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SIMPLE *IN VITRO* CULTURE OF EMBRYOS OF THE GIANT FRESHWATER PRAWN *(MACROBRACHIUM ROSENBERGII)*

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ABSTRACT

Embryos of the giant freshwater prawn were cultured in vitro in deionized water and in 15%, 30% and 45% artificial seawater (ASW), respectively. Deionized water and 45% ASW did not support development to hatching whereas 15% and 30% ASW supported normal development and provided 25.6% ± 2.3 and 27.1% ± 1.2 hatching rates on day 18.5, respectively. Addition of 100, 200 or 300 μg/ml streptomycin to the 15% or 30% ASW did not improve the survival or hatching rate. On the other hand, the presence of 0.3 part per million (ppm) formalin in 15% ASW improved the hatching rate to 47.1 ± 6.1 on day 18.5. Higher formalin levels of 0.6 or 0.9 ppm were detrimental to the developing embryos.

INTRODUCTION

In externally brooding decapods, females carry the embryos on the pleopods beneath the abdomen till hatching. The embryo's vestments protect it from external condition. The major problem for embryonic survival is the heavy contamination of microorganisms, e.g. bacteria, fungi and protozoa[1]. The "preening" behavior[2], cleaning of the abdomen by the first pair of the pereiopods, and maternal secretion[3] from the brooding mother reduce growth of the microorganisms.

Adequate O_2 supply, replenishment of optimal nutrient and/or growth factor at an optimal physiological condition, e.g. temperature and pH, are essential in supporting embryonic groth[3]. High levels of nutrient, in contrast, retard growth of the embryos due to increase in bacterial contamination[3] and in turn reduces O_2 supply reaching the embryos.

The condition for *in vitro* culture of developing embryos, in general, simulates the natural condition supporting embryonic growth. Development for an *in vitro* culture of the embryos is essential for experimental investigation concerning the manipulation of fertilized eggs or developing embryos. The present report describes an attempt to develop simple embryo culture of the giant freshwater prawn *(Macrobrachium rosenbergil) in vitro.*

MATERIALS AND METHODS

Mature giant freshwater prawns, *Macrobrachium rosenbergii,* were maintained in separate chambers of aerated, recirculating water at 26°C Natural mating was allowed by placing a female which had completed premating molt with a mature male. After spawning, clusters of 6-24 hr embryos were removed from the brooding female. The clusters were teased and separated into individual embryos or into small pieces (a maximum of 30 embryos per piece).

In vitro culture was performed and carried out at room temperature (28-29°C). An average of about 400 embryos were placed in each of a 100 ml evaporating dish containing 40 ml of culture medium. Various culture media were tested: deionized water (DW), 15%, 30% and 45% artificial seawater (ASW). Different salinities of ASW were diluted from stock ASW as described by Cavanaugh[4] with the following composition: 423 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 23 mM $MgCl_2$, 25.5 mM $MgSO_4$ and 2.1 mM $NaHCO_3$.

The effect of streptomycin was investigated at concentrations of 100, 200 or 300 μg/ml in each of the culture media. Addition of 0.3, 0.6 or 0.9 part per million (ppm) of formalin solution (37%) in 15% ASW was also conducted for the effect on survival rate of the embryos.

Developmental stages of the cultured embryos were compared with those of the embryos carried in the laboratory-maintained brooding female and photographs were taken with bright-field microscopy. Survival rate (percentage of surviving embryos) or hatching rate (percentage of hatched larvae) was scored from a total of 100 random counts. Degenerated embryos were aspirated out and fresh media were replaced every 2 days. Student's t test was used to evaluate statistical significance.

RESULTS

Since spawning usually occured at night whereas examination of development and determination of survival rates were performed at daytime, the developmental time of the day following spawning was, therefore, considered as day 0.5.

Embryos cultured in DW either in the absence or presence of streptomycin did not survive to hatching. In the absence of streptomycin, survival rate of the embryos drastically dropped to 47.7 ± 4.0 (mean ± SEM) on day 4.5, and none survived to day 8.5 (Fig. 1A). In the presence of 100 μg/ml streptomycin, the survival rate was 56.7 ± 7 on day 4.5 and dropped to 8.0 ± 7.0 on day 8.5 (Fig. 1B). Increasing streptomycin concentrations to 200 or 300 μg/ml did not significantly ($P > 0.05$) improve the survival rates at all developmental stages examined (cf. Fig. 1B, 1C and 1D).

Embryos cultured in 15% or 30% ASW showed similar results. In the absence of streptomycin, the survival rates in 15% and 30% ASW on day 4.5 were 82.7 ± 1.2 and 83.7 ± 2.5, respectively (Fig. 1A). The survival rates gradually declined to the final of 25.6 ± 2.3 and 27.1 ± 1.3 respectively, on day 18.5 (Fig. 1A). Addition of 100, 200 or 300 μg/ml streptomycin to 15% or 30% ASW did not significantly ($P > 0.05$) alter the survival rate throughout the course of development (Fig. 1A, 1B, 1C and 1D). Survival after day

8.5 of embryos cultured in 45% ASW either in the absence or presence of streptomycin were lower than those cultured in 15% or 30% ASW.

Addition of 0.3 ppm formalin in 15% ASW significantly ($P < 0.05$) improved the survival rates of the embryos cultured beyond day 8.5 (Fig. 2). The survival and hatching rates on day 13.5 and 18.5 were 47.1 ± 5.0 and 27.9 ± 4.8, respectively, in the control, whereas those with the addition of 0.3 ppm formalin were 63.2 ± 6.7 and 47.1 ± 6.2, respectively. However, higher formalin concentrations of 0.6 or 0.9 ppm significantly ($P < 0.05$) reduced the hatching rates to 2.9 ± 4.1 and 1.1 ± 2.3, respectively, on day 18.5.

Despite differences in the survival rate of the embryos cultured in various media there were no striking variations in developmental progress. Furthermore, developmental stages of the cultured embryos were comparable to those of the embryos carried in the brooding mother. By 18 hr after spawning, the embryos developed to about the 32-cell stage (Fig. 3A). Increase in cell number was observed in the first 2 days and on day 4.5, a clear region at one pole of the embryonic mass was easily discernable (Fig. 3B). The clear region extended lengthwise forming the trunk of the growing embryo by day 6.5 (Fig. 3C). As the clear region developed further, the yolky mass lessened. By day 8.5, a pair of small dark eye spots was developed on the yolky mass (Fig. 3D). By day 10.5, the clear region which developed into trunk and caudal portion occupied about 2/3 of the embryo mass; the eye spots were enlarged and oval-shaped (Fig. 3E). Three days later, appendages had formed beneath the clear trunk region; the eyes were large and surrounded by striation and the translucent globules at the dorso-caudal portion of the yolky mass clearly exhibited rhythmic contraction (Fig. 3F). By day 17.5, the eyes were dark rounded and striation was obvious; the translucent globules became enlarged and occupied most of the dorsal area of the yolky mass (Fig. 3G). The newly hatched larvae on day 18.5 showed closed approximation of the eyes (Fig. 3H) and the eyes were large and extended laterally by day 19.5, one day after hatching (Fig. 3I).

DISCUSSION

Mature and juvenile giant freshwater prawns can survive in freshwater or in slight salinity, but newly hatched larvae cannot survive except in slight salinity. In freshwater, brooding females carrying developing embryos must provide an environment around the embryo cluster under the abdomen which presumably differs from the external freshwater condition. Maternal secretion, which has antimicrobial activity,[3] can possibly increase the salinity around the embryos. The present studies indicated that the embryos survived and developed normally in 15% or 30% ASW. Distilled water or high salinity (45% ASW) was not compatible with survival of the embryos. Developmental stages and hatching day of the cultured embryos were comparable to those carried by the brooding mother.

There has been no report to date of an *in vitro* culture of the decapod crustraceans. The technique reported here has eliminated air bubbling and required separation of large embryo clusters into either individual embryo or small cluster so as to expose large portion of the embryo surface to the medium for oxygen exchange. Medium was changed every 2 days.

Addition of streptomycin at 100, 200 or 300 μg/ml did not improve the survival rate of the embryos. It could be that fungal or protozoan infection overcame the bacterial infection or that the dose of the streptomycin used was still not sufficient. Ineffectiveness of penicillin and streptomycin against bacterial infection in hatchery rearing of marine bivalve larvae has been reported[5].

Formalin has been used extensively in fish[6, 7] and crustacean[8, 9] hatchery to reduce fungal and protozoan infections. The effectiveness of formalin treatment is the result of the interplay of treatment duration, dose and the organic load in the water[9]. All previous reports involve short period of treatment at high formalin level in the pond or tank environment. In the present studies *in vitro* system using defined culture solution was used and therefore microorganism contaminations were expected to be low at the beginning of the culture. Embryos were exposed throughout the course of development of low formalin level of 0.3 ppm with frequent change of the medium of effectively reduce contaminations. However, formalin at higher levels of 0.6 and 0.9 ppm were detrimental and no hatching was obtained.

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บทคัดย่อ

เอมบริโอของกุ้งก้ามกรามหลังตกจากตัวแม่ 6-24 ชม. นำมาเลี้ยงในน้ำทะเลเทียมที่ความเข้มข้น 15% หรือ 30% จะมีการเจริญและฟักเป็นตัวในวันที่ 18.5 ได้ตามปกติ โดยมีอัตราการฟักเป็น 25.6 ± 2.3 และ 27.1 ± 1.2 ตามลำดับ ส่วนเอมบริโอที่เลี้ยงในน้ำกรองอย่างเดียว หรือในน้ำทะเลเทียมที่มีความเข้มข้น 45% จะไม่เจริญจนฟักเป็นตัว ยาปฏิชีวนะสเตรบโตไมซิน ความเข้มข้น 100, 200 หรือ 300 ไมโครกรัม/มิลลิลิตร ที่เติมในสารละลายใช้เลี้ยงเอมบริโอไม่มีผลต่ออัตราการรอดหรือการฟักของเอมบริโอ แต่ฟอร์มาลินความเข้มข้น 0.3 ส่วนในล้านส่วนที่เติมในน้ำทะเลเทียม 15% ช่วยให้อัตราการฟักของเอมบริโอสูงขึ้นเป็น 47.1 ± 6.1 ฟอร์มาลินที่ความเข้มข้นสูงขึ้นคือ 0.6 หรือ 0.9 ส่วนในล้านส่วนเป็นอันตรายต่อเอมบริโอ

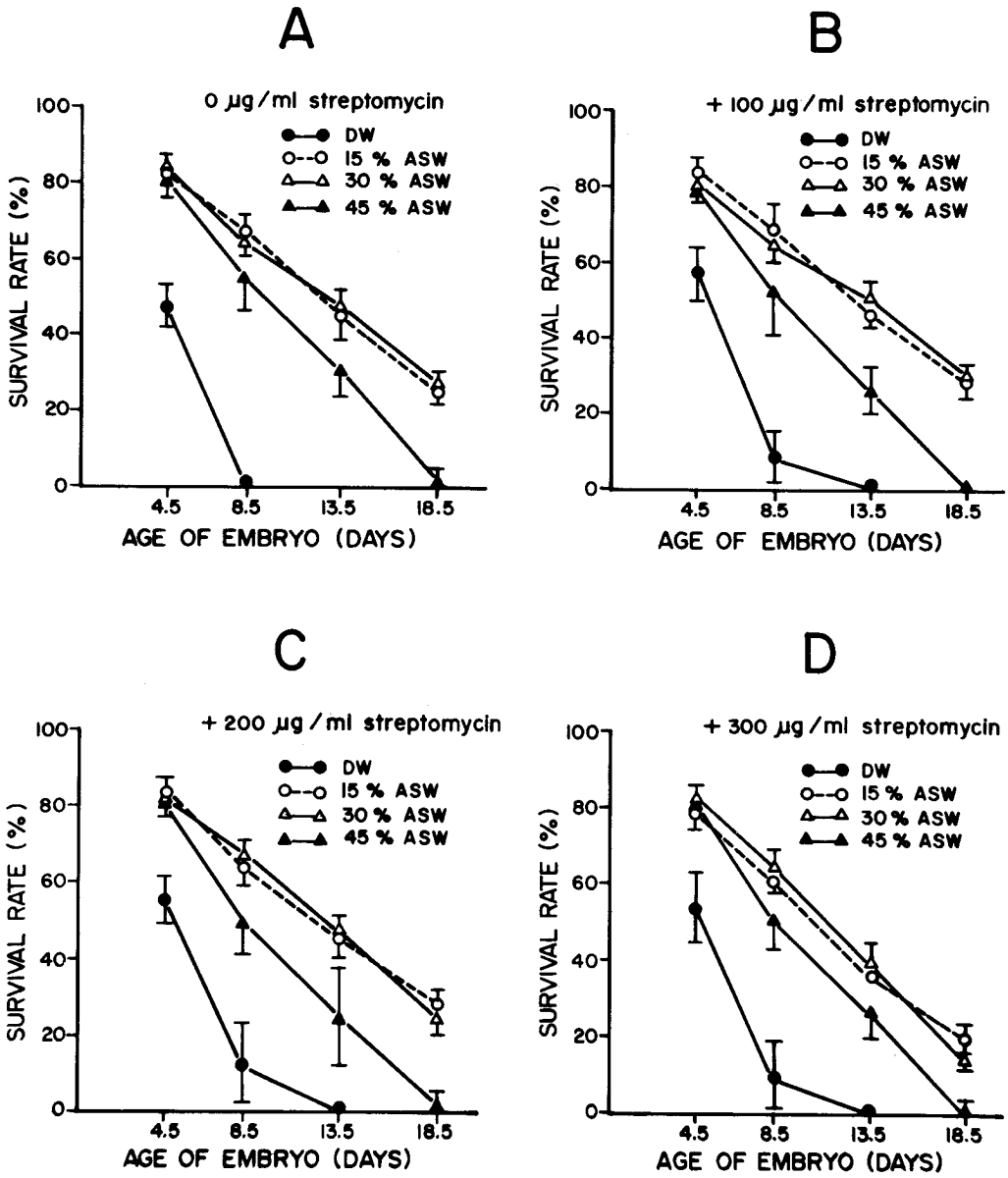

Fig. 1 Survival rates at various developmental stages of *Macrobramium rosenbergii* embryos cultured in DW, 15%, 30% or 45% ASW in the absence (a) or presence of 100 (b), 200 (c) or 300 (d) µg/ml streptomycin.

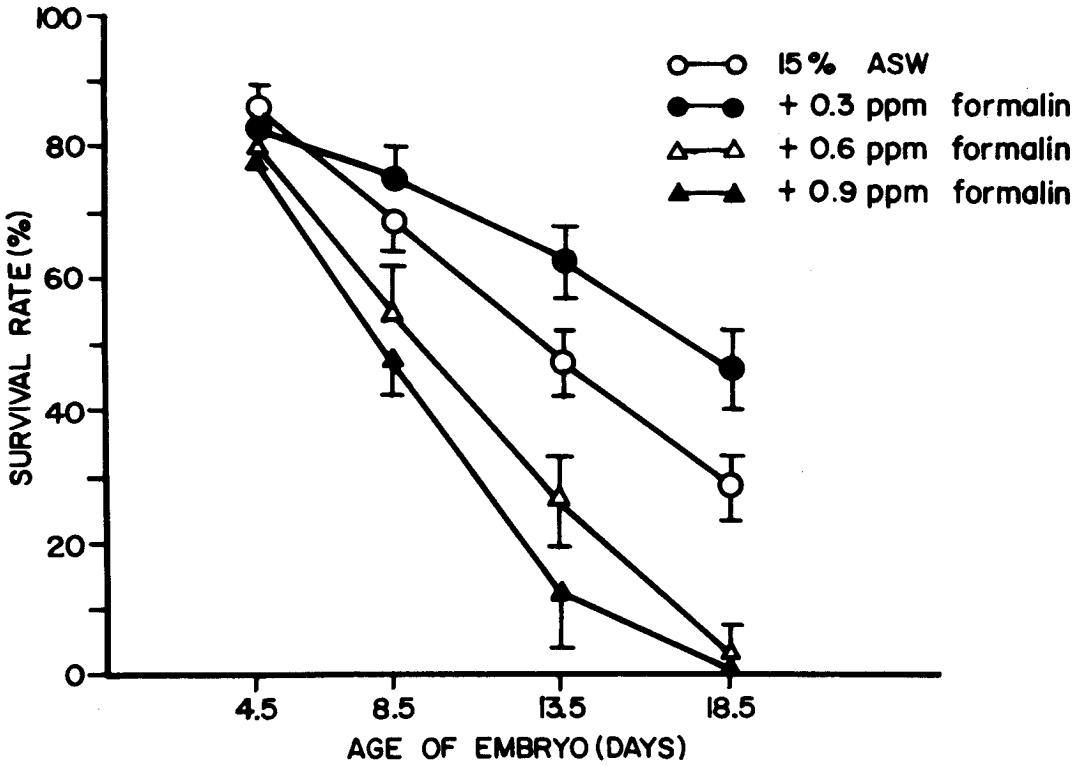

Fig. 2 Survival rates at various developmental stages, 4.5, 8.5, 13.5 and 18.5 days, of embryos cultured in 15% ASW in the absence or presence of 0.3, 0.6 or 0.9 ppm formalin.

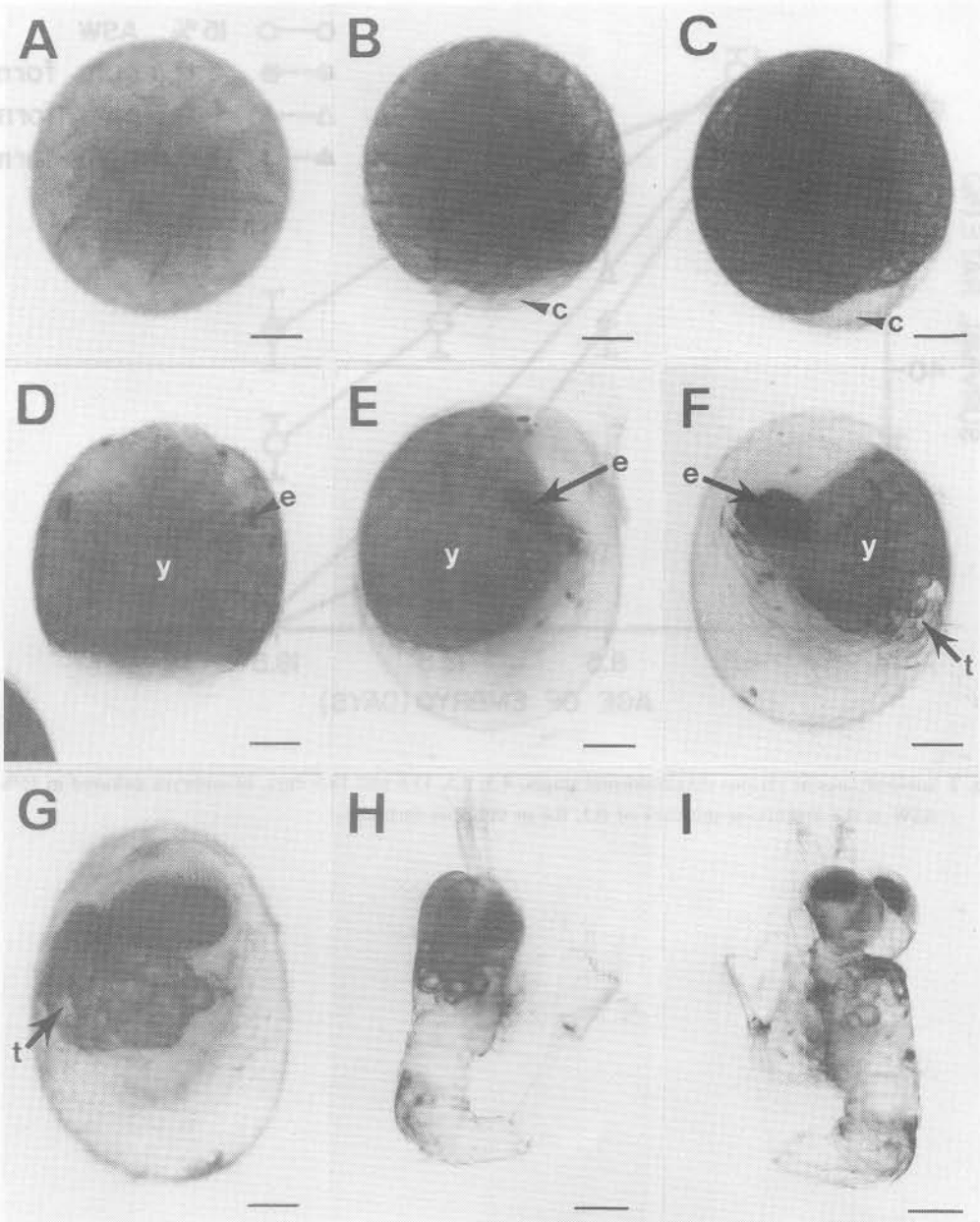

Fig. 3 Bright-field micrographs of developing embryos cultured in 15% ASW. A, 18 hr-old embryo of about 32 cells; B, 4.5 day-old embryo showing a clear region (c) at one pole; C, 6.5 day-old embryo; D, 8.5 day-old embryo developing 2 eye spots (e) in the yolky mass (y); E, 10.5 day-old embryo showing enlarged oval-shaped eyes and the yolky portion (y); F, 13.5 day-old embryo showing large, oval-shaped eyes (e) surrounded by striation and the translucent globules (t) at the dorso-caudal region of the yolky mass (y); G, 17.5 day-old embryo with prominent, dark, relatively rounded eyes and the translucent globules (t) enlarged; H, newly hatched larva on day 18.5; I, hatched larva on day 19.5. Scale bars = 100 μm in A-G, and 200 μm in H and I.