Chung Shan Medical Journal 2018; 29:81-92

Original Article

Effects of UVC-Irradiated Leanness-Enhancing Agents on *Daphnia magna*

Ming-Wei Lee[1], Algui K. F. Chen[2], Shih-Hsien Chang[2], Kai-Sung Wang [2,3,*]

[1] *School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*
[2] *School of Public Health, Chung Shan Medical University, Taichung, Taiwan*
[3] *Department of Family and Community Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan*

In this study, the potential impact of leanness-enhancing agents on the aquatic environment was investigated. The effects of UVC-irradiated ractopamine and clenbuterol on growth, reproduction, and embryonic development of laboratory batches of *Daphnia magna* were examined. UVC irradiation simulated the effect of sunlight on these chemical substances. Adverse effects of ractopamine on the embryos of daphnids occurred mainly within the first 24 h, and intensified from 24 to 48 h. Clenbuterol showed no acute toxic effect on embryos of or young daphnids within 48 h. While ractopamine demonstrated more significant adverse effects on daphnids than clenbuterol, opposing results were observed after UVC irradiation. Chronic toxicity test revealed that β-agonists induce teratogenic effects on daphnids following 72 h exposure. The effects of UVC-irradiated ractopamine on gravid daphnids were less marked than those of non-irradiated ractopamine. Opposing results were observed for clenbuterol. Maternal abnormality, embryo retardation, and offspring fatality rate in gravid daphnids increased and embryo hatch rate decreased, with prolonged UVC irradiation and higher concentrations of clenbuterol. The mechanism of the biological effects of UVC irradiation on β-agonists merits further investigation.

Keywords: β-agonists, *Daphnia magna*, embryo toxicity, teratogenics, UVC irradiation

Introduction

Phenethanolamines, commonly called β-adrenergic agonists (β-agonists) or repartitioning agents, are compounds that alter the ratio in which dietary energy intake is partitioned between lean tissue and fat tissue, resulting in a favorable shift in the lean-to-fat ratio of growing animals [1]. Ractopamine and clenbuterol are β-agonists with similar chemical structures and functions. They were originally developed for treating diseases, but side effects of reduced fat levels and increased muscle protein anabolism were found when administered to animals [2, 3]. They have been illegally applied as nutrient-repartitioning agents in livestock to enhance the production of muscle tissues. Owing to their potential adverse effects on cardiovascular and central nervous systems of consumers, many countries have prohibited the use of ractopamine and clenbuterol on animals reared for meat production [4]. Taiwan banned the use of all β-agonists as leanness-enhancing agents in farm animals in October 2006. However, in August 2012, the Taiwanese government agreed to adopt the maximum residue limit (10 ppb), set by the Codex Alimentarius Commission, for ractopamine

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

in imported beef [5]. In pigs, treatment during finishing with repartitioning doses of β-agonists has been associated with modifications of vascular microcirculation characterized by ulcerative lesions of distal extremities. Moreover, elevated heart rate and increased peripheral catecholamine concentrations have been reported in pigs fed ractopamine [6-8]. β-agonists applied at high dosage as nutrient-repartitioning agents in livestock are retained in animal bodies. They have been detected in pig feed, pig urine and pig liver [9,10]. Therefore, β-agonist-containing effluents from pig farms and slaughterhouses may be an important source of contaminants, especially in rural regions.

Prior research has demonstrated that ractopamine is eliminated through urine or feces either unchanged or after conjugation as glucuronides or sulfates [11, 12]. These metabolites can be converted into the parent compound by hydrolysis [11, 13]. In animal waste, ractopamine can be transferred to the soil by manure or through contamination of water sources. Wastewater-contaminated rivers affect the aquatic ecosystem. Ractopamine has previously been quantified in wastewater from a treatment lagoon, as well as in groundwater associated with a concentrated animal-feeding operation [14].

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, with special reference to aquatic crustaceans, have recently been of interest as test model organisms for developing non-mammalian test systems to evaluate toxicities of chemicals, heavy metals, and pesticides, as well as for ecotoxicological assessment of the risk of environmental pollutants to aquatic organisms [15]. *Daphnia magna* (*D. magna*) embryos have also been used in the study of environmental hormones. Our recent study demonstrated that plant growth regulators (PGRs) may cause both acute toxicity to and teratogenic effects on young *D. magna* and *D. magna* embryos. The parthenogenetic developmental stages of *D. magna* embryos have the potential for evaluating the effects of synthetic compounds on aquatic ecosystems [16].

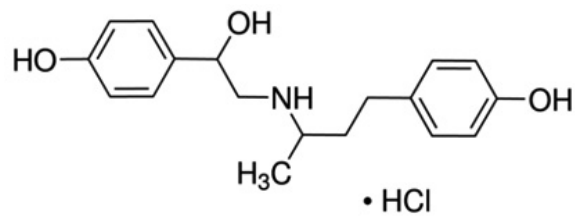
Efficient methods for detecting ractopamine include high-performance liquid chromatography with fluorescence detection (HPLC-FD) [4, 17], liquid chromatography-mass spectrometry (LC-MS) [18], gas chromatography-mass spectrometry (GC–MS) [19], enzyme-linked immunosorbent assay (ELISA) [20], electrochemical detection [9], immune-chromatographic test strip assay [21], and biomimetic enzyme-linked immunosorbent assay [22].

To the best of our knowledge, the effects of UVC-irradiated ractopamine or clenbuterol on freshwater crustacean *D. magna* have not been reported. The aims of this study were to investigate the biological acute toxicities and teratogenic effects of ractopamine and clenbuterol on *D. magna* and to examine the impact of UVC-irradiated ractopamine and clenbuterol on growth, reproduction and embryonic development of *D. magna*.

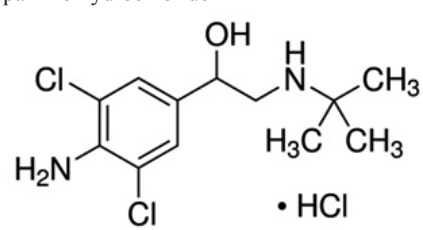
Materials and Methods

Chemicals

Ractopamine-hydrochloride (CAS-No 90274-24-1), IUPAC name 4-[3-[[2-Hydroxy-2-(4-hydroxyphenyl) ethyl] aminobutyl phenol hydrochloride, molecular formula $C_{18}H_{23}NO_3$•HCl, molecular weight 337.84 g/mol, white microcrystalline powder, solubility in water 4100 mg/ , and clenbuterol-hydrochloride (CAS-No 21898-19-1), IUPAC name 4-Amino-alpha-[(tert-butylamino) methyl]-3,5-dichlorobenzyl



(a) ractopamine-hydrochloride



(b) clenbuterol-hydrochloride

Fig.1. Molecular structures of β-agonists

alcohol hydrochloride, molecular formula $C_{12}H_{19}$ O Cl_3•HCl, molecular weight 313.65 g/mol, colorless microcrystalline powder, solubility in water 46.5 μg/mL were both purchased from Sigma Aldrich Co., USA. (Fig.1)

Chlorella vulgaris strains and culture conditions

Green algae, *Chlorella vulgaris* (*C. vulgaris*), have been cultured in our laboratory at Chung Shan Medical University since 2001. Algae were cultured in BG11 medium: 75 mg/L $MgSO_4$•$7H_2O$, 40 mg/L K_2HPO_4•$3H_2O$, 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 $MnCl_2$•$4H_2O$, 0.39 mg/L Na_2MoO_4•$2H_2O$, 0.222 mg/L $ZnSO_4$•$7H_2O$). The culture medium was autoclaved at 121.5°C for 30 min.

C. vulgaris was cultivated in 2000-mL flasks plugged with perforated rubber stoppers. 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 placed 6 cm away 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 provided 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* can reach 1.0 x 10^6 cells/mL. The actual number of *C. vulgaris* cells was counted with a hemocytometer under a dissecting microscope [23].

Acute toxicity tests on neonates of *D. magna*

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

Simulated high-hardness medium was employed to prepare the series of concentrations of dilution water for the acute toxicity tests on daphnid neonates [16, 24]. Tests were performed in 50 mL of medium in 100 mL glass beakers. The 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 toxicity of ractopamine and clenbuterol, *D. magna* neonates, randomly selected from laboratory cultures (< 24 h old, ≥ third brood), were exposed to the simulated high-hardness medium (control) or to different concentrations of ractopamine (range from 2.5 to 100 μg/L) or clenbuterol (range from 0.625 to 20 mg/L). Four replicates with five *D. magna* neonates per replicate were used according to US EPA procedure [25]. Both 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 showing no movement within 15 s after gentle stirring were defined as immobile. EC50 values were calculated on probit analysis [26] according to nominal concentrations.

Acute toxicity tests on embryos of *D. magna*

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 [16, 27]. 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). Twenty replicates per concentration were used. 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 the antennae, helmet, rostrum, Malpighian tube, and sensory bristles and unextended tail spine. For the analysis of toxicity to the embryos, the 48 h EC50 values and 95% confidence limits were calculated on probit analysis [26] on the basis of nominal concentrations.

Teratogenic assay

The series concentrations for teratogenic assaywere based on the 48 h LOECs for the two β-agonists for acute toxicity tests on embryos. Ten replicates per concentration were used. After being washed, embryos were placed in individual wells of tissue culture plates with 300 mL of control medium or with disinfectant at the desired concentrations. Embryos (8 h-old eggs; stage 2) were incubated at a constant temperature (20 ± 2°C) with a photoperiod of 16 h (light):8 h (dark). Embryos were subjected to microscopic examination every 24 h to determine developmental stage and abnormalities. The developmental time of every *D. magna* neonate that hatched was recorded, as were morphological abnormalities in the formation of carapace, first and second antennae, eyes, brood chamber, abdominal protuberance, Malpighian tube, sensory bristles, tail spine, and pigmentation, regardless of whether it survived or not [28-30].

Comparisons of biological effects of β-agonists after UVC irradiation

To compare biological degradation of β-agonists after UVC (Ultraviolet subtype C, wavelength 254 nm, irradiation 0.5-10 joules/m) irradiation, serial concentrations of ractopamine and clenbuterol were individually irradiated by UVC for 0.5, 1.0, and 2.0 h, respectively. The toxicity impact assessment of UVC-irradiated ractopamine and clenbuterol included acute toxicity mortality rate and developmental abnormality rate. The experimental details were the same as those mentioned above with the exception that the solutions were exposed to UVC prior to being tested on daphnids.

Chronic toxicity tests

The series concentrations were based on the results of the acute toxicity tests on embryos. In addition, hatched daphnids were inspected for gross morphological abnormalities under a low-magnification microscope (10-63 times magnification, Nikon SMZ800, Japan). The observation time was extended to 168 h. The development of embryos was observed using embryonic development inhibition rate (EDI) at different teratogenic rates [15, 16], and the body lengths and widths of maternal daphnids and hatched neonates were recorded daily..

Results and Discussion

Table 1. Toxicities of ractopamine and clenbuterol on embryos of and neonate *D. magna* after 24 and 48 h (n = 20).

β-agonists	Embryo (stage 2)		Neonate (< 24-h old)	
	24 h EC_{50}	48 h EC_{50}	24 h EC_{50}	48 h EC_{50}
Control	-	-	-	-
Ractopamine [a]	16.6 ± 1.6	10.9 ± 0.9	-	-
Clenbuterol [b]	-	-	-	-

[a]: Serial concentrations of ractopamine are 3.125, 6.25, 12.5, 25 and 50 μg/L
[b]: Serial concentrations of clenbuterol are 1.25, 2.5, 5.0 ,10 and 20 mg/L
−: No effect in this study

(a) 0 μg/L (b) 5 μg/L (c) 7.5 μg/L (d) 10 μg/L

Control **Embryonic growth retardation**

Fig.2. Toxic effects of ractopamine at different concentrations on embryos of *D. magna* after 24-h (magnification x500).

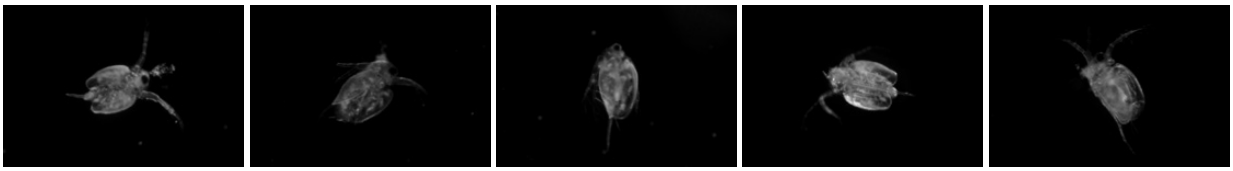
Acute toxicities of ractopamine and clenbuterol on *D. magna* embryos and neonates

In this study, the acute toxicities of β-agonists, as defined by 24 and 48 h EC50 values, were determined using *D. magna* embryos and neonates, while the control groups were cultured in simulated high-hardness medium (Table 1). Results of 24-h exposure indicated that ractopamine is toxic to embryos of *D. magna* (24 h EC50 = 16.6 ± 1.6 μg/L). Prolonged ractopamine exposure of 48 h resulted in a decline in EC50 value of *D. magna* embryos, implying increase in ractopamine toxicity over time (48 h EC50 = 10.9 ± 0.9 μg/L). The toxicity ratio of 24 h EC50/48 h EC50 was 1.5, indicating that acute toxic effects of ractopamine on embryos of daphnids mainly occur within the first 24 h, and continue to worsen from 24 to 48 h. However, ractopamine showed no toxic effect on young daphnids. Clenbuterol demonstrated no acute toxicity to daphnid embryos or neonates within 48 h.

Teratogenic effects of ractopamine and clenbuterol on *D. magna*

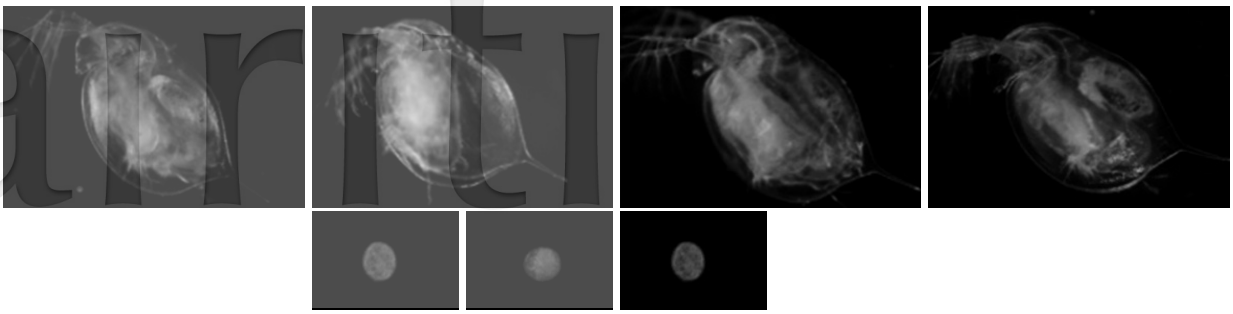
Figure 2 illustrates the toxic effects of ractopamine on embryos of *D. magna*. After 24 h exposure, growth retardation was observed. The higher the ractopamine concentration, the more severe the retardation. Ractopamine continued to induce teratogenic effects between 48 and 72 h of exposure during which embryos were developing into neonates. Fig. 3 shows representative teratogenic effects of neonates of *D. magna* induced by ractopamine at different concentrations after 72-h. They included albinism, open carapace, protein loss and visceral atrophy. Death of *D. magna* neonates occurred after exposure to 15 μg/L of ractopamine for 72 h. Fig. 4 shows representative embryonic growth retardation in brood chamber of gravid *D. magna* induced by ractopamine at different concentrations after 72-h. In addition, albinism, visceral atrophy and abortion were observed. Brood chamber death of gravid *D. magna* occurred after exposure to 100 μg/L of ractopamine for 72 h.

As mentioned above, clenbuterol showed no acute toxic effects on embryos of or young daphnids within 48 h. However, toxic effects could be observed in *D. magna* after exposure to clenbuterol for 96 h at various concentrations (2.5, 5.0, and 10 mg/L). From Fig. 5, juvenile *D. magna* exposed to 5 mg/L of clenbuterol showed albinism, open carapace, protein loss and abnormal ecdysis. Moreover, clenbuterol induced embryonic growth retardation in brood chamber, and brood chamber death of gravid *D. magna* occurred after exposure



(a) 0 μg/L albinism, open carapace and protein loss
(b) 5 μg/L albinism and open carapace
(c) 7.5 μg/L albinism and visceral atrophy
(d) 10 μg/L albinism and open carapace
(e) 15 μg/L albinism and death

Fig.3. Toxic effects of ractopamine at different concentrations on neonates of *D. magna* after 72-h (magnification x100).



(a) 12.5 µg/L (b) 25µg/L (c) 50 µg/L (d) 100 µg/L

Embryonic growth retardation in brood chamber **Death in brood chamber**

albinism, visceral atrophy and abortion abortion

Fig.4. Toxic effects of ractopamine at different concentrations on gravid *D. magna* after 72-h (magnification x100).

to 10 mg/L of clenbuterol for 72 h.

Comparisons of ractopamine- and clenbuterol-induced teratogenic effects revealed that ractopamine affected the early stage of embryonic development and had more of a serious impact than clenbuterol. Gravid *D. magna* were more sensitive to ractopamine than to clenbuterol. In addition, ractopamine caused more abortions, earlier brood chamber death and visceral organ atrophy in gravid *D. magna* than clenbuterol.

Toxic effects of UVC-irradiated ractopamine and clenbuterol on *D. magna*

Contrasting results were observed when β-agonists were subjected to UVC irradiation. Table 2 shows the effects of UVC-irradiated β-agonists on survival of embryos of, neonate and gravid *D. magna* after 24 and 48 h exposure. The 48-h survival rates of embryos of, neonate and gravid daphnids exposed to UVC-irradiated ractopamine were higher than for those exposed to non-irradiated ractopamine (90 ± 10, 80 ± 10 and 80 ± 10 vs. 50 ± 10, 50 ± 20 and 50 ± 20, respectively). On the contrary, the 48-h survival rates of embryos of, neonate and gravid daphnids exposed to UVC-irradiated clenbuterol were lower than for those exposed to non-irradiated clenbuterol (10 ± 5, 0 and 40 ± 25 vs. all 100% survival, respectively). In other words, UVC-irradiated ractopamine had weaker toxic effect on daphnids, while UVC-irradiated clenbuterol had stronger toxic effect on daphnids. Although the toxic effect of ractopamine on daphnids was 1000 times that of clenbuterol, UVC irradiation reduced the negative impact of ractopamine on embryos of, neonate and gravid daphnids.

Effect of irradiation duration on toxicity of ractopamine and clenbuterol to *D. magna*

Figures 6 and 7 illustrate the effects of UVC-

(a) 2.5mg/L
albinism, open carapace, and protein loss

(b) 5.0mg/L
albinism and abnormal ecdysis

(c) 10 mg/L
albinism, open carapace, and abnormal ecdysis

Fig.5. Toxic effects of clenbuterol on embryos of *D. magna* after 96-h (magnification x100).

Table 2. Effects of UVC-irradiated ractopamine and clenbuterol on survival of embryos of and neonate and gravid *D. magna* after 24 and 48 h (n = 20).

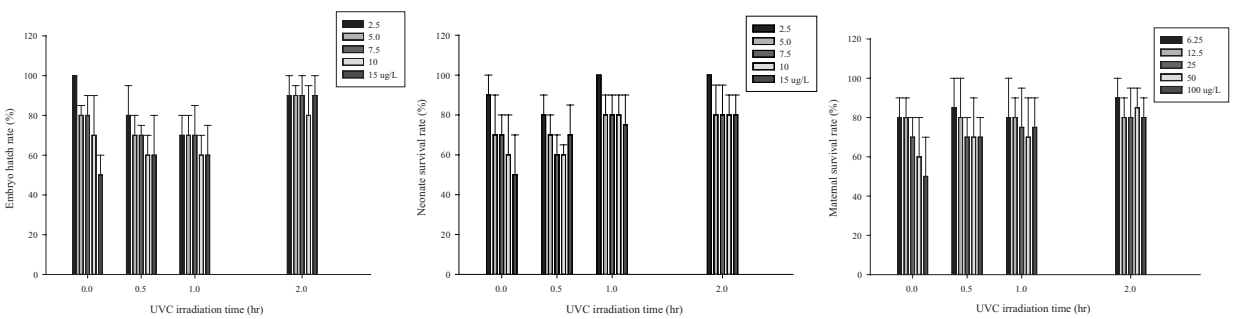
β-agonists	Embryo (stage 2)		Neonate (< 24-h old)		Maternal (gravid) [c]	
	24 h	48 h	24 h	48 h	24 h	48 h
Control	100	100	100	100	100	100
Ractopamine [a]	70 ± 15	50 ± 10	60 ± 10	50 ± 20	80 ± 20	50 ± 20
Ractopamine [R]	100	90 ± 10	100	80 ± 10	100	80 ± 10
Clenbuterol [b]	100	100	100	100	100	100
Clenbuterol [R]	10 ± 5	10 ± 5	0	0	40 ± 25	40 ± 25

[a]: Concentration of ractopamine is 15 μg/L
[b]: Concentration of clenbuterol is 20 mg/L
[c]: Gravid maternal *D. magna* are, respectively, exposed to 100 μg/L of ractopamine and 10 mg/L of clenbuterol.
[R]: Exposed to UVC irradiation (Ultraviolet subtype C, wavelength 254 nm, irradiation 0.5-10 joules/m) for 2 h

irradiated β-agonists on hatch rate of embryos and survival rates of neonate and gravid *D. magna*. After 2 h of UVC irradiation, ractopamine had significantly less toxic effect on daphnids than after 0.5 or 1.0 h of UVC irradiation (Fig. 6a, b, and c, respectively). In contrast, as shown in Fig. 7, UVC-irradiated clenbuterol showed greater adverse effects on daphnids. This increased with increasing clenbuterol concentration and irradiation time.

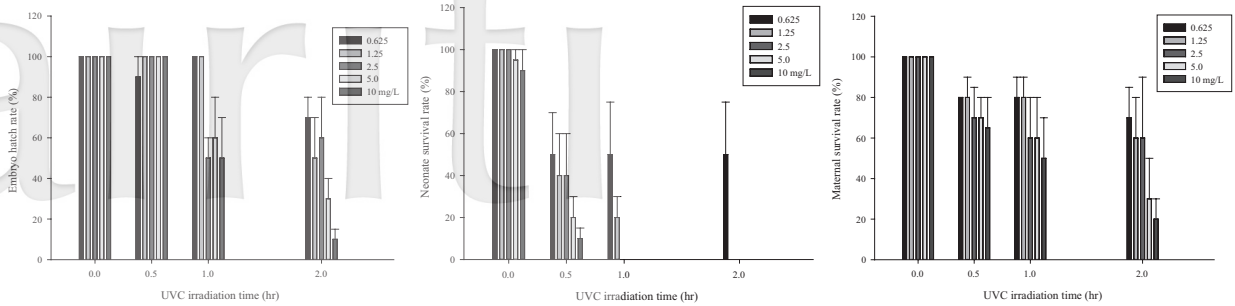
As mentioned above, embryonic growth retardation was observed in daphnids after 24-h exposure to non-irradiated ractopamine (Fig. 2), while embryos of daphnids exposed to UVC-irradiated ractopamine showed open carapace and reduced activity after 72 h (Fig. 8a). In other words, the emergence of teratogenic effects caused by exposure to ractopamine was delayed by UVC irradiation. Similarly, albinism, visceral organ atrophy, open carapace and death, indicative of toxic effect of ractopamine on gravid daphnids, were observed after 120-h exposure (Fig. 8b). In summary, the toxic effects of 2-h UVC-irradiated ractopamine are less significant than those of ractopamine without UVC irradiation or ractopamine irradiated for a shorter duration.

While similar teratogenic effects were observed in daphnids exposed to UVC-irradiated clenbuterol for 72 h (Fig. 9a) and 120 h (Fig. 9b), the adverse effects of 2-h UVC-irradiated clenbuterol were more marked than those of clenbuterol without UVC irradiation or irradiated for a shorter duration. That is to say, UVC irradiation turns clenbuterol



(a) 48-h exposure (n = 20) (b) 48-h exposure (n = 20) (c) 96-h exposure (n = 5)

Fig.6. Effects of UVC-irradiated ractopamine on (a) hatch rate of embryos, (b) survival rate of neonates and (c) gravid *D. magna*



(a) 48-h exposure (n = 20) (b) 48-h exposure (n = 20) (c) 96-h exposure (n = 5)

Fig.7. Effects of UVC-irradiated clenbuterol on (a) hatch rate of embryos, (b) survival rate of neonates and (c) gravid *D. magna*

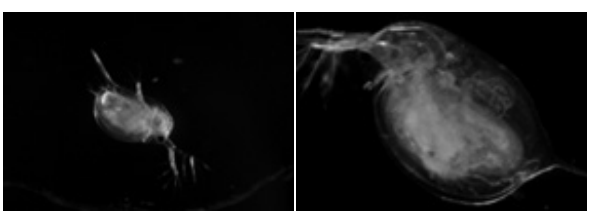
toxic and prolonged irradiation further enhances this toxicity.

Chronic toxicity of ractopamine and clenbuterol to *D. magna*

Table 3 compares the chronic toxic effects of ractopamine and clenbuterol with and without UVC irradiation on gravid maternal *D. magna* after exposure for 168 h. In general, long-term exposure of gravid daphnids to β-agonists leads to gross morphological abnormalities (endpoints) including embryonic retardation and intra brood chamber death. Comparisons revealed that UVC irradiation ameliorates the toxic effects of ractopamine, resulting in lower maternal abnormality, embryo retardation, embryo hatch rate and offspring fatality rate in gravid daphnids. In contrast, UVC-irradiated clenbuterol showed greater adverse effects on gravid daphnids, as evidenced by higher maternal abnormality, embryo retardation and offspring fatality rates, as well as lower embryo hatch rate.

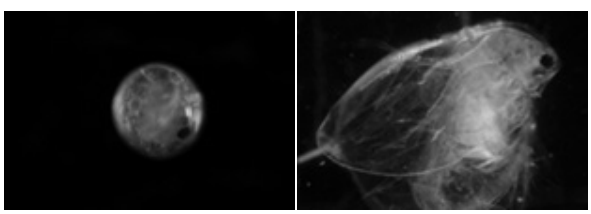
Taken together, these results indicated that UVC irradiation lowers ractopamine toxicity to daphnids and delays the emergence of teratogenic features. The longer the irradiation duration, the lower the toxicity. On the contrary, UVC irradiation enhanced the toxicity of clenbuterol to daphnids, which increased with higher concentration and prolonged irradiation.

In view of β-agonist toxicities, particularly those of clenbuterol, there are approximately 160 countries, including China, those of the European Union, and Russia, that have banned the usage of β-agonists as growth promoters. Recent trade restrictions on import of American beef and/or pork by China, Russia and Taiwan [31-33] have made the detection of this drug in meat a high priority. Many countries have enacted laws to prohibit or restrict the use of ractopamine as feed additive in animal production [34]. So far, there is no legislation prohibiting or restricting the use of ractopamine or clenbuterol as feed additive



(a) Embryo grows to juvenile with reduced activity (b) Gravid representative low activity

Fig.8. Toxic effects of 10 µg/L 2-h UVC-irradiated ractopamine on embryos of and gravid *D. magna* after 72-h and 120-h, respectively (magnification x100).



(a) Embryo growth retardation (b) Gravid representative death of embryos in brood chamber, visceral atrophy, embryo abortion, open carapace and death

Fig.9. Toxic effects of 10 mg/L 2-h UVC-irradiated clenbuterol on embryos of and gravid *D. magna* after 72-h and 120-h, respectively (magnification x100).

Table 3. Effects of ractopamine and clenbuterol with and without UVC irradiation on gravid *D. magna* after 168-h (n=10).

Effect of exposure to β-agonists (%)	Ractoapmine [a]		Clenbuterol [b]	
	Non-irradiated	2-h UVC-irradiated	Non-irradiated	2-h UVC-irradiated
Maternal abnormality	72.0	43.3	0	97.1
Embryo retardation	50.0	30.0	29.3	45.0
Embryo hatch rate	90.0	66.0	96.0	48.0
Offspring fatality rate	59.6	44.8	36.8	80.0

[a]: Concentration of ractopamine is100 μg/L
[b]: Concentration of clenbuterol is10 mg/L

in aquatic animal production. The published literature contains no data related to the behavior (sorption, leaching, and degradation) of β-agonists in freshwater. Ractopamine and clenbuterol, with water solubilities of 4100 mg/L and 46.5 mg/L, respectively, can enter the water from animal waste and pose a threat to organisms in the aquatic environment. Moreover, animal manure is frequently spread onto fields, serving as a vehicle for entry of β-agonists into the environment or the food chain. Ractopamine has been detected in an agricultural watershed [35], in effluent-dominated streams [36], in swine lagoon wastewater and in groundwater removed from a well [14]. Non-target species such as quail, duck, trout, bluegill, Daphnia, green algae and earthworms may be affected by β-agonists in the environment. β-agonists can also impact on seed germination. Results of toxicological studies have indicated lethality and overt physical signs of toxicity at concentrations not expected in runoff [37]. However, negative consequences of ractopamine on more sensitive endpoints, such as decreased brood size and reduced locomotion, have recently been described in nematodes, and chronic exposure to 10 μg/L of ractopamine has been found to reduce nematode lifespans [38]. The present findings on *D. magna* are consistent with previously reported results.

Conclusions

To our knowledge, the effects of UVC-irradiated ractopamine or clenbuterol on freshwater crustacean *D. magna* have not been reported. The results of this study showed that ractopamine is acutely and biologically toxic to *D. magna*. The toxicity ratio of 24 h EC50/48 h EC50 was 1.5, indicating that adverse effect of ractopamine on embryos of *D. magna* occurs mainly within the first 24 h with continuous increase from 24 to 48 h. In contrast, clenbuterol posed no toxic effect on embryos of or young *D. magna* within 48 h. Furthermore, toxic effects of UVC-irradiated ractopamine on daphnids were less significant than those of non-irradiated ractopamine. In contrast, UVC-irradiated clenbuterol showed adverse effects on daphnids which increased with prolonged exposure and length of irradiation. In other words, UVC irradiation photodegraded or photolyzed ractopamine, thus ameliorating its toxic impact on daphnids. On the contrary, clenbuterol was polymerized or concentrated with UVC irradiation, thus enhancing its adverse effects on daphnids. The mechanism of the biological effects of UVC on β-agonists merits further investigation.

Acknowledgment

This work was supported by a research grant from Chung Shan Medical University under project CSMU-INT-103-5.

Conflict of interest

All authors declare that they have no conflicts of interest.

Reference

1. Watkins LE, Jones DJ, Mowrey DH, Anderson DB, Veenhuizen EL: The effect of various levels of ractopamine hydrochloride on the performance and carcass characteristics of finishing swine. Journal of Animal Science 1990; 68: 3558–95.
2. Crescenzi C, Bayoudh S, Cormack PAG, Klein T, Ensing K: Determination of clenbuterol in bovine liver by combining matrix solid-phase dispersion and molecularly imprinted solid-phase extraction followed by liquid chromatography/electrospray ion trap multiple-stage mass spectrometry. Analytical Chemistry 2001; 73: 2171–77.
3. Wang H, Zhang Y, Li H, Du B, Ma H, Wu D, Wei Q: A silver–palladium alloy nanoparticle-based electrochemical biosensor for simultaneous detection of ractopamine, clenbuterol and salbutamol. Biosensors and Bioelectronics 2013; 49: 14–19.
4. Wu C, Sun D, Li Q, Wu K: Electrochemical sensor for toxic ractopamine and clenbuterol based on the enhancement effect of graphene oxide. Sensors and Actuators B 2012; 168: 178–184.
5. Wu M, Deng J, Chen Y, Chu W, Hung DZ, Yang CC: Late diagnosis of an outbreak of leanness-enhancing agent–related food poisoning. American Journal of Emergency Medicine 2013; 31: 1501–03.
6. Marchant-Forde JN, Lay DC, Pajor EA, Richert BT, Schinckel AP: The effects of ractopamine on the behavior and physiology of finishing pigs. Journal of Animal Science 2003; 81: 416–22.
7. Poletto R, Rostagno MH, Richert BT, Marchant-Forde JN: Effects of a "step-up" ractopamine feeding program, sex, and social rank on growth performance, hoof lesions and Enterobateriaceae shedding in finishing pigs. Journal of Animal Science 2009; 87: 304–13.
8. Catalano D, Odore R, Amedeo S, Bellino C, Biasibetti E, Miniscalco B, Perona G, Pollicino P, Savarino P, Tomassone L, Zanatta R, Capucchio MT: Physiopathological changes related to the use of ractopamine in swine: Clinical and pathological investigations. Livestock Science 2012; 144: 74–81.
9. Wang W, Zhang Y, Wang J, Shi X, Ye J: Determination of β-agonists in pig feed, pig urine and pig liver using capillary electrophoresis with electrochemical detection. Meat Science 2010; 85: 302–05.
10. Du W, Zhao G, Fu Q, Sun M, Zhou H, Chang C: Combined microextraction by packed sorbent and high-performance liquid chromatography–ultraviolet detection for rapid analysis of ractopamine in porcine muscle and urine samples. Food chemistry 2014; 145: 789–95.
11. Smith DJ, Giddings JM, Feil VJ, Paulson GD: Identification of ractopamine hydrochloride metabolites excreted in rat bile. Xenobiotica 1995; 25: 511–20.
12. Smith DJ, Feil VJ, Paulson GD: Identification of turkey biliary metabolites of ractopamine hydrochloride and the metabolism and distribution of synthetic [14C] ractopamine glucuronides in the turkey. Xenobiotica 2000; 30: 427–40.
13. Smith DJ, Shelver WL: Tissue residues of ractopamine and urinary excretion of ractopamine and metabolites in animals treated for 7 days with dietary ractopamine. Journal of Animal Science 2002; 80: 1240–49.
14. Bartelt-Hunt S, Snow DD, Damon-Powell T, Miesbach D: Occurrence of steroid hormones and antibiotics in shallow groundwater impacted by livestock waste control facilities. Journal of Contaminant Hydrology 2011; 123: 94–103.
15. 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.
16. 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.
17. Wu YL, Yang T, Shan JH, Huangfu WG, Wu YL: Determination of residual clenbuterol enantiomers in swine urine by high performance liquid chromatography. Chinese Journal of

Analytical Chemistry 2010; 38: 833–37.
18. Juan C, Igualada C, Moragues F, León N, Mañes J: Development and validation of a liquid chromatography tandem mass spectrometry method for the analysis of b-agonists in animal feed and drinking water. Journal of Chromatography A 2010; 1217: 6061–68.
19. He L, Su Y, Zeng Z, Liu Y, Huang X: Determination of ractopamine and clenbuterol in feeds by gas chromatography–mass spectrometry. Animal Feed Science Technology 2007; 132: 316–23.
20. Shelver WL, Smith DJ: Application of a monoclonal antibody-based enzyme-linked immunosorbent assay for the determination of ractopamine in incurred samples from food animals. Journal of Agricultural and Food Chemistry 2002; 50: 2742–47.
21. Gao HF, Han J, Yang SJ, Wang ZX, Wang L, Fu ZF: Highly sensitive multianalyte immunochromatographic test strip for rapid chemiluminescent detection of ractopamine and salbutamol. Analytica Chimica Acta 2014; 840: 68–74.
22. Fang GZ, Lu JP, Pan MF, Li WW, Ren L, Wang S: Substitution of antibody with molecularly imprinted film in enzyme-linked immunosorbent assay for determination of trace ractopamine in urine and pork samples. Food Analytical Methods 2011; 4: 590–97.
23. Chen Algui KF, Chang SH, Wang KS: Evaluation of acute toxicity and teratogenic effect of female hormone by *Daphnia magna* embryo assay. Asia-Pacific Academic Consortium for Public Health. The 45th Annual Conference Wuhan, China, 2013. p. 182–83.
24. 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.
25 USEPA: 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.
26. USEPA: Probit Analysis Software, Ver. 1.3. US Environmental Protection Agency, Cincinnati, OH. 2001.
27. 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.
28. 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.
29. 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.
30. 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.
31. Bauer J: Science-based trade decisions vs. Russia/China ractopamine actions. Available at: http://nationalhogfarmer.com/ business/ science based trade decisions vs Russia China ractopamine actions. (accessed September, 2014).
32. Flynn D: Lost meat sales to Russia over ractopamine costing millions, not billions. Available at: http://www.foodsafetynews. com /2013/12/ millions but not billions lost in beef and pork sales to Russia over ractopamine. (accessed September, 2014).
33. Anonymous: Taiwan will not import US ractopamine pork: COA. Available at: http:// www.chinapost.com.tw/taiwan/ national / national news /2014/04/02/ 404275/Taiwan-will. htm (accessed September, 2014).
34. Nielen M, Lasaroms J, Essers M, Oosterink J, Meijer T, Sanders M. et al.: Multiresidue analysis of beta-agonists in bovine and porcine urine, feed and hair using liquid chromatography electrospray ionisation tandem mass spectrometry. Analytical

and Bioanalytical Chemistry 2008; 391: 199–210.
35. Jaimes-Correa JC, Snow DD, Bartelt-Hunt SL: Seasonal occurrence of antibiotics and a beta agonist in an agriculturally-intensive watershed. Environmental Pollution 2015; 205: 87–96.
36. Brown D, Snow D, Hunt GA, Bartelt-Hunt SL: Persistence of pharmaceuticals in effluent-dominated surface waters. Journal of Environmental Quality 2015; 44: 299–304.
37. Elanco Animal Health: Environmental assessment for the use of Paylean® type A medicated article (ractopamine hydrochloride) in the feed of swine. NADA 1995: 140-863.
38. Zhuang Z, Zhao Y, Wu Q, Li M, Liu H, Sun L. et al.: Adverse effects from clenbuterol and ractopamine on nematode *Caenorhabditis elegans* and the underlying mechanism. PLoS One 2014; 9: 1–11.