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THE RETARDED GASTRULATION INDUCED IN DEVELOPING SEA URCHIN EMBRYO BY REARING IN D_2O-SEA WATER

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ABSTRACT

Sumitro, S.B. 1997. The Retarded Gastrulation Induced in Developing Sea Urchin Embryo By Rearing in D2O - Sea Water. ***Biology*, 2(4): 175-191**

The process of gastrulation was lingeringly proceeded in developing sea urchin embryo reared in 40% D_2O-sea water (D_2O-SW). In general, these embryo possessed large blastocoel, and occasionally developed with evaginated gut. The micro surgical experiments confirmed that the postulated higher hydrostatic pressure of blastocoel was not responsible for gut evagination. Thus the alternative hypothesis, "stabilization" of micro tubular cytoskeleton in the filopodia of the secondary mesenchyme cells, and disharmony of cellular development remain the possible cause of gut evagination. This hypothesis, however are coincided with the fact that D_2O strongly inhibited cell division and archenteron formation.

Key words : retarded gatrulation, sea urchin, D_2O

INTISARI

Sumitro, S.B. 1997. Penghambatan gastrulasi embrio landak laut dalam air laut yang mengandung D2O. ***Biologi*, 2(4): 175-191**

Proses gastrulasi pada embrio landak laut berjalan amat lambat bila mereka dipelihara dalam air laut mengandung D_2O (Deuterium Oxide) 40%. Secara umum, mereka menampakkan bentuk blastula dengan rongga besar dan kadang-kadang berakhir dengan gejala exogastrulasi. Penelitian ini membuktikan bahwa pembesaran volume blastocoel dan exogastrulasi bukanlah karena peningkatan tekanan hidrostatik

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cairan di dalamnya. Karena D_2O dikenal menghambat dinamika polimerisasi mikrotubules dan menghambat pembelahan sel, maka pembesaran ukuran blastula dan exogastrulasi lebih mungkin diakibatkan oleh efek D_2O tersebut di atas.

Kata kunci : penghambatan gastrulasi, landak laut, D_2O

INTRODUCTION

If developing sea urchin embryo was reared in 40% D_2O-SW at normal temperature, embryonic cells managed to divide, at slower rate, and the biological clock set for normal development was prolonged (Hoshi, 1979; Sumitro & Sato, 1989). For instance, D_2O affected the micrometers formation and delayed further development of cells derived from micrometers (Sumitro & Sato, 1989). This could cause disorganized pace of differentiation amongst embryonic cells. Present observation showed that the process of gastrulation was extended when embryo was continuously reared in 40% D_2O-SW beginning from an early stage of development. The gastrulation was less extended when D_2O was applied at later stage of development. This cumulative effect of D_2O resulted "animalization" in *Hemicentrotus pulcherrimus* but was not clear in *Clypeaster japonicus*.

When embryos were continously reared in D_2O-SW, they developed to larger blastulae with larger blastocoel and some of them proceeded gut evagination (Hoshi, 1979). In general, larger blastocoel was thought to be associated to the phenomenon of embryonic swelling (Ishihara *et al.* 1982), and was oftenly considered to have resiprocal relationship with the process of gut evagination (Dan & Okazaki, 1956; Ettenshon, 1984). Thus, it was possible that the resulted gut evagination in D_2O-treated embryo was due to the increased hydrostatic pressure in the blastocoel pushing the invaginating endodermal plate outward.

Other report suggested that the gut evagination was a result of the absence of force pulling inward by secondary mesenchyme cells (Dan & Okazaki, 1956). Because microtubules and stress fibers were involved in the formation of the filopodia of the secondary mesenchyme cells (Tilney & Gibbin, 1969), possibly the absence of inward pulling was due to the "stabilization" (Itoh & Sato, 1984; Marsland & Zimmer-man, 1985; Sato *et al.* 1989; Sumitro *et al.* 1989; Takahashi & Sato, 1984) of microtubules along with other cytoskeletal components by D_2O. To clarify these standing hypothesis, we examined the induction of gut evagination in the developing sea urchin embryos culturing in D_2O-SW. Data obtained are des-cribed below.

MATERIAL AND METHODS

Obtaining gamets and rearing

Mature eggs of *H. pulcherrimus* or *C. japonicus* were obtained by injecting isotonic (0.55 M) KCl into the coelom of an adult. Dry sperm was obtained by electric stimulation. Eggs were inseminated and reared fallowing the methods which was previously reported (Sumitro & Sato, 1989). Temperature was maintained at 17^0 to 18^0C for *H. pulcherrimus* and 20^0 to 23^0C for *C. japonicus*. D_2O (99.86 M percent purity; Bio-Rad Laboratories, Richmond, U.S.A.) was eluted through resin, chelex 100 (Bio-Rad, Laboratories, Richmond, U.S.A.) before using, to avoid from possible metals contamination attributed as the cause of abnormal development of sea urchin embryo (Mitsunaga *et al.* 1983; Nemer, 1985). Normal artificial sea water (NASW) and 99.86 M percent D_2O-SW (stock solution) were prepared according to the M.B.L. formula (Ostrow, 1956). Rearing medium was 40% D_2O- SW.

Obtaining the timetable of embryonic development

Starting from a few hours before the hatching stage, small amounts of embryos from each series of D2O treatment were removed every 1 hrs, and fixed with 2.5 & glutaraldehyde in NASW. The number of embryo in each sample, then were examined and counted with a DIC-microscope (gut evaginated embryos or larvae excluded). When more than a half of embryos had gone through a particular stage, we designated that time as the point at which the embryos had reached to that stage. The timetable of embryonic development of both control and D_2O-treated embryos were drawn based on the data obtained from three time repeated observations.

Cell counts of macerated embryo or larva

A modified methods for cell counts (Stephen *et al.* 1986) was used for determining the number of

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cell per embryo or larva at respective stage. We used Kane's medium (1M glycine, 2mM EDTA; Kane, 1973) which completely dissociated cells of embryos of *H. pulcherrimus*. Observations with DIC-microscope revealed no sign of lysed-cell formation, thus we used this dissociation medium to estimate total cell number per individual embryo or larva.

Sucking and injecting blastocoelomic fluid

A drop of sea water (0.02 ml) containing two or three blastulae was put on a clean slide glass, then placed onto the mechanical stage of an inverted microscope (IMT-2, Olympus, Japan). Microsurgical operation then was done using a set of micromanipulator (MO-103 M, Narashige Co.,Ltd., Japan). A holding pipet (tip diameter: 40-60 um) was used to immobilize the blastula and a micropipet (tip diameter 1.5 to 3 um) was used to either suck or inject the blastocoellomic fluid. Silicon oil with viscosity of 500 centistokes (Shin-Etsu Chemical Co., Ltd., Tokyo) was used for the precise controlling action of the micropipet. After the operation, blastulae were released in NASW or 40% D_2O-SW, and maintained in moist microchamber (Lutz & Inoue, 1986) to develop up pluteus.

RESULTS AND DISCUSSION

Retarded gastrulation in D_2O-SW

The process of development of embryos from fertilization up to the hatching stage, in which the blastomeric division was the marked activity of developing embryo (Dan *et al.* 1980; Masuda, 1979; Takahashi & Okazaki, 1979), was extended by D_2O. For instance, continuous rearing from an early stage of development with 40% D_2O, at 17^0C, the hatching stage of *H. pulcherrimus* embryo showed 3 hrs lag compared to the control. The pronounced inhibitory effect of D_2O on the embryonic morphogenesis appeared to occur at the gastrulation stage. When the control embryo reached late gastrula, about 24 hrs after fertilization, embryos which were transfered to D_2O-SW at 8-cell stage were still in the onset of gastrulation. These treated embryos completed gastrulation more than 18 hrs later as compared to control. The process of gastrulation was faster on embryos transfered to D_2O-SW at later stage as compared to those transfered at earlier stage. (Fig 1). Shows that morphogenic retardation proceeded along with the inhibition effects of D_2O on cell divisions. This may indicate a contributive effect of slow rate cell division on the abnormal development of embryos reared in D_2O-SW.

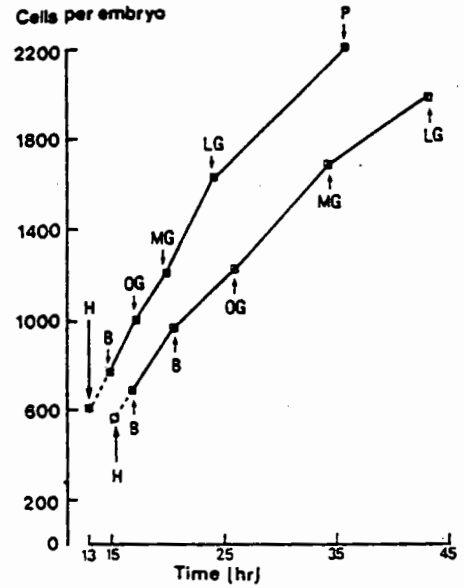

Figure 1. Number of cell per embryo of developing H. pulcherrimus embryo reared in 40% D2O-SW since 8 cell stage (open rectangle) and in NASW (solid rectangle) was plotted against time. H, B, OG, MG, LG, and P indicates the stages of hatching, blastula, onset of gastrulation, mid-gastrulation late gastrulation and prism respectively.

The process of gastrulation has been subdivided into two phases (Dan & Okazaki, 1956; Gustafson & Kinnander, 1956): The first phase is known as primary invagination or endodermal plate invagination. At this time the archenteron extends 1/4 to 1/2 of the way across the blastocoel. The second phase is marked by appearence of filopodia of secondary mesenchyme cells. The archenteron elongates while it's diameter decreases completing the gastrulation Fig 2. These two phases also could be distinguished in the D_2O-treated embryos which exhibited sucessful gastrulation.

Fig 3 shows that *H. pulcherrimus*, continuously reared in D_2O-SW extended the time required for primary invagination (first phase of gastrulation) approximately 4 hrs longer than control. However, the process to second step of gastrulation was slightly extended, when the embryo were introduced to D_2O at the begining of endodermal plate invagination. D_2O also did not prolong the second step of gastrulation when embryos were introduced to D_2O-SW at mid-gastrula stage after the completion of the first step of gastrulation.

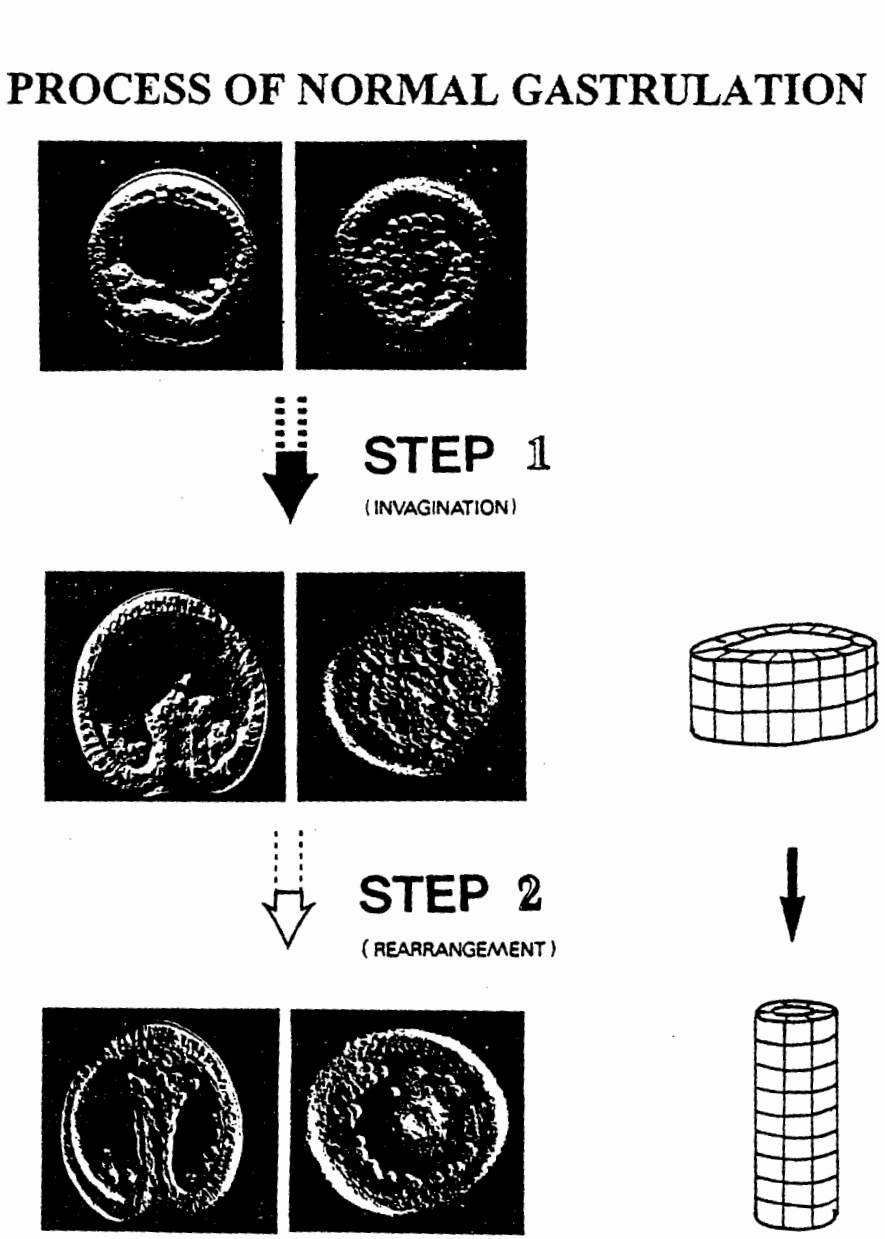

Figure 2. The Gastrulation Process is indicated by archenteron formation. The process is started by invagination of endodermal plate at vegetal region (step 1), and fallowed by elongation of archenteron by mean of cellular rearangement (see diagram) and filopodial rearangement (small arrow).

Some embryos exhibited gut evagination when they were reared in D_2O-SW (Table 1). Continuous observation on the process of gut evagination of an embryo of *H. pulcherrimus* showed that embryo

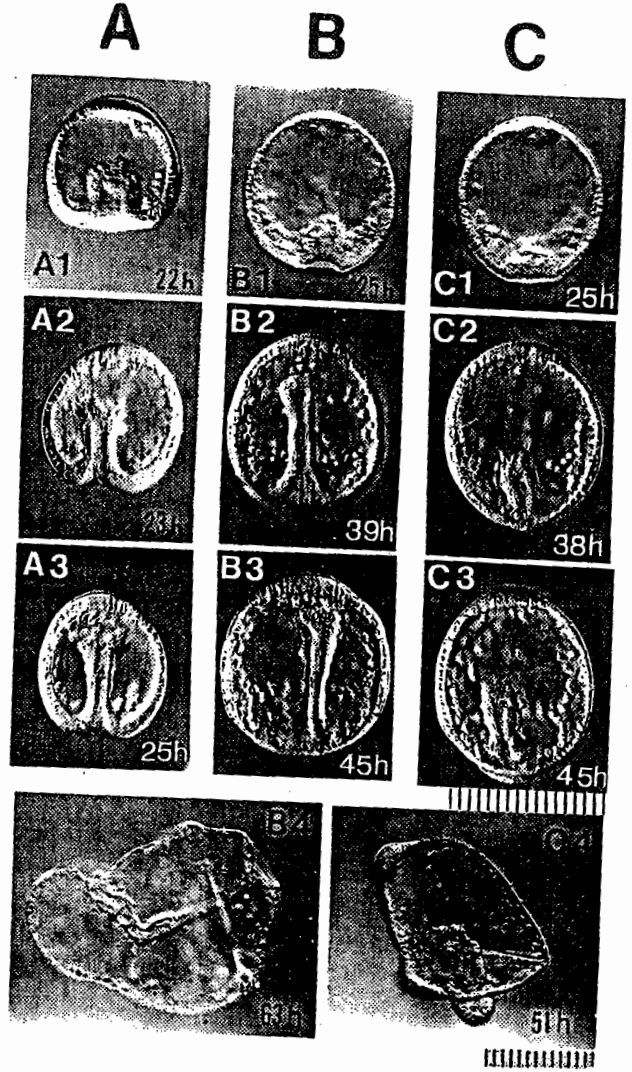

Figure 3. A : Normal gastrulation of H. pulcherrimus ; B&C : Delayed process of gastrulation producing abnormal larvae (B4 & C4)
The embryonic age 1s indicated at lower right of each picture (h = hour)

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began to exhibit endodermal protrusion at vegetal region when it's slim archenteron reached less than a half way across the blastocoel (Fig.3). While the slim archenteron elongated, the endodermal protrusion was more prominent. Embryos reared in D_2O-SW mostly identified as typical incomplete gut evagination (entoexogastrulation). Complete gut evagination (exogastrulation) however was ocasionally occured.

Gut evagination occured along with "animalization". We preserved the term "animalization" for embryo which exhibited ever-grown ectoderm, and repressive endodermal development (see Fig. 3B4). For *H. pulcherrimus*, significant number of gut evaginated larvae only appeared when they were continuously reared in D_2O-SW from an early stage of development. For *C. japonicus*, significant number of gut evaginated larvae could also be found when the embryos were continuously reared in D_2O-SW since the later stage, namely, the hatching stage. In both species, gut evaginated larvae did not appear if the embryos were introduced to D_2O-SW at primary mesenchymal blastula stage or later (Table 1).

Table 1. Percentage of gut evaginated larvae reared in D2O-SW (observed at 65 hrs after fertilization)

		H. pulcherrimus		*C. japonicus*	
A. Stage when D_2O was introduced	No. of exp.	Control	D_2O	Control	D_2O
8-cell	1	0	11.3	0	28.3
	2	0	8.9	0	29.1
	3	0	7.2	0	21.4
Hatching	1	0	0	0	10.2
	2	0	0	0	2.3
	3	0	0	0	15.0
Primary mesenchymal blastula	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
B. Embryo reared in D_2O-SW since 8-cell stage was recovered to NASW at stage of					
Hatching	1	0	0	0	0
	2	0	0	0	0
Large primary mesenchymal blastula	1	0	0	0	1.2
	2	0	0.7	0	0
	3	0	14	0	0

Rearing temperature was 17°C for *H. pulcherrimus* and 23°C for *C. japonicus*. The concentration of D2O was 40% in NASW

Higher hydrostatic pressure developed inside the blastocoel did not induce gut evagination.

In *C. japonicus*, D_2O induced larger blastulae but did not induce either larger gastrulae or "animalization". Thus, the D_2O effect was different as compared with *H. pulcherrimus*. Further observations indicated that during 4th division, 8 blastomeres of *C. japonicus* embryos synchronously divided even if they were reared in 40% D_2O-SW. It was also note that the number of exogastrulation was higher in the species. One of the interpretations for the induction of larger blastulae might be the phenomenon of embryonic swelling induced by D_2O. If this was the case, the hydrostatic pressure in the large blastocoel should be higher than control. Since treatments inducing gut evagination also induced large blastocoel (Dan & Okazaki, 1956; Hashi, 1979; Okazaki, 1956; Takahashi *et al.* 1977), it might be true that gut evagination induced by rearing in D_2O-SW was due to the higher hydrostatic pressure in the blastocoel. The following experiments, then, was done to clarify this issue.

Eleven large primary mesenchymal blastulae of *C. japonicus* which were continuously reared in D_2O-SW, were sucked by micromanipulation to reduced the hydrostatic pressure inside their blastocoel. These embryos, then, were allowed to develop in D2O-SW in a moist microchambers (see

Table 2. The gastrulation in the embryo having increased and reduced coelomic pressure.

	Normal	Gut evagination
A. Number of Injected blastula		
9	9	0
B. Number of sucked blastula		
11	7	4

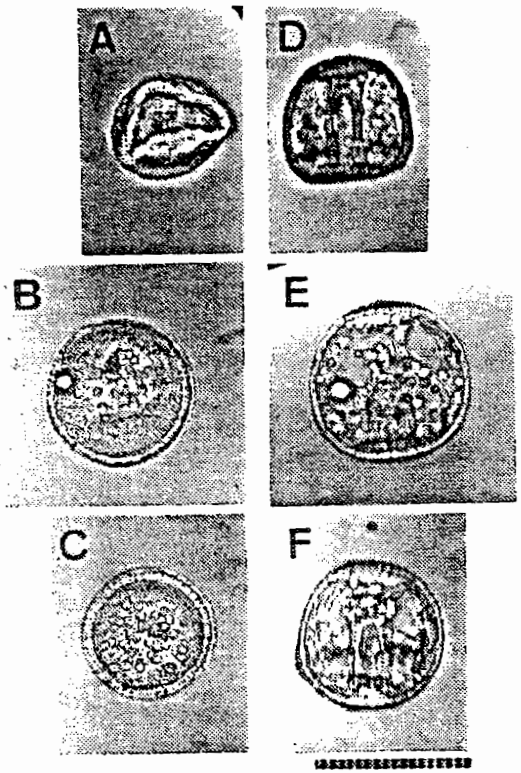

Figure 4. Microsurgical experiment
A & B : Larger blastula was sucked it's blastocoelic fluid
C & D : Exogastuilated embryo developed from respective sucked blastula

also material and method). A few hours after sucking, four out of eleven sucked embryos exhibited endodermal protrusion. These embryos definitely identified as Table 2 gut evaginated larvae when they developed into pluteus stage (Fig. 4).

On the otherwise, fresh blastocoelomic fluid obtained from control primary mesenchymal blastula was injected into the blastocoel of other control primary mesenchymal blastula, untill match it's volume equal to the average large size of primary mesenchymal blastulae reared in D_2O-SW. This blastula, then, was marked by injecting a droplet of silicon oil (500 centistokes). After all, this enlarged blastula, were let to develop in NASW (in a moist microchamber)

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up to pluteus stage. Result showed that the artificially injected larger blastula developed into larger gastrula. On the contrary, the blastula in which some of it's blastocoelomic fluid was removed by micromanipulation, developed into smaller gastrula. As the coelomic pressure increased, the shape of gastrula became round (Fig.5). In general, embryo with higher coelomic pressure proceeded slower gastrulation as compared to the embryo possessed lower coelomic pressure. However, the results obtained from nine experiments confirmed that none ofthem developed into gut evagination (Tabel 2).

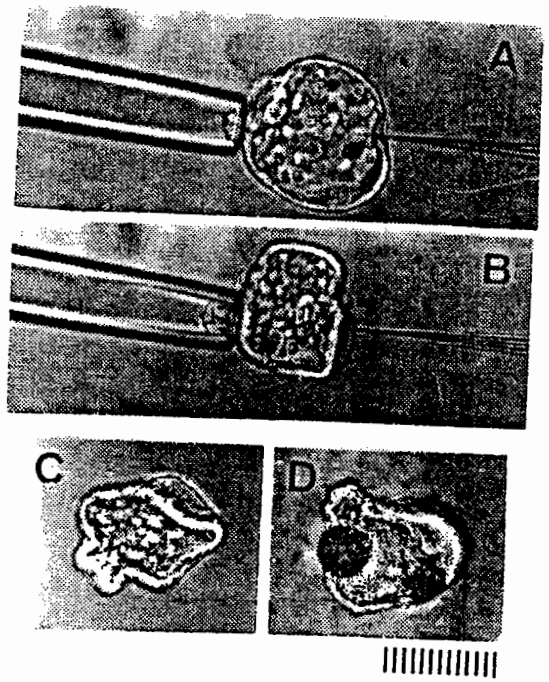

Figure 5. Effet of hydrostatic pressure in the blastocoel to the process of gastrulation in C. japonicus embryos.

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"Animalization"

The division rate of blastomeres in developing sea urchin embryo was slowdown in the presence of D_2O, cause extension of each process of further development. Observations on the time course of the embryonic development of both in control and D_2O-SW revealed that continuous rearing in 40% D_2O from the early stage of development clearly extended the process of gastrulation. The gastrulation process was less disturbed if D_2O was introduced at later stages, and gastrulation was completed on time if D_2O was introduced at mid-gastrulation. Thus, the extension of gastrulation in D_2O-SW should be a result of cummulative effect of D_2O throughout development. Based on our previous observation (Sumitro & Sato, 1989), we found D_2O disturbed the micromere formation and the development of cells derived from micromeres as compared to control. Because small micromeres participate in the archenteron formation (Amemiya, 1989; Pehrson & Cohen, 1986), It is possible to assume, especially in *H. pulcherrimus* embryo, that the delay of micromere formation and the slow pace of development of micromeric cells contributed to the slodown of gastrulation. Larger gastrula of *H. pulcherrimus* in D2O-SW should be an early sign of "animalization"

On the contrary, embryos of *C. japonicus* did not develop into animalized larvae when they were continuously reared in D_2O-SW. Indeed, embryos developed into larger primary mesenchyme blastulae in D_2O-SW, but later development showed normal size gastrulae. This kind of phenomenon, therefore, was frequenly associated to the embryonic swelling (Ettenshon, 1984; Ishihara *et al.* 1982). However, the fact which was illustrated in figure 3, might showed an other explanation. The volume of large primary mesenchymal blastula reared in D_2O-SW, at 21 hrs after fertilization, was about equal to the volume of those control gastrula, at 20 hrs after fertilization, rising a suggestion that the embryonic volume of this D_2O-treated blastula remained to increase when it's development to enter gastrulation was inhibited or delayed by D_2O.

It was not clearly known why *C. japonicus* embryos in D_2O-SW which exhibited slow gastrulation, did not develop into large gastrulae and animalized larvae. However, the fact that D_2O did not disturb the micromere formation, suggesting that D_2O might not disturb the animal-vegetal balance of the developing *C. japonicus* embryos.

Gut evagination

D2O might "stabilized" microtubules assembled in the filopodia of secondary mesenchyme cells, but embryos transferred into D2O-SW at mid-gastrula stage could complete the gastrulation on time. Thus, suggesting that D2O did not directly disturb the second phase of gastrulation, supporting to the conclusion saying that the second phase of gastrulation is microtubule-independent process (Hardin, 1987).

Our observation indicated that the gut evagination did not correspond to the higher coelomic pressure pushing endodermal plate outward. The facts that gut evagination occured only in embryos which were transferred into D_2O-SW at hatching stage or earlier, suggested that gut evagination was due to the cumulative effect of D_2O disturbing the original program set in blastomeres each throughout the development.

It was clear from our observations that vegetal protrusion of gut evaginating embryos progressed along with the elongation of slim archenteron. This fact suggested that vegetal protrusion occured when the archenteron had already entered the second phase of gastrulation in which the archenteron elongates due to the cell rearrangement (Amemiya *et al.* 1956; Ettenshon, 1984; Hardin & Cheng, 1963). It was probable that vegetal protrusion existed because primary ivagination, first phase of gastrulation, failed to complete. Some of presumptive archenteron cells should remain on the periphery of the lip of the blastopore. These remainders might protrude out-ward during their rearrangement.

Observations of the early gastrulation of the developing embryo of *Lytechinus pictus* (Ettenshon, 1984) indicated that cell division, in the invaginating endodermal plate, might be important for successful primary invagination. Thus, the inhibitory effect of D_2O on cell division might be a considerable reason for the failure of the primary invagination. However, it could not be the main reason, since the pronounced inhibitory effect of D_2O on the primary invagination only appear-ed when embryos were continuously

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reared in D_2O-SW from an early stage of development.

Futher studies clarifying this problem is continued focusing on the fate of 8 small micromeres of 32-cell stage under continuous culture in D_2O-SW. This is a necessity since 8-small micomeres was suggested to be involved in archenteron formation (Pehrson & Cohen, 1986), and hold the trigger of the gastrulation proccess (Amemiya, 1989).

CONCLUSION

1. The inhibition effect of D_2O on cellular division of developing embryos caused disharmony of cellular development which is responsible for abnormal embrionic development such as animalization and gut evagination.
2. The micro surgery experiments showed any indication that D_2O indused higher blastularcoelomic pressure presumed to be responsible for either producing larger blastulae or gut evagination.

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