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EFFECTS OF MOLTING ON THE GROWTH OF PRIMARY CULTURED MUSCLE CELLS FROM *MACROBRACHIUM NIPPONENSE* AND *PENAEUS VANNAMEI*

Wei-Na Wang[1]*, An-Li Wang[1], Dong-Hong Nu[2], Yi-Chen Liu[2], and Ru-Yong Sun[1]

[1] College of Life Science, South China Normal University, Guangzhou 510631, People's Republic of China

[2] College of Life Science, Hebei University, Baoding 071002, People's Republic of China

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Abstract

This study examined the effects of molting stage on the growth of primary-cultured cells from the shrimps *Macrobrachium nipponense* and *Penaeus vannamei*. Muscle cells in different molting stages were cultured in L-15 medium, supplemented with 1g/l glucose,1g/l $MgCl_2$,1 g/l $CaCl_2$,100 IU/ml penicillin, and 100 µg/ml streptomycin. Results showed that cells cultured in the early postmolt stage grew best. They had an unfolding cell shape, a compact monolayer, a high rate of coverage, and the highest RNA:DNA ratio. Differences among early postmolt, premolt, and intermolt cells were significant (p<0.05).

Introduction

With the expansion of intensive aquaculture, viral diseases have threatened the shrimp aquaculture industry. Shrimp cell culture has therefore gained recent attention for the development of diagnostic reagents and probes for use in the shrimp aquaculture industry. A number of researchers succeeded in initiating primary cell cultures from lymphoid, hepatopancreas, muscle, and ovarian tissues of a range of economically important freshwater and marine shrimps (Chen et al., 1988; Hu et al., 1990; Fraser and Hall, 1999; Itami et al., 1999; Wang et al., 2001). Muscle growth in crustaceans is intermittent (Whiteley and El Haj, 1997) and closely associated with the molt cycle due to the presence of a rigid

* Corresponding author. E-mail: weina63@yahoo.com.cn

calcified exoskeleton. The focus of the present study was to discover which molt stage of shrimp provides the most suitable material for cell culture.

Biochemical indicators reflecting muscle growth could be an interesting alternative to the classic methods of evaluating growth rates. The relationships between somatic growth and biochemical variables have been studied in fish and crustaceans (Grant, 1996; Buckley, 1997). Promising biochemical indicators are the activity of metabolic enzymes and the RNA:DNA ratio. Growth also requires the cellular machinery for protein synthesis, i.e., ribosomal RNA, which represents 85-94% of cellular RNA (McMillan and Houlihan, 1988). Therefore, RNA is a sensitive indicator of growth (Bulow, 1970) and the concentration of DNA is an indicator of cell number (Buckley, 1997), and neither is sensitive to environmental changes. In short, RNA:DNA is an indicator of metabolism that is not influenced by cell number or cell size. In this study, we carried out primary culture of muscle tissue of *Macrobrachium nipponense* and *Penaeus vannamei* in different molt stages by using a selected and suitable medium. Further, we compared the effect of different molt stages on the RNA:DNA ratio, shape, and growth of cultured cells in order to supply references for shrimp muscle cell culture.

Materials and Methods

Shrimp. Juvenile shrimp of *M. nipponense* (avg wt 0.5 g) from Baiyangdian Lake in Heibei Province, China, and *P. vannamei* (avg wt 0.8 g) from a commercial shrimp hatchery in Hebei Province, China, were transferred and acclimated for two weeks prior to the experiment. The shrimp were held individually in small glass aquaria. The shrimp were observed and the ecdysis time was recorded. Molt stages were determined as in Peebles (1977) and Chan et al. (1988).

Experimental materials. Leibovitz medium (L-15; Sigma) supplemented with 1 g/l glucose, 1 g/l $MgCl_2$, 1 g/l $CaCl_2$, 100 IU/ml penicillin, and 100 µg/ml streptomycin was used. The medium and other heat-labile solutions were filter sterilized using sterile 0.22 µm pore size filters. After filtration, the medium was added to 15% heat-inactivated fetal bovine serum (FBS). Heat stable salt solutions and glassware were sterilized in an autoclave for 30 min at 115°C and 15 psi. The experiments were conducted in a Biohazard cabinet which was surface sterilized for 24 h with a germicidal UV light source.

Primary culture. Shrimps in different molt stages were anesthetized in cold water (4°C) for 20 min and then surface sterilized by submersion in 70% alcohol for 10 min. Subsequently, muscle tissue from the abdomen was excised and placed into a cold holding medium (4°C) for 10 min. The holding medium consisted of 9.5g/l L-15 medium with 1000 IU/ml penicillin and 1000 µg/ml streptomycin. Tissues were washed several times in cold PBS and then cut into small pieces (1 mm^3). Fragments were inoculated into 24 well culture plates and fed with the media containing L-15 medium plus 15% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The plates were incubated with 5%CO_2 at 26°C in a CO_2 incubator. Cultured cells were observed daily under an inverted microscope.

Muscle cell cultures formed a monolayer within 3-5 days. After 5 days, the cells were washed with distilled water, trypsinized, washed three times with distilled water, and their RNA:DNA ratios were immediately measured.

Subsequent cell cultures. Muscle cell cultures in the early postmolt stage formed monolayers within 3-5 days and subcultures were made by trypsinization (trypsin, 0.25% at 25°C, 10 min gentle shaking). Cells were counted and cell suspensions were transferred to other culture plates.

Measurement of RNA:DNA. Nucleic acids were extracted and purified from 0.7 ml samples using a modification of the Schmidt-Thannhauser method described by Wang et al. (2001). RNA and DNA were estimated from the absorbency at 260 nm of the appropriate hydrolysate. Because of the susceptibility of nucleic acid to enzymatic and heat degradation, homogenization and extraction were carried out on fresh material at temperatures of 0-4°C.

Results

Effect of molt stage on shape and growth of primary muscle cells. The molt cycle of both shrimp species was repeatedly 6-7 days. We observed the morphology of the cultured muscle cells in the early postmolt (A; 1-12 h after ecdysis), intermolt (C; 2-3 days after ecdysis), and premolt (D; 6-7 days after ecdysis) stages. The attachment and growth of the cultured cells differed in each stage. Cells in the early postmolt stage were best for unfolding shape, intact construction, 90% rate of coverage, and rapid attachment and growth. The described method of tissue processing had a good effect on well-dissociated attaching cells, and cell cultures were subcultured. However, the cultured cells in the intermolt stage had slim fibrillar cells, 50% rate of coverage, and a slow growth rate. The morphology of the *M. nipponense* cells in the primary culture differed from that of the *P. vannamei* cells (Table 1; Figs. 1, 2); the fibrillar muscle cells of the *M. nipponense* were thicker than those of the *P. vannamei* (Fig. 3).

Effect of molt stage on RNA:DNA of primary muscle cells. The RNA:DNA ratio was highest during the early postmolt stage and next highest in the premolt stage while there were no differences between intermolt periods (C_1; C_2; C_3; C_4; Table 2). Therefore, muscle cells in early postmolt and premolt are the most suitable for culture.

Discussion

The morphology, attachment, and growth of the cultured cells differed among molt stages. The best cell growth appeared to be in the early postmolt and premolt stages while the worst was in the late postmolt and intermolt stages. Muscle growth in crustaceans is a complex process involving the interplay of synthesis and degradation rates in a tissue-dependent manner that is closely coordinated with the molt cycle. Muscle growth in Crustacea occurs over the molt and involves a corresponding increase in fractional rates of sarcomeric protein synthesis (Whiteley and El Haj, 1997). The protein synthesis rate in the leg, claw, and abdominal muscles of the lobster, *Homarus americanus*, also increases during late premolt and early postmolt (El Haj et al., 1996). The variations in muscle protein content are reflected in differences in the levels of amino acid incorporation into protein during the course of the molt cycle (Chang, 1995). The variation in muscle growth in different molt stages may be related to the regulation of some hormones, although the mechanism behind this relationship is still uncertain and may be controlled at the molecu-

Table 1. Comparison of the morphology of primary-cultured muscle cells of *Macrobrachium nipponense* and *Penaeus vannamei* in different molting stages.

Stage (after ecdysis)	*M. nipponense*		*P. vannamei*	
	Shape	*Coverage (%)*	*Shape*	*Coverage (%)*
A - postmolt (1-12 h)	Wide fibrillar cells, even coverage, even monolayer	85-90	Slim fibrillar cells, compact coverage	85-90
C - intermolt (2-3 days)	Slim fibrillar cells, low rate of attachment, uneven coverage	45-50	Thin and short fibrillar cells, sparse coverage	50-55
D- premolt (6-7 days)	Slim fibrillar cells, even coverage	70-80	Slim fibrillar cells, even coverage	80-85

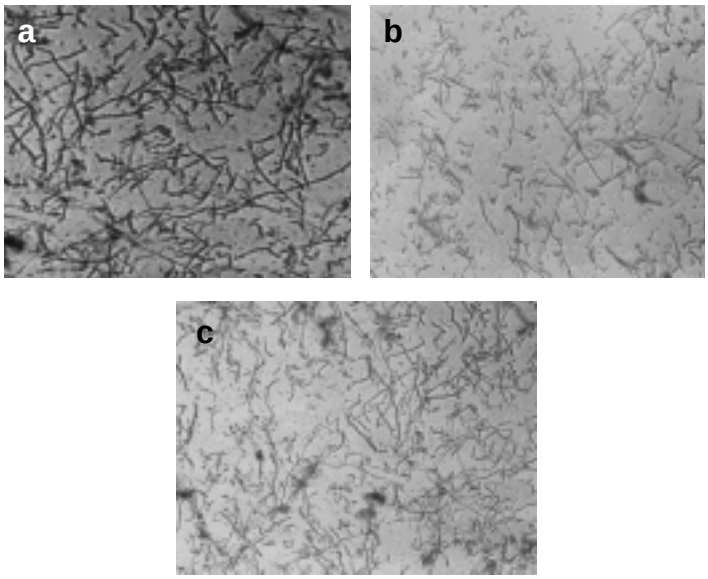

Fig. 1. The morphology of primary-cultured muscle cells of *Macrobrachium nipponense* in different molting stages: (a) postmolt (stage A); (b) intermolt (stage C); and (c) premolt (stage D; x 500).

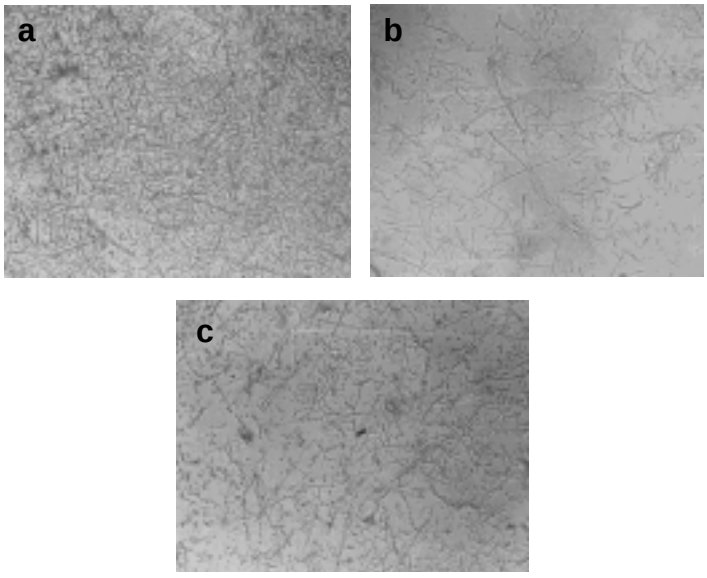

Fig. 2. The morphology of primary cultured muscle cells of *Penaeus vannamei* in different molting stages: (a) postmolt (stage A); (b) intermolt (stage C); and (c) premolt (stage D; x 500).

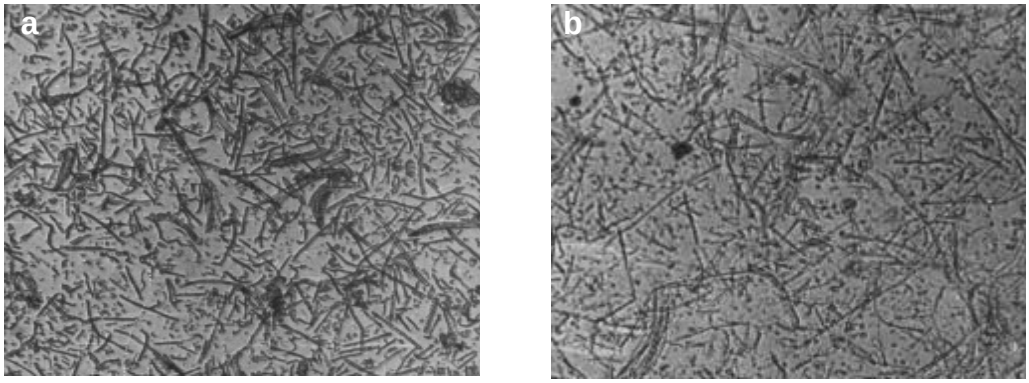

Fig. 3. The morphology of subcultured muscle cells of (a) *Macrobrachium nipponense* and (b) *Penaeus vannamei* in postmolt stage (x 500).

lar level. El Haj and Whiteley (1997) reported that crustacean growth occurs just after the formation of the exoskeleton and is related to hormone regulation. It follows that the main period of growth occurred during the postmolt period after new exoskeleton recalcification. Hartnoll (2001) showed that the hyperglycemic hormone plays a role in molt control in crustaceans, but this role needs clarification. Initial studies in *Carcinus* revealed that actin mRNA levels increased in the leg muscles during premolt and postmolt (El Haj et al., 1992).

The RNA:DNA ratio may be useful as an index of the cultured condition for fish and shrimp. Clemmesen (1989) showed that the RNA:DNA value increased with increasing age and length of juvenile herring. Stuck et al. (1996) showed that the RNA:DNA ratio is useful as a condition index for *P. vannamei.* In the present study, the variations of RNA:DNA of the primary cultured muscle cells of both *M. nipponense* and *P. vannamei* in different molt stages are consistent with the variations of morphology, attachment, and cell growth. Therefore, variation of the RNA:DNA ratio can be regarded as an indicator of protein metabolism and growth rate and this index can be used to measure the growth rate of cultured cells.

The morphology of the muscle cells of the two species differed. These differences, perhaps due to species, should be further researched.

In summary, the RNA:DNA ratio of primary cultured muscle cells of *M. nipponense* and *P. vannamei* was much higher during the early postmolt and premolt stages than during the intermolt and late postmolt stages, conforming results of earlier researchers that cells have high vitality and reproductive ability in view of strong growth and metabolic regulation that agree with the shape and growth of cultured cells. Therefore, according to the results of our experiment, muscle cell samples from shrimp in the early postmolt and premolt periods can achieve better culture effects and be subcultured.

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Table 2. The RNA:DNA of primary-cultured muscle cells of two shrimp species in different molting stages.

Macrobrachium nipponense							
Molting stage	A	B_1	B_2	C_1	C_2	D	
Period after ecdysis	1-12 h	16-20 h	1-2 d	2-3 d	3-4 d	6-7 d	
RNA:DNA	14.49±0.33^{a}	4.39±0.98^{c}	3.67±0.72^{c}	4.80±0.49^{c}	3.55±0.47^{c}	10.38±0.85^{b}	
Penaeus vannemei							
Molting stage	A	B	C_1	C_2	C_3	C_4	D
Period after ecdysis	1-12 h	1-2 d	2-3 d	3-4 d	4-5 d	5-6 d	6-7 d
RNA:DNA	15.48±0.40^{a}	3.60±0.31^{c}	4.93±0.23^{c}	3.46±0.29^{c}	5.05±0.09^{c}	5.73±0.23^{c}	9.78±0.11^{b}

Values in a row with different superscripts are significantly different (p<0.05).
A = early postmolt, B = late postmolt, C = intermolt, D = premolt

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