

1-1-2013

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PEKMEZEKMEK, AYPER BOĞA; BİNOKAY, UĞUR SEÇİL; AKILLIOĞLU, KÜBRA; and SERTDEMİR, YAŞAR (2013) "Evaluation of E330-induced developmental toxicity using FETAX," *Turkish Journal of Biology*. Vol. 37: No. 3, Article 3. <https://doi.org/10.3906/biy-1204-24>
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Evaluation of E330-induced developmental toxicity using FETAX

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Received: 12.04.2012 • Accepted: 09.10.2012 • Published Online: 16.05.2013 • Printed: 17.06.2013

Abstract: The present study evaluates the teratogenic and toxicological effects of citric acid (E330), a food additive, on *Xenopus laevis* embryos. The embryos were exposed to a range of E330 concentrations, from stage 8 to 11 of development, for 96 h under static renewal test conditions. The median lethal concentrations, no-observed adverse effect concentration (NOAEC), lowest observed adverse effect concentration (LOAEC), and minimum concentration to inhibit growth (MCIG) values were calculated. The lethal concentration (LC) values LC_{10} , LC_{20} , LC_{30} , LC_{40} , and LC_{50} determined for E330 exposure were 0.0113, 0.0117, 0.0119, 0.0122, and 0.0124 g/L, respectively. NOAEC and LOAEC values were calculated as 0.001 and 0.01 g/L. Since the effective concentration (EC_{50}) value could not be determined, the E330 teratogenic index (TI), which is LC_{50}/EC_{50} , was not calculated. MCIG was calculated to be 0.010 g/L. The anomaly rate in *Xenopus* embryos treated with E330 was quite low. Therefore, the endpoint for the *Xenopus* embryos was whether they were alive or dead at the end of the study. It can be concluded from these observations that using unlimited amounts of E330 may result in preterm birth or abortion in humans, and E330 usage must be reevaluated and, potentially, limited. Moreover, our results confirm that the Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) can be a useful pretest for integrated biological hazard assessment.

Key words: Citric acid, E330, *Xenopus*, FETAX, teratogenicity

1. Introduction

Patterns in the purchasing of food have changed dramatically over the past 50 years. There is an increased consumption of packaged and processed foods. The trend to purchase semiprepared or fully prepared food is driven by an increase in the number of the people working outside the home, changes in eating habits, time constraints limiting how much time can be spent on food preparation, and a desire to spend less time on food preparation. Inevitably, there is an increased use of food additives. Due to the increased production and consumption of food additives, technology is becoming an integral part of the food industry (1). One topic at the forefront of the important issues faced by the world today is food safety. The food industry faces the challenge of increasing food production while assuring the safety of the food produced, preventing the loss of nutrients, and maintaining food quality over an extended shelf life (2). Although food additives (FAs) have many benefits, as mentioned above, some consumers believe that FAs are harmful to human health. The main reasons for concern are that FAs may mask poor quality or damaged food, food that was processed incorrectly, or imitation food products, and may also reduce the nutritional value of the product.

Additionally, there is a concern that FAs may be used as a substitute for good food processing and packaging techniques (2).

The food additive used in this study was citric acid (E330). Citric acid is found at high concentrations in lemons, oranges, limes, and in all sour fruits. Citric acid is a weak organic acid. It is a natural preservative, and it is used as a flavoring in acidic or sour food and in soft drinks (3,4). The citric acid cycle is also an important biochemical pathway that is utilized by all living organisms (4,5). E330 is a versatile chemical with a wide range of applications in the food, textile, pharmaceutical, cosmetic, and detergent industries. It is also increasingly used in order to remove toxic and corrosive gases in the air.

In 1917, the American food chemist James Currie discovered that the mold *Aspergillus niger* had certain strains that could be used for the production of citric acid. *Aspergillus niger* fermentation is "generally recognized as safe" (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act (6), and compared to other fermentation products, citric acid has one of the highest production levels in the world. Global production of citric acid in 2007 was over 1.6×10^6 t (7). The food industry consumes

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about 70% of the total E330 produced, while other industries consume the remaining 30%, as it is necessary to use inexpensive and readily available raw materials for industrial production processes (8). The European Union allows citric acid as well as lemon juice to be added as an acidic regulator into fruit juice and fruit nectar. The maximum dose of citric acid that can be added is 3 g/kg for fruit juice and 5 g/kg for fruit nectar (9).

The acceptable daily intake (ADI) value quantifies the daily amount of a particular substance in food that a person can ingest over a lifetime without a health risk. Based on the results of toxicological studies, the harmless dose of E330 and the ADI value is defined as not specified (NS, unlimited). Other similar NS substances include sodium carbonate, sodium citrate, caramel color, monosodium glutamate, carrageenan, and emulsion constructive additives (10). However, it is uncertain if ADI values are valid for infants (0–12 months) and children (1–12 years). The ADI values may not provide adequate protection for these groups (10).

According to INCHEM, which provides chemical safety information from intergovernmental organizations, frequent or heavy consumption of E330 may lead to dental erosion. Additionally, muscle twitching or cramps, swelling or weight gain, fatigue, mood changes, and rapid and shallow breathing may occur. It is not known whether E330 and sodium citrate can pass into breast milk or harm a nursing baby. It has been emphasized that taking E330 as a medication may be dangerous in some diseases. For instance, people with kidney problems, heart damage, Addison's disease, high potassium levels, heat cramps, and severe dehydration should not take E330 as a medication (11).

Dickens (12), who studied the distribution of E330 in animals, found that cancerous tissues contain greater amounts of E330 in the associated bodily fluids than normal. Another study examined the electrocardiographic

parameters of pulmonary hypertensive chickens that were orally exposed to E330 for 45 days. It was found that E330 may modulate pulmonary hypertension and the effect could be observed through electrocardiography (13). Aktaç et al. investigated the effects of E330 on rat liver and observed that there was an unexpected increase in enzyme activity and organ weights, especially for the kidney, and a decrease in overall body weight compared to the control group (14).

The most recent assessment of E330 consumption was prepared in 1973 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (5) (Table 1). The use of E330 today is based on the 1973 guidelines, which are usually considered safe by the US Food and Drug Administration (15). However, some countries describe E330 as 'the most dangerous/cancerous' additive and have given E330 the title 'E poison in food'. These concerns arose in France in 1976, in Germany in 1986, and then in Turkey in 1996. Increasing concerns about E330 have recently been displayed on the Internet (9). Nonetheless, E330 is still considered harmless and the use of this substance is not restricted. We designed this study to help further elucidate the safety of E330. We used the frog embryo teratogenesis assay *Xenopus* (FETAX) to assess the health impacts of E330. The FETAX test was standardized in the 1980s and no literature could be found regarding the use of FETAX in determining E330 teratogenicity.

The FETAX is a 96-h whole-embryo static renewal assay providing a rapid, simple, and cost-effective method for preliminary evaluation of the mechanisms of developmental toxicity using *Xenopus laevis* embryos (16). It is essentially an organogenesis test, and organogenesis is highly conserved across amphibians and mammals. The first 96 h of the embryonic development of *Xenopus* parallels many of the major processes observed in human organogenesis. Thus, FETAX may be useful for predicting potential human developmental toxicants and teratogens.

Table 1. Evaluations performed by the Joint FAO/WHO Expert Committee on E330.

INS	330
JECFA No.:	218
Chemical names:	2-Hydroxy-1,2,3-propane-tricarboxylic acid; 2-hydroxy-1,2,3-propane-tricarboxylic acid, monohydrate
Functional class:	Acidulant; synergist for antioxidant; sequestrant; flavoring agent
Latest evaluation:	1973
ADI:	Not limited
Comments:	Group ADI for E330 and its calcium, potassium, sodium, and ammonium salts
Report:	NMRS 53/TRS 539-JECFA 17/35

The short-term FETAX test, used in various studies, was found to have an 89% concordance rate with results obtained in rodents (16–23) and provides information concerning the embryotoxic effects of a particular substance based on larva length (24). Therefore, FETAX data may play a role in determining ADI values.

The aim of the present study was to investigate the mortality, teratogenicity, and growth inhibition of E330, a substance widely used in the food, beverage, textile, pharmaceutical, cosmetic, and detergent industries, in *Xenopus laevis* embryos using the FETAX system. The results of this study may have implications for human health.

2. Materials and methods

The toxicity test used in this study was performed in strict accordance with the procedures of the American Society for Testing Materials (ASTM) Standard Guide (25).

2.1. Test substances

E330 was purchased from Sigma (Catalog No: C1909, St. Louis, MO, USA). Follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG, Pregnyl, 5000 IU) were obtained from Serono and Organon, respectively (İstanbul, Turkey). The FETAX solution and De Boers Tris (DBT) reagents were supplied by Sigma.

2.2. Test organism and bioassay

The African clawed frog (*Xenopus laevis*) is a species commonly used as a model organism for frog research (18). The *Xenopus laevis* used in this study were purchased from the United States and from China in the early part of the 1990s. Adult *Xenopus* breeding and embryo collection were performed as described in ASTM E1439-98 (25). Adult *Xenopus laevis*, bred in the Physiology Department of Çukurova University (Adana, Turkey), were housed in aquaria (95 × 60 × 44 cm) at 23 ± 1 °C under a 12-h light–dark cycle and were fed ad libitum twice weekly (26).

2.3. In vitro fertilization

In vitro fertilization was performed using the method described by Lindi et al. (27). In each experiment, 2–3 *Xenopus* females were injected with 700–1000 IU of hCG 16 h before the females laid their eggs in plastic petri dishes. The eggs were immediately inseminated using a sperm suspension obtained by mincing the testes of *Xenopus* males into 1–2 mL of cold DBT (all units in mM: NaCl, 119; KCl, 2.5; CaCl₂, 1.8; and Tris/HCl, 15, at pH 7.5). The osmolarity of the DBT solution was higher than that of the FETAX solution in order to keep the spermatozoa from engaging in flagellar movement (16). After 1 min, 10 mL of FETAX solution (containing in mg/L: NaCl, 625; NaHCO₃, 96; KCl, 30; CaCl₂, 15; CaSO₄·2H₂O, 60; and MgSO₄, 70, at pH 7.8–8.0) was added to each petri dish. Successful insemination was detected after a few minutes when all of the eggs were oriented with the dark side (animal pole) up.

All irregularly segmented eggs were eliminated. Normally developing embryos were selected up until the late blastula stage (stages 8–11) and were used for the FETAX assay (28).

2.4. FETAX procedure

The experiment was performed in order to determine the effects of E330 using the FETAX test. Approximately 2500 (N = 1200 control, N = 1300 assay) embryos were used and each experiment was replicated 3–5 times. For each assay, 20–25 embryos were randomly placed in covered 100-mm glass petri dishes in 50 mL of different concentrations of citric acid, ranging from 0.0001 to 0.0140 g/L. Each concentration was tested in 4 separate petri dishes, with a total of 80–100 embryos. In the control groups, only the FETAX solution was used, and during the assays, the embryos were grown in a tissue culture incubator at 23 ± 1 °C. All solutions were replaced daily over a 4-day test period and the E330 stock solution was prepared fresh each day. Dead embryos were removed and fresh solution was added. After 96 h of exposure to E330 (stage 46 embryos), living larvae were fixed in 3% (w/v) formalin and the number of malformed embryos was determined using a dissection microscope (29). All embryos were scored by measuring the length from head to tail using a millimeter ruler. A length lower than normal was indicative of growth inhibition (14). For flexed larvae, measurements were taken along the curvature of the notochord (30).

2.5. Statistical analysis

The data were analyzed using SPSS 18.0. A probit analysis was used to measure the effect of the lethal concentration (LC) values of citric acid on *Xenopus* embryos. The probabilities that the embryos were normal, abnormal, or dead were given in counts and percentages for each E330 concentration. The no-observed adverse effect concentration (NOAEC) and the lowest observed adverse effect concentration (LOAEC) values were determined using Dunnett's multiple comparison procedure. In order to determine the minimum concentration to inhibit growth (MCIG), the head-to-tail lengths at different concentrations of E330 were compared using analysis of variance (ANOVA). P < 0.05 was considered significant.

3. Results

3.1. Incidence of normal, abnormal, and dead *Xenopus* embryos

Citric acid was investigated using FETAX because data concerning its teratogenicity and harmful effects are very limited. FETAX is a 96-h whole-embryo static renewal assay using *Xenopus laevis* embryos (14). Figure 1 shows the mean mortality and malformation rates in the control and citric acid-exposed groups during the 96-h test, from stages 8–9 to stage 46 in the embryonic developmental period, based on the FETAX results. During the experimental period, the mean mortality rate in the control

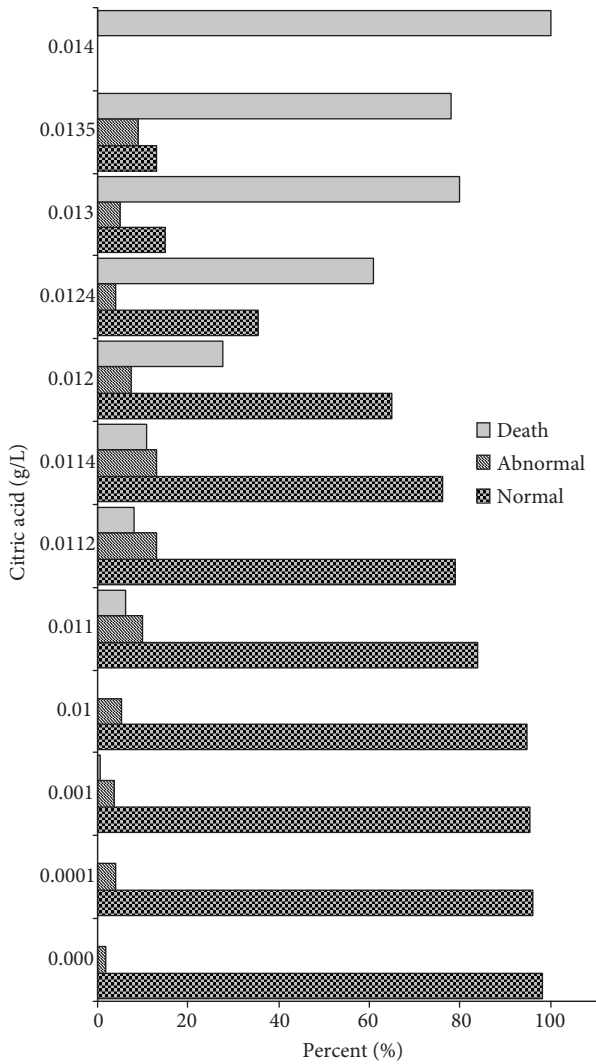


Figure 1. The total percentage within a given dose (0.0001–0.140 g/L) of E330 normal, abnormal, and mortality rates in the control and treatment groups, based on the FETAX assay.

group treated with FETAX solution was 0%. The control results met the criteria established by the ASTM standard for test acceptance (25). The rate of embryo malformations in the treatment group was 6.6%, the death rate was 31.9%, and death started at a concentration of 0.0100 g/L. With only a slight increase in E330 concentration to 0.0140 g/L, 100% of the citric acid-exposed group died. The anomaly and death rates were dependent on the dosage and the numbers of deaths or malformed embryos were determined using a dissection microscope. These results show that E330 concentrations used as food additives in industry should be carefully reexamined.

3.2. Lethal concentration values

The LC values observed in this study are presented in Table 2. According to the probit analyses, the LC₁₀, LC₂₀, LC₃₀, LC₄₀, and LC₅₀ values were 0.0113, 0.0117, 0.0119, 0.0122, and 0.0124 g/L, respectively. The teratogenic index (TI) value (LC₅₀ / EC₅₀) could not be determined because the EC₅₀ value could not be obtained due to the very low number of anomalies (about 6.6%). According to our results, whether the *Xenopus* embryos were alive or dead at the end of the study depended on the E330 concentration.

3.3. NOAEC and LOAEC values

The NOAEC and LOAEC values are shown in Table 3. The NOAEC and the LOAEC values were 0.001 g/L and 0.01 g/L, respectively. There was a statistically significant difference in the NOAEC and LOAEC values compared to the control (P < 0.05). These values show in which concentration range E330 should be used.

3.4. MCIG values

In Figure 2, the MCIG values of the exposed and control groups are shown. The MCIG value was determined to be between 0.010 g/L (P = 0.011) and 0.011 g/L (P < 0.001). The total mean length of the control embryos (N = 1090) was 9.409 ± 0.029 mm and the average values of the upper and lower limits of the 95% confidence interval (CI) were

Table 2. The LC (lethal concentration × 100) values for E330, based on the FETAX assay.

Probit	95% confidence		Limits mean for death	Limits × 100 concentration (g/L)
	Mean	Lower (95% CI)	Upper (95% CI)	
LC ₁₀	1.13	1.11	1.14	
LC ₂₀	1.17	1.15	1.18	
LC ₃₀	1.19	1.18	1.20	
LC ₄₀	1.22	1.21	1.23	
LC ₅₀	1.24	1.23	1.25	

Table 3. E330 (citric acid) NOAEC and LOAEC values based on FETAX assay.

	Values	N	Estimated concentrations (g/L)
	Probit	NOAEC	200
	LOAEC	111	0.01*

NOAEC and LOAEC are expressed as g/L (*P < 0.05).

N: Total number of embryos exposed to the estimated concentrations during the 96-h exposure period.

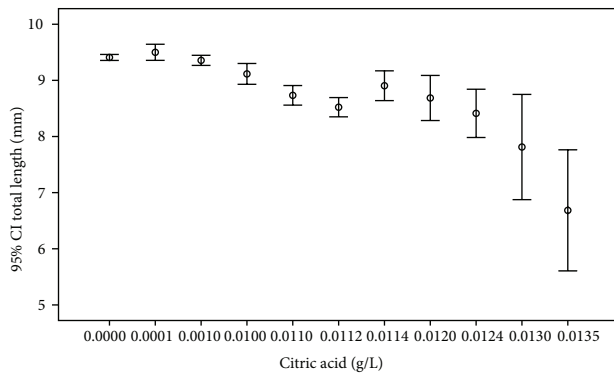


Figure 2. Total lengths (mm) of *Xenopus laevis* according to E330 dosage. 95% CI error bars are presented.

determined to be 9.352 mm and 9.465 mm, respectively. The total mean length of the exposed embryos (N = 751, because 351 embryos died) was found to be 8.572 ± 0.134 mm. The average values of the upper and lower limits of the 95% CI were determined to be 8.309 mm and 8.836 mm, respectively (P < 0.001). The calculated MCIG values indicated that embryonic development was impaired within the 0.010–0.011 g/L concentration range. Moreover, at the end of 24 h, the growth of *Xenopus* embryos was halted at the 22nd developmental stage at a 0.1–0.2 g/L concentration, suggesting that the unlimited use of E330 may result in some problems such as preterm birth or abortion in humans.

We observed several types of malformations in *Xenopus laevis* that were caused by E330. Gut malrotation, eye malformations including microphthalmia, body malformations including kinked and somite abnormalities, and dermal blister or bullae were observed at a rate of less than 1% for each abnormality (N = 73) at concentrations of E330 ranging from 0.0001 g/L to 0.0135 g/L (Figure 3). However, this result was not as critical as the death ratio, since the anomaly rate was very low (*vide infra*).

4. Discussion

The most dangerous aspect of environmental pollutants is described by the phrase 'delayed effects'. Avoiding substances with delayed effects can be difficult because this may mean foregoing a standard convenience. The general trend when determining the safety of a particular

substance is to detect the harmless dose and determine the maximum concentration at which the substance can be safely used (28).

Many previous studies concerning the safety of E330 support our results that citric acid may have harmful effects on living organisms. In one study, more than 5% E330 was added to the diet of young rats and guinea pigs. The abnormality seen was a decrease in the packed cell volume (31). In another study in which E330 was added continuously to rats' water, decreases in continuous water intake, the ratio of liquid-to-food intake, and body weight were observed. Due to the lack of water intake, dehydration ensued. These data suggest that substances decreasing the quality of the taste of the water do not exert a unitary influence on fluid intake, and further underscore the complexity of ecological factors involved in regulating drinking behavior (32). Aktaç et al. observed tissue degeneration, cytoplasmic vacuolation, nuclear membrane breakdown, pyknotic nuclei, and necrosis of hepatocytes using microscopic examination of mouse liver upon E330 exposure. These data suggested that E330 has a hepatotoxic effect and that long-term E330 exposure may cause destruction of mouse liver. The researchers concluded that additional detailed studies were needed to explain the mechanism by which E330 was causing the harmful effects (14). Tuttle et al. (33) tried to reduce the density of the Puerto Rican frog (*Eleutherodactylus coqui*) using citric acid administered in the air. Citric acid is approved for controlling *Eleutherodactylus coqui*, which is an invasive pest in Hawaii. At the end of the study it was found that 16% and 11% citric acid treatments were able to reduce the density of *Eleutherodactylus coqui* and that the effects of the treatment could last for 5 months. They also noted that repeated treatments appeared to be more effective for reducing the density than single applications.

In this study, the embryotoxicity of E330 was detected using the FETAX test. The LC_{50} value was determined; however, the EC_{50} could not be elucidated, and consequently the teratogenicity index could not be determined. Using microscopic examination at the end of 24 h, we observed that the growth of *Xenopus laevis* embryos was halted at the 22nd developmental stage at

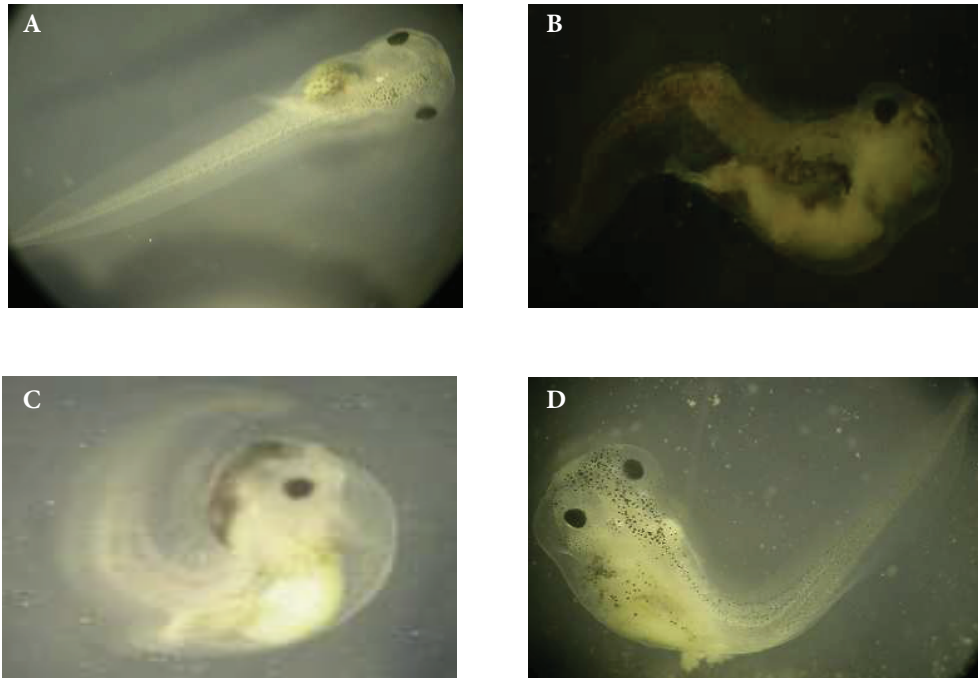


Figure 3. *Xenopus* larvae at 5 days old. A: Anterior view of a control larva. B, C, D: Larvae exposed to 0.01 g/L E330 concentration. B: Plurimalformed larva affected by generalized edema, abnormal gut coiling, kinked tail. C: Larva affected by tail flexure and microphthalmia. D: Tail flexure.

a certain threshold concentration of E330 (0.1–0.2 g/L). Thus, the survival of the embryos was dose-dependent, and in addition to developmental delays, we observed a low rate of different types of malformations in *Xenopus laevis* embryos caused by E330. The treated embryos were frequently extremely swollen and full of fluid in the tissue spaces. Edema occurred in the head, torso, and/or fin of the affected embryos. Most of these embryos exhibited structural abnormalities as described above prior to the appearance of the edema, indicating that swelling, in itself, was not the primary cause of the other defects. The only exception to this may be in the case of secondary displacement of visceral organs by edema. This effect was also observed in one of the head anomalies, namely microphthalmia. The third prevalent malformation from exposure to E330 was kinked tail. Macroscopic observations indicated that the appearance of kinks was preceded by the formation of what seemed to be fluid-filled cysts in the region between the axial skeleton and the epidermis. The occurrence of more than one kink per animal was not unusual, although these kinks were not lethal in laboratory-raised animals. However, all the malformed embryos had abnormal notochords, suggesting that dysfunction of this organ may have been the primary factor leading to the pathogenesis of the other tissues (34). The dose-dependent increases in the incidence of edema, tail flexure, microphthalmia, and edema-related anomalies observed in this FETAX assay

study could not be compared with similar studies using amphibians and other vertebrates due to lack of available data.

With our FETAX analysis, we observed that the ADI concentration, which is currently unlimited (not specified), may be more dangerous than was previously realized for human health. Our study showed that at the 0.1–0.2 g/L E330 range, *Xenopus* embryos did not further develop and they were all dead at the 22nd stage.

The ADI values for E330 were determined by the JECFA and were based on a wide variety of research studies, including extensive toxicological studies that were overseen and evaluated by international commissions. However, it was implied that the ADI values could be changed based on the results of new research (10).

The potential risk of E330 use on human embryogenesis, and thus E330 teratogenicity, is not known for certain. Although assessing the risk of exposure to E330 solely based on this *in vitro* study is not sufficient, our results highlight the importance of using different animal models to improve risk assessment and suggest a need for more research on the industrial use of food additive concentrations.

Acknowledgment

This work was supported by the Scientific Research Office of Çukurova University (I.U. BAP), project no. TF2009BAP7.

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