Twin *Xenopus laevis* embryos appearing from flattened eggs

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Abstract: Remarkable progress has recently been made in molecular biology of double axis formation in *Xenopus laevis*. Leaving aside, for the time being, the problem of the gene expressions regulating *Xenopus laevis* development, here I show that pulse treatment could induce formation of a secondary axis in a fertilized *Xenopus laevis* egg. At 3 min after insemination, metal oxides were added to *Xenopus* fertilized eggs, and then twin embryos appeared. *Zirconium oxide* (ZrO_2) was the most effective metal oxide for producing twin embryos. ZrO_2 was added to the fertilized eggs, and 30 sec later, the eggs were dejellied with cysteine solution and washed within 7 min after insemination. The fertilized eggs began flattening at around 15 min after insemination. When the degree of flattening (the vertical length of the egg divided by the horizontal length) of the eggs at the 16- and 32-cell stages became less than 0.4 degrees, production of twin embryos occurred. Many flattened eggs at less than 0.4 degrees formed twin embryos. The third cleavage of eggs treated with metal oxides was meridional, while the normal third cleavage was horizontal.

Keywords: *Xenopus laevis*, twinning, flattened eggs, *Zirconium oxide*, pulse treatment

Introduction

Experiments on twin embryo production in frogs can be classified into two types. The first type consists of inserting foreign tissues or genes into fertilized eggs.^{1)–4)} The second type consists of experiments to change the environment of fertilized eggs.^{5)–7)} The gravity change in Gerhart's experiment is a typical case of the second type of twin embryos production.⁶⁾ Gerhart concluded that twin embryo production requires cytoplasmic determinant displacements. If we accept the existence of a determinant factor, then the second type of experiments becomes related to first type. In other words, determinants separate and move in two directions in response to gravity change, and a twin embryo is produced. Several reports have described that the twin formation is related to the flattening of the eggs,^{8)–10)} but the degree of flattening has not been reported. I conjecture that the production of twin embryos depends on the flattening of the fertilized egg in the early development stages. Here, the

dejellying method was different from the usual method.¹¹⁾ The fertilized eggs were treated with cysteine solution earlier than in the typical dejellying experiments.

It is well known that lithium chloride is effective for axis perturbation of *Xenopus laevis*.¹²⁾ However, many of the metal compounds that might be useful for this purpose have not been examined. Accordingly, I also examined dozens of metal oxide compounds for their potential in axis perturbation of *Xenopus laevis*. I focused on metal oxides because they are generally stable and common materials in the environment. Finally, ZrO_2 was found to be the most effective reagent, and produced twin embryos. In this study, I examine two process of *Xenopus laevis* embryo development—namely, the processes of egg flattening and twin embryo production.

Materials and methods

Xenopus laevis eggs were artificially fertilized. A 100 μ l suspension of metal oxide (5 mg/ml Steinberg solution) was added to a mass of eggs at 3 min after insemination by pulse treatment. The mass was swirled at a rate of approximately 3 rotations/sec in a clean beaker for 30 sec, and then 1 or 2 ml of 5% (W/V) cysteine solution (adjusted to pH 7.5) was rapidly added for dejellying. All dejellying and washing procedures (10 times with 100% Steinberg

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solution) could be finished within 7 min after insemination. The eggs were released to orientate freely in gravity. Control eggs were also obtained using the usual dejellying procedure.¹¹⁾ Measurement of the length of an egg was read from a photograph of an egg under the ruler on the bottom of the dish. Metal oxides were of practical or special grade and purchased from Wako Chemical Industries.

Results

Table 1 shows the percentages of twin embryos treated with metal oxide compounds at 3 min after insemination. Variations of the percentage of twin formation seemed to be large as a whole. Normal developmental rates of embryos treated with Fe_2O_3 , Cu_2O or Ag_2O are more than 90% (data not shown). The eggs treated with ZrO_2 or Y_2O_3 showed a high percentage of twin embryo production. The eggs treated with other metal oxides also formed a twin embryo, but the percentage was not as high as by ZrO_2 or Y_2O_3 treatment. ZrO_2 was found to be the most effective for producing twin embryos, and Y_2O_3 was sometimes toxic (data not shown). I therefore used ZrO_2 for the next experiment.

At steps in the cleavage process of the eggs treated with ZrO_2 , first and second cleavages were vertical like a normal cleavage. Although a normal third cleavage is horizontal, in this experiment the third cleavage was meridional (Fig. 1a). From the perspective of the animal pole, the 8 cells treated with ZrO_2 were observed equal cell size of blastomeres (Fig. 1a, Fig. 2, St. 4). Figure 1b shows that the flattened 16-cell embryos had a dent at the animal pole. However, the blastula was spherical like normal embryos (Fig. 1c). The spherical shape began to change form and adopt a fig-fruit shape between the gastrula and neural stages (Fig. 1). Two neural tubes appeared clearly at the neural stage (Fig. 1d, St. 13). Fertilization envelopes were apparent at each stage. Twin embryos clearly appeared in the tail buds and tadpoles (Fig. 1e, f, Fig. 2, St. 13). Fig. 2 shows schematic representation of the stages of *Xenopus laevis* development about the control and metal oxides treated eggs. At 2 cell stage (St. 2), almost same shape is shown. When the eggs were treated with only cysteine solution without metal oxides at 3 min after insemination, the cleavage of eggs did not proceed (Fig. 2). Metal oxide treatments at 3 min after insemination rescue the cleavage defects caused by this unusual dejellying method. Most of the eggs treated with Fe_2O_3 , Cu_2O or Ag_2O developed normally (Table 1, Fig. 2). At 8 and 16 cell stage

Table 1. Effect of metal oxide compounds for twinning of embryos. Each sample was prepared as suspension of metal oxide 50 mg/ml Steinberg solution. Addition of each metal oxide to a mass of eggs was done at least more than 5 times.

Inorganic compounds	Percentage conjoined twins (%)
BeO	5–28
MgO	2–30
Sc ₂ O ₃	13–23
V ₂ O ₅	10–36
CrO ₃	8–32
Fe ₂ O ₃	0–0.1
Co ₃ O ₄	10–22
Cu ₂ O	0–0.2
ZnO ₂	0–1
Ga ₂ O ₃	10–24
GeO ₂	3–21
Y ₂ O ₃	22–78
ZrO ₂	23–98
Nb ₂ O ₃	13–45
MoO ₂	8–31
Ag ₂ O	0–0.1
In ₂ O ₃	10–34
SnO ₂	2–20
TeO ₂	18–24
Ta ₂ O ₅	7–21
WO ₃	1–13

(St. 4 and St. 5), from the animal pole view, ZrO_2 treated eggs show to spread than control egg (Fig. 2, St. 4 and St. 5). In this experiment, twin embryos have second axis at least. I scarcely observed three or more heads were formed. I could not observe twin embryo metamorphosed. FITC-dextran is non-toxic and useful as a cell lineage tracer,¹³⁾ but I did not use, because I wanted to avoid artifact about inside of an egg at least.

Figure 3b shows a side view of a 16-cell embryo with ZrO_2 treatment. Until the early blastula stage the embryos continued to be flattened. Flattened eggs appeared by dejellying procedure at unusual timing with existence of ZrO_2 . Here, unlike in the usual method,¹¹⁾ dejellying was done before the egg rotation period. In the usual method the dejellying starts after egg rotation, at 8 min after insemination.¹¹⁾ ZrO_2 solution was added to the mass of eggs between 2 min 30 sec and 4 min after insemination, and then dejellying and washing were finished within 7 or 8 min after insemination, and the flattening of eggs occurred and double axis formed. The same treatment of the eggs with ZrO_2 after insemination

Fig. 1. Successive change of *Xenopus* embryos treated with ZrO_2 at 3 min after insemination and schematic process of development. a, 8-cell; b, 16-cell; c, blastula; d, neurula; e, tail bud; f, tadpole; Bar length: 1.0 mm.

7 min later did not show flattening. Additional factors may be required in flattening process, but are still unknown. From my observation, when there is a dent on an animal pole at the 16-32 cell stages, the incidence of twin embryos production may be up.

The degree of flattening was measured from the horizontal length and vertical length of an egg. Figure 4a shows the measurement of the horizontal length and vertical length of 32-cell embryos treated with ZrO_2 at 3 min after insemination. The flattening degree was calculated from the horizontal and vertical lengths. The flattening degree was calculated

as the vertical length divided by the horizontal length at the 32-cell embryo stage (Fig. 4b). Flattening degrees of embryos treated with ZrO_2 were calculated as 0.36 ± 0.03 ($n = 31$). Many twin embryos appeared when the flattening degree of eggs at the 32-cell stage was less than about 0.4. Degrees of control embryos indicate 0.7 ± 0.08 ($n = 19$). Here I show that the twin embryo production was dependent on the flattening degree. The degrees of flattening after 7 min of ZrO_2 treatment were not measured, because the spherical shape of eggs and cleavage patterns showed normal development.

Fig. 2. Schematic representation of top view about normal and metal oxides treated eggs development, pictures cited and modified from the textbook.¹⁷⁾ Usual dejellinging procedure is done at 10–40 min after insemination. My unusual dejellinging started at 3 min and 30 sec after insemination. Abbreviations, St. 2: two cell stage, St. 4: eight cell stage, St. 5: sixteen cell stage, St. 13: initial neural plate stage, St. 22: early tail bud stage, ap: animal pole, vp: vegetative pole, nf: neural fold, eyv: eye vesicle, cg: cement gland.

Fig. 3. Side view of flattening egg at 16-cell stage. White arrowhead indicates top of egg and black arrowhead indicates bottom of egg. Bar length: 1.0 mm a. Control egg. The eggs are supported by forceps to show vegetal half of eggs and measure length of egg on the ruler. b. ZrO_2 treated egg is supported by forceps to show vegetal half of egg and measure length of egg.

The percentages of twin formation according to the ZrO_2 treatment time are shown in Fig. 5. Each point represents an average of data from 21 to 73 embryos from one frog. The percentages of twin

embryos at the normalized treatment times of 0.025, 0.03 and 0.04 (2 min 30 sec, 3 min and 4 min) after insemination indicate a high frequency of twinning. The incidence of twin embryos showed a decrease at the normalized treatment times of 0.07, 0.14, 0.2, 0.4, and 0.6 (7, 14, 20, 40 and 60 min) after insemination. In addition, metamorphosis of twin *Xenopus* tadpoles could not be observed in this experiment.

Discussion

Several researchers in classical embryological studies have recognized that twin embryo production is related to the flattening of amphibian eggs. However, there has been no discussion about the flattening process in fertilized eggs and twin embryo appearance. Gerhart and colleagues also showed that twin embryo production requires two cytoplasmic displacements in the eggs.⁶⁾ They mentioned that twin formation was related to the flattening of the

Fig. 4. Vertical and horizontal length of eggs. a. The open circles represent control eggs. The filled circles represent flattened eggs treated with ZrO_2 at 3 min after insemination. b. The ratios of vertical length/horizontal length are shown, control eggs ($n = 19$) and ZrO_2 treated eggs ($n = 31$). The values are expressed as the means $\pm$ SD.

eggs, although they did not discuss the relationship between twin production and flattening of the eggs.

If a fertilized egg has been flattened to a flattening degree of less than 0.4, this can affect a determinant of morphogenesis moving. The results of this experiment suggest that the movement of a determinant is related to the flattening degree of eggs. Alternatively, another determinant may be induced by the flatness of the fertilized egg during development. In any case, the flattening degree of the egg is crucial for twin production.

Fig. 5. The abscissa records the time of ZrO_2 treatment for fertilized eggs, normalized to first cleavage and real time after insemination. Twinning frequency is reduced after 0.07. The open circles represent average twinning frequencies of egg batches from different frogs. The data for each circle are from 21–73 eggs from one frog.

Gerhart explained the twin embryo production by two directions of gravity. The gravity changes influence the inside of the fertilized egg. Gerhart interpreted that the gravity change induced determinant moving in the fertilized egg in the implantation experiment by Curtis, and the twinning in Curtis' experiments took place by inattentive rotation of cytoplasmic contents and extreme flattening of the egg.^{1),5)}

In Curtis' experiment, a flattened egg was observed at the 16- and 32-cell stages,¹⁾ and I also suspect that this egg had a flattening degree of less than 0.4.

From another point of view, twinning is well known to occur in eggs that have been forced to undergo multiple cortical rotations along different animal-vegetal meridians. Normally, cortical rotation activates the Nieuwkoop center of the vegetal hemisphere, which will thereafter in the blastula induce the formation of the Spemann organizer region in the marginal zone.¹⁴⁾ Here, ZrO_2 treatment may provoke multiple cortical rotation and two-separate meridians according to the flattening. In my experiment, when the egg became flattened, two neural tubes occurred in the neurula, and then a twin embryo was produced. In any case, however the *Xenopus* spherical shape of the eggs is maintained, no twin formation occur.

ZrO_2 has a high ionic character. According to the chemical bond theory of Pauling, material that

has the ability to form a second axis may be more likely to have a high ionic bonding property.¹⁵⁾ When material with a high ionic character coexists at the short dejellying process, the very early period in development, the *Xenopus* embryo can become flattened. Mechanism of molecular basis of inorganic substance is still unknown, although gene expressions by microinjection technique show many important factors.^{13),16)} Role of dent formation on an animal pole may relate unknown cytoplasmic determinant movement during embryo flattening. In addition, flattened embryo by physical force developed normally in my preliminary experiment. Further examination is necessary to elucidate the relationship between the flattening mechanism and gene expression. In general, I consider that the shape change itself is meaningful for gene expressions. In addition, dejellying procedure in my experiment is different from usual dejellying procedure. By my dejellying procedure, cleavage defect of fertilized egg was rescued by addition of metal oxides at 3 min after insemination and successive cysteine treatment. It may be a new method for dealing with *Xenopus* fertilized egg.

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