

Dicrocoeliosis of Old Mouflon Ewes - Effect on Biotransformation Enzymes and Metabolism of Anthelmintics *In Vitro*

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Abstract: Parasitic infections can modify the host's ability to metabolize drugs by altering the biotransformation enzymes. Mouflon (*Ovis musimon*), the wild sheep, is very sensitive to parasitic diseases. In the present study, *in vitro* activities of thirteen hepatic biotransformation enzymes and the *in vitro* metabolism of albendazole and flubendazole were compared in old mouflon ewes, either non-infected or infected with lancet flukes (*Dicrocoelium dendriticum*). The most pronounced decline in 6 β -testosterone hydroxylase, glutathione-S-transferase and UDP-glucuronosyl transferase activities was observed in *Dicrocoelium*-infected animals. In addition, dicrocoeliosis caused a decrease in both albendazole and flubendazole phase I hepatic metabolism. However, the changes were very slight therefore any undesirable alterations in albendazole and flubendazole pharmacokinetics are not expected. Decreased activities of 6 β -testosterone hydroxylase, glutathione-S-transferase and UDP-glucuronosyl transferase can impair drug elimination from the infected animals. This should be taken into consideration in pharmacotherapy or pharmacoprophylaxis of mouflons and sheep.

Keywords: *Dicrocoelium dendriticum*, lancet fluke, albendazole, flubendazole, drug metabolism.

1. INTRODUCTION

Mouflon (*Ovis musimon*) represents a popularly hunted and food-producing animal species in Central Europe. In the Czech Republic, the mouflon population is comparable to that of domestic sheep (*Ovis aries*). Mouflon is very sensitive to many parasitic diseases (muelleriosis, trichostrongylosis, fasciolosis or dicrocoeliosis). These diseases cause health problems, untimely death of animals and last but not least economic losses for breeders. Therefore anthelmintics are used frequently in the breeding of mouflon, especially in game-parks and farms.

Dicrocoeliosis belongs among most important and frequent pasture helminthoses in sheep [1] and mouflons. It is caused by *Dicrocoelium* spp. (Platyhelminthes, Trematoda, Dicrocoeliidae), commonly known as "lancet fluke" or "small liver fluke".

Despite its widespread distribution (Europe, Asia, North Africa and North America), dicrocoeliosis is not very well examined and is often underestimated by researchers and practitioners in many countries. This is because of the multiple parasitic infections and the difficulties encountered in diagnosing it [2]. The diagnosis of dicrocoeliosis is still based mainly on the detection of the eggs in the host feces [3] and on the postmortem examination of liver and bile [4]. Immunodiagnostic methods [5] are useful too.

Control of the small liver flukes infection comprises restrictive husbandry practices and treatment of hosts. Methods directly affecting the intermediate host populations are not

feasible because of the high cost and ecological reason. Correct treatment (in terms of timing and dosage) plays a fundamental role in anthelmintic resistance development. The strategic treatment of all animals exposed to infection by free grazing is the most common option for dicrocoeliosis control [6]. Several benzimidazoles, probenzimidazoles, praziquantel, closantel, oxclosanid and diamphenetid have been verified for treatment of dicrocoeliosis [7].

Albendazole (ABZ; methyl-[5-(propylsulphonyl)-1*H*-benzimidazole-2-yl]-carbamate) is a benzimidazole anthelmintic with broad-spectrum activity. In domestic sheep [8] or mouflon [9], ABZ is rapidly metabolized through a two-step S-oxidation. In the first step, chiral albendazole sulfoxide (ABZSO) is formed, which is further oxidized to albendazole sulfone (ABZSO₂). The structure of ABZ and its main metabolites are shown in [10]. While ABZSO is responsible for the anthelmintic activity, ABZSO₂ is pharmacologically inactive [11]. Successful treatment of dicrocoeliosis in domestic sheep requires a repeated administration of therapeutics [12] or a higher single dose of ABZ [13]. In both ways, the total therapeutic dosage exceeds the dosage commonly used in other more common helminthoses.

Flubendazole (FLU; methyl-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl]-carbamate) is another benzimidazole compound with anthelmintic activity. The biotransformation of FLU is extensive and follows similar metabolic pathways in various animal species. Ketoreduction and carbamate hydrolysis are the major phase I metabolic pathways in birds, pigs or sheep [14, 15]. The structure of FLU and its reduced (FLU-R) and hydrolyzed (FLU-H) metabolites are shown in [16]. Identical biotransformation pathways have been described for mebendazole-another benzimidazole anthelmintic (FLU-like structure lacking the fluorine atom). Reduction of the carbonyl group is generally considered a deactivation pathway protecting organisms against the toxic effect of re-

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active aldehydes and ketones. Because reduced mebendazole lacks any anthelmintic activity [17, 18], FLU reduction probably also represents deactivation. However, no exact information about the anthelmintic activity of reduced flubendazole is available.

Pathological consequences of dicrocoeliosis may significantly alter the detoxification of anthelmintics due to changes in expression and biotransformation enzyme activities. The present study was focused on the determination and comparison of activities of the main biotransformation enzymes, ABZ and FLU hepatic metabolism in non-infected mouflons and in mouflons with dicrocoeliosis. The aim of this study was to evaluate the pharmacological and toxicological consequences of dicrocoeliosis in the liver tissue of mouflon. Furthermore, ultimate knowledge of drug biotransformation is necessary for a safe consumption of animal products.

2. MATERIALS AND METHODS

2.1. Chemicals

Albendazole was purchased from Sigma-Aldrich (Prague, Czech Republic). Albendazole sulfoxide and albendazole sulfone were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Flubendazole and its two main metabolites (reduced flubendazole, hydrolysed flubendazole) were provided by Janssen Pharmaceutica (Prague, Czech Republic). All other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich.

2.2. Animals and Biological Material

Old mouflon ewes (*Ovis musimon*, 5-7 years-old) bred in Janovice and Vlkov game-parks in Czech Republic were used. At the beginning of winter season (2-3 months before the experiment), all mouflons in game-parks were collectively orally treated with ivermectin in order to eliminate the round worms totally, according to our previous experience [19]. The animals were not subjected to any other pharmacological administrations. Animals in the first group (non-infected, $n = 3$) were coprologically negative, and the second group (D-infected, $n = 4$) comprised fluke positive animals (EPG ranged between 480 and 1020 and adult flukes between 150 and 400); measurements carried out as described in [20]. No other endoparasitic infection was detected in the D-infected group. Mouflons were culled according to Czech slaughtering rules for farm animals (stunning, exsanguination) in February and March. The liver of mouflons from both groups were taken out immediately, sliced (1 cm in thickness), washed repeatedly and pressed gently in physiological solution to remove blood and flukes (infected group). Slices of the left liver lobes were cut into smaller pieces and stored in liquid nitrogen during the transport to laboratory. The study was approved by Ethical Committee of Charles University, Czech Republic.

2.3. Preparation of Subcellular Fractions

Frozen pieces of liver were thawed at laboratory temperature (up to 15 minutes) and homogenised at the w/v ratio of 1:6 in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandeline, Germany). The microsomal and cytosolic fractions were isolated by fractional ultracentrifugation of the

tissue homogenate in the same buffer. A re-washing step (followed by a second ultracentrifugation) was included at the end of the microsomes preparation procedure. Microsomes, finally resuspended in a buffer containing 20% glycerol (v/v), and cytosol were stored at -80°C . Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

2.4. Enzyme Assays

Each enzyme assay was performed in triplicate for each animal. The amount of organic solvents in the final reaction mixture did not exceed 0.1% (v/v).

The cytochrome P450 (CYP1A and 3A), 7-ethoxyresorufin (EROD), 7-methoxyresorufin (MROD) and benzyloxyresorufin (BROD) *O*-dealkylase activities were determined using fluorimetric determination of resorufin [21] at 37°C . Each substrate (dissolved in dimethylsulfoxide, DMSO) was added at a final concentration of $2.5\ \mu\text{M}$. The amount of microsomal protein in the reaction mixture ranged between 0.30-0.35 mg. Assays were conducted using a Perkin-Elmer luminescence spectrophotometer LS 50B, $\lambda_{\text{EX}}=530\ \text{nm}$ and $\lambda_{\text{EM}}=585\ \text{nm}$. The product formation was monitored continuously during 3 minutes. The EROD, MROD and BROD activities were calculated using the standard amount-addition technique.

6β -Testosterone hydroxylase activity (6β -TOH), ascribed mainly to CYP3A, was assayed using high performance liquid chromatography (HPLC) [22]. The final concentration of substrate (dissolved in methanol) was 1 mM and the amount of microsomal protein was 1.2-1.4 mg.

The 7-methoxy-4-trifluoromethylcoumarin demethylase (MFCD) activity and activity of flavine monooxygenases (FMO) were determined as described in our previous study [10].

The activities of reductases of carbonyl group were tested using the following substrates: 4-pyridincarboxaldehyde, metyrapone (dissolved in redistilled water) and DL-glyceraldehyde (dissolved in dimethylsulfoxide). Activity towards all of these substrates were measured in cytosolic samples, in microsomes were tested only activity towards 4-pyridincarboxaldehyde and metyrapone. The concentrations of substrates were 1 mM (for DL-glyceraldehyde it was 10 mM), concentrations of NADPH were 0.1 mM (0.3 mM for DL-glyceraldehyde). Potassium phosphate buffer pH 6.0 was used. 50 μL (10 μL for 4-pyridincarboxaldehyde) of enzyme volume (protein content 116-900 μg) was added into 1 mL reaction mixture. Spectrophotometric determination (detection wavelength 340 nm, 25°C) of NADPH consumption in the reaction mixture served for the assessment of reductase activities [23-26].

The enzyme activities for the model substrate 1-acenaphthenol (substrate of aldo/keto reductases/dehydrogenases) were determined using methods described by Palackal *et al.* [25] with modifications. The velocity of substrate dehydrogenation was determined spectrophotometrically by measuring the change in absorbance of the cofactor nicotinamide adenine dinucleotide phosphate oxidized form (NADP⁺) at 340 nm. The final 1.0 mL system contained 1 mM acenaphthenol dissolved in DMSO (1% of organic sol-

vent in final mixture), 1.0 mM NADP⁺, 50 µl of cytosol and 0.1 M TRIS-HCl buffer (pH 8.9).

Oracin reductases were assayed as described [27]. Briefly, cytosolic and microsomal fraction (100 µL) were incubated with 0.33 mM oracin and 1 mM NADPH in total buffer volume of 0.3 mL. Incubations (37°C, 30 min) were terminated by cooling and alkalization, the incubates were liquid-liquid extracted into ethyl acetate. The extracts were evaporated to dryness and residues were dissolved in the mobile phase prior to the HPLC injection. The HPLC separation of oracin and dihydrooracin was performed using a 250 x 4mm BDH Hypersil column. The mobile phase was prepared by mixing the buffer (10 mM, hexansulphonane, containing 0.1 M triethylamine, pH 3.27 set by H₃PO₄) with acetonitrile (ratio 3:1, v/v). HPLC separation was performed at 25°C with a flow rate of 1.5 mL/min. Dihydrooracin was detected with fluorescence detector using λ_{EX}=340 nm and λ_{EM}=418 nm. Oracin was detected with UV-VIS detector at a detection wavelength of 280 nm.

The microsomal UDP-glucuronosyltransferase (UGT) activity was assayed following the method by Mizuma *et al.* [28]. Microsomes were preincubated with a Slovasol detergent at 4°C for 20 minutes. The reaction mixture (total volume of 0.1 mL) contained 10 µL of microsomes (0.12-0.14 mg of protein), 0.33 mM UDP-glucuronic acid, 166.8 µM *p*-nitrophenol (dissolved in re-distilled water) in 0.1 M Tris/HCl buffer (pH 7.4). After 20 minutes of incubation at 37°C, the reaction was stopped by addition of 50 µL of 3% trichloroacetic acid (v/v). After shaking and centrifugation (3 min., 5000 rpm), 50 µL of the supernatant were mixed with 50 µL of 1M NaOH. The absorbance was measured using a BioRad microplate reader (detection wavelength of 415 nm).

The cytosolic glutathione-S-transferase (GST) activities were assayed using 1-chloro-2,4-dinitrobenzene as a substrate (dissolved in ethanol). The reaction mixture (total volume 1 mL) contained 10 µL of cytosol (0.14-0.18 mg of protein), 1 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M Na-phosphate buffer (pH 6.5). The reaction mixture was shaken and then the absorbance was measured spectrophotometrically at 340 nm four times in minute intervals.

2.5. Incubation of Microsomes with ABZ and ABZSO

The hepatic microsomal fractions were incubated with either ABZ (0.5-25 µM) or *rac*-ABZSO (0.5-25 µM). The reaction mixture (total volume of 0.3 mL) contained 100 µL of microsomal suspension containing 1.1-1.4 mg of proteins, NADPH (1 mM) and 0.1 M Na-phosphate buffer (pH 7.4). The blank samples contained 100 µL of 0.1 M sodium phosphate buffer (pH 7.4) instead of microsomes. The incubations were carried out at 37°C for 60 min under aerobic conditions. The product formation was linear up to 60 minutes. The incubation was stopped by cooling and by an addition of 30 µL of concentrated ammonium solution. In the next step, 700 µL of ethyl acetate were added, shaken (2 min., vortex) and centrifuged (3 min., 5 000 rpm). Six hundred µL of the organic layer were taken up, evaporated using an Eppendorf 5310 concentrator and analysed using HPLC.

2.6. Incubation of Microsomes and Cytosol with FLU

The cytosolic and microsomal fractions were incubated with FLU (0.5-25 µM) under the same conditions as de-

scribed in paragraph 2.5. The cytosolic fraction contained 1.4 -1.8 mg of proteins in reaction mixture.

2.7. HPLC Analysis of ABZ and its Metabolites

Achiral HPLC analysis was carried out using a Shimadzu LC-10ADvp solvent delivery module, a Shimadzu SIL-10ADvp autoinjector, a Shimadzu RF-10AxI fluorescence detector (λ_{EX}=290 nm, λ_{EM}=320 nm), a Shimadzu CTO-10ACvp column oven fitted with a LiChroCART 250-3 (LiChrospher 60 RP-select B, 250 mm x 3 mm, 5 m) reverse-phase HPLC column (Hewlett Packard, USA) equipped with LiChroCART 4-4 (LiChrospher 60 RP-select B, 4 mm x 4 mm, 5 m) guard column (Merck, Germany). The mobile phase A consisted of acetonitrile-25 mM potassium phosphate buffer (pH 3.0; 1:2, v/v). The flow rate was 0.5 mL/min in isocratic mode. All experiments were carried out at 25°C. Data were processed using the Shimadzu Class VP integrator software, version 6.12 SP2. The compounds were identified according to the retention times of respective reference standards. Under these chromatographic conditions, the retention times were 5.3 min (ABZSO), 7.4 min (ABZSO₂), and 17.3 min (ABZ).

2.8. HPLC Separation of ABZSO Enantiomers

During the reverse phase HPLC analysis, the ABZSO fractions were collected into vials. The collected fractions were evaporated to dryness using Eppendorf 5310 concentrator and redissolved in 200 µL of 0.2% 2-propanol in water (v/v). The mobile phase B consisted of 2-propanol-0.01 M phosphate buffer solution (pH 6.9; 0.2:99.8, v/v).

One hundred µL of each sample were injected into a Shimadzu HPLC system fitted with a Chiral-AGP column (150 mm x 4 mm, 5 m), equipped with Chiral-AGP guard column (10 mm x 4 mm, 5 m; both ChromTech, Hägersten, Sweden). The flow rate of the mobile phase B was 0.9 mL/min. All experiments were carried out at 25°C. This chiral chromatographic method was adapted from that described previously by Delatour *et al.* [8]. The retention time was 6.3 min for (-)-ABZSO and 18.6 min for (+)-ABZSO. The ABZSO enantiomers were determined according to reference above mentioned [8].

2.9. HPLC Analysis of FLU and its Metabolites

Chromatographic analyses were performed with the same chromatograph and column as described for analysis of ABZ metabolites. The mobile phase was a mixture of acetonitrile and 25 mM potassium phosphate buffer (pH 3.0; 3:7, v/v) delivered at a flow rate of 0.7 mL/min in isocratic mode. The temperature was 25°C. Using the photodiode array detector, chromatograms were recorded at 246 nm and 300 nm (scan 195-380 nm), spectrofluorimetric detector (λ_{EX}=290 nm, λ_{EM}=320 nm). FLU-H, FLU-R, albendazole (I.S.) and FLU were detected with the photodiode array detector. The spectrofluorimetric detector served for improving the sensitivity of FLU-R detection (FLU-H and FLU are not fluorescent). Under these chromatographic conditions, the retention times were 5.2 min (FLU-H), 6.2 min (FLU-R), 17.5 min (ABZ as IS) and 23.3 min (FLU).

2.10. HPLC Separation of FLU-R Enantiomers

The chromatographic method used for the determination of flubendazole and its two metabolites has been described

previously [16]. The chromatographic system was composed of an SCM1000 solvent degasser, a P4000 quaternary gradient pump, an AS3000 autosampler with a 100 μ L sample loop, an UV6000 LP photodiode array detector (UV143 PDA) with Light Pipe Technology, an SN4000 system controller and a data station with the ChromQuest 4 analytical software. Daicel chiral column (250 mm x 4.6 mm) packed with Chiralcel OD-R (Daicel Chemical Industries Ltd., Japan) and mobile phase consisting of acetonitrile - 1M aqueous NaClO₄ (4:6, v/v) were employed for chiral chromatographic separations. The flow rate was 0.5 mL/min. UV detection was performed in dual wavelength mode (246 and 300 nm). The retention time was 13.9 min for (-)-FLU-R and 16.5 min for (+)-FLU-R.

2.11. Statistical Analysis

Student's t-test and Mann-Whitney test was used for the statistical evaluation of differences between non-infected animals and animals with dicrocoeliosis. A probability of p 0.05 was considered significant.

3. RESULTS

3.1. Effect of dicrocoeliosis on *In Vitro* Activities of Hepatic Biotransformation Enzymes

Several enzyme activities corresponding to the main isoforms of cytochrome P450 (CYPs) were measured in hepatic microsomes isolated from the non-infected and D-infected animals (Table 1). Microsomes from the infected animals exhibited lower MROD and MFCD activity when compared with microsomes from the non-infected animals. These changes were only slight and the statistical significance was not proved due to large inter-individual differences. A significant difference was observed in 6 β -TOH activity: the mean decrease in hepatic microsomes of the infected animals was 42%. Both experimental groups did not differ in other CYP activities (EROD and BROD) that were tested.

TBSO activity is mainly ascribed to flavine monooxygenases (FMO). The values of TBSO activity in the non-infected and infected mouflons are presented in Table 1. The TBSO microsomal activities of infected and non-infected mouflons did not differ significantly from each other.

As for the conjugation enzyme activities, glucuronidation of p-nitrophenol and glutathione-S-transferase activity towards 1-chloro-2,4-dinitrobenzene were assayed. Both activities showed significant decrease in infected mouflons (37% in UGT and 27% in GST, respectively).

Measured activities towards substrates of reductases/dehydrogenases are summarized in Table 2. Slight but significant decrease (16%) in cytosolic acenaphtenol dehydrogenase activity was found in D-infected animals. On the other hand, dicrocoeliosis caused a mild increase in activity of microsomal pyridincarboxaldehyde reductase and cytosolic oracin reductases. The activities of microsomal oracin reductases were almost two-fold higher in the infected group than in the non-infected animals.

3.2. Effect of Dicrocoeliosis on *In Vitro* Metabolism of ABZ

To compare the velocity of ABZ oxidative biotransformation, ABZ (at various concentrations) was incubated with nicotinamide adenine dinucleotide phosphate (NADPH) and hepatic microsomes of non-infected or D-infected mouflons. The concentrations of ABZ, ABZSO and ABZSO₂ were determined using HPLC (Fig. 1A,B). As ABZSO exists in two enantiomeric forms, a chiral stationary phase was used for the separation of the ABZSO enantiomers. In blank samples (without microsomes), no ABZ metabolite was detected. In mouflon liver, ABZ oxidation was stereospecific, with preferential formation of (+)-ABZSO. Comparing the both experimental groups, an insignificant shift in the ABZSO enantiomeric ratio ((+)-ABZSO/(-)-ABZSO) was observed: from 1.5 in the non-infected animals to 1.7 in the infected animals. In both animal groups, the ABZSO to ABZSO₂ conversion exhibited practically the same velocity.

Table 3 shows apparent kinetic parameters (calculated by the GraphPad Prism program, according to Michaelis-Menten kinetics). The apparent kinetic parameters, maximal velocity (V'_{max}) and Michaelis constant (K'_m), of the ABZ conversion (both first and second step of sulfoxidation) were significantly lower in the D-infected group than in the non-infected group. Although the reaction of non-infected animals displayed higher maximal velocity, the microsomal metabolizing enzymes from D-infected animals showed

Table 1. Specific Activities of Biotransformation Enzymes Tested in Hepatic Microsomes or Cytosole from Non-Infected Mouflons or Mouflons with Dicrocoeliosis

| Specific Activity | Non-Infected | Dicrocoelium-Infected |
|------------------------------------------|-----------------------------------|------------------------------------|
| EROD (pmol/min/mg of proteins) | 41.2 \pm 1.0 | 37.3 \pm 8.2 |
| MROD (pmol/min/mg of proteins) | 47.9 \pm 12.3 | 35.5 \pm 16.9 |
| BROD (pmol/min/mg of proteins) | 11.6 \pm 1.8 | 13.3 \pm 1.1 |
| MFCD (nmol/min/mg of proteins) | 0.40 \pm 0.12 | 0.27 \pm 0.14 |
| 6 β -TOH (nmol/min/mg of proteins) | 0.36 \pm 0.11 | 0.21 \pm 0.08* |
| TBSO (nmol/min/mg of proteins) | 2.01 \pm 0.53 | 2.04 \pm 0.37 |
| pn-UGT (nmol/min/mg of proteins) | 7.13 \pm 0.59 | 4.52 \pm 0.76* |
| GST (μ mol/min/mg of proteins) | 0.72 \pm 0.12 | 0.53 \pm 0.14* |

Data represent the mean \pm SD from 3 non-infected animals and 4 D-infected animals. Each sample was made in triplicate (n=9, resp.12).

*Significant difference (p < 0.05) between non-infected and D-infected animals.

Table 2. Specific Activities (nmol/min/mg of Proteins) of Reductases/Dehydrogenases Tested in Hepatic Microsomes or Cytosol from Non-Infected Mouflons or Mouflons with Dicrocoeliosis

| Substrate | Specific Activity [nmol/min/mg of Protein] | | | |
|-------------------------|--------------------------------------------|--------------|---------------|---------------|
| | Cytosol | | Microsomes | |
| | Non-Infected | Infected | Non-Infected | Infected |
| DL-glyceraldehyde | 0.33 ± 0.02 | 0.32 ± 0.05 | ND | |
| 4-pyridincarboxaldehyde | 0.89 ± 0.03 | 0.90 ± 0.14 | 0.20 ± 0.04 | 0.23 ± 0.04* |
| 1-acenaphthenol | 1.50 ± 0.33 | 1.27 ± 0.24* | ND | |
| metyrapon | nd | | 0.08 ± 0.05 | 0.09 ± 0.03 |
| oracin | 0.6 ± 0.02 | 0.7 ± 0.09* | 0.006 ± 0.001 | 0.01 ± 0.005* |

Data represent the mean ± SD from 3 non-infected animals and 4 D-infected animals. Each sample was made in triplicate (n=9, resp.12).

ND: Not determined.

nd: Not detected.

*Significant difference (p < 0.05) between non-infected and D-infected animals.

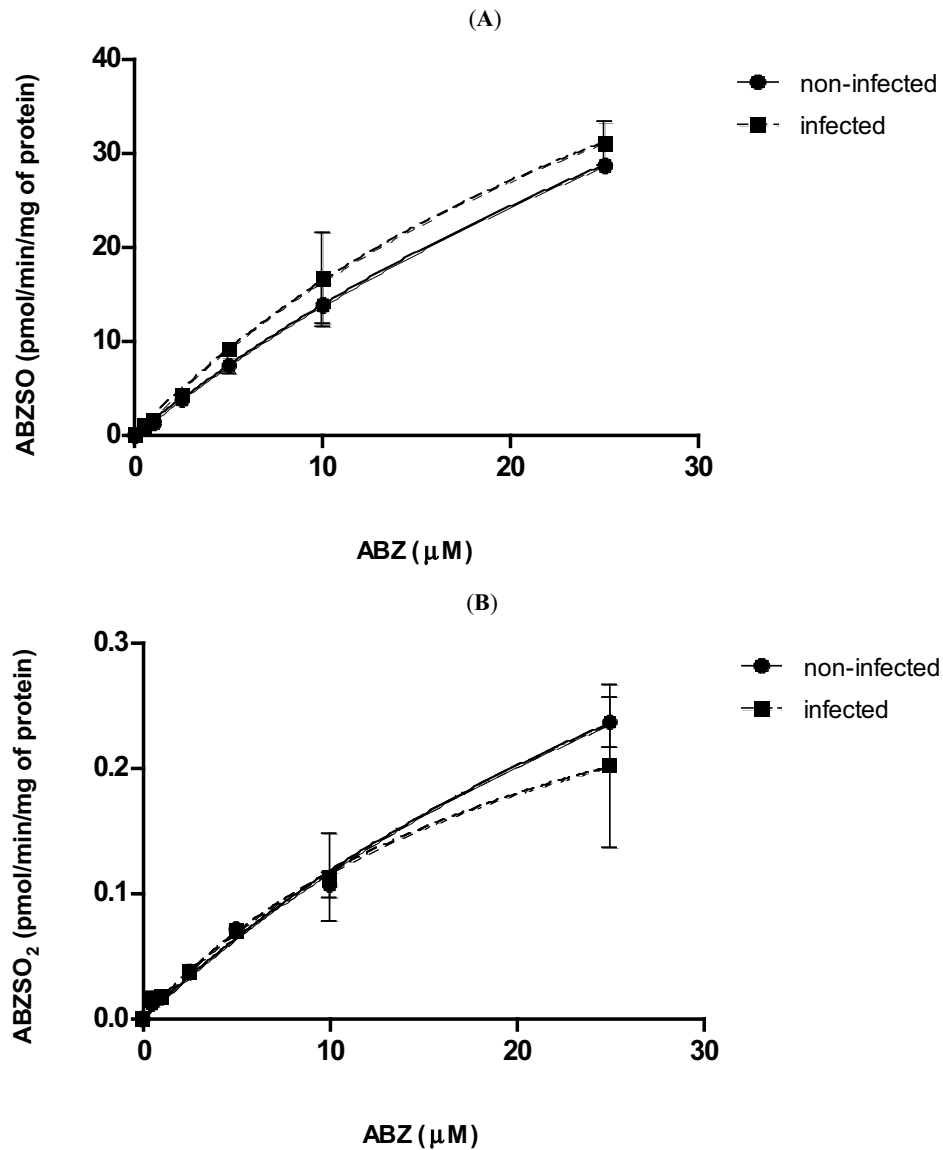


Fig. (1). Velocity of formation of ABZSO (A) or ABZSO₂ (B) in dependence of substrate ABZ concentration in hepatic microsomes from non-infected mouflons and mouflons with dicrocoeliosis. Mixtures of microsomes from 3 non-infected animals and 4 D-infected animals were used. Each sample was made in triplicate.

Table 3. Apparent Kinetic Parameters V'_{max} (nM/min) and K'_m (μ M) of ABZ, ABZSO, and FLU Biotransformation Determined in Hepatic Microsomes (m) and Cytosole (c) of Non-Infected Mouflons and Mouflons with Dicrocoeliosis

| Substrate | Localisation | Reaction | Parameters | Non-Infected | Infected |
|-----------|--------------|------------------------------------------------------------|----------------------|--------------------------------------|--------------------------------------|
| ABZ | m | ABZ \rightarrow ABZSO | V'_{max} K'_m | 458.0 \pm 13.5 63.5 \pm 2.5 | 323.1 \pm 19.1* 37.6 \pm 3.3* |
| | | ABZ \rightarrow (ABZSO) \rightarrow ABZSO ₂ | V'_{max} K'_m | 3.2 \pm 0.9 51.4 \pm 15.1 | 1.7 \pm 0.4* 23.5 \pm 2.7* |
| ABZSO | m | ABZSO \rightarrow ABZSO ₂ | V'_{max} K'_m | 9.0 \pm 1.4 30.2 \pm 7.5 | 9.1 \pm 2.5 35.7 \pm 14.3 |
| FLU | c | FLU \rightarrow FLU-R | V'_{max} K'_m | 388.0 \pm 103.9 45.4 \pm 17.2 | 238.4 \pm 36.2* 31.8 \pm 7.5 |
| | m | FLU \rightarrow FLU-R | V'_{max} K'_m | 7.7 \pm 0.5 15.6 \pm 2.2 | 6.6 \pm 0.8 25.7 \pm 4.9* |

higher substrate affinity. The velocity of ABZSO formation was considerably higher compared with ABZSO₂ formation.

No metabolites were detected in the cytosolic incubations with either ABZ or ABZSO.

3.3. Effect of Dicrocoeliosis on *In Vitro* Metabolism of ABZSO

To study the ABZSO sulfoxidation in the microsomal incubations, *rac*-ABZSO was used as a substrate. In blank samples (without microsomes), no ABZSO metabolite was detected and no changes of ABZSO enantiomeric ratio were found. No difference in the velocity of ABZSO₂ formation in the microsomal incubations of D-infected and non-infected mouflons was observed (Fig. 2). Also the kinetic parameters remained unchanged (see Table 3). The conversion of ABZSO to ABZ was not detected.

3.4. Effect of Dicrocoeliosis on *In Vitro* Metabolism of FLU

For the study of FLU biotransformation, FLU (at various concentrations) was incubated with NADPH and each of hepatic subcellular fractions of non-infected or infected mouflons. The concentration of FLU and its metabolites was determined using HPLC. Only one metabolite, FLU-R, was detected. Comparing both groups, the velocities of FLU-R formation were similar and a significant difference was observed at higher substrate concentrations only (Fig. 3A,B). As FLU-R exists in two enantiomeric forms, a chiral stationary phase was used for their separation. In the *in vitro* incubation of cytosol with FLU, (+)-FLU-R was the prevailing enantiomer and only traces of (-)-FLU-R were detected. In microsomal incubations, the extent of FLU-R formation was significantly lower than in cytosol. The maximal FLU-R formation velocity in cytosole differed significantly between the non-infected and the infected animals: the FLU metabo-

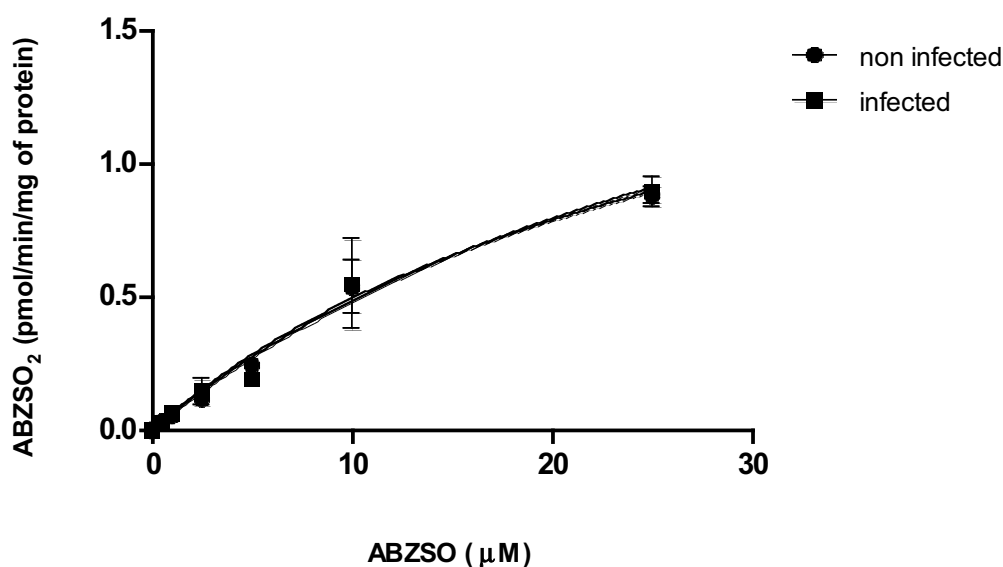


Fig. (2). Velocity of formation of ABZSO₂ in dependence of substrate *rac*-ABZSO concentration in hepatic microsomes from non-infected mouflons and mouflons with dicrocoeliosis. Mixtures of microsomes from 3 non-infected animals and 4 D-infected animals were used. Each sample was made in triplicate.

lization in the infected group was less intensive (see Table 3).

4. DISCUSSION

Parasitic infections can modify host’s ability to metabolize drugs by altering the biotransformation enzymes, and these changes may have various pharmacological, toxicological or physiological consequences. As drug metabolism occurs predominantly in the liver, those parasites that occupy sites in this organ, such as *Fasciola* spp. or *Dicrocoelium* spp., tend to be the ones with the greatest effects on the host’s ability to metabolize drugs. These effects can modify the host’s response to substances unrelated to the infection and to drugs that are administered under a chemotherapeutic regime. However, most pharmacokinetic and biotransformation studies have been performed in healthy animals and a potential of infection to change the condition of the organism has not been considered [29, 30].

Regarding parasitism, a significant decrease in the activities of all drug metabolizing enzymes in the infected animals was proposed [31]. Later studies revealed that the effect of parasitism is not very unambiguous. Although in rats the single infection caused by *Fasciola hepatica* decreased all activities of drug metabolizing enzymes that were tested, most of these activities did not change under repetitive fluke infections. Moreover, CYP3A and CYP1A activities increased [32]. In sheep, *Fasciola hepatica* infection (single or bi-infection) downregulated the CYP3A expression and activity in hepatic microsomes, but the GST activity in hepatic cytosol remained unchanged [33, 34]. *Schistosoma mansoni* infection increased the production of aflatoxin B-1 metabolites formed by the action of human microsomal monooxygenases [35]. Moreover, *Schistosoma mansoni* infection caused a significant enhancement of CYP1A activity in the liver microsomes in mice [36, 37]. On the other hand, alterations of murine hepatic CYPs in early (acute) *Schistosoma*

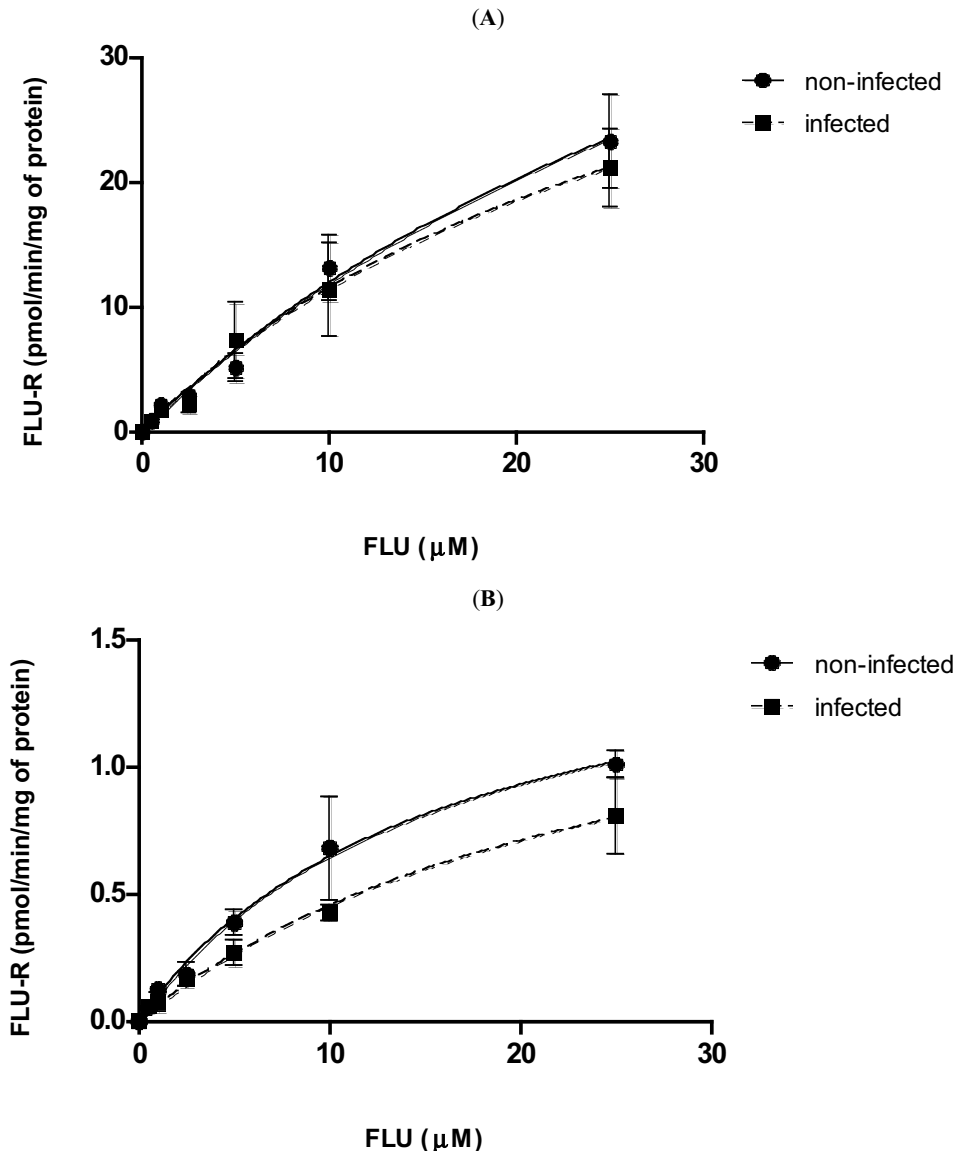


Fig. (3). Velocity of formation of FLU-R in dependence of substrate FLU concentration in hepatic cytosol (A) and microsomes (B) from non-infected mouflons and mouflons with dicrocoeliosis. Mixtures of cytosole from 3 non-infected animals and 4 D-infected animals were used. Each sample was made in triplicate.

mansoni infection were mild, or even nonexistent [38]. Infection of mice with low levels of *Schistosoma mansoni* cercariae increased GST activity while severe infection significantly diminished such activity [39].

Not only the extent of parasitoses, but also the age and sex of animals and the duration of parasitoses may affect the changes in biotransformation enzymes evoked by parasitic infection. With respect to these facts, two consecutive studies comparing the metabolism of anthelmintics and biotransformation enzyme activities in animals with dicrocoeliosis and in the non-infected ones were carried out. In our previous study [10], young (11-13 months old) mouflon rams were used while in the present study, two groups of old mouflon ewes - non-infected and D-infected-were compared. In young mouflons only two enzyme activities were significantly affected by dicrocoeliosis (increased TBSO activity and decreased GST activity) but in old mouflons further changes in enzyme activities caused by the long term exposure to parasitic diseases were observed.

In the present study, cytochromes P450, flavin monooxygenases, reductases/dehydrogenases and conjugation enzymes which participate in biotransformation of ABZ, ABZSO and FLU were investigated. Comparing the oxidising enzymes activities in both experimental groups of old mouflons, a significant decrease in 6 β -TOH activity, corresponding mainly to the activity of CYP3A, was observed. In our previous study, dicrocoeliosis did not affect this activity in young animals. On the other hand, dicrocoeliosis did not cause the changes in TBSO activity (corresponding mainly to FMO) in old mouflons while in young infected mouflons a significant increase in TBSO activity was found. In old mouflons with dicrocoeliosis, the UGT as well as GST activities significantly decreased. The reduction in GST and UGT activities could cause a significant decrease in biotransformation of drugs which are predominantly metabolized by conjugation with glutathione or glucuronic acid. It may result in a slowdown in drug elimination, in prolonged drug presence in the body, thus increasing the risk of animal products drug contamination. This fact should be considered during the pharmacotherapy of infected food-producing animals.

Only slight differences were found when reductase/dehydrogenase activities in non-infected and D-infected animals were compared. An increase in conversion of oracin to dihydrooracin was observed in both subcellular fractions from D-infected animals as well as the activity of microsomal reductases towards 4-pyridincarboxaldehyde. A significant decrease in activities of acenaftenol dehydrogenases was observed in cytosol from the infected animals (compared with the non-infected animals). Such changes in expression and activity of the biotransformation enzymes may be caused by toxic effects of parasite excretion products or inflammation mediators.

From the pharmacological point of view, the study of the effect of parasitism directly on biotransformation of anti-parasitic drugs is particularly important. In gerbils infected by *Echinococcus granulosus*, a higher plasma concentration of ABZSO₂ after ABZ administration and an increased ABZ sulfoxidation in hepatic microsomes were found (compared with the control animals) [40]. On the other hand, a significant decrease in ABZ sulfonation during experimental fascioliosis in sheep *in vivo* as well as *in vitro* was reported

[41]. In our experiments, with ABZ as a substrate, dicrocoeliosis reduced the apparent maximal velocity (V'_{max}) of ABZSO and ABZSO₂ formation in incubations with microsomes. Generally, the first step of ABZ oxidation (sulfoxidation) proceeded much faster than the second step (sulfonation). The ABZ sulfoxidation is carried out by the FMO and CYP3A while sulfonation is catalyzed by CYP1A. Thus after the administration of ABZ to animals, the anthelmintically active ABZSO arises quickly and is only slowly metabolized by CYP1A to the anthelmintically inactive ABZSO₂. V'_{max} of the ABZSO to ABZSO₂ conversion was the same in non-infected animals as in fluke infected animals.

Stereospecificity of ABZ biotransformation was studied because two different enantiomeric forms of ABZSO, namely, (-)-ABZSO and (+)-ABZSO, may be formed by liver microsomes. Multiple enzymes participate in ABZSO enantiomer formation: CYP3A generates mainly (-)-ABZSO while the FMO produce (+)-ABZSO [10]. In domestic sheep and mouflon, (+)-ABZSO formation predominates [42, 43]. In our experiments, the (+)-ABZSO/(-)-ABZSO ratio shifted from 1.5 in the non-infected animals to 1.7 in the D-infected animals. This shift was probably caused by a decrease in (-)-ABZSO formation due to a lowered CYP3A activity. This result is in a good agreement with the decreased 6 β -TOH activity found.

Ketoreduction and carbamate hydrolysis represent the major phase I metabolic pathways of FLU in all species investigated [14]. Although no exact information about anthelmintic activity of FLU metabolites is available, reduction as well as hydrolysis of FLU probably results in its deactivation. In our *in vitro* study, only FLU-R was detected after incubation of hepatic subcellular fractions with FLU. In contrast, formation of both FLU metabolites (FLU-R, FLU-H) was described after intravenous or intraruminal administration of FLU to sheep [15]. Hydrolytic activity toward FLU may occur in tissues such as blood, plasma, lungs and kidney. Particularly the ruminal microflora is very active in hydrolytic metabolic reactions and may contribute to the FLU conversion into FLU-H. Formation of FLU-R occurred mainly in cytosolic samples and this reaction is almost highly stereospecific with (+)-FLU-R dominance. The velocity of FLU reduction was slightly (but significantly) lower in the D-infected animals compared with the non-infected animals.

The apparent maximal velocities (V'_{max}) of product formation were lower in infected than non-infected animals in all enzymatic reactions tested. The apparent Michaelis constants (K'_m) were also decreased due to infection. K'_m changes may indicate an alteration in proportional participation of anthelmintics metabolising enzymes in microsomal and cytosolic multienzymatic systems. As both kinetic parameters similarly decreased, dicrocoeliosis did not affect the intrinsic clearance (V'_{max} / K'_m) and thus a similar extent of biotransformation may be expected in the infected as well as non-infected animals.

5. CONCLUSION

Dicrocoeliosis in mouflons caused only slight (not pharmacologically important) changes in ABZ and FLU hepatic biotransformation. However, activities of both tested conjugation enzymes, namely glutathione-S-transferase and UDP-

glukuronosyl transferase, were significantly decreased in infected mouflon ewes. This fact can be problematic because of the potential to impair elimination of many drugs, and should be taken into consideration in pharmacotherapy and pharmacoprophylaxis. Mouflon and domestic sheep are genetically very close. Genetic data provided a strong evidence for domestication of sheep from several mouflon subgroups [44]. Therefore the experimental data found in mouflon should be also valid for domestic sheep.

ACKNOWLEDGEMENT

Financial support of this project was provided by Grant Agency of Czech Republic, Grant No. 524/07/0611.

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Received: January 9, 2008

Revised: February 8, 2008

Accepted: February 27, 2008