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Impact of Oxytetracycline on *Rana tigrina*

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Abstract

Oxytetracycline (OTC) is a popular antimicrobial agent for the prevention and treatment of bacterial diseases of cultured frogs (*Rana tigrina*) in Thailand. Doses used are similar to those recommended for fish. Tadpoles ages 5, 15 and 25 days were fed with 1, 3, 5 gram OTC/kilogram of feed, or bathed with OTC at a concentration of 10, 20 or 30 ppm for five days. Residues of the drug were eliminated from 5 and 15 day old tadpoles three days post treatment, but 25 day old tadpoles needed four days to eliminate OTC.

Frogs weighing 120 g were treated once with OTC by natural or forced feeding at doses of 1, 3 or 5 g/kg of feed or were injected with OTC at 10 or 50 mg/kg body weight. OTC were measured in the serum and various internal organs. Elimination of OTC from the tissues of frogs treated by injection was longer than that of OTC fed frogs.

Histological studies revealed that tadpoles and frogs treated with OTC showed microvesicular fatty change and necrosis in the liver and kidneys. Mucosa of the intestinal tract in the injected group exhibited focal hyaline degeneration. These results indicate that long term OTC treatment has an effect on the histology of tadpoles and frogs.

Introduction

Bacterial infections, especially red leg disease, cause important economic losses in frog culture in Thailand. Mass mortalities of tadpoles and frogs associated with bacterial infections are common. To minimise these losses, tadpoles and frogs are treated with antibacterial drugs. Oxytetracycline (OTC), a Food and Drug Administration (FDA) approved antibiotic, is the most commonly

used broad-spectrum prophylactic and therapeutant used in the control of these diseases, in both hatcheries or rearing ponds. Frog farmers use the same dose rates of OTC as those recommended for the treatment of fish. Application dose rates normally range between 10-30 ppm for long baths and 1-5 g/kg of feed per day for five to seven days for oral treatments (Tonguthai & Chanratchakool 1992). Studies on tissue residues resulting from treatment with OTC have been reported in trout (Jacobsen 1989), hybrid catfish (Somsiri et al. 1997), striped bass (Xu and Rogers 1994), black tiger shrimp (Chanratchakool et at. 1994) and juvenile blue shrimp (Mohney et al. 1997). However, no information concerning residues and effect of OTC on frog tissue is available.

The objectives of these studies were to evaluate OTC residues in frog tissues and examine any associated histopathological change.

Materials and Methods

Experimental animals

Tadpoles, ages 5, 15 and 25 days and frogs of average weight, 120 $\pm$ 2.5 g were obtained from the Aquatic Animal Health Research Institute's hatchery. The tadpoles were maintained in 200 l aquaria with dechlorinated water and aeration. The frogs were maintained in 500 l fiberglass tank. The animals were fed daily with commercial feed at 2% of their total body weight. Water temperature in both aquaria and tank ranged from 28 to 30°C.

Administration of OTC

OTC was applied to different ages of tadpoles either in bath form at 10, 20 and 30 ppm or in feed at 1, 3 and 5 g/kg of pellet for five days. Two types of OTC bath treatments were investigated : 1) water was not changed during the experimented period and 2) a complete water change was carried out daily with the OTC treatment being renewed each time. Two hundred tadpoles were treated in each group. Five tadpoles were sampled from each experimental tank every day until no OTC could be detected to determine levels of drug residues in the tissues and for histopathological examination.

A group of 60 frogs were fed with OTC at 1, 3 and 5 g/kg of pellet or were injected intramuscularly with a concentration of 10 or 50 mg/kg of frog. Samples of blood, muscle, liver, spleen and kidney from three treated frogs from each group were taken at 30 minutes, 1, 6, 12, 24 hours and then every day until no OTC could be detected. Tissues were also examined for signs of histopathological change.

OTC determination

A bioassay technique described by Bennett et al. (1966) was used to measure OTC concentrations in both tadpole and frog tissue. This technique utilized, *Bacillus cereus* as the indicator organism.

Histopathological studies

Both tadpole and frog samples were fixed in 10% buffer formalin for 24 hours, tissue samples were then dehydrated, embedded in parafin, sectioned to 5 mm and stained with hematoxylin and eosin (Humason 1972).

Statistical analysis

Randomized complete block design and complete block design were used to statistically compare the residues of OTC in various treatment groups and Duncan's multiple range test was used to identify differences, if there is any, between treatment pairs.

Results and Discussion

Residues of OTC in both tadpole and frog tissues are presented in Tables 1-5. The groups of tadpoles treated with OTC for five days showed accumulation of OTC in tissues during this period. After feed or bath treatment with OTC had stopped, the concentration of OTC in tissues declined very quickly and OTC could not be detected by day 9 post treatment. The OTC level in frogs fed with a single dose at 1, 3 and 5 mg OTC/kg of food reached the maximum level in the stomach and intestine within 1 hour after feeding and no drug was detected at 3 days post feeding. This is similar to the results seen in the studies on residues of OTC in cultured juvenile blue shrimp (*Penaeus stylirostris*), fed with medicated feed for 14 days (Mohney et al. 1997). The frogs injected with a single dose of OTC showed maximum levels of OTC within 6 hours in most tissues examined and no drug could be detected after nine days post injection. OTC levels were found to be higher in the kidney than in other tissues. After feeding of medicated feed and bath treatments were stopped, OTC concentrations in tadpoles and frogs declined rapidly. In contrast, OTC residues in frogs injected with the drug declined very slowly. Residues of the drug were eliminated from 5 and 15 day old tadpoles 3 days post treatment, but 25 day old tadpoles needed four days. Frogs on the other hand needed only two days to eliminate OTC when the drug was administered orally, but nine days was required when the drug was injected into the frogs. It appeared that 25 day old tadpoles needed a longer time to eliminate OTC from their body than frogs, possibly because the tadpoles at this stage are in the last stage of metamorphosis. Therefore, they may spend more energy on growth activity rather than detoxification. The minimum inhibitory concentration (MIC) of OTC against 14 strains of *Aeromonas hydrophila* and 20 strains of *A. sobria* isolated from cultured frogs were 0.15-80 and 0.3-320 mg/ml, respectively (Somsiri et. al., 1995). The OTC level in both tadpoles and frogs in most of the experiments is lower than the MIC for the Aeromonads. Thus, these OTC concentrations would not effectively inhibit *Aeromonas* spp. growth.

Statistical analysis of OTC residues in treated tadpoles showed no significant difference among the age groups of tadpoles but showed significant difference among the OTC application methods used. The residues of drug in frogs

Table 1. Tissue levels of OTC in tadpoles bathed in OTC for five days with daily water changes.

Time (Days)	OTC Conc.dose (ppm)	Residue of OTC in tadpoles (µg/g) 5 days old	15 days old	25 days old
1	10	9.90	18.70	19.00
	20	18.73	25.00	37.00
	30	64.33	50.30	76.00
2	10	15.43	37.00	37.00
	20	25.00	39.00	72.26
	30	70.33	75.00	78.00
3	10	18.20	49.90	50.00
	20	38.33	64.00	78.00
	30	78.00	77.00	99.08
4	10	18.73	60.71	76.00
	20	39.00	78.00	78.70
	30	78.67	102.51	156.00
5	10	19.00	78.70	80.00
	20	41.67	103.70	103.70
	30	80.00	154.30	303.00
6	10	1.33	4.77	9.60
	20	1.50	4.97	9.93
	30	4.90	6.92	19.00
7	10	0.00	1.10	1.30
	20	0.00	1.20	4.40
	30	1.17	1.26	4.70
8	10	0.00	0.00	0.00
	20	0.00	0.00	1.00
	30	0.00	0.00	1.10
9	10	0.00	0.00	0.00
	20	0.00	0.00	0.00
	30	0.00	0.00	0.00

Table 2. Tissue levels of OTC in tadpoles bathed in OTC for five days with no water change.

Time (Days)	OTC Conc.dose (ppm)	Residue of OTC in tadpoles (µg/g) 5 days old	15 days old	25 days old
1	10	11.53	18.20	18.20
	20	17.00	27.38	39.00
	30	70.33	80.00	80.00
2	10	16.90	18.20	18.20
	20	37.70	51.00	75.00
	30	76.00	80.00	80.00
3	10	18.73	18.20	19.00
	20	37.70	75.00	75.00
	30	77.00	80.00	80.00
4	10	18.73	18.20	18.20
	20	37.00	75.00	76.06
	30	80.00	80.00	80.00
5	10	18.73	18.20	18.20
	20	37.00	78.00	78.00
	30	78.00	80.00	80.00
6	10	1.30	1.20	4.70
	20	1.33	4.70	4.90
	30	4.83	4.90	9.90
7	10	0.00	0.00	1.10
	20	0.00	0.00	1.20
	30	1.20	1.20	4.55
8	10	0.00	0.00	0.00
	20	0.00	0.00	0.00
	30	0.00	0.00	1.00
9	10	0.00	0.00	0.00
	20	0.00	0.00	0.00
	30	0.00	0.00	0.00

Table 3. Tissue levels of OTC in tadpoles fed with OTC medicated pellets for five days.

Time (Days)	OTC Conc.dose (ppm)	Residue of OTC in tadpoles (µg/g) 5 days old	15 days old	25 days old
1	1	2.67	2.67	2.70
	3	4.77	4.83	4.70
	5	7.63	9.30	6.98
2	1	3.27	2.70	3.80
	3	4.97	4.90	4.90
	5	9.50	9.60	9.50
3	1	3.83	3.83	4.50
	3	9.00	9.30	9.30
	5	9.63	12.70	12.70
4	1	4.03	4.50	4.90
	3	12.77	12.70	12.88
	5	15.43	15.43	15.42
5	1	4.70	4.70	9.00
	3	15.43	18.22	18.20
	5	18.47	19.10	19.00
6	1	0.70	0.29	1.00
	3	1.17	1.27	1.40
	5	1.27	1.54	3.30
7	1	0.00	0.00	0.00
	3	0.00	0.00	0.00
	5	0.00	0.00	1.20
8	1	0.00	0.00	0.00
	3	0.00	0.00	0.00
	5	0.00	0.00	0.00

Table 4. Tissue levels of OTC in frogs after feeding with OTC medicated pellets in a single dose.

Time	OTC Conc.dose (g/kg)	Conc of OTC (ppm) Liver	Spleen	Kidney	Muscle	Stomach	Blood	Intestine
1 hr	1	0.00	0.10	0.30	0.10	50.00	0.00	0.00
	3	0.00	0.50	1.35	0.20	70.00	0.10	68.40
	5	0.00	0.90	2.00	1.00	80.00	0.10	70.00
3 hr	1	0.00	1.44	1.00	0.40	28.00	0.00	0.00
	3	1.00	2.07	2.20	0.50	32.00	0.00	33.40
	5	0.50	4.01	4.86	1.30	34.00	0.20	48.60
6 hr	1	0.70	1.62	2.34	1.00	14.50	0.00	0.00
	3	0.50	2.70	3.33	0.60	27.00	0.00	20.50
	5	1.00	5.25	5.02	1.80	30.00	0.00	28.80
1 Days	1	0.00	0.00	0.00	0.00	5.50	0.00	0.00
	3	0.00	0.00	1.125	0.00	14.25	0.00	10.00
	5	0.00	0.00	1.80	0.00	25.00	0.00	26.10
2 Days	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00	4.00	0.00	2.35
	5	0.00	0.00	0.00	0.00	0.00	0.00	12.60
3 Days	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 5. Tissue levels of OTC in frogs after a single injection of OTC.

Time	OTC Conc.dose (mg/kg)	Residue of OTC in tissue (ppm) Liver	Spleen	Kidney	Blood	Muscle
30 min	10	1.85	2.70	6.12	1.80	18.50
	50	4.50	3.78	16.20	3.30	26.25
1 hr	10	2.70	3.33	12.60	2.30	13.00
	50	10.00	8.82	33.30	6.80	34.00
6 hr	10	5.50	5.40	18.00	2.90	9.00
	50	15.00	14.40	37.80	6.80	26.25
12 hr	10	6.25	6.84	16.20	2.30	7.00
	50	15.00	20.70	33.30	5.25	21.00
24 hr	10	3.00	2.07	4.23	0.87	3.00
	50	13.00	18.45	23.40	4.70	18.50
2 Days	10	2.35	1.80	3.33	0.68	1.85
	50	10.25	10.35	18.45	4.20	13.50
3 Days	10	1.00	1.44	2.34	0.54	0.90
	50	3.40	5.40	9.90	2.60	3.40
4 Days	10	0.80	0.00	1.80	0.37	0.40
	50	3.00	3.33	6.80	2.05	2.70
5 Days	10	0.00	0.00	0.00	0.23	0.00
	50	2.70	1.80	4.86	1.80	0.90
6 Days	10	0.00	0.00	0.00	0.20	0.00
	50	1.60	1.44	4.32	0.42	0.625
7 Days	10	0.00	0.00	0.00	0.14	0.00
	50	0.00	0.00	2.34	0.33	0.50
8 Days	10	0.00	0.00	0.00	0.00	0.00
	50	0.00	0.00	2.07	0.23	0.00
9 Days	10	0.00	0.00	0.00	0.00	0.00
	50	0.00	0.00	0.00	0.00	0.00

fed with OTC showed no significant difference among the OTC application methods but OTC residues in the stomach and intestine showed a highly significant difference (99%) to levels in the liver, spleen, kidney, muscle and blood at 24 hours post feeding. After 24 hours, no significant OTC residues were detected in any tissue. The residues of drug in frogs injected with OTC revealed highly significant differences among the injection doses from 30 minutes to five days but at six to nine days post injection, no significant difference was observed at 95% level. Duncan's multiple range test indicated that OTC residues in the muscles were different from the other tissues and blood at 30 minutes post injection; drug residues in the muscles and kidney were not significantly different at six hours post injection and residues in the muscle, kidney and spleen were not significantly different at 12 hours. Thereafter no significant difference at 95% level was observed.

Livers from all ages of tadpoles in the groups bathed with OTC at 10, 20 and 30 ppm without water changes for five days showed no histopathological change compared with the control group. However the OTC bath groups with daily water changes had microvesicular fatty change in the liver. Five day old tadpoles bathed at 10 ppm OTC showed this change within 96 hours and the groups bathed at 20 and 30 ppm showed the same change within 48 hours. Fifteen day old tadpoles bathed with 10 ppm OTC had no microvesicular

change during the treatment period but in groups bathed in 20 ppm OTC microvesicular change was observed within 96 hours. The 15 day old tadpoles bathed in 30 ppm OTC and 25 day old group of tadpoles bathed in 10-30 ppm OTC showed individual hepatocytic necrosis in the liver but no microvesicular fatty change. Five day old tadpoles fed with 1 and 3 OTC mg/kg of feed had no microvesicular fatty change in the liver but the group fed with 5 OTC mg/kg showed individual hepatocytic necrosis and microvesicular change at 120 hours post treatment (Fig. 1).

Kidneys of 5, 15 and 25 day old tadpoles bathed in 10 ppm OTC without changing the water exhibited glomerular degeneration at 5, 1 and 1 day post treatment, respectively. Similarly, in the groups bathed in 20 and 30 ppm without changing the water, at five days post treatment, all ages of tadpoles showed glomerular degeneration, and the epithelial cell lining of the proximal tubules exhibited a variety of changes including: cloudy swelling, pyknotic nuclei, necrotic cell and microvesicular fatty change in the cytoplasm. The group of tadpoles bathed in OTC with daily water changes showed the same histopathological change as observed in the unchanged water group but these changes occurred more rapidly; the changes were observed within two days in 5 and 15 day old animals but in 25 day old tadpoles these changes were observed at 3 days post treatment. The group of tadpoles which were fed with OTC showed these changes at 24 hours post treatment.

Frogs fed with 1 OTC mg/kg showed no histopathological change. Frogs fed with 3 and 5 OTC mg/kg of food showed microvesicular fatty change in the liver at 3 days and 6 hours, respectively but exhibited necrosis of epithelial cell lining of proximal tubules at 24 and 6 hours, respectively (Fig. 2) . The 10 and 50 mg/kg OTC injected frogs showed kidney necrosis at 6 and 2 hours, respectively; microvesicular fatty change in the liver at 24 hours and 10 minutes, respectively; and focal hyaline degeneration of mucosa of the intestinal tract at 24 hours post injection.These histopathological changes also occured in humans treated with OTC for a long period (Kumar et. al., 1997).

Residues of drugs in tadpole tissue showed no statistical difference between the no water change group and daily water change group. However the histopathological examination indicated that tadpoles in the no water change group showed kidney necrosis only while tadpoles in the daily water change group exhibited both liver and kidney necrosis. Because OTC in the group with daily water changes was renewed every day, the tadpoles in this group encountered more active substances than in the other treatment group. This may have been due to the degradation of OTC in water. Choo (1984) reported that the half-life of OTC in freshwater was 58 hours out of direct sunlight, at an average pH of 7.3 and at a temperature of 27°C. More necrosis was observed in groups receiving higher doses of OTC and in older tadpoles indicating a relationship between age and OTC dose. When both bath and oral treatments were compared, higher OTC residues were observed in animals which were bath treated. This may be due to better absorption of the OTC through the skin and gills of tadpoles than through the intestinal tract. The unequal ability on feeding of individual tadpole or frog may also affect the amount of OTC ingested.

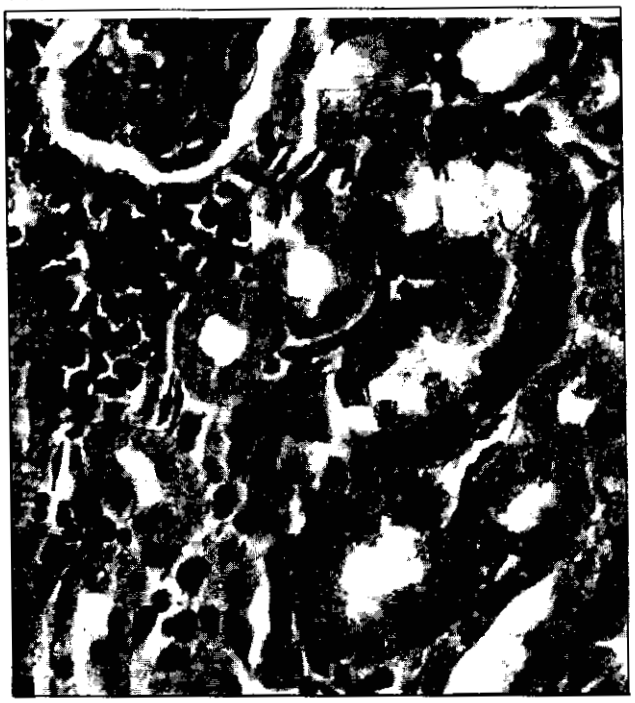

Fig. 1. Liver of tadpoles showed hepatocytic necrosis and microvesicular changes H&E x 264.

Fig. 2. Kidney of frog exhibited necrosis of epithelial cell lining of proximal tubules. H&E x 264.

Conclusion

Internal organs from tadpoles bathed with OTC at 10-30 ppm or fed with medicated feed at 1, 3 and 5 OTC g/kg of food, and from frogs fed with 1, 3 5 OTC g/kg of food or injected with 10 or 50 OTC mg/kg body weight were

darker in colour than normal; liver and/or kidney necrosis was observed. Drug residues in both tadpoles and frogs were lower than established MICs for OTC against *Aeromonas* species. Thus, these OTC concentrations would not effectively inhibit Aeromonads infection. OTC is therefore not recommended for bacterial disease prevention in frogs.

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