Effects of 4-tert-Octylphenol on the Incubation of Eggs in Japanese Brown Frogs (*Rana japonica*)

HIROAKI KAWAGUCHI, YOSHIHISA UMEKITA, MASAKAZU SOUDA and HIROKI YOSHIDA

Department of Tumor Pathology, Field of Oncology, Kagoshima University, Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, 890-8544, Japan

**Abstract.** 4-tert-Octylphenol (OP), is an endocrine disruptor or surfactant widely used in herbicides. Its effects (0, 1 and 10 mg/l) on the incubation of eggs were examined using wild Japanese brown frogs (*Rana japonica*). In 10 mg/l OP, all the eggs were corrupted and no eggs developed. In 1 mg/l OP, 9.8% eggs developed and systemic edema, malformations such as crooked vertebrae and atrophy of the systemic muscles were observed in all the surviving tadpoles. These results suggested that OP use in paddy fields may affect the survival rate of wild frogs and induce malformation.

Paddy fields have been decreasing in Japan the past few years, and a rapid decrease in the number of frogs has also been noted. A farmer, who produces rice under an organic farming culture without agrichemicals in Kihokuchou, Kagoshima, Japan, observed that frogs, including the Japanese brown frog (*Rana japonica*), wrinkled frog (*Rana rugosa*), tree frog (*Hyla japonica*) and black-spotted pond frog (*Rana nigromaculata*), inhabiting his own and surrounding paddy fields have been decreasing, and that malformations such as miniaturization (about 1/5 size) and decreased legs have been seen in these species since 1998 when new throw-in-type formulations of herbicides were used in paddy fields. These new agrichemicals float on the water surface until completely diffused into the water (1, 2).

4-tert-Octylphenol (OP) is one of the alkylphenols which are widely used in herbicides, and an endocrine disruptor or non-ionic surfactant (3). It has been reported that OP affects the mortality of tadpoles and accelerates the onset of sexual differentiation in frogs (4, 5). In Japan, OP has been detected at lower concentrations (<0.01 ~ 0.09 ng/ml) in rivers (6).

In this study the effects of OP on egg incubation were examined using wild Japanese brown frogs.

**Materials and Methods**

Wild eggs (n: 1,200) of the Japanese brown frog (*Rana japonica*), collected from a paddy field in Kihokuchou, Kagoshima in March, were incubated in distilled water (DW) with air at 18-20°C for 24 hours for acclimatization at the Institute of Laboratory Animal Sciences, Frontier Science Research Center, of the Kagoshima University (7). The use of animals in this research complied with all relevant guidelines set by Kagoshima University.

The eggs were divided into three groups of 400 eggs. In group I, the eggs were maintained in DW, in groups II and III, the eggs were maintained in 1 or 10 mg/l OP (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA) dissolved in DW, respectively.

After various incubation times, the tadpoles were fixed in 10% phosphate-buffered formalin at room temperature, embedded in paraffin, sectioned, routinely stained with hematoxylin and eosin (H&E) and examined histopathologically.

The percentages were tested by means of the Four-fold Contingency Table.

**Results**

On day 1 after the beginning of examination, the percentage of addled eggs in groups I, II and III was 0, 51.8 and 100%, respectively. All the addled eggs died and became gray and opaque with a stench. On day 4, the percentage of surviving tadpoles in groups I, II and III was 100, 9.8 and 0%, respectively (Table I).

On day 7, the percentage of tadpoles with edema in groups I and II was 0 and 100%, respectively. All the addled eggs died and became gray and opaque with a stench. On day 4, the percentage of surviving tadpoles in groups I and II was 100, 9.8 and 0%, respectively (Table I).

On day 7, the percentage of tadpoles with edema in groups I and II was 0 and 100%, respectively. On day 14, the percentage of surviving tadpoles in groups I and II was 67.0 and 0%, respectively (Table II).

Grossly, systemic edema (head and body) and malformation, such as crooked vertebrae (tail), were observed in all the surviving tadpoles of group II (Figure 1). Histopathologically, systemic subcutaneous edema and atrophy of systemic muscles were noted in the tadpoles of group II (Figure 2).
Discussion

It has previously been reported that the LC50 value of OP was 1.36 μM in tadpoles (*Rana pipiens*) (4), and that 10^{-5} M of 17β-estradiol (E2) induced embryo (*Xenopus laevis*) death (8). In the present study, 1 (low dose) and 10 (high dose) mg/l of OP were selected for testing the effect on mortality and malformation. The high dose of OP corrupted the frog eggs and they could not survive.

It has also been reported that OP demonstrated weak estrogenic activities in rats (9), and that E2 induced malformations of tadpoles (*Xenopus laevis*) such as crooked vertebrae and small heads (8). In the present study, it was considered that the occurrence of crooked vertebrae may have been due to estrogenic activity with the low dose of OP. In turn, the present study added a new finding: the low dose of OP could induce the systemic edema and atrophy of systemic muscles. The precise mechanisms of these results are unknown, but it was speculated that these results may have been due to direct toxicity of the low dose of OP.

These results suggested that OP, if used in paddy fields, may affect the survival rate of wild frogs and induce malformation such as crooked vertebrae.

---

**Table I. Effects of OP on the incubation of frog eggs.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment#</th>
<th>Day 0 (No. of eggs)</th>
<th>Day 1 [No. of addled eggs (%)]</th>
<th>Day 4 [No. of surviving tadpoles (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DW</td>
<td>400</td>
<td>0 (0%)</td>
<td>400 (100%)</td>
</tr>
<tr>
<td>II</td>
<td>OP, 1 mg/l</td>
<td>400</td>
<td>207 (51.8%)**</td>
<td>39 (9.8%)**</td>
</tr>
<tr>
<td>III</td>
<td>OP, 10 mg/l</td>
<td>400</td>
<td>400 (100%)**</td>
<td>0 (0%)**</td>
</tr>
</tbody>
</table>

*Frog eggs incubated in distilled water (DW), 1 or 10 mg/l of 4-tert-octylphenol (OP). **p<0.01: significantly different from group I.

**Table II. Effects of OP on the growth of tadpoles.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment#</th>
<th>Day 4 (No. of tadpoles)</th>
<th>Day 7 [No. of tadpoles with edema (%)]</th>
<th>Day 14 [No. of surviving tadpoles (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DW</td>
<td>400</td>
<td>0 (0%)</td>
<td>268 (67.0%)</td>
</tr>
<tr>
<td>II</td>
<td>OP, 1 mg/l</td>
<td>39</td>
<td>39 (100%)**</td>
<td>0 (0%)**</td>
</tr>
</tbody>
</table>

*Frog eggs incubated in distilled water (DW) or 1 mg/l of 4-tert-octylphenol (OP). **p<0.01: significantly different from group I.

**Figure 1. Gross findings of tadpoles. Tadpole (A) in group I (distilled water, DW), no abnormal changes seen and (B) in group II (OP, 1 mg/l), systemic edema and crooked vertebrae (tail) evident. Bar = 2 mm.**
Figure 2. Histological findings of tadpoles. Cross-section of head (A) tadpole in group I (distilled water, DW), no abnormal changes seen and (B) tadpole in group II (OP, 1 mg/l), systemic edema and atrophy of muscles evident. Bar = 100 μm. Cross-section of abdomen (C) tadpole in group I (DW), no abnormal changes seen and (D) tadpole in group II (OP, 1 mg/l), atrophy of muscles evident. Bar = 100 μm. Cross-section of abdomen (E) tadpole in group I (DW), no abnormal changes were seen and (F) tadpole in group II (OP, 1 mg/l), atrophy of muscles evident. Bar = 25 μm. S, spine; OC, oral cavity; Ed, edema; M, muscles. H&E stain.
Acknowledgements

This work was supported in part by Kodama Memorial Fund Medical Research. We are grateful to Mr. S. Miyata, Mr. M. Samejima and Mr. T. Kodama for their valuable assistance.

References


Received August 21, 2007
Revised November 9, 2007
Accepted November 28, 2007