Repeated Treatment with Furazolidone Induces Multiple Cytochrome P450-Related Activities in Chicken Liver, but Not in Rat Liver

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ABSTRACT. The nitrofuran antimicrobial agent, furazolidone (FZ), is still used in veterinary medicine in some countries in the Middle and Far Eastern countries. The present study aimed to investigate the effects of successive bolus doses of FZ and its metabolite 3-amino-2-oxazolidinone (AOZ) on cytochrome P450 (CYP)-related activities in the livers of rats and chickens. Female Wistar rats and white Leghorn chickens were orally administered FZ once a day for 4 consecutive days. FZ-treated chickens showed an increase in multiple CYP-related activities, however, rats treated with FZ did not show these changes. In chickens, treatment with FZ also induced production of microsomal CYP2C6-like apoprotein. The present study demonstrated that FZ caused a multiple-type induction of CYP-related activities in chickens, but not in rats.

KEYWORDS: antimicrobial substance, chicken, cytochrome P450, drug metabolism.

The nitrofuran antimicrobial drug, N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone (furazolidone, FZ), has been used for more than forty years to treat certain bacterial and protozoal infections in humans and animals [3]. The use of FZ in food-producing animals has been forbidden in European Union countries, the U.S.A. and Japan as well as many other countries owing to its mutagenic and carcinogenic activities [1, 3]. However, FZ remains as an antibacterial and antiprotozoal feed additive for poultry, cattle and farmed fish in some Middle and Far Eastern countries [3, 14]. It is also used to treat infectious diseases in humans, especially for eradication of Helicobacter pylori [24]. Therefore, further findings concerning the pharmacological and toxicological properties of this drug can be anticipated [28].

A considerable number of studies have reported the undesirable and toxicological effects of FZ. One of the major side effects of FZ is its effect on drug-metabolizing enzymes. Alterations in drug-metabolizing enzyme activity induced by FZ may influence the pharmacological or toxicological action of some drugs and pollutants [27].

In the rat, successive administration of FZ in the diet has been shown to result in increased cytochrome P450 (CYP) content, and depending on the substrate used, an increase or decrease of CYP-related activities [11]. Successive oral administration of FZ was also reported to cause induction of hepatic CYP1A1 isozymes [32]. In addition, successive bolus doses of FZ in rats were shown to decrease the metabolic rate of two kinds of drugs in in vivo and increase the duration of barbital anesthesia [3]. In contrast, there are only a small number of conflicting reports on the effect of FZ on drug-metabolizing enzymes in chickens, one of the common animals treated with FZ. Treatment with FZ (0.04%, for 10 days) in feed caused a decrease in the duration of barbital anesthesia, but had no such effect when administered as a bolus dose of 200 mg/kg FZ [5]. Recently, we have demonstrated that FZ treatment in chickens induced facilitation of its metabolic rate that was dependent on increased activity of NADPH cytochrome P450 reductase in the liver [31].

FZ is generally reduced at the nitro group at the initial step of its biotransformation and then metabolized successively into metabolites containing a 3-amino-2-oxazolidinone (AOZ) side-chain, which bind covalently to proteins [3, 38]. AOZ inhibits monoamine oxidase (MAO) activity [35] and may be metabolized into irreversible MAO-inhibitors, 2-hydroxy ethyl hydrazine (HEH) in rats [34]. Although some MAO inhibitors suppress several CYP-related catalytic actions in human [26] and rat [9], there is little investigation of the effect of AOZ and HEH on microsomal CYP-dependent actions in chickens.

The aim of this study was to investigate the effect of successive bolus doses of FZ and its metabolites, AOZ and HEH, on CYP-related activities in rat and chicken livers. The current study demonstrated that chickens treated with FZ had an increase in CYP-related activities and also enhanced induction of CYP2C6-like apoprotein.

The reagents were obtained as follows: Nicotinamide adeninedinucleotide (NADPH), glucose 6-phosphate (G6P) and glucose 6-phosphate dehydrogenase (G6PDH) from Oriental Yeast Co. Limited (Tokyo, Japan); furazolidone (N-(5-nitro-2-furfurylidene)- 3-amino-2-oxazolidone, ©2013 The Japanese Society of Veterinary Science
Table 1. Effects of FZ, AOZ, HEH and 2% acacia solutions on the hepatic microsomal CYP content and CYP-related activities in rats and chickens

| Animal | Treatment | CYP content (nmol/mg microsomal protein) | HXOH (nmol/mg/min) | PROD (pmol/mg/min) | APND (nmol/mg/min) | EROD (nmol/mg/min) | MROD (nmol/mg/min) | PNPH (nmol/mg/min) |
|--------|-----------|------------------------------------------|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Rat    | Control   | 0.67 ± 0.11                              | 2.92 ± 0.46        | 28.7 ± 10.8       | 1.38 ± 0.18       | 0.54 ± 0.09       | 0.22 ± 0.03       | 8.11 ± 3.98       |
|        | FZ (62.5) | 0.71 ± 0.06                              | 2.17 ± 0.56        | 24.3 ± 7.5        | 1.53 ± 0.12       | 0.58 ± 0.13       | 0.21 ± 0.06       | 7.68 ± 0.37       |
|        | FZ (125)  | 0.80 ± 0.18                              | 2.02 ± 0.55        | 31.7 ± 9.9        | 1.95 ± 0.53       | 0.72 ± 0.26       | 0.28 ± 0.10       | 10.41 ± 3.06      |
|        | AOZ       | 0.72 ± 0.06                              | 2.04 ± 0.48        | 41.9 ± 13.3       | 1.57 ± 0.33       | 0.65 ± 0.15       | 0.29 ± 0.04       | 10.53 ± 4.28      |
|        | HEH       | 0.66 ± 0.12                              | 2.12 ± 0.73        | 22.9 ± 9.8        | 1.50 ± 0.33       | 0.47 ± 0.12       | 0.17 ± 0.05       | 10.08 ± 1.85*     |
| Chicken| Control   | 0.20 ± 0.01                              | 3.32 ± 0.64        | 1.9 ± 3.3         | 3.15 ± 1.26       | 0.20 ± 0.02       | 0.32 ± 0.12       | 0.57 ± 0.29       |
|        | FZ (62.5) | 0.44 ± 0.16                              | 4.57 ± 2.13        | 11.6 ± 14.6       | 7.72 ± 2.23*      | 0.54 ± 0.23       | 0.57 ± 0.21       | 1.64 ± 0.56*      |
|        | FZ (125)  | 0.65 ± 0.04*                             | 9.99 ± 3.95*       | 11.4 ± 14.6       | 12.29 ± 3.99*     | 0.64 ± 0.10*      | 0.77 ± 0.24*      | 2.71 ± 0.94*      |
|        | AOZ       | 0.28 ± 0.06                              | 3.73 ± 1.88        | 10.2 ± 13.5       | 4.09 ± 0.35       | 0.32 ± 0.18       | 0.41 ± 0.04       | 1.11 ± 0.15*      |
|        | HEH       | 0.32 ± 0.02                              | 4.54 ± 1.92        | 20.9 ± 17.8       | 6.15 ± 0.34       | 0.51 ± 0.23       | 0.53 ± 0.09       | 1.71 ± 0.50*      |

FZ) and 3-amino-2-oxazolidinone (AOZ) from Ueno Fine Chemical Industry Co. Limited (Osaka, Japan); 2-hydroxyethyl hydrazine (HEH), 7-ethoxyresorufin, 7-methoxyresorufin and 7-pentoxyresorufin from Sigma-Aldrich (St. Louis, MO, U.S.A.); 4-nitrocatechol from Wako Pure Chemical Industries, Limited (Osaka, Japan); p-nitrophenol from Kanto Kagaku Co. Limited (Tokyo, Japan); and hexobarbital from Nagase & Co. Limited (Itami, Japan). Polyclonal anti-rat CYP1A1, 2B1, 2E1, 2C6 antibodies were obtained from Daiichi Pure Chemicals Co. Limited (Tokyo, Japan) and horseradish peroxidase-labeled anti-goat IgG from Nagase & Co. Limited (Itami, Japan). The respective drugs were administered to the animals once a day.

The animals treated with the respective drugs were euthanized by decapitation under deep anesthesia with carbon dioxide 24 hr after the last administration of the drug. Liver microsomes were prepared according to the method of Omura and Sato [25] and the procedure of the preparation followed those described in a previous report [31]. The protein concentration of the microsome fraction was determined by the method of Lowry et al [21] using a spectrophotometer (Hitachi U-3000, Hitachi Ltd., Tokyo, Japan). Total hepatic microsomal CYP content was measured according to the method of Omura and Sato [25].

Hexobarbital hydroxylation: The incubation and extraction procedures described by Farrell and Correia [10] were used to measure hexobarbital hydroxylase (HXOH) activity. After 15 min of incubation, the remaining hexobarbital was extracted with 4 ml heptane containing 1.5% isomyl alcohol and then transferred to 1 ml of 0.8 M K2HPO4 buffer (pH 11). The amount of remaining hexobarbital in the aqueous phase was measured as the difference in absorbance between 280 nm and 245 nm using the spectrophotometer.

Alkoxiresorufin O-dealkylation: Microsomal O-dealkylation of 7-ethoxyresorufin (EROD), 7-methoxyresorufin (MROD) and 7-pentoxyresorufin (PROD) were measured according to the method of Clark et al [7]. After 10 min of incubation, formation of resorufin in methanol phase was measured using a spectrophotofluorometer (EP777, JASCO Corporation, Tokyo, Japan) at 528 nm excitation and 590 nm emission wavelengths.

Aminopyrine N-demethylation: Aminopyrine N-demethyle (APND) activity was determined by measuring the rate of formaldehyde formation according to the methods of Cooper and Brodie [8] and Nash [23]. After 10 min of incubation, the rate of formaldehyde formation in the supernatant solution was determined spectrophotometrically at 415 nm.
**Western blot analysis**: The analysis was performed on the hepatic microsomal protein obtained from the chickens treated with FZ (125 mg/kg) or 2% aqueous acacia solution as a control. Microsomal protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Laemmli [18] and Towbin et al. [36] and then transferred to a nitrocellulose membrane (Toyo Roshi Kaisha Ltd., Tokyo, Japan). Anti-rat CYP1A1, 2B1, 2C6, 2E1 and 3A2 antibodies were used to detect each CYP isoform. The membrane was immunostained using diaminobenzidine, and the staining patterns were analyzed using NIH Image v. 1.63 [20].

CYP content and related activities in rats and chickens with FZ, AOZ and HEH treatment are shown in Table 1. CYP content was slightly increased (but not significant statistically) in the FZ-treated rats. This result is consistent with a previous report [30]. In chickens, treatment with 62.5 mg/kg or 125 mg/kg of FZ resulted in a 2-fold and 3-fold increase in CYP content respectively, compared with controls. This increase in CYP content occurred in a dose-dependent manner.

FZ-treated rats showed no alteration in the CYP-related activities investigated in this study. These activities included CYP2B-related HXOH, CYP2B1-related PROD, CYP2D1-related APND, CYP1A-related EROD, MROD and CYP2E1-related PNPH [12]. These data are in contrast to the findings of Fukuhara and Takabatake [11] who demonstrated that rats fed FZ at a dose of 600 ppm for 7 days had a significant increase in aniline hydroxylase (CYP 2E1-related) activity and significant decrease in APND activity. This discrepancy may have been caused by the different method of FZ-dosing used in that study. Our results were also inconsistent with those of a previous study that showed FZ treatment in rats caused induction of CYP1A1 estimated using the Western blot analysis [32]. In contrast, treatment of chickens with 125 mg/kg of FZ was associated with a significant increase in the CYP-related activities (i.e., HXOH, APND, EROD, MROD and PNPH), except for PROD (Table 1). In addition, UDP-glucuronyl transferase activity also increased 2.3-fold (data not shown). These results partly corresponded to those of Bartlet et al. [5].

We also investigated the metabolites of FZ, AOZ and HEH in order to clarify the effect on CYP content and CYP-related activity (Table 1). In rats, no alterations in CYP content and CYP-related activities were produced by AOZ treatment. Treatment with HEH caused no change in CYP content and CYP-related activities, with the exception of PNPH. In chickens, AOZ and also HEH treatments caused a slight increase in CYP content, but no alteration in CYP-related activity with the exception of PNPH. This finding indicates that AOZ may not contribute to the augmentation of the multiple-types CYP-related activities induced by FZ in chickens. Treatment with HEH in both animals resulted in a significant increase in PNPH activity, and this finding was consistent with a previous report by Akin and Norred [4] who demonstrated that a hydrazine derivative increased aniline hydroxylase activity. Although HEH increased PNPH activity, AOZ had no such effect in rat. This result indicates that AOZ is not biotransformed to HEH in rats, in contrast to the suggestion of Stern et al. [34], but consistent with the findings of Timperio et al. [35].

Figure 1 shows the effects of administration of FZ on sleep time in the animals. Although it was not significant, FZ-treated rats showed a tendency of increased sleep time. This finding is inconsistent with previous studies that reported a significant increase in sleep time with FZ at multiple doses of between 100 – 400 mg/kg [3, 30]. This discrepancy may be attributable to the dose and frequency of FZ administration. In contrast to rats, FZ-treated chickens showed a significant decrease in sleep time. Our finding is consistent with the results of Bartlet et al. [5] who demonstrated that 400 ppm of FZ in feed provided for 10 days also caused a significant decrease in sleep time in the chickens. In the current study, chickens treated with 125 mg/g of FZ had a decrease in sleep time associated with a significant increase in HXOH activity, whereas those given 62.5 mg/kg of FZ showed a similar...
decrease in sleep time, but no significant increase in HXOH activity. The decrease in sleep time may therefore be attributable to an increase in HXOH activity, in combination with a significant increase in relative liver weight and/or NADPH P450 reductase activity [31]. Figure 2 shows the result of the Western blot analysis of hepatic microsomes obtained from chickens treated with either FZ (125 mg/kg) or 2% acacia solution. Microsomal protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The results of staining were analyzed using NIH Image v.1.63. A). Anti-rat CYP 2C6 antibody reacted with microsomal apoprotein. The arrow indicates the position of CYP2C6 isofoms. B) Expression level of CYP 2C6 apoprotein. Each column represents the mean of three animals, and the range bars indicate SD. The asterisk indicates a significant difference from controls (Student’s t-test, $P<0.05$).

Our study demonstrated that there were species differences between rats and chickens regarding the effects of FZ on drug-metabolizing enzymes. A number of diverse factors that may cause species differences in the effect of CYP inducers on drug-metabolizing enzymes have been investigated [6]. Basically, variability in the effect of FZ on CYP induction between species depends on differences in the induction mechanism, which in birds include xenobiotic-sensing nuclear receptors of chicken X receptor, which might have a broader substrate spectrum than those of mammalian receptors of pregnane X receptor and constitutive androstane receptor for detoxification [13]. Differences in the type of isozyme induced by FZ may also contribute to species variability.

It was reported that lipid peroxidation of hepatic microsomes decreased CYP content in rats [19], and FZ causes lipid peroxidation of hepatic microsomes in both chickens [29] and rats [2]. Studies have shown that FZ causes greater lipid peroxidation of hepatic microsomes in rats compared...
with chickens, as the effectiveness of the antioxidant system is superior in chickens [16, 22]. Accordingly, FZ may cause a greater decrease in the amount of CYP content in rats than that in chickens. As a consequence, the differences in hepatic CYP content between rats and chickens treated with FZ may be magnified by this reduction.

In conclusion, the current study demonstrated that successive treatments of FZ given orally to chickens resulted in a significant increase in hepatic CYP content and multiple CYP-related activities. FZ treatment also induced CYP2C6-like apoprotein in hepatic microsomes in chickens. AOZ administration at an equimolar dose to FZ (125 mg/kg) caused no alteration in CYP-related activities in both rats and chickens with the exception of PNPH activity. This study confirmed that FZ treatment in chicken causes induction of drug-metabolizing enzymes observed previously in rats and pigs [32, 37]. FZ may therefore induces multiple CYPs in humans and numerous animal species. Compounds, including FZ, which induce multiple CYP isozymes, have various toxic actions in animals, such as increasing the toxic effect of some drugs and pollutants [27]. It is therefore important that more attention is paid to the usage of FZ.

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