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An Assay System for Developmental Toxicity Using Embryos and Larvae of *Xenopus laevis*

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Summary

Xenopus laevis embryos and larvae were exposed to seventeen chemicals for the evaluation of *Xenopus laevis* embryo and larva system as one of alternatives of developmental toxicity of chemicals. All of the chemicals induced some developmental toxicity in a dose-dependent manner. Based on our data and those of other investigators, it is considered that *Xenopus* embryo and larva system is a good candidate for a simple and effective test system to evaluate developmental toxicants. Furthermore, advantages and disadvantages of this system are discussed.

Introduction

A large number of chemicals have been introduced to our life in succession. The currently applied means for detecting teratogens require evaluation in mammals. Since developmental toxicity tests using mammals are so time consuming and expensive, and furthermore due to the recent great concern for animal welfare, the inexpensive and rapid screening system for detecting potential developmental toxicants, especially teratogens, have been developed to reduce the problems in mammals. Mammalian development consists of a sequence of developmental processes, and many of these processes have not been fully clarified yet. Furthermore the mechanism of teratogenesis and the species difference in teratogenic response have not been clearly understood. So there is no complete alternative system for teratogens today. However, it would be possible to predict the teratogenic potential of chemicals with a battery system including various developmental processes, which could reduce the number of mammalian tests needed.

Efforts to use non-mammalian organisms for such a test system have been made (see reviews[1,2]). *Xenopus laevis* embryo and larva system is one of the most advantageous candidates because of a number of advantages as described in our previous report[3]. Among the advantages, the most important one is that development of *Xenopus* embryo includes cleavage, gastrulation, neurulation, and organogenesis following to fertilization, and that these developmental events are mechanistically comparable to those of mammals. *Xenopus* embryos and larvae have been shown to be sensitive to a number of chemicals, and have been used as a test system for environmental pollutants[4]. As a teratogenesis assay system, Dumont et al.[5] initially developed and standardized the procedure, afterwards many investigations have followed[6-16]. We examined developmental toxicity of several kinds of chemicals for validation of *Xenopus* embryo and larva system. Based on the results, we discuss the possibility and problems of this system.

Outline of procedures

Freshly laid fertilized eggs were obtained from *Xenopus laevis* by injecting 200 and 300 i.u. of human chorionic gonadotropin (Teikoku Zoki, Co.), respectively into the dorsal lymph sac of sexually mature males and females. Larvae were staged according to Nieuwkoop and Faber's normal table of

Xenopus laevis[17]. For culture, an acrylic plate with 80 hemispherical holes was used in order to exclude the influence from other embryos and/or larvae. Each hole contained 1.5 ml of test solutions and one embryo or larva. Test chemicals, lead (lead acetate, lead nitrate), cadmium (cadmium chloride, cadmium sulfate), lithium (lithium chloride, lithium carbonate), mercuric chloride, sodium selenite, caffeine, trypan blue, hydroxyurea, ethidium bromide, ethanol, amaranth, saccharin sodium, vitamin C and acetone, were dissolved in dechlorinated tap water filtered through activated carbon. Normally developing embryos and larvae at a specific developmental stage were selected manually with the aid of a dissecting microscope. Blastula, early gastrula and newly hatched larvae were continuously exposed to the solution containing graduated concentrations of test chemicals at 22–23°C for 72, 72 and 48 hr, respectively. Control larvae were maintained in dechlorinated tap water. Forty larvae per dose level were examined. Observations were made daily, and dead and abnormal embryos were checked. Test solutions were renewed every 24 hr. Death was determined by skin pigmentation, structural integrity, responsiveness to physical stimulation by forceps and absence of a heartbeat. At the termination of the tests, surviving larvae were fixed with 10% neutral formalin. External abnormalities and the developmental stage of the larvae were examined using a dissecting microscope. Some of abnormal embryos and larvae were examined morphologically using light microscope, scanning and transmission electron microscopes.

Developmental toxicity of seventeen chemicals

All of the chemicals examined induced some developmental toxicity on *Xenopus* embryos and larvae in a dose-dependent manner. Summary of the results is shown in Table 1. The lowest concentrations to induce developmental toxicity of metals, Pb, Hg, Cd, Li and Se, were low (0.1–10 mg/l). On Pb, Cd and Li, two kinds of salts of these metals were examined and similar results were obtained, i.e., the two salts induced lethality and abnormality at nearly the same concentration and the same type of abnormalities were found. These results suggest that the effects are attributable to the metal itself. The lowest concentrations to induce developmental toxicity of caffeine, trypan blue, hydroxyurea and ethidium bromide, which are teratogenic in mammals or DNA synthesis inhibitors, were 100–500 mg/l. On the other hand, those of ethanol (weak mammalian teratogen), amaranth (non-teratogen), saccharin sodium (non-teratogen) and acetone (teratogenicity has not been determined) were very high (>10g/l). In the case of vitamine C (non-teratogen), the concentration to induce abnormalities was low (250 mg/l), which might be related to acidic pH of the test solution and further investigation on the mechanism involved is needed. Because all chemicals are potential teratogens if administered in appropriate doses at sensitive stages of development[18], the concentration to induce abnormality or lethality is

Table 1. Lowest developmentally toxic concentration in *Xenopus laevis* system

Concentration (mg/l)	Lethality	Abnormality
≧10,000	saccharin sodium acetone amaranth	acetone ethanol
≧1,000	caffeine hydroxyurea	
≧100	trypan blue vitamin C ethidium bromide lithium carbonate lithium chloride sodium selenite	hydroxyurea vitamin C caffeine
≧10	cadmium chloride cadmium sulfate	lithium chloride lithium carbonate sodium selenite
≧1		cadmium chloride cadmium sulfate
<1	mercuric chloride lead nitrate lead acetate	mercuric chloride lead acetate lead nitrate

important to assess the developmental toxicity of chemicals. For example, lithium induced abnormalities at the concentration of 10 mg/l, which is considerably high compared to the blood serum level of lithium of normal humans (0.002–0.01 mg/l), but nearly comparable to the blood serum level of lithium effective for mania (3.5–8.5 mg/l)[19]. Since teratogenicity of lithium in humans is discussed[20], the results obtained in this system is noteworthy. In the case of caffeine, abnormalities were induced at the concentration of 100 mg/l. On the teratogenicity of caffeine for humans, Sullivan et al[21] concluded that the teratogenic activity depends on the production of high peak blood levels probably around 60 μg/ml or greater. Since blood plasma level of caffeine after the intake of 250 mg/l caffeine, which nearly corresponds to the intake of three cups of coffee, is about 10 mg/l[22], continuous exposure to caffeine at the concentration of 100 mg/l and more would not occur in adult humans. However, since it is reported that caffeine readily crosses the placenta and accumulates in fetal tissues[23,24], and that the fetus lacks the enzymes necessary for caffeine metabolism[25], it is considered that one must pay attention on the potential teratogenicity of caffeine.

One of the advantages of this system is that large numbers of embryos are available, which allows the construction of concentration-response curves and LC_{50}, EC_{50} and LC_{50}/EC_{50} (Teratological Index: TI) can be calculated based upon the curves. Dumont et al.[5] used TI for comparisons of diverse compounds with regard to their inherit teratogenic risks. They set that a TI values of 2.0 or greater indicate the need for the further testing in higher-level assays. TI values between 1.5 and 2.0 suggest that materials should be treated with suspicion and caution as potential teratogens and tested further in other screening systems. TI values below 1.5 reflect compounds whose lethality may be more pertinent to risk assessment than teratogenicity. Dawson et al.[26,27] reported that generally, TI values <1.5 indicate low teratogenic potential, and that greater TI values signify an increased potential for teratogenesis.

Developmental toxicity may also be assessed by considering the effects of chemicals on growth. Head-tail length, dorsal fin length, ventral fin length, height at cloaca and head breadth were measured using Nikon profile projector V-12 at the end of each test, and it was shown that the head-tail length is the most sensitive and easily measured endpoint on growth in this system. Growth was more sensitive parameter than abnormalities. For example, in the case of acetone, abnormalities were induced at the concentration of 8%, but a significant reduction in growth occurred at the concentration of 4%. Dawson and Bantle[9] showed that the amount of growth inhibition in *Xenopus* embryos in the 96 hr tests was positively correlated with the degree of teratogenicity of the compound. Furthermore, Dawson et al.[27] suggested that compounds with significant teratogenic potential generally inhibit growth at concentrations <30% of the respective LC_{50} values.

Abnormalities induced in *Xenopus*

The type and severity of abnormalities are two other important factors to assess the results. The main abnormalities induced by the exposure to these chemicals are shown in Table 2. Main abnormalities in the embryonic stage were abnormal neurulation, which were found in embryos exposed to Pb, Hg, Cd, caffeine, ethanol and vitamin C. Embryos with the neural plate region degenerated widely died during neurulation. Some of the affected embryos developed to the early larval stage, but died afterward within 24 hours. Lightly affected embryos with only slight groove at the fusion area of neural tube, recovered from the damage and developed to larvae with normal features, although their development was considerably delayed, as in the case of 4% ethanol. To elucidate pathogenesis of the neural tube defects,

Table 2. Abnormalities induced in *Xenopus* embryos and larvae

Treatment	Abnormalities induced
Pb	AN, edema in the thorax
Cd	AN, many small edemas in the abdomen and tail
Li	SLW, AF, edema
Hg	AN, AF, eye abnormality, edema
Se	SLW, AF, eye abnormality, edema, abnormal swimmng
Caffeine	AN, SLW, AF, eye abnormality, edema
Hydroxyurea	AF, abnormal dorsal fin, edema
Ethanol	AN
Vitamin C	AN, SLW, AF, weak pigmentation
Acetone	AF, abnormalities in the tail

AF: abnormal body flexure
AN: abnormal neurulation
SLW: shortened body length with wavy fin

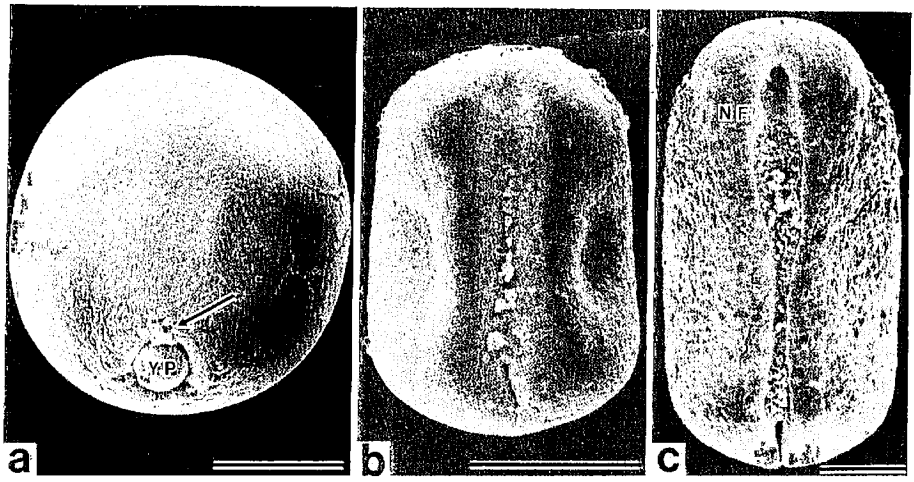

Fig. 1. Embryos exposed to 1 mg/l lead acetate from blastula stage. The cephalic sides of embryos are at the top of the photographs. (a) Embryos after 17.5 hr exposure. Note the spherical-shaped degenerating cells (arrow) on the dorsal side of blastopore. YP: yolk plug. Bar=500 μm. (b) Embryos after 20.5 hr exposure. Note the many degenerating cells observed in the neural groove, comparatively in large numbers at the caudal region. Bar=500 μm. (c) Embryo after 26.5 hr exposure. The neural fold (NF) is adjacent to each other, but not touched. Note the many degenerating cells in the neural groove and neural plate region. Bar=250 μm.

embryos after 17.5, 20.5 and 26.5 hr exposure to 1 mg/l lead acetate from blastula stage were examined morphologically. Delay of gastrulation as well as degenerating and desquamating cells on the dorsal side of blastpore was observed (Fig. 1a). Embryos during neurulation had many degenerating and desquamating cells in the neural groove, comparatively in larger numbers at the caudal region (Fig. 1b). Degenerating cells were also observed in the neural plate region in more severely damaged embryos (Fig. 1c). Therefore, the neural tube defects induced by lead acetate were attributable to delay of gastrulation accompanied with damaged cells locating at the dorsal side of blastopore, followed by degeneration and desquamation of epithelium of the neural groove and neural plate region. It has been shown that, during gastrulation, the surface cells in the vicinity of the blastopore groove change shape as they invaginate[28]. It has also been shown that, during neurulation, extensive shape changes occur in cells of neural groove and neural plate[29]. Therefore, it is supposed that cells during the extensive shape change are easily

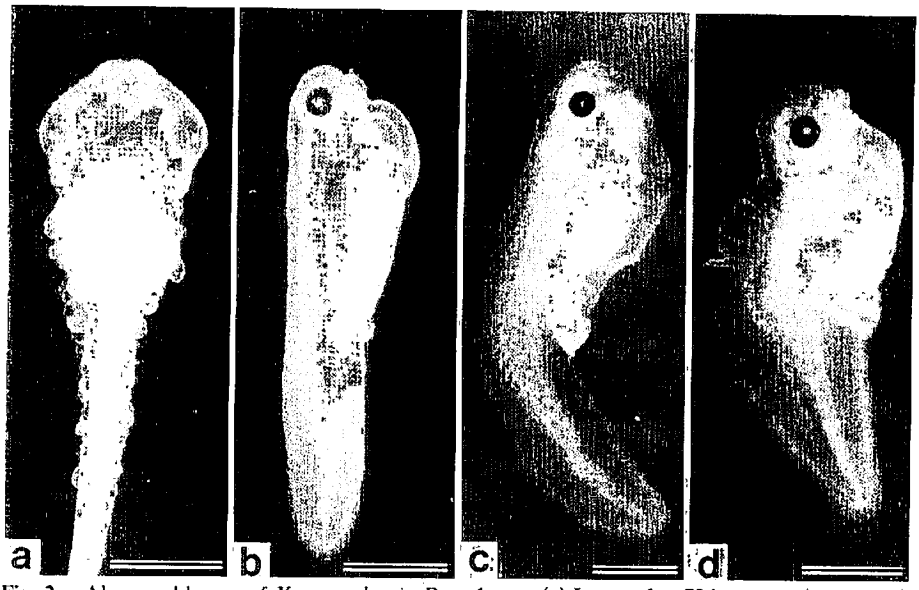

Fig. 2. Abnormal larvae of *Xenopus laevis*. Bar=1 mm. (a) Larva after 72 hr exposure to 1 mg/l cadmium chloride from gastrula stage. Note the many small edemas in the abdomen and tail. (b) Larva after 72 hr exposure to 1 mg/l lead acetate from blastula stage. Note the edema in the pericardium and thorax. (c) Larva after 72 hr exposure to 500 mg/l hydroxyurea from blastula stage. Note the abnormal body flexure and edema in the thorax and abdomen. (d) Larva after 48 hr exposure to 500 mg/l caffeine just after hatching. Note the short body length accompanying wavy fin with abnormal body flexure.

affected by the chemicals.

Main abnormalities observed in larval stage were edema in various body parts, abnormal body flexure, eye abnormality and shortened body length with wavy fin (Fig. 2). Shortened body length with wavy fin was found in larvae exposed to Se, Li, caffeine and vitamin C. The external features looked similar among them. To elucidate the pathogenesis of the abnormality, histological study of caffeine treated larvae were done, which showed clear dose-dependency in the severity of the abnormality. Ultrastructural study of the myotomal cells suggested that the effects on the larvae were attributable to the pharmacological action of caffeine associated with the translocation of intracellular calcium[30].

Another example for mechanism study of chemicals is dioxins (unpublished data). Severe edema mainly in the head around eye, thorax and abdomen were induced in larvae exposed to 2, 3, 7, 8-TCDD (TCDD) at the embryonic and/or early larval stages until 8 days after fertilization. Most of these larvae generated edema died within several days. Although 17 chemicals above mentioned induced abnormalities and/or lethality during the exposure period, TCDD did not induce the abnormality and lethality during the exposure period, but several days after transferring to the dechlorinated water from the TCDD containing water. Delayed effects of TCDD are similarly observed in mammals. In addition, edema was observed in mouse[31], chickens[32], great blue heron embryos[33], rainbow trout[34] and pike[35] after exposure to TCDD. Therefore, it is important to clarify the pathogenesis of edema formation to understand the action of TCDD. *Xenopus laevis* may be useful to elucidate the toxicological mechanisms of TCDD.

Advantages and disadvantages of the system

Summary on the advantages of this system as the screening system of developmental toxicity are as follows. 1. A number of endpoints (LC_{50}, EC_{50}, TI as expressed LC_{50}/

EC_{50}, developmental stage, growth, motility, and type and severity of abnormalities) can be available. By integrating them, comparatively precise and detailed evaluation of chemicals on the potential developmental toxicity is possible. 2. Since there are no maternal effects and placental relationship which make the interpretation of data complicated, precise assessment of the active form of the chemicals can be made. 3. For the chemicals of which metabolites have teratogenic effects, metabolic activation system has been developed by Fort et al.[13,15]. They reported that the predictability of FETAX is more than 90% including the chemicals tested using their metabolic activation system. 4. This system possibly generate informations concerning mechanisms of teratogenesis. The disadvantage of this system are as follows. 1. Depending on the batch, incidence of lethality and/or abnormality in controls is occasionally high. 2. This system is inadequate to detect the effects on bone, limb, digit and/or palate development. The former three can be examined, but it is time consuming and requires much labor. The development of the palate in *Xenopus laevis* is considerably different from that of mammals. 3. There is a difficulty to use the data obtained in this system directly for human risk assessment due to phylogenetic difference. Concerning to the disadvantages described in 1 and 3, future studies may be able to resolve the problems.

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