Chung Shan Medical Journal 2020; 31:73-80

Original Article

Effects of benzoic acid and sodium nitrite toxicity on *Daphnia magna* embryos

Shih-Hsien Chang[1], Cheng-Yih Liaw[1], Ken K. S. Wang[1, 2,*]

[1] *School of Public Health, Chung Shan Medical University, Taichung, Taiwan*

[2] *Department of Family and Community Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan*

Background/Purpose: An assay system using *D. magna* embryos was applied to the investigation of the adverse effects of food additives.

Methods: Immobilization test and embryonic development assay procedures were utilized to evaluate the toxic effects of benzoic acid and sodium nitrite on *D. magna* embryos according to US EPA procedures. The 48h-EC_{50} values and 95% confidence limits were calculated on Probit analysis, based on nominal concentrations.

Results: Long-term exposure of *D. magna* to preservatives led to delayed first brood and changes in number of hatchlings, as well as differences in offspring sex ratio, number of offspring I hatchlings, number of living generations, and survival rate. The impact of sodium nitrite on embryos of *D. magna* was greater than that of benzoic acid ($p<0.001$).

Conclusion: Daphnid embryos are markedly more sensitive to benzoic acid and sodium nitrite than juveniles. In addition, sodium nitrite has higher toxicity to *Daphnia* embryos than benzoic acid. The results of the present study clearly demonstrated that benzoic acid and sodium nitrite cause acute toxicity to and have teratogenic effects on *D. magna* neonates and embryos, respectively.

Keywords: preservatives, *Daphnia magna*, embryo toxicity, teratogenic

Introduction

Preservatives have been widely used in food, drugs, and cosmetics to prevent aging and decay [1, 2]. Some are naturally occurring compounds, for example those found in prunes, cinnamon, tea, and berries [1]. Some preservatives act as antimicrobial agents and/or antioxidants. Benzoic acid (BA) is a specific inhibitor of bacteria [3].

* Corresponding Author: Ken K.S. Wang, Ph.D.
School of Public Health, Chung Shan Medical University, Taichung, Taiwan
Address: No. 110, Sec. 1, Jianguo N. Rd., Taichung 402, Taiwan
Tel: +886-4-24730022 ext. 11890
E-mail: kenwang@csmu.edu.tw

BA and benzoate salts have been used as direct food and beverage additives for decades. These compounds effectively prevent microbial growth, as benzoates exert a high toxicological specificity toward bacteria and other food contaminants [4, 5]. These are classic preservatives that contain aromatic structures that naturally occur in small, organic, antimicrobial, bimolecular, hydrophobic form [6]. BA is used in a variety of foods, such as sauces, wine, and canned fruit. Among the most heavily benzoate-exposed populations in Taiwan, the highest daily dose is 3.1 mg/kg(bw)/day, or 62% of the acceptable daily intake [7]. Excessive amounts of this preservative can be harmful to human health [8, 9]. Sodium nitrite ($NaNO_2$) is widely used as a food preservative and color fixative in fish and meat

[10, 11]. Sodium nitrate and nitrite positively affect the flavor, appearance, safety, and quality of cured meats. In particular, addition of nitrite imparts the characteristic color and flavor to cured meats and prevents rancidity by inhibiting lipid peroxidation [12, 13, 14]. Moreover, nitrite improves the safety of meat by inhibiting the growth of microorganisms, particularly *Clostridium botulinum* [15]. People are exposed to nitroso compounds through nitrite/nitrate preserved foods and beverages, such as beer, or they are formed in the stomach following uptake of the precursor nitrates, nitrites, and secondary amines [16]. When administered to organisms in excessive amounts, these food additives cause toxic reactions [17]. Sodium nitrate and nitrite produce potential carcinogenic substances such as N-nitroso compounds (NOCs) during cooking, smoking, or drying. Several studies have reported that meat-related factors, such as NOCs, heme iron, and heterocyclic amines contribute to cancer risk [18, 19, 20].

Daphnids are often employed to investigate the toxicity of chemicals in aquatic ecosystems due to their high sensitivity, short generation time, and ease of manipulation. In addition, invertebrates, especially aquatic crustaceans, have recently gained attention as model organisms for non-mammalian test systems to evaluate the toxicities of chemicals, heavy metals, and pesticides, as well as for ecotoxicological assessments of environmental pollutants [21, 22, 23]. *Daphnia magna* (*D. magna*) embryos have also been used in the study of environmental hormones. Our recent study demonstrated that plant growth regulators (PGRs) cause acute toxicity to and have teratogenic effects on *D. magna* juveniles and embryos [24]. The parthenogenetic developmental stages of *D. magna* embryos are of potential benefit in the evaluation of the effects of synthetic compounds on aquatic ecosystems [25].

Numerous studies have been conducted on the antibacterial, antifungal, and antiviral activities of natural and synthetic phenolic compounds including various substituted BAs and sodium nitrite [26, 27]. Until recently, most studies on the acute toxicity of food additives and LD_{50}, LC_{50} and IC_{50} values of individual toxins have been on monkeys, rats, mice, rabbits, fish (*Gambusia affinis*), bacteria (*Salmonella typhimurium, Escherichia coli*), and human cells (HepG2, BEAS-2B)[28, 29, 30, 31, 32]. In contrast, studies on the effects of BA and sodium nitrite, as well as other carboxylic acids, on freshwater crustaceans have been limited. To the best of our knowledge, the effects of BA and sodium nitrite on freshwater crustacean *D. magna* embryos have not been reported. The aims of this study were to investigate the impact of BA and sodium nitrite on growth, reproduction, and embryonic development of *D. magna*.

Materials and methods

Chemicals

BA (CAS.no. 00065-85-0), IUPAC name Benzenecarboxylic acid, molecular formula C_6H_5COOH, molecular weight 122.123 g/mol, colorless crystalline solid, solubility in water 3.44 g/L (25 °C), and sodium nitrite (CAS no. 07632-00-0), IUPAC name sodium nitrate, molecular formula $NaNO_3$, molecular weight 84.9947 g/mol, colorless crystals, solubility in water 91.2 g/100 mL (25 °C), were both purchased from Sigma Aldrich Co., USA. (Fig.1)

Chlorella vulgaris strains and culture conditions

Stocks of green algae, *Chlorella vulgaris* (*C. vulgaris*), have been cultured in our laboratory at Chung Shan Medical University since 2001. For this study, BG11 medium (75 mg/L $MgSO_4 \cdot 7H_2O$, 40 mg/L $K_2HPO_4 \cdot 3H_2O$, 27 mg/L $CaCl_2$, 20 mg/L Na_2CO_3,1500 mg/L $NaNO_3$, 6 mg/L citric acid monohydrate, 6 mg/L ammonium ferric citrate, 1 mg/L Na_2EDTA) with 1 mL trace metal solution (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2 \cdot 4H_2O$, 0.39 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.222 mg/L $ZnSO_4 \cdot 7H_2O$) was used. Culture medium was autoclaved at 121.5°C for 30 min.

C. vulgaris was cultivated in 2000-mL flasks plugged with perforated rubber stopper. A glass tube was pushed through the stopper with one end placed close to the flask bottom for gas supply. Three Philips straight fluorescent tubes (40 W/tube) were positioned 6 cm from the flasks. Illumination intensity was about 6000 lux with photoperiodicity of 16:8 (light for 16 h and dark for 8 h). The

temperature was maintained at 25 ± 2°C with air-conditioning. The gases were sterilized using 0.2 μm PTFE gas filter diaphragm Midisart-2000 (SRP65, Sartorius, Germany) with 4% CO_2 as carbon source at a rate of 0.5 v/v/min. The solution pH was 6-8. After seven days of culture, the concentration of *C. vulgaris* reached 1.0 x 10^6 cells/mL. The actual number of *C. vulgaris* cells was counted with a hemocytometer under a dissecting microscope [25].

Acute toxicity tests on *D. magna* neonates

D. magna populations have been maintained parthenogenetically at Chung Shan Medical University since 2001. *D. magna* used in this study were kept at approximately 20°C in 10 L tanks on a windowsill of the laboratory. The tanks generated enough green algae (*C. vulgaris*) to sustain a colony of several hundred for at least 6 months. The tanks were topped up alternately with dechlorinated and conditioned tap water to replenish water loss from evaporation and aerated with filtered air.

Simulated high-hardness medium was employed in the preparation of a series of concentrations of dilution water for acute toxicity tests [33, 34]. These tests were performed in 50 mL of medium in 100 mL glass beakers. Beakers containing *D. magna* neonates were placed in a growth chamber (temperature 20 ± 2°C, under a 16/8 h light/dark cycle). To evaluate the acute toxicities of BA and sodium nitrite, *D. magna* neonates, randomly selected from laboratory cultures (< 24 h old, ≥ third brood), were exposed to the simulated high-hardness medium (control) or varying concentrations of BA (range 500 to 1000 mg/L) or sodium nitrite (5 to 100 mg/L). Four replicates of five *D. magna* neonates each were used according to US EPA procedures [35]. Immobilization and mortality of *D. magna* neonates in each beaker were assessed under a low-magnification microscope (10-63 times magnification, Nikon SMZ800, Japan) every 24 h during the test period. Immobility was the endpoint for determining acute toxicity. *D. magna* neonates that showed no movement, within 15 s of gentle stirring, were defined as immobile. EC_{50} values were calculated on probit analysis [36] according to nominal concentrations.

Acute toxicity tests on *D. magna* embryos

Immediately after the release of the third brood, females were isolated from the culture and observed until the passage of new embryos from the ovaries to the brood chamber. This time point was taken as time zero of embryonic development. Eight hours after time zero, females were placed under a dissecting microscope and embryos were removed by introducing a small pipette with simulated high-hardness medium into the brood chamber to create a low flow to push the embryos to the microscope slide [24, 37, 38]. After being washed, embryos were placed in individual wells of tissue culture plates with 300 mL of control medium or with β-agonist at desired concentration (according to the lowest observed effect concentration (LOEC) for neonates at 48 h). For each concentration, there were 20 replicates. Embryos were incubated at a constant temperature (20 ± 2°C) with a photoperiod of 16 h (light):8 h (dark). Embryonic development was observed at 24, 48 and 72 h under a low-magnification microscope. The percentage of embryos that exhibited developmental abnormalities was determined when the development of daphnids in the control group was complete. Abnormalities included incomplete development of antennae, helmet, rostrum, Malpighian tube, and sensory bristles, as well as bent tail spine. For the analysis of toxicity to embryos, 48 h EC_{50} values and 95% confidence limits were calculated on probit analysis [36] based on nominal concentrations.

Teratogenic assay

The series of test concentrations for teratogenic assay were based on the 48 h LOECs for the two β-agonists. Ten replicates per concentration were used. Embryos (8 h-old eggs; stage 2) were washed and placed in individual wells of tissue culture plates with 300 mL of control medium or with preservative at the desired concentrations. They were incubated at a constant temperature (20 ± 2°C) with a photoperiod of 16 h (light):8 h (dark) and subjected to microscopic examination every 24 h to determine developmental stage and abnormalities. The development time of each *D. magna* neonate that hatched was recorded, in addition

to morphological abnormalities in the carapace, first and second antennae, eyes, brood chamber, abdominal protuberance, Malpighian tube, sensory bristles, tail spine, and pigmentation, regardless of survival status [25, 39, 40, 41].

Statistical analysis

EC_{50} values were calculated on Probit analysis using SAS statistical software (SAS Institute, Inc., Cary, NC). One-way ANOVA and Tukey test were used for comparisons with controls.

Results

Acute toxicities of benzoic acid and sodium nitrite to *D. magna* embryos and neonates

In this study, the acute toxicities of preservatives, as defined by 24 and 48 h EC_{50} values, were determined using *D. magna* embryos and neonates. Control groups were cultured in simulated high-hardness medium (Table 1). Results of 24-h exposure indicated that *D. magna* embryos are less sensitive to BA and sodium nitrite. Prolonged sodium nitrite exposure of 48 h resulted in a decline in EC_{50} value of *D. magna* embryos, implying increase in sodium nitrite toxicity over time (48 h EC_{50} = 57.9±3.5 mg/L). The toxicity ratio of 24 h EC_{50}/48 h EC_{50} indicated that acute toxic effects of sodium nitrite on daphnid embryos occur after the first 24 h and worsen from 24 to 48 h. *D. magna* neonate 48 h EC_{50} values (95% CI) were 135.2±4.7 mg/L for BA and 55.8±6.2 mg/L for sodium nitrite. Acute toxicity increased with duration of exposure for both preservatives. Moreover, the toxicity of sodium nitrite to *D. magna* neonates was 2.4 times that of BA.

Teratogenic effects of benzoic acid and sodium nitrite on *D. magna*

Table 2 illustrates the toxic effects of BA and sodium nitrite on *D. magna* embryos. After 72 h exposure, differing degrees of teratogenic effects were observed, such as growth retardation, forelimb deformities, heart defects, Malpighian tube malformation, visceral atrophy, carapace deformities, tail spine defects, and albinism, when compared with control group (Fig. 2).

For a preliminary test to determine dose toxicity, sodium nitrite was added at nominal concentrations from 5 to 40 mg/L at the beginning of culture. Four replicates of five stage 1 or 2 embryos each were exposed to sodium nitrite at each concentration. Incubation continued for 4 days, with medium change after 2 days. In the control group without sodium nitrite treatment, all the embryos developed and hatched neonates were observed on the second day after the start of culture. After 48 hours and comparison with the control group, the test group showed poor development with shrunken antennae and forelimbs, slightly atrophied helmet, deformed rostrum, poorly formed digestive caeca, atrophied brood chamber, undifferentiated Malpighian tube, incompletely differentiated splanchnic cells, deformed and open carapace, shrunken sensory bristles, and bent tail spine (Fig. 3a). After exposure for 72 hours, test group showed immobility and activity was lower than that of control group. Moreover, antennae had not stretched out fully, compound eyes had not closed and were distorted and swollen, heart showed deformities, brood chamber was shrunken, carapace showed corrugations, sensory bristles were shrunken, tail spine was bent, and growth retardation was observed (Fig. 3b). Following exposure for 96 hours, sodium nitrite induced teratogenic effects on the formation of internal organs and carapace or death of *D. magna*, when compared with exposure for 72 hours (Fig. 3c). The results showed that a higher concentration of sodium nitrite and long-term exposure cause severe teratogenic effects on *D. magna* embryos.

Chronic toxicities of benzoic acid and sodium nitrite on *D. magna* embryos

To ensure the feasibility of chronic toxicity test of preservatives on *D. magna* embryos, concentrations of BA and sodium nitrite were based on 48h-EC_{10} value ± 20% (i.e. 80 and 120 mg/L for BA and 20 and 30 mg/L for sodium nitrite) for 72 h pre-test. The results are shown in Fig. 4. Fig. 4a and 4b illustrate the toxic effects of BA on *D. magna* embryos. After 72 h exposure,

deformities were observed. The higher the BA concentration, the more severe the deformities. Similar results were obtained for sodium nitrite. Sodium nitrite continued to induce teratogenic effects between 48 and 72 h of exposure during which embryos developed into neonates. After 72 h exposure, deformities were observed. The higher the sodium nitrite concentration, the more severe the deformities including those that were fatal (Fig. 4c and 4d).

As mentioned above, we attempted to observe the chronic toxicity of BA and sodium nitrite to *D. magna* embryos for 21 days. Concentrations of BA and sodium nitrite were based on 48h-EC_{10} values of neonate acute toxicity. The results are shown in Table 3. In general, long-term exposure of *D. magna* to preservatives led to delayed first brood and changes in number of hatchlings, as well as differences in offspring sex ratio, number of offspring I hatchlings, number of living generations, and survival rate. The impact of sodium nitrite on *D. magna* embryos was greater than that of BA (p<0.001) and both food additives affected *D. magna*.

Discussion

There has been increasing interest in the use of invertebrates, especially aquatic crustaceans, as model organisms due to the need for non-mammalian systems to evaluate toxicities of chemicals and environmental pollutants [42]. The results of this study indicated that daphnid embryo assay is more sensitive than acute daphnid neonate immobilization assay for BA and sodium nitrite. Material Safety Data Sheet (MSDS) provided by Merck Taiwan rarely records the LC_{50} values of daphnids or only shows EC_{50} values. In this study, the LC_{50} and EC_{50} values of daphnids were lower than the LD_{50} value of rats [43], indicating that the EC_{50} value of daphnids is more sensitive than the LD_{50} value of rats [44, 45]. In animal models of chronic toxicity, EC_{50} of *D. magna* cannot be compared with LD_{50} of rats. Based on MSDS data, BA and sodium nitrite show no toxic effects on daphnids. However, in this study, 100 mg/L of BA and 25 mg/L of sodium nitrite produced toxic effects on *D. magna* embryos after 72 h, respectively. Therefore, the results of this study can be used to supplement MSDS data.

Muccini et al. [46] reported that the ionized and un-ionized forms of halobenzoic acids contribute differently to toxicity. Zhao et al. [47] reported acute toxicities of BA to *D. magna* at three different pH values (6.0, 7.8, and 9.0). Toxicities decreased as pH increased. Kamaya et al. [27] reported that hydroxylated BA has lower pKa value, although with higher toxicity than *meta*- and/or *para*-hydroxylated BA, indicating that hydroxyl-benzoate derivatives behave differently from halo-benzoates. In this study, during the immobilization toxicity test, BA was substituted with hydroxyl (OH) and/or methoxyl (OMe) groups on the benzene ring under neutralized conditions. BA 48h- EC_{50} has been reported to be 447 mg/L [27], which was higher than in this study (120.4±3.2 mg/L, embryo 48 h EC_{50}), pointing to the precision of this study. BA induced morphological abnormalities and delayed embryonic development. Similar results have been noted for phenols [37]. In both studies, 2 to 6-h-old cleaved eggs (between stage 1 and 2) were used. Eggs in earlier stages of development might be more sensitive to embryonic development and to detection of malformation. However, it is difficult to remove eggs less than 2-h old uninjured from adult females. Additionally, treatment of immature eggs is difficult to control due to rapid developmental processes. More advanced handling techniques will be needed to overcome these challenges.

Inhibition test of *D. magna* embryo immersion using sodium nitrate showed no change within the first 24 hours. This may have been due to the shell covering of the embryo, which reduced drug permeability [42]. After incubation for 48 hours, the embryo became a neonate. At that time, the outer shell disappeared, allowing the drug to permeate and causing deformity. When food additives are given to organisms in excessive amounts, they may cause toxic reactions. For example, nitrite-nitrate containing foods react with endogen amines to form carcinogenic and mutagenic cells. It is well documented that certain types of foods and beverages for human consumption pose toxic, genotoxic, or carcinogenic hazards. Sarıkaya and

Çakır [17] suggested that sodium nitrate is toxic to and causes deformation of human neural cells, which is consistent with the results of this study. Sodium nitrate at concentrations below 12.4 mg/L demonstrated toxic effects on *D. magna* embryos, indicating that the toxic effects on organisms is significant. The results of this study are consistent with those of previous studies. Thus, it is necessary to be concerned about dosage. This study revealed that there are relationships among concentration, exposure duration, and deformities of *D. magna* embryos. Deformities positively correlated with duration. As the concentration increased, the rate of deformity increased. Moreover, at a constant concentration, the rate of deformity increased as the duration increased. These results were confirmed on chronic toxicity test (Table 3).

Conclusion

An assay system using *D. magna* embryos was applied to the investigation of the adverse effects of food additives. Data was compared with that of the literature. *Daphnia* embryos were markedly more sensitive to BA and sodium nitrite than juveniles. In addition, sodium nitrite had higher toxicity to *Daphnia* embryos than BA, suggesting that sodium nitrite holds potential risk and that its use as a food additive should be restricted. The present study clearly demonstrated that BA and sodium nitrite cause acute toxicity to and teratogenic effects on *D. magna* neonates and embryos, respectively. In this study, *D. magna* embryos were used to detect the toxicity of food additives as indicators of sensitivity and teratogenic effects.

References

1. Korotkova E I, Avramchik O A, Angelov T M, Karbainov Y A: Investigation of antioxidant activity and lipophilicity parameters of some preservatives. Electrochimica Acta 2005; 51: 324-332.
2. Lord H, Pawliszyn J: Microextraction of drugs. Journal of Chromatography A 2000; 902: 17-63.
3. Soni MG, Burdock GA, Taylor SL, et al.: Safety assessment of propyl paraben: a review of the published literature. Food and Chemical Toxicology 2001; 39: 513-532.
4. Warth AD: Mechanism of action of benzoic acid on *Zygosaccharomyces bailii*: effects on glycolytic metabolite levels, energy production, and intracellular pH. Applied and Environmental Microbiology 1991; 57 (12): 3410–3414.
5. Hoffman TE, Hanneman WH: Physiologically-based pharmacokinetic analysis of benzoic acid in rats, guinea pigs and humans: Implications for dietary exposures and interspecies uncertainty. Computational Toxicology 2017; 3: 19-32.
6. Brul S, Coote P: Preservative agents in foods: Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 1999; 50: 1-17.
7. Ling MP, Lien KW, Wu CH, Ni SP, Huang HY, Hsieh DP: Dietary exposure estimates for the food preservatives benzoic acid and sorbic acid in the total diet in Taiwan. Journal of Agricultural and Food Chemistry 2015; 63 (7): 2074–2082.
8. Ling MP, Lien KW, Wu CH, Ni SP, Huang HY, Hsieh DP: Dietary exposure estimates for the food preservatives benzoic acid and sorbic acid in the total diet in Taiwan. Journal of Agricultural and Food Chemistry 2015; 63 (7): 2074–2082.
9. Shu Y, Yu B, He J, Yu J, Zheng P, Yuan Z, Chen D, Mao X: Excess of dietary benzoic acid supplementation leads to growth retardation, hematological abnormality and organ injury of piglets. Livestock Science 2016; 190: 94-103.
10. De Roos AJ, Ward MH, Lynch CF, Cantor KP: Nitrate in public water supplies and the risk of colon and rectum cancers. Epidemiology 2003; 14: 640-649.
11. Özena H, Kamberb U, Karamana M, Gülc S, Atakisi E, Özcane K, Atakisi O: Histopathologic, biochemical and genotoxic investigations on chronic sodium nitrite toxicity in mice. Experimental and Toxicologic Pathology 2014; 66: 367–375.
12. Kurt Ş: The effects of grape seed flour on the quality of Turkish dry fermented sausage (Sucuk) during ripening and refrigerated storage. Korean Journal for Food Science of Animal Resources 2016; 36(3): 300-308.
13. Choi YS, Hwang KE, Kim HW, Song DH, Jeon

KH, Park JD et al.: Replacement of pork meat with pork head meat for frankfurters. Korean Journal for Food Science of Animal Resources 2016; 36(4): 445-451.
14. Richards MP: Redox reactions of myoglobin. Antioxidants & Redox Signaling 2013; 18(17): 2342-2351.
15. Sofos JN, Busta FF, Allen CE: Botulism control by nitrite and sorbate in cured meats: A review. Journal of Food Protection 1979; 42(9): 739-770.
16. Mukherjee J, Sarkar S, Ghosh A, et al.: Immunotherapeutic effects of T11TS/S-LFA3 against nitrosocompound mediated neural genotoxicity. Toxicology Letters 2004; 150: 239-257.
17. Sarıkaya R, Çakır Ş: Genotoxicity testing of four food preservatives and their combinations in the *Drosophila* wing spot test. Environmental Toxicology and Pharmacology 2005; 20: 424-430.
18. Bingham SA, Hughes R, Cross AJ: Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. The Journal of Nutrition 2002; 132(11): 35225-35255.
19. Bastide NM, Chenni F, Audebert M, Santarelli RL, Taché S, Naud N, et al.: A central role for heme iron in colon carcinogenesis associated with red meat intake. Cancer Research 2015; 75(5): 870-879.
20. Sinha R, Cross A, Curtin J, Zimmerman T, McNutt S, Risch A, et al.: Development of a food frequency questionnaire module and databases for compounds in cooked and processed meats. Molecular Nutrition & Food Research 2005; 49(7): 648-655.
21. Emmanuel E, Keck G, Blanchard JM, et al.: Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environment International 2004; 30: 891-900.
22. Villegas-Navarro A, Rosas LE, Reyes JL: The heart of *Daphnia magna*: effects of four cardioactive drugs. Comparative Biochemistry and Physiology Part C 2003; 136: 127-134.
23. Ton SS, Chang SH, Hsu LY, Wang MH, Wang KS: Evaluation of acute toxicity and teratogenic effects of disinfectants by *Daphnia magna* embryo assay. Environmental Pollution 2012; 168: 54-61.
24. Wang KS, Lu CY, Chang SH: Evaluation of acute toxicity and teratogenic effects of plant growth regulators by *Daphnia magna* embryo assay. Journal of Hazardous Materials 2011; 190: 520-28.
25. Lee MW, Chen Algui KF, Chang SH, Wang KS: Effects of UVC-Irradiated Leanness-Enhancing Agents on *Daphnia magna*. Chung Shan Medical Journal 2018; 29:83-94.
26. Friedman M, Henika PR, Mandrell RE: Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 2003; 66:1811-1821.
27. Kamaya Y, Fukaya Y, Suzuki K: Acute toxicity of benzoic acids to the crustacean *Daphnia magna*. Chemophere 2005; 59: 255-261.
28. Blair M, Chappel CI: 4-week range-finding and 1-year oral toxicity studies of sucrose acetate isobutyrate (SAIB) in cynomolgus monkey. Food and Chemical Toxicology 1998; 36: 121-126.
29. Walton K, Walker R., van de Sandt JJM, et al.: The application of in vitro data in the derivation of acceptable daily intake of food additives. Food and Chemical Toxicology 1999; 37: 1175-1197.
30. Hurtt ME, Cappon GD, Browining A: Proposal for a tiered approach to developmental toxicity testing for veterinary pharmaceutical products for food-producing animals. Food and Chemical Toxicology 2003; 41: 611-619.
31. McKean C, Tang L, Tang M, et al.: Comparative acute and combinative toxicity of aflatoxin B1 and fumonisin B1 in animals and human cells. Food and Chemical Toxicology 2006; 44: 868-876.
32. Fujii M, Sun B, Nishika H, et al.: Evaluation of the safety and toxicity of the oligomerized polyphenol oligonol. Food and Chemical Toxicology 2007; 45: 378-387.
33. Wang KS, Lu CY, Chang SH: Evaluation of acute toxicity and teratogenic effects of plant growth regulators by *Daphnia magna* embryo assay. Journal of Hazardous Materials 2011; 190: 520–28.
34. Baer KN, Goulden CE: Evaluation of a high-

hardness COMBO medium and frozen algae for *Daphnia magna*. Ecotoxicology and Environmental Safety 1988; 39: 201–06.

35. US EPA: Ecological Effects Test Guidelines, OPPTS 850.1010 Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids, Cornelius I. Weber. Environmental Monitoring and Support Laboratory, US Environmental Protection Agency, Cincinnati, OH, EPA, 1996. 712-C-96-114: p. 1–8.
36. US EPA: Probit Analysis Software, Ver. 1.3. US Environmental Protection Agency, Cincinnati, OH. 2001.
37. Abe T, Saito H, Niikura Y, et al.: Embryonic development assay with *Daphnia magna*: application to toxicity of aniline derivatives. Chemosphere 2001; 45: 487-495.
38. Sobral O, Chastinet C, Nogueira A, Soares AMVM, Gonc-alves F, Ribeiro R: In vitro development of parthenogenetic eggs: a fast ecotoxicity test with *Daphnia magna*. Ecotoxicology and Environmental Safety 2001; 50: 174–79.
39. Baird DJ, Barber I, Soares AMVM, Calow P: An early life-stage test with *Daphnia magna* Straus: an alternative to the 21-day chronic test? Ecotoxicology and Environmental Safety 1991; 22: 1–7.
40. Liu H, Yu H, Giesy JP, Sun Y, Wang X: Toxicity of HC Orange No. 1 to *Daphnia magna*, zebrafish (*Brachydanio rerio*) embryos, and goldfish (*Carassius auratus*). Chemosphere 2007; 66: 2159–65.
41. De Schamphelaere KAC, Stubblefield W, Rodriguez P, Vleminckx K, Janssen CR: The chronic toxicity of molybdate to freshwater organisms. I. Generating reliable effects data. Science of Total Environment 2010; 408: 5362–71.
42. Ohta T, Tokishita SI, Shiga Y, et al.: An assay system for detecting environmental toxicants with cultured cladoceran eggs *in vitro*: malformations induced by ethylenethiourea. Environmental Research A 1998; 77: 43-48.
43. Budavari, S: The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals. Ed11. Rahway, N.J., U.S.A.: Merck and Co., Inc. 1989: p.170, 1101, 1365, and 8599.
44. MSDS: Benzoic acid. (https://www.cdhfinechemical.com/images/product/msds/ 19_308110588_BenzoicAcid-CASNO-65-85-0-MSDS.pdf)
45. MSDS: Sodium nitrite. (https://www.cdhfinechemical.com/images/product/ msds/ 101_562335722_SodiumNitrite-CASNO-7632-00-0-MSDS.pdf)
46. Muccini M, Layton AC, Sayler GS, et al.: Aquatic toxicities of halogenated benzoic acids to *Tetrahymena pyriformis* B. Archives of Environmental Contamination and Toxicology 1999; 62: 616-622.
47. Zhao YH, Ji GD, Cronin MTD, et al.: QSAR study of the toxicity of benzoic acids to *Vibrio fischeri, Daphnia magna* and carp. Science of the Total Environment 1998; 216: 205-215.