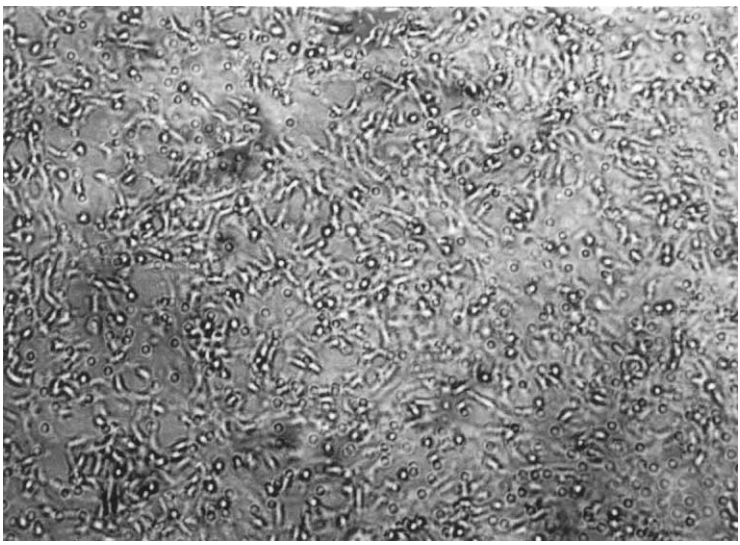


Fig. 3. Growing status of embryonic cells induced by IGF-II and bFGF (250 ×). IGF-II- and bFGF-induced embryonic cells grew and proliferated more rapidly than those not treated by these growth factors. The concentration of IGF-II used was 80 ng/ml and that of bFGF is 20 ng/ml.

中国科技论文在线 http://www.paper.edu.cn

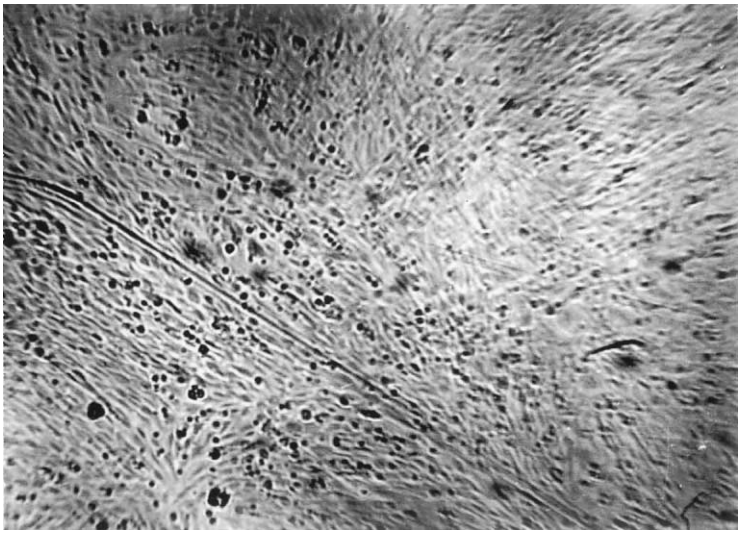


Fig. 4. The confluent monolayer formed by embryonic cells (250 ×). At the appearance of IGF-II and bFGF, the shrimp embryonic cells formed a confluent monolayer at day 7 after culture initiation. These cells has been subcultured for 10 passages until now on condition that these cells were exposed continuously to the above growth factors.

condition that these cells were exposed continuously to the above growth factors (Fig. 4).

3.3. Contamination in shrimp embryonic cell culture

The cells from shrimp embryos attached to the surface of cell culture vessels within 24 h. However, heavy contamination with yeasts, fungi and protozoa was the main problem encountered continually throughout the culture experiments, even though the shrimps were pretreated with high concentrations of penicillin (1000 IU/ml), streptomycin (1000 μg/ml), Fungizone (25 μg/ml) and Nystatin (100 μg/ml) over night.

The eggs treated with a 1:10 buffered iodophore and malachite green (0.01 mg/ml) as described in Section 2 were less contaminated, even though contaminations of yeast and protozoa still occurred in some cultures.

4. Discussion

A number of researchers have succeeded in initiating primary cell cultures from hepatopancreas, lymphoid and ovarian tissues of a range of economically important marine penaeid shrimps in the past decade (Chen et al., 1986, 1988; Luedeman and Lightner, 1992; Nadala et al., 1993; Tong and Miao, 1996; Itami et al., 1999; Kasornchandra et al., 1999; Toullec, 1999; West et al., 1999). Advancement to established cell line status through serial subculturing, however, has not yet been reported. In the present

中国科技论文在线 http://www.paper.edu.cn

study, attempts to develop a cell line from the shrimp, *P. chinensis*, have proved hopeful. Embryonic tissue was selected for culture since these undifferentiated and rapidly multiplying cells from developing life stages were expected to adapt to in vitro growth more readily than the highly differentiated cells of mature shrimp. The described method of tissue processing gave good yields of well-dissociated suspensions of viable cells, and early problems with aquatic bacterial and fungal contamination were partially overcome by including buffered iodophore disinfectant and malachite green at all but the final preparative stages.

In previous reports on establishment of primary cultures, cell proliferation was most successful from tissues where tissue fragments adhered to the flask surface. It would appear that dissociated crustacean cells, like normal cells from vertebrate tissues, are anchorage-dependent, and failure to attach to the vessel surface on subculture may have been the most important single factor in thwarting cell line development. Further studies into chitosan and other substances on the attachment of shrimp cells to the vessel surface are needed. Chitosan was used to improve the attachment of embryonic cells to the vessel surface in this study. In this study, it was observed that 2.0 chitosan used at a final concentration of 2 mg/ml of culture media substantially improved cell attachment. be However, even if a methodology for establishing crustacean cell lines can be developed, it may still be of concern that the rate of growth of the cultured cells may prove impracticably slow for routine virological requirements. Therefore, the effects of growth stimulating factors also need to be investigated.

In this study, the growth factors IGF-II and bFGF were employed to stimulate the proliferation of embryonic cells from *P. chinensis*. A significant stimulation effect on embryonic cell proliferation of IGF-II and bFGF simultaneously added to supplement MPS medium was observed. It was also found that this stimulating effect on cell proliferation differed with different concentrations, with the most effective concentrations being 80 ng/ml of IGF-II plus 20 ng/ml of bFGF. It is reasonable that higher concentrations of these factors could not achieve a better effect, since the number of their membrane receptors may well be a major limiting factor in their stimulation of signal transduction.

The successful subculture of embryonic cells induced by IGF-II and bFGF in this study implies that coinduction of growth factors may be a very hopeful way for establishing rapidly proliferating cell lines of *P. chinensis* and the other crustaceans as well, and may serve as an alternative to transgenic technology with introduced oncogenes.

5. Conclusions

From the results obtained in this study, it can be concluded that (1) the antibiotics- and serum-supplemented MPS, with a osmolality of 2.4% and a pH value of 7.0–7.2, is a suitable medium for in vitro culture of embryo cells from *P. chinensis*. (2) The addition of chitosan (2.0 g/L) has a positive effect on cell attachment and growth improvement. (3) IGF-II and bFGF simultaneously added into the culture medium can induce the embryonic cells to multiply much more rapidly with the induced cells having been subcultured for 10 passages until now, and still being in good growing and dividing

中国科技论文在线 http://www.paper.edu.cn

status on condition that these cells were exposed continuously to the above growth factors.

Acknowledgements

The skilled technical assistance of Shang-Liang Tong and Zhen-Ping Shi is gratefully acknowledged. This work was supported and funded by the China Science and Technology Committee as the "Marine 863 Hitech Project."*[SS]*

References

Chen, S.N., Kou, G.H., 1989. Infection of cultured cells from the lymphoid organ of *Penaeus monodon* Fabricius by monodon-type baculovirus (MBV). J. Fish Dis. 12, 73–76.

Chen, S.N., Wang, C.S., 1999. Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. Methods Cell Sci. 21 (4), 199–206.

Chen, S.N., Chi, S.C., Kou, G.H., Liao, I.C., 1986. Cell culture from tissues of grass prawn *Penaeus monodon*. Fish Pathol. 1, 161–166.

Chen, S.N., Jong, K.J., Kou, G.H., 1988. Cell cultures from hematopoietic tissue and ovary of penaeid shrimp, *Penaeus monodon*. In: Kuroda, Y., Kurstak, E., Maramorosch, K. (Eds.), Invertebrate and Fish Tissue Culture. Springer-Verlag, Berlin, pp. 195–198.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiamg, H.C., Lo, C.F., 1995. Pathogenity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org. 23 (3), 165–173.

Fraser, C.A., Hall, M.R., 1999. Studies on primary cell cultures derived from ovarian tissue of *Penaeus monodon*. Methods Cell Sci. 21 (4), 213–218.

Frerichs, G.N., 1996. In vitro culture of embryonic cells from the freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 143, 227–232.

Hu, K., Wang, L.P., Duan, Y.M., 1990. Studies on a cell culture from the hepatopancreas of the oriental shrimp *Penaeus orientalis* Kishinouye. Asian Fish. Sci. 3, 299–307.

Itami, T., Aoki, Y., Hayashi, K.I., Yu, Y., Takahashi, Y., 1989. In vitro maintenance of cells of lymphoid organ in kuruma shrimp *Penaeus japonicus*. Nippon Suisan Gakkaishi 55, 2205.

Itami, T., Maeda, M., Kondo, M., Takahashi, Y., 1999. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, *Penaeus japonicus*. Methods Cell Sci. 21 (4), 237–244.

Kasornchandra, J., Khongpradit, R., Ekpanithanpong, U., Boonyaratpalin, S., 1999. Progress in the development of shrimp cell cultures in Thailand. Methods Cell Sci. 21 (4), 231–235.

Lightner, D.V., 1992. Shrimp virus diseases: diagnosis, distribution and management. In: Wyban, J. (Ed.), Proceedings of the Special Session on Shrimp Farming. Orlando, Florida. World Aquaculture Society, Baton Rouge, LA, pp. 238–253.

Lu, Y., Nadala Jr., E.C., Brock, J.A., Loh, P.C., 1991. A new virus isolated from infectious hypodermal and hepatopoietic necrosis virus (IHHNV) infected penaeid shrimps. J. Virol. Methods 31, 189–196.

Luedeman, R.A., Lightner, D.V., 1992. Development of an in vitro primary cell culture system from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei*. Aquaculture 101, 205–211.

Nadala, E.C., Loh, P.C., Lu, P.C., 1993. Primary culture of lymphoid, nerve, and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei*. In Vitro Cell. Dev. Biol. Anim. 29A (8), 620–622.

Rosenthal, J., Diamant, A., 1990. In vitro primary cell cultures from *Penaeus semisulcatus*. In: Perkins, F.O., Cheng, T.C. (Eds.), Pathology in Marine Science. Academic Press, San Diego, CA, pp. 7–13.

Tapay, L.M., Lu, Y., Gose, R.B., Nadala Jr., E.C., Brock, J.A., Loh, P.C., 1997. Development of an in vitro quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. J. Virol. Methods 64 (1), 37–41.

Tong, S.L., Miao, H.Z., 1996. Attempts to initiate cell cultures from *Penaeus chinensis* tissues. Aquaculture 147, 151–157.

中国科技论文在线 http://www.paper.edu.cn

Toullec, J.Y., 1999. Crustacean primary cell culture: a technical approach. Methods Cell Sci. 21 (4), 193–198.
Wang, C.H., 1995. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis. Aquat. Org. 23 (3), 239–242.
West, L., Mahony, T., McCarthy, F., Watanabe, J., Hewitt, D., Hansford, S., 1999. Primary cell cultures isolated from *Penaeus monodon* prawns. Methods Cell Sci. 21 (4), 219–223.