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DEVELOPMENT OF MUD CRAB (*SCYLLA OLIVACEOUS* HERBST) OOCYTE AFTER *IN VITRO* CULTURE WITH THORACIC GANGLION EXTRACTS OF ESTUARINE CRABS (*NEOEPISESARMA LAFONDI* JACQUINOT AND LUCAS)

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ABSTRACT

Thorax ganglion was reported as a source of stimulating hormone for gonad development. The aim of this research was to study the development of mud crab (*Scylla olivaceous*) oocytes cultured in *in vitro* medium supplemented with thoracic ganglion extracts of *Neoepisesarma lafondi*. Immature crabs with 110 mm carapace width were collected from the mouth of Bawana Marana Rivers, Maros Regency of South Sulawesi, Indonesia. Pieces of ovarian tissues from those crabs were incubated within 24 hours in culture medium (Medium-199 with 100 IU/ml Penicillin-G) supplemented with 2 mg/ml thoracic ganglion extract. Result showed that the diameter of mud crab oocytes was increased in congruent with the incubation time. The development was prominent after *in vitro* incubation for 8 hours ($P<0.05$). Early development of oocyte entered the previtellogenic phase after 8 hours of incubation.

Key words: mud crab-oocyte, *in vitro*, thoracic ganglion-extracts

INTRODUCTION

Each organism grows and develops accordingly through its unique way within the environmental limitation. All organisms develop as a result of metabolism process. They take up all necessary nutrients and break down into its chemical components to produce energy and recompose into other components suitable for growth and development.

Oogenesis is a process development of sprout cells into oocyte. During oogenesis, there are two important events, development and maturation. This process is stimulated by gonadotropine hormone. According to John & Sivadas (1979), development of mud crab ovarium consists of three levels, immature, maturing and ripe. The process of

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crustacean ovarium development is divided into four stages namely immature, previtellogenesis, vitellogenesis and maturation. Wallace & Selman (1981) proposed that in general development of oocyte comprises four stages i.e. early development, yolk vesicle, vitellogenesis and maturation. Yolk vesicle stage is assumed as initial stage dependant on gonadotropine hormone. Lee & Walker (1995) divided the alteration during vitellogenesis stage into primary vitellogenesis and secondary vitellogenesis.

As a source of stimulating hormone for gonad development, thoracic ganglion extract (TGE) from non-economic crabs have been proven in stimulating development of oocyte of mud crab after 24 hr *in vitro* culture (Fujaya *et al.* 2005). However, less is known on how the oocyte development process occurs. This study was conducted to search the development events every 4 hr during 24 hr culture in terms of oocyte size and internal development.

MATERIALS AND METHODS

Animal

Mud crab (*Scylla olivaceous*) and estuarine crab (*Neoepisesarma lafondi*) were collected from mangrove swamp and mudflats of Bawana Marana River, South Sulawesi. Animal target used in this research were one immature female of mud crab *S. olivaceous* (110 mm carapace width) as a source of ovarian which had been cultured *in vitro*, and thirty estuarine crabs (*N. lafondi*) with 40 mm carapace width were used as a donor of thorax ganglion.

Ganglion Collection, Preservation, and Extraction

Estuarine crabs were rinsed and anaesthetized in cool box (8°C). The thoracic ganglion was dissected out and kept into acetone until further processing (Fujaya *et al.* 2000a). Ganglion tissues (0.25 g) were homogenized in distilled water. Following centrifugation, the supernatant was removed into a small tube and kept at -20°C until further use.

Ovarian Collection and *In Vitro* Incubation

Mud crabs were rinsed with clean seawater and anaesthetized in a refrigerator (4°C). The ovary was dissected from the cephalothorax, washed in physiological saline (0.82% NaCl) containing 600 IU/ml Penicillin-G and cut into small pieces (5-6 mm in length). Ovary refers herein to all the ovarian tissues in one mud crab. Pieces of ovarian tissues were incubated in culture vials containing 2 ml of culture medium (Medium -199 with 100 IU/ml Penicillin-G and 2 mg/ml thoracic ganglion extract). The last research found that 2 mg/ml of thoracic ganglion extract from estuarine Crabs (*N. lafondi*) was the best (Fujaya *et al.* 2005). The pH medium was adjusted to 7 by addition of $NaHCO_3$ (Fujaya *et al.* 2002) and the ovarian tissues were incubated for 24 hr at 25 ± 1°C in orbital shaker (50-60 rpm) darkness.

Ovarian Histology

The ovarian explants were fixed for 24 hr in aqueous Bouin's fluid. Thereafter, the tissues were dehydrated in alcoholic series, and embedded in paraffin (m.p. 56-58°C). Five mm sections were cut and stained with Delafield's haematoxylin followed by counterstaining in alcoholic eosin (Kiernan 1990).

Experimental Design and Data Analysis

The experiment was designed with a completely randomized design with seven incubation times (0, 4, 8, 12, 16, 20, 24 hours) and four replicates. One hundred of oocytes in each culture ovarian explants were measured by an ocular micrometer. Statistical treatment of the experimental data followed the analysis of variance at significant level of 0.05 ($P<0.05$).

RESULTS AND DISCUSSION

In the *in vitro* culture, there was no necrosis of ovary. This indicated that the cells were alive and metabolism occurred under this *in vitro* condition. Initial diameter of oocyte was 42.68 ± 5.71 μm and developed to 53.08 ± 8.01μm at 24 hr culture (Figure 1). The oocyte diameter was increased about 10.40 μm. While for the cultured oocyte without supplementation of thoracic ganglion extracts, the diameter increase was only ± 5.69 μm (Figure 2). This result showed that the thoracic ganglion extract was able to stimulate oocyte development although the extract of ganglion thorax was taken from other crab species.

Based on variance analysis and Bonferroni test, the oocyte diameter was significantly increased after 8 hr of incubation in congruent with the longer period of incubation time. In this research, the biggest oocyte diameter was found at 24 hr of incubation. This change indicates that after 8 hr of incubation, early development phase started to take place. Accretion of oocyte size caused by accumulation of a number of substances is necessary for embryo growth.

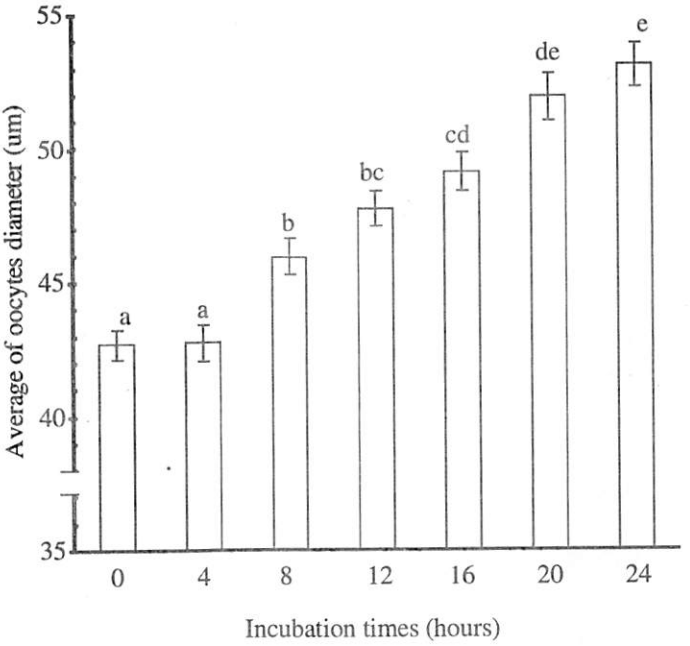

Figure 1. Oocyte diameter of mangrove crab during *in vitro* culture with 2 mg thoracic ganglion extract (TGE) of *Neoepisesarma lafondi* per ml medium. Significance ($P<0.05$) shown by different legends (a-e).

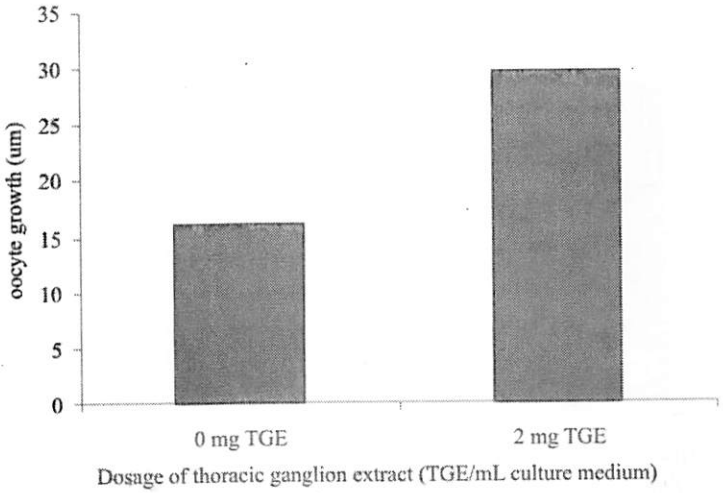

Figure 2. Comparison of oocyte diameter growth of mangrove crab within 24 hr *in vitro* culture supplemented with and without thoracic ganglion extract (TGE)

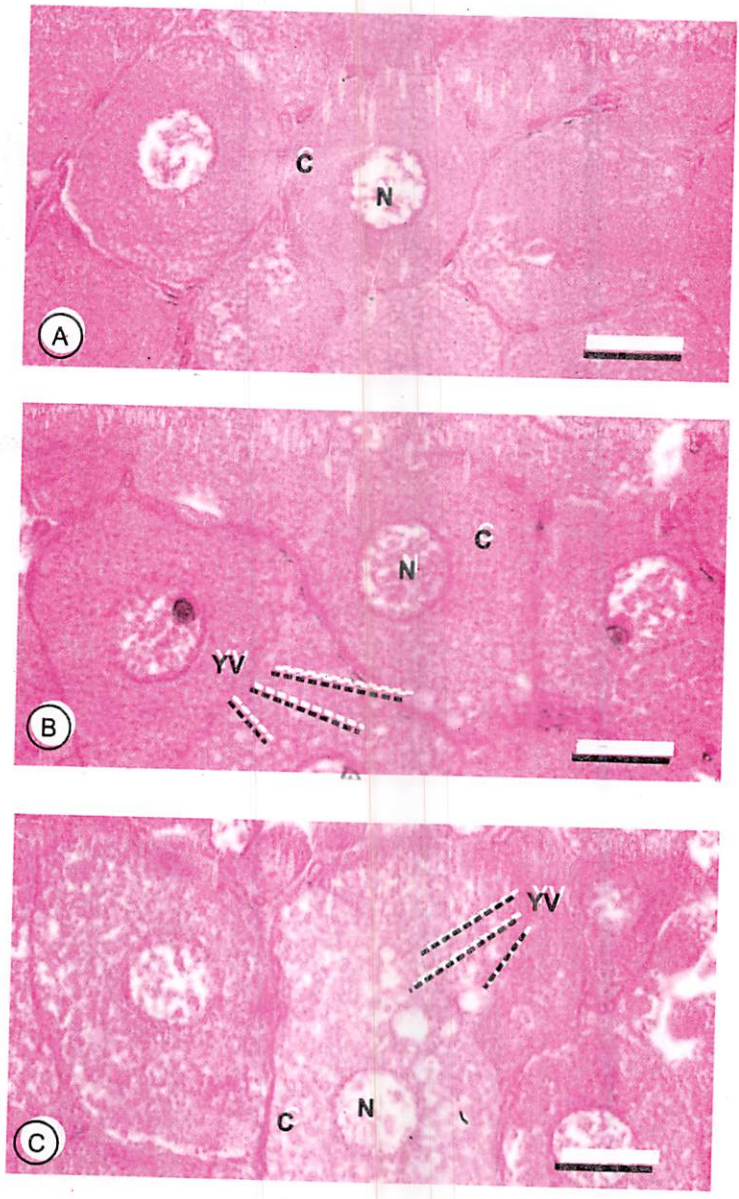

Figure 3. Histological illustration of mud crab oocyte cultured *in vitro* in the medium supplemented with 2 mg of thoracic ganglion extract of *Neoepisesarma lafondi*. A. Before culture; B. 8 hr of culture: oocyte diameter increased and initial forming of yolk vesicle; C. 24 hr of culture: oocyte diameter increased. C: cytoplasm; N: nucleus. Dashed lines represent yolk vesicle (YV). Bar = 20 um

According to Wallace & Selman (1981), early development phase is characterized by increase of nucleus size and in the number of nucleolus, accumulation of DNA complex in various structures, and storing of particles responsible for the formation of basophile cells by cytoplasm such as RNA. At this phase, a large number of RNA is stored in the oocyte cytoplasm. This condition enables the embryo to produce protein for its development. After the early development phase, the oocyte will enter the yolk vesicle phase which is assumed as the beginning of dependency on gonadothropine hormone. At this phase, the oocyte size increases without accumulation of egg yolk materials but formation of yolk vesicle. This vesicle does not contain true egg yolk. Therefore, Chan (1995) proposed to use the term previtellogenic phase instead of yolk vesicle phase. In this research, formation of vesicle was observed after 8 hr of incubation and the vesicle continuously increased after 24 hr (Figure 3). It is likely that the oocyte may enter maturing phase if the treatment would exceed more than 24 hr.

John & Sivadas (1979) reported on histological changes in the oocytes of the estuarine crab *Scylla serrata* (Forsskal) after eyestalk ablation, and found that the size of oocytes ranged from 0.04 – 0.12 mm (average 0.074 mm) have entered the maturing phase. Maturing phase is a process of accumulation of egg yolk into the oocyte. In this research, the size of oocyte was around 0.05 mm (53.08 mm) at 24 hr incubation, but it has not entered the maturing phase. Nevertheless, the growth of the oocyte in this study was faster compared to the result as reported by John & Sivadas (1979). Studies have shown that the oocyte entered the maturing phase 18 days after eyestalk ablation. Eyestalk ablation was conducted to eliminate Gonad Inhibiting Hormone (GIH) which is located in organ-X and sinus gland. When the GIH decreased in circulation, Gonad Stimulating Hormone (GSH) will increase; therefore maturation of oocyte will take place (Adiyodi & Adiyodi 1970; Meusy & Payen, 1988).

Oocyte will enter vitellogenesis phase or maturing after immature and previtelogenic phases in the *in vivo* culture. (Chan 1995; John & Sivadas 1979; Fujaya *et al.* 2000b). However, this further development hardly occurred in the *in vitro* culture without enhancing raw material of egg yolk into the culture medium as conducted in this research.

Vitelline is a specific protein in the egg yolk which accumulates into oocytes during vitellogenesis. The pigment of crustacean vitelline contains lipoprotein (Meusy & Payen 1988). Vitelline and lipid droplets are minor components during immature phase, but concentration of these components increase and become the major component on mature oocyte. Consequently, accumulation of egg yolk does not only cause the oocyte grows larger but also its color changes from yellow till orange. In this research, oocyte color did not change; the color remained white during the whole study period. This indicated that the oocyte has not developed into maturing phase.

Lipovitelline started to accumulate in the developing oocyte as a response to hormonal signal (Lee & Walker 1995). According to several researchers, gonad-stimulating hormone (GSH) which is synthesized by thorax ganglion (Adiyodi & Adiyodi 1970; Sarojini *et al.* 1995, Chan 1995, Zapata *et al.* 2003) is an important hormone in accumulation of lipovitelline process. GSH stimulates other hormone secretions mainly steroids which are secreted by follicular cells, like estradiol and progesteron. Consequently, the estradiol plays a role in the stimulation of vitellogenin synthesis by hepato-

pancreas, body fat, and oocyte. Warrier *et al* (2001) explained that estradiol 17b could function as a part of multihormonal system, by activating vitellogenin synthesis related to metabolic pathway. In addition, it could also have a cumulative effect with other hormones influencing vitellogenesis. These include the neuropeptide hormones, such as the GSH and the vitellogenin inhibiting hormone (VIH) which has an agonist-antagonist effect. The terpenoids, such as methyl farnesoate (Laufer *et al.* 1990) and the polyhydroxylated keto steroids such as the ecdysteroids (Gunamalai *et al.* 2003; Chan 1995) have a stimulatory effect on vitellogenesis.

CONCLUSION

From this study, it can be concluded that the development of the mud crab oocyte was increased by longer period of culture in *in vitro* medium supplemented with thoracic ganglion extracts of *N. Lafondi*. The oocytes incubated in culture medium supplemented with 2 mg/ml thoracic ganglion extracts of *N. Lafondi* were developed into previtellogenesis phase after 8 hours of incubation.

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