Effects of calcium ionophore on the morphogenesis in horseshoe crabs

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Introduction

The transplantation of center cells into early gastrula embryos induced secondary embryos in horseshoe crabs (Itow and Mochizuki, 1988; Itow *et al.*, 1991a). Besides, the microinjection of several chemical reagents induced secondary embryos in horseshoe crabs. Especially calcium ionophore A23187 (Calbiochem-Behring Co.) induced secondary embryos at a high rate (Itow *et al.*, 1991b).

The calcium ionophore is known to stimulate the influx of calcium ion into cells. We examined the effects of calcium ionophore on horseshoe crab embryonic developments by treatments at different developmental stages. Four types of malformations were induced by the short time treatment with calcium ionophore. In the present paper, these malformations are characterized.

Materials and Methods

Adult horseshoe crabs, *Limulus polyphemus* were sent from Woods Hole Marine Laboratory, Massachusetts. They were transferred to Shizuoka University, where the present study was conducted.

Fertilized eggs were obtained with the artificial insemination. Embryos at different developmental stages were treated with calcium ionophore A23187 (Calbiochem-Behring Co.). Each interval of a developmental stage and the next stage is about half a day. Calcium ionophore was firstly dissolved in dimethylsulfoxide (DMSO), then dissolved in sea water. The final concentration of DMSO was less than 1%. The final concentration of calcium ionophore and the duration of treatment are shown in the results. Many groups of 40 embryos each were treated for a short time in small laboratory dishes filled with 1 ml sea water containing calcium ionophore. After treatment, the embryos were returned to normal sea water which was renewed every days.

Normal and treated embryos were vitally stained with 1/20,000-1/400,000 neutral red in sea water and then observed under a stereomicroscope. The embryos were observed at Stages 20, 21 (hatching stage) and the stages after hatching.

Results and Discussion

Conditions of treatments with calcium ionophore

The conditions for formation of deformed embryos were as follows. (1) Developmental Stage. Four types of malformations were induced after the treatment at each stage from Stage 1 (the stage immediately after insemination) to Stage 13 (the stage of appearance of body segment structures). Embryos at earlier stages were considerably affected by treatments of calcium ionophore. Many embryos at earlier stages failed to develop after treatments of calcium ionophore, while the same treatment at later stages could permit embryos to develop to the advanced stages (Fig. 1). (2) Duration of treatment. Deformed embryos were induced by the treatment of the ionophore for only 5 min. No embryos treated before Stage 7 (early gastrula) developed by the treatment for more than a few hours at more than 50μg /ml calcium ionophore. Some embryos after Stage 7 could develop after exposure to 100μg /ml calcium ionophore for even 12 h. The abnormal embryos were obtained by the treatment of various duration from 5 min to 12 h, mainly for 10 to 30 min at those stages (Fig. 2). (3) Concentration of calcium ionophore. The embryos before Stage 7 were not developed by the treatment at 100μg /ml calcium ionophore for more than 10 min. Deformed embryos were obtained efficiently by the treatment at 50μg /ml calcium ionophore for a few ten minutes before Stage 7. The embryos at each

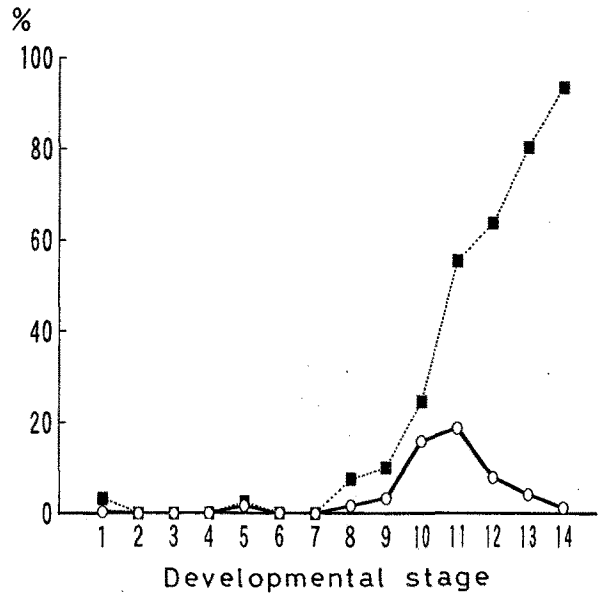

Fig. 1 Results of treatments with 100μg /ml calcium ionophore for various durations from 5 min and 1 h at different developmental stages. 120 to 639 embryos were treated at each stage. ■ : rate of developed embryos, ○ : rate of malformed embryos against all treated embryos.

stage from Stage 8 to Stage 13 often developed as deformed embryos after the treatment for 10 to 30 min at 100μg /ml calcium ionophore.

One percent DMSO did not affect on the development of horseshoe crab.

Characteristics of the malformations after the treatments with calcium ionophore (Fig. 3)

1. Embryos with big head (Fig. 4a)

The embryos at each stage from Stage 1 (immediately after insemination) to Stage 6 (late blastula stage) developed with big heads after the treatment with calcium ionophore. This type of malformations had the normal anterior part and the extremely incomplete posterior part of embryos. The degree of malformation and other characteristics did not differ among the monsters obtained by treatments at different stages. The normal anterior part of monsters with big heads was the prosegmental part and the first segment. The right and left appendages of the 2nd and 3rd segments often fused at the middle line of bodies in these monsters (Fig. 5).

2. Multiple embryos (Fig. 4b)

The multiple embryos were induced after the treatment of calcium ionophore at blastura stage and early gastrula stage, especially early gastrula stage (Stage 7). The multiple embryo had two or more bodies in an egg.

Calcium ionophore and calcium ion may affect on the embryonic development of horseshoe crabs as follows: (1) induction of the differentiation of center cells, (2) production of inducers from the center cells, (3) increase of cell number accepting the inducer, and/or (4) indirect stimulation of these systems. The analysis of the induction system is expected in the future.

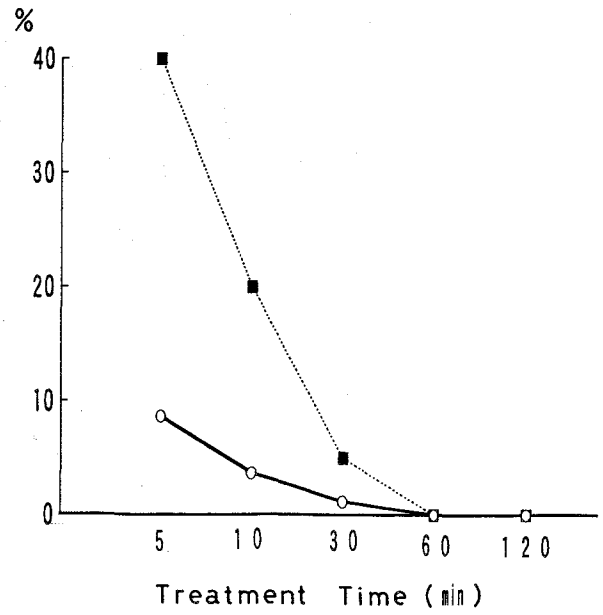

Fig. 2 Results of treatments at 50 μg/ml calcium ionophore for different times at Stage 7. 80 embryos were treated for each treatment. ■ : rate of developed embryos, ○ : rate of malformed embryos among all treated embryos.

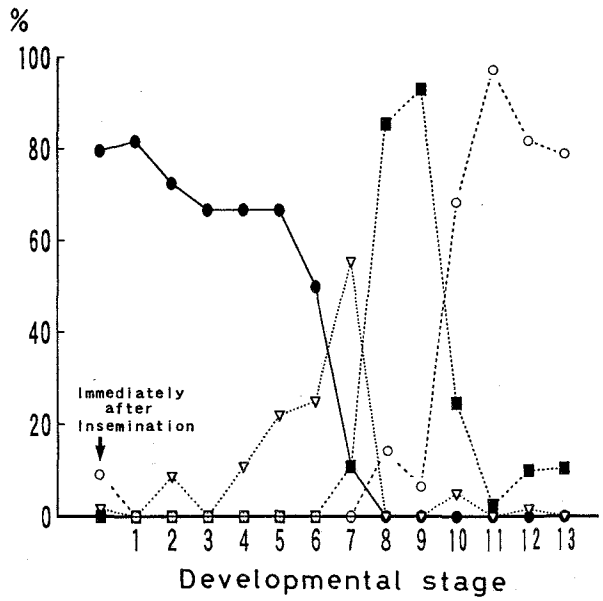

Fig. 3 Percentage of each malformation induced by treatments with calcium ionophore at different stages. ● : monsters with big heads, ▽ : multiple monsters, ■ : monsters with defects and fusions of segments and appendages, ○ : monsters with multiple eyes.

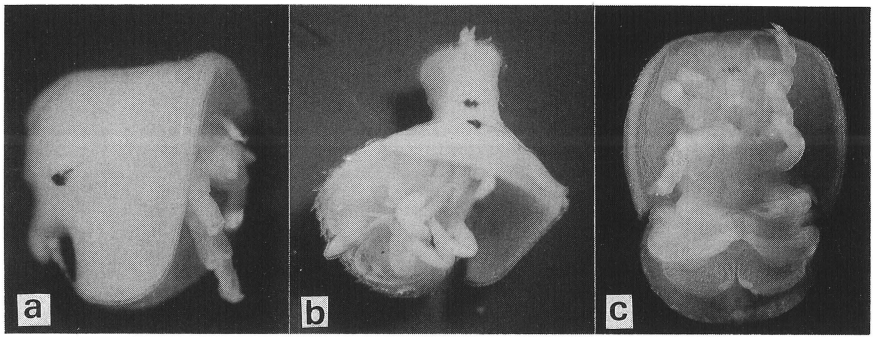

Fig. 4 Malformations induced by treatments with calcium ionophore. a: monster with big head, b: multiple monster, c: monster with fusions of segments and appendages.

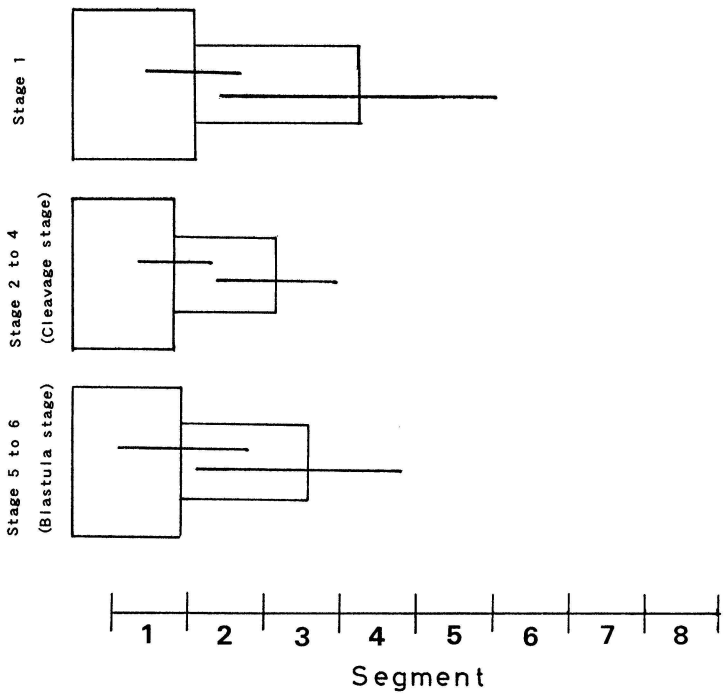

Fig. 5 Monsters with big heads induced by treatments at different stages. The parts with appendages are shown in this figure. The segments with a pair of normal appendages and with only fused appendages are shown as large and small squares, respectively. Bars show the standard deviations (n = 9 to 27).

3. Embryos with defects and fusions of segments and appendages (Fig. 4c)

After the treatments with calcium ionophore at late gastrula stage (from Stage 8 to Stage 10) embryos developed with defects and with fusions of segments and appendages. The position of defects and fusions was mainly the 4th and 5th segments (Fig. 6).

4. Embryos with multiple eyes (Fig. 7)

When embryos at each stage between Stage 10 (the stage of late gastrula or the stage of completion of

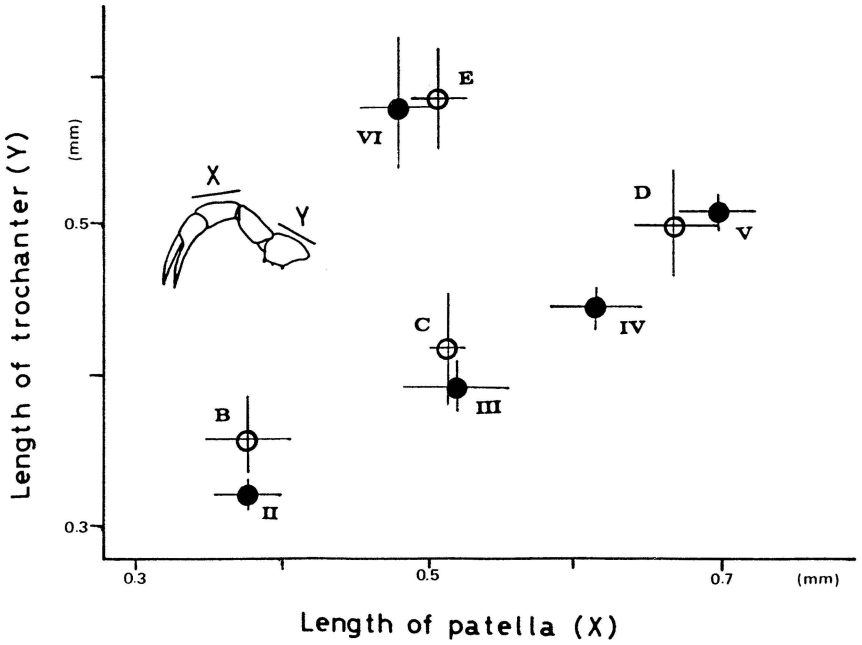

Fig. 6 Comparison of the length of two portions of the appendages in normal embryos and segment defective ones having five pairs of prosomal appendages. ● : normal embryos, ○ : segment-defective embryos. Bars show the standard deviation. II to VI : 2nd to 6th appendages of normal embryos. B to E: 2nd to 5th appendages of segment-defective embryos.

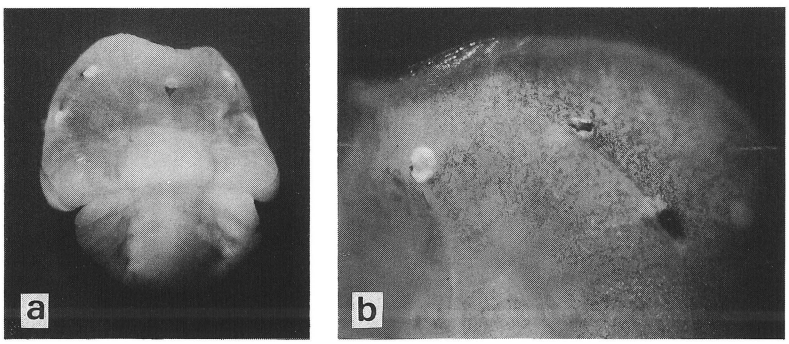

Fig. 7 Monster with multiple eyes. a: whole feature, b: enlargement of eye portion.

germ disc) and Stage 13 (the stage of appearance of segment structures) were treated with calcium ionophore, the lateral eyes were increased in number by the treatment of calcium ionophore. A normal embryo had a pair of eyes, but the monster with multiple eyes often had two pairs of eyes. The position of the posterior eyes in these monsters was the same as or similar to that of normal eyes. The anterior eyes of these monsters were situated at abnormal anterior positions.

The embryos treated at Stage 10 often had the both malformations of defective segments and multiple eyes. We classified these monsters into the monster with multiple eyes. The position of defects of these embryos was at the 4th and 5th segments. Most of the embryos treated at each stage from Stage 11 to Stage 13 had only one abnormality, that is, the multiple eyes (Fig. 8).

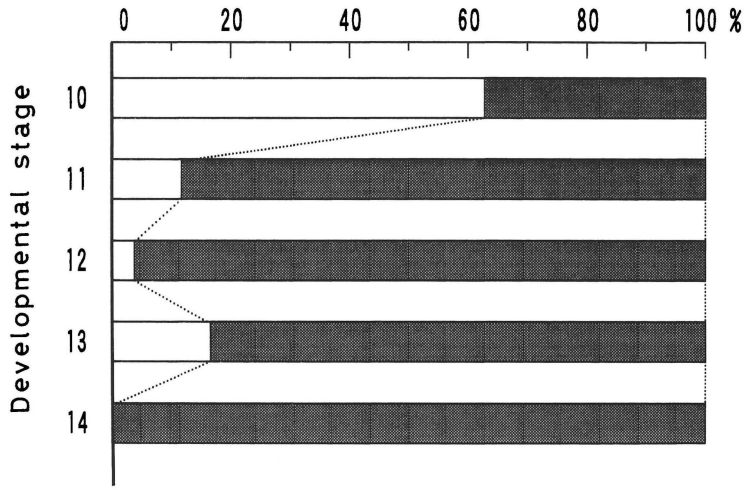

Fig. 8 Abnormality of the monsters with multiple eyes induced after treatments at different stages. The abnormality was the defects and fusions in or near the 4th and 5th segments. Solid square: percentage of monsters with only multiple eyes, open square: percentage of monsters with multiple eyes and other abnormalities.

The monsters with multiple eyes must be the experimental system for the analysis of eye formation and nervous development.

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