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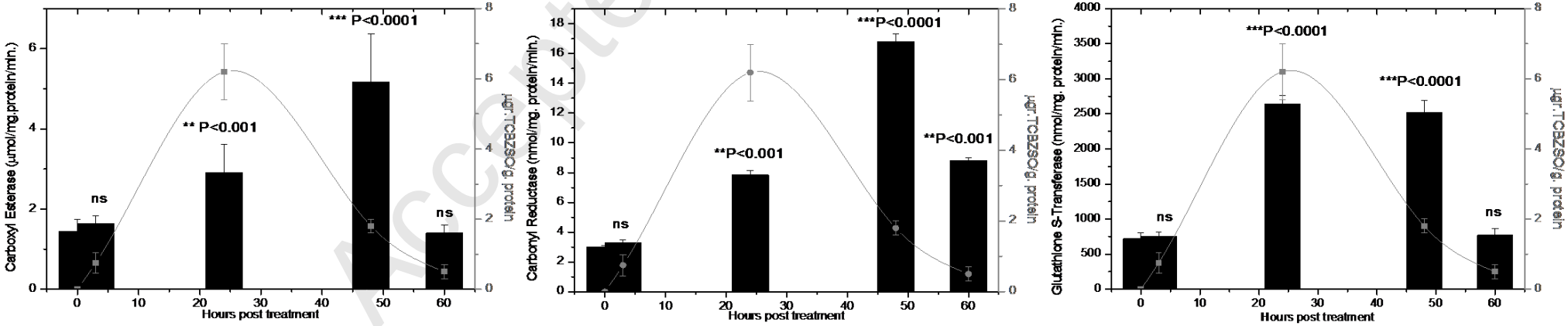

Enzymatic activities of Carboxylesterase (CE) Glutathione S-Transferase (GST) and Carbonyl Reductase (CBR) measured in *F. hepatica* recovered from sheep treated with triclabendazole (10 mg/Kg.).

- *In vivo* assessment of CE, GST and CBR in *F. hepatica* recovered to TCBZ treated sheep
- Increase of the metabolite sulfoxide (TCBZSO) and sulfone (TCBZSO2)
- Increase in enzymatic activity at 24 and 48 h PT of the three enzymes tested.
- The highest enzymatic activity was observed after peak of TCBZSO (active metabolite)
- Return of enzyme activities to basal values to 60h PT

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ABSTRACT

Fasciolasis is a zoonotic parasitic disease caused by *Fasciola hepatica* and its control is mainly based on the use of triclabendazole (TCBZ). Parasite resistance to different anthelmintics is growing worldwide, including the resistance of *F. hepatica* to TCBZ. In the present work we evaluate "*in vivo*" the activity of xenobiotic metabolizing enzymes of phase I (Carboxyl esterases) and phase II (Glutathione S-transferases and Carbonyl reductases) recovered of flukes from sheep treated with TCBZ . All three enzymes showed increased activity in TCBZ flukes returning 60 hours post-treatment at similar to baseline unexposed flukes. TCBZ action may induce secondary oxidative stress, which may explain the observed increment in activities of the analyzed enzymes as a defensive mechanism. The enzymes analyzed are candidates to participate actively in the development of resistance at TCBZ in *F. hepatica*.

KEYWORDS

Fasciola hepatica, triclabendazole, anthelmintics resistance, xenobiotics metabolizing enzymes

Fasciolasis is a zoonotic parasitic disease caused by the trematode *Fasciola hepatica*. Its control is mainly based on the use of triclabendazole (TCBZ), a halogenated benzimidazole thiol derivative which shows excellent efficacy against both juvenile (immature) and adult stages. In the case of anthelmintics, the induction of anthelmintics metabolizing enzymes could increase anthelmintics deactivation in parasites bodies and by this way facilitate the surviving of some helminthes individuals exposed to anthelmintic therapy [1]. This process can start anthelmintic resistance phenomenon. Parasite resistance to different anthelmintics is growing worldwide, including the resistance of *F. hepatica* to TCBZ. The xenobiotic metabolizing enzymes (XME) of parasitic helminthes may protect these organisms against toxic effects of anthelmintics, and the ability to inactivate anthelmintics via biotransformation processes can represent an advantageous defense strategy of the parasites [2].

The carboxylesterases (CEs) are members of XME and are α, β- serine hydrolase multigene family that hydrolyzes esterified xenobiotics to alcohol and carboxylic acid products. In the liver fluke eight esterases were distinguishable [3]. Others XME, the Glutathione S-transferase (GST) appear to be the major phase II detoxification system present in parasitic worms. In the liver fluke, GST account for as much as 4% of the total soluble protein, with a widespread distribution in the parasites tissues suggesting important physiological roles. [4]. The XME Carbonyl reductase (CBR) constitutes the family of the aldo-keto reductases in human and other mammalian tissues [5] is a cytosolic monomeric, NADPH-dependent oxidoreductase reducing a wide variety of endogenous and xenobiotic carbonyl compounds, which exert toxic effects on biological systems [6]. The most of studies concerning the metabolic response of liver fluke against the anthelminthic TCBZ only have referred to "*in vitro*" or "*ex vivo*" test models. The increased activity of Flavin monooxigenase (XME phase I) [7] and GST (XME phase II) [8] in the flukes TCBZ resistant (Sligo strain) provides an understanding of the phenomenon of resistance. This overexpression confirms that manifestation is a multienzymatic response involving more than one metabolic pathway [8]. In *F. hepatica* at present is unknown if CE and CBR are inducible enzymes from the action of TCBZ, if such action is confirmed, this enzymes would be likely candidates to participate actively in the phenomenon of resistance to this drugs. In this work, we evaluate, "*in vivo*", the action of XME of phase I (CEs) and phase II (GST and CBRs) of *F. hepatica* recovered from TCBZ treated sheep.

Ten parasite-free Corriedale weaned lambs were orally inoculated with 200 metacercariae of *F. hepatica* TCBZ-susceptible (Cullompton strain). This metacercariae were kindly provided by

Professor I. Fairweather, School of Biology and Biochemistry, The Queens University of Belfast, Northern Ireland, UK. For details of the history of this fluke strain, see [9]. The infection was confirmed 16 weeks later by the presence of eggs in faeces and indirect estimation of liver damage after determination of high levels of Glutamate Dehydrogenase and Gamma Glutamyl Transferase activities. The animals were treated orally with TCBZ Novartis® (10 mg/kg) and stunned and exsanguinated immediately at 0, 3, 24, 48 and 60 h post-treatment (PT). Animal procedures and management protocols were approved by the Ethics Committee according to Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, UNCPBA, Argentina http://www.vet.unicen.edu.ar , and to internationally accepted animal welfare guidelines [10]

The parasites were rinsed extensively with NaCl 0.9%, at 37 ºC to remove bile and/or adhering materials according to method previously described [11]. Flukes were transported to the laboratory in flasks filled with phosphate buffer (PB) (0.1 M, pH 7.4) at 4 ◦C. All subsequent operations were performed between 0 and 4 ◦C. Each parasite was cut into small pieces and washed several times with PB. Samples were homogenized (1:1) in PB, pH 7.4, centrifuged at 10,000×g for 20 min and the resulting supernatant centrifuged at 100,000×g for 60 min [12]. The supernatant obtained (cytosolic fraction) was collected and stored at −80 ºC until assay. The pellets, (microsomal fraction), was suspended in 0.1 M PB, collected and stored at −80 ◦C until assay. Protein content was determined using the Lowry method with bovine serum albumin as standard [13].

GST enzymatic activity in cytosolic fractions was monitored by a continuous spectrophotometric method [14] using 1-chloro- 2,4 -dinitrobenzene as substrate, the analyzed protein samples varied from 0.005 to 0.05 mg. CE enzymatic activity in microsomal fractions of *F. hepatica* was determined using 0.33 mM p-nitrophenyl acetate as substrate in phosphate buffer saline (PBS) pH. 7.2 according to a previously described method [15], the analyzed protein samples varied from 0.003 to 0.010 mg. The amount of p-nitro phenol released by the enzymatic reaction was measured spectrophotometrically at 405 nm. Absorbance values were converted to µmol of hydrolyzed substrate/minute/mg. protein. The CBR activity, was measured using menadione as substrate according to a previously published procedure [16], the analyzed protein samples varied from 0.0025 - 0.005 mg. Ten repetitions (n = 10) for each time and enzymatic activity were done.

The samples were analyzed by HPLC to determine the concentration of TCBZ and its metabolites following the methodology previously described [17]. Data were compared statistically by two-way ANOVA using the Bonferroni test as the post-ANOVA analysis with

Graph Pad Instant® 3.0 software. The three enzymes tested (CE, GST and CBR) exhibited an increase activity in the TCBZ treated flukes. These increases within the flukes was followed behind the peak of TCBZSO concentration (Table 1B). The highest activities for CE were observed at 48 h. PT (5170 nmol/ min. / mg. protein) compared to that measured in the cytosolic fraction obtained from control flukes (1450 nmol/min/mg.protein) which were not exposed to the drug. The highest GST activities were observed at 24 h. PT (2644 nmol/min.mg protein) and 48 h. PT (2519 nmol/min.mg protein). Both enzymes resulted 3.5-3.7 fold higher compared to that measured in the cytosolic fraction from control flukes. The activity for both enzymes returned at 60 h PT to basal levels similar to non-exposed flukes (Table 1A).The highest activities for CBR (16.82 nmol/min/mg protein), were observed at 48 h PT. This values resulted significative higher (5.6 fold higher) compared to that measured in the cytosolic fraction (3 nmol/min/mg protein) from control flukes. At 60 h PT the CBR activity (8.8 nmol/min/mg protein) resulted 2.9 fold higher than obtained in those measured in non-exposed flukes (Table 1A).

TCBZ is metabolized into TCBZSO by the host liver but also by the parasites subcellular fractions [18] which exhibits significantly higher sulfoxidative activity as compared to nematode and cestode parasites [11]. The liver flukes showed efficient oxidative biotransformation of the anthelmintic TCBZ into its sulfoxide derivative (TCBZSO) form, which exerts most of the toxic potential to the parasite. In the present work, TCBZSO concentration determination in fluke tissues showed a concentration peak of 6.35 nmol/100 mg of fluke protein at 24 h PT which was consistent with previous reports [11]. TCBZ action may induce secondary oxidative stress in *F. hepatica,* which may explain the observed increment in activities of the analyzed enzymes as a defensive mechanism. In fact, the highest activities of the enzymes analyzed in this work were observed when the peak TCBZSO concentration was measured within the flukes recovered from treated sheep. These preliminary results may be useful to further understand the mechanisms underlying the drug metabolism/disposition and activity in target helminthes parasites. The enzymes analyzed are candidates to participate actively in the development of resistance at TCBZ in *F. hepatica.*

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Table 1

A) Enzymatic activities of Carboxylesterase (CE) Glutathione S-Transferase (GST) and Carbonyl Reductase (CBR) measured in cytosolic (GST, CBR) and microsomal (CE) fractions of parasite specimens recovered from sheep treated with the flukicidal compound.
Statistical significance: ns (not significative), ** (P< 0,001) and *** (P<0,0001) post ANOVA Bonferroni test (n=5).

B) Concentrations (µg/g. of protein) of triclabendazole (TCBZ) and its metabolites TCBZSO (TCBZ sulphoxide) and $TCBZSO_2$ (TCBZ sulphone) measured in parasite specimens recovered from sheep treated with TCBZ.
References: PT: post-treatment, nd: not detected, na: not applicable

Time PT *(h)*	**A**			**B**		
	Enzymatic activities *(nmol/mg. protein/min)*			**Concentrations of TCBZ and its metabolites** (µg/g. of protein)		
	CE	**GST**	**CBR**	**TCBZ**	**TCBZSO**	**$TCBZSO_2$**
0	1450	719	3	***na***	***na***	***na***
3	1640 ***ns***	756 ***ns***	3.3 ***ns***	***nd***	0.80	0.08
24	2910**	2644***	7.85**	0.14	6.35	13.9
48	5170***	2519***	16.82***	***nd***	1.73	11.1
60	1400 ***ns***	769 ***ns***	8.8**	***nd***	0.71	8.09