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A Multienzyme Response is involved in the Phenomenon of *Fasciola hepatica* Resistance to Triclabendazole

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Abstract

The trematode *Fasciola hepatica* is the causative agent of a parasitic zoonosis known as fasciolosis, disease that affects humans and most species of domestic animals. Triclabendazole (TCBZ) is the most widely used fasciolicide, however, its indiscriminate use has led to the expression of anthelmintic resistance in the liver fluke.

In the present work we evaluated "*in vitro*" the microsomes activity of different xenobiotic metabolizing enzymes of Phase I Carboxylesterases (CE) and Phase II the cytosolic activity of Glutathione S-Transferase (GST), Glutathione peroxidase (GPx) and Glutathione Reductase (GSR) in adults of *F. hepatica* susceptible (Cullompton strain) and resistant (Sligo and Oberon strains) to triclabendazole. In this work is detected a multienzyme response involving at all the family of enzymes glutathione dependent. Carboxylesterases activity did not differ between the different strains tested not being involved in the resistance phenomenon. These results contribute to the understanding of the mechanisms referred to the phenomenon of resistance to TCBZ.

Keywords: *Fasciola hepatica*; Triclabendazole; Carboxilestearases; Glutathione peroxidase

Introduction

Fasciolasis is considered a major neglected tropical disease by the World Health Organization, affecting between 2.4 and 17 million people around the globe [1], it is caused by *Fasciola hepatica*, a parasitic digenean trematode that is also a major pathogen for domestic and wild ruminants, fasciolasis is attributed to economic losses estimated in US $2000-$3000 million yearly, associated to lower weight gains, impared fertility, reduced milk and wool production or fatalities in the livestock industry [2]. Anthelmintics are currently the most used method of control of parasitic helminthes both in humans and livestock [3]. Anthelmintic control of fascioliasis is based mainly on the use of Triclabendazole (TCBZ), halogenated benzimidazole thiol derivative that shows efficacy against both juvenile (immature) and adult stages of *F. hepatica* [4]. Fasciolasis has been considered a major emerging disease in the last few years, due to a rise in reported human and livestock cases in several countries, which is believed to be due to weather change determining a different distribution of the snail, Galba truncatula, and a required intermediate host [3].

Anthelmintic remain the only accessible means in the struggle against helminthic parasites, which cause significant morbidity and mortality in man and farm animals [3]. Parasite resistance to different anthelmintic is growing worldwide including triclabendazole (TCBZ) resistant fasciolasis previously reported [2]. The induction of anthelmintics metabolizing enzymes related to xenobiotic metabolizing enzymes (XME) could increase anthelmintic metabolic deactivation within parasites facilitating the survival of resistant helminthes exposed to anthelmintic therapy [5]. General biological roles of helminth GSTs include xenobiotic detoxification and ligand binding/transport functions [6]. In *F. hepatica*, GSTs are found in the tegument, muscular tissues, parenchymal cells, and the intestine [7] GSTs account for as much as 4% of the total soluble protein [8] and are major detoxification enzymes in adult helminths [8]. This property has been assessed in cytosolic GSTs (cGST) , [9] were as much as eight classes of GST isozymes are present in *F. hepatica* . Glutathione dependent enzymes are classified within phase II of the XME, these include certain enzymes that add reduced glutathione to xenobiotics, increasing its water solubility and facilitating their excretion, this process involves Glutathione Peroxidase (GPx) the Glutathione Reductase (GSR) and Glutathione S-Transferase (GST) [10]. During this study we assessed "*in vitro*", microsomal activity of different XME of Phase I Carboxylesterases (CE) and Phase II cytosolic activity of Glutathione S-Transferase (GST), Glutathione peroxidase (GPx) and Glutathione Reductase (GSR) in adults of *F. hepatica* susceptible (Cullompton strain) and resistant (Sligo and Oberon strains) to triclabendazole.

Materials and Methods

Parasite material

Fifteen parasite-free corriedale weaned lambs were orally parasitized each with 200 *F. hepatica* metacercariae contained in a gelatin capsule and kindly provided by Professor I Fairweather, school of biology and biochemistry, Queen's University Belfast, Northern Ireland, UK. Five animals were parasitised with the cullompton strain, TCBZ-susceptible five with the TCBZ-resistant Sligo strain and five

with the TCBZ-resistant Oberon strain. For details of the history of the three strains [6]. Fasciolasis was confirmed 16 weeks later by the presence of eggs in faeces and liver damage was indirectly estimated by the determination of liver enzymes (glutamate dehydrogenase and gamma glutamyl transferase). Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to internationally accepted animal welfare guideline [11].

Collection and processing of adult flukes

Adult flukes were collected from the bile ducts and liver, and processed to obtain the cytosolic (Cyt) and microsomal (Ms) fractions and the incubation assay conditions were as described earlier [12].

Preparation cytosolic and microsomal fractions

Parasite specimens (10 g-15 g) of the TCBZ-susceptible or TCBZ–resistant isolates of *F. hepatica* were rinsed with cold KCl (1.15%) and then transported to the laboratory in flasks filled with phosphate buffer (0.1 M, pH 7.4) at 4°C. All subsequent operations were performed between 0 and 4°C. Each sample was cut into small pieces and washed several times with a phosphate buffer. Samples were homogenized (1:1) in phosphate buffer, pH 7.4 with Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, USA), centrifuged at 10.000 g for 20 minutes and the resulting supernatant centrifuged at 100.000 g for 60 min.

The supernatant (Cyt) and the pellets (Ms) suspended in 0.01 M phosphate buffer, were collected and stored at -80°C until incubation assays analysis. Proteins content from the Ms and Cyt fractions were determined using bovine serum albumin as a standard [13].

Enzymatic in cytosolic and microsomal fractions

Carboxylesterase enzymatic activity in microsomal fractions of *F. hepatica* was determined using 0.33 mM p-nitrophenyl acetate in phosphate buffer saline pH. 7.2 (PBS) as substrate, and analyzed protein samples varied from 0.003 mg 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 of protein according to a previously described method [14].

Glutathione Peroxidase enzymatic activity in cytosolic fractions of *F. hepatica* was determined using witch monitor NADPH consumption by a spectrophotometrically at 340 nm for three minutes method. Absorbance values were converted to nmol of hydrolyzed substrate/minute/mg of protein according to a previously described method [15].

Glutathione Reductase enzymatic activity in cytosolic fractions of *F. hepatica* was determined using a calibration curve by assaying cytosols *Fasciola hepatica* protein analyzed samples varied concentration from 0.0002 mg to 0.02 mg mM GSH and then determining enzyme activity was performed for both determinations after incubation for two minutes at 30ºC and reading were taken at 412nm after 5 minutes. Absorbance values were converted to nmol of hydrolyzed substrate/minute/mg of protein according to a previously described method [16].

Data analysis

The data on the amount of nmol GSPx/min/mg.protein and that of the GR/min/mg.protein the cytosolic fraction (n=13) and of umol CE/min/mg.protein of the different trematodes were statistically compared using analysis of variance (ANOVA). Statistical comparisons were carried out by two-way ANOVA using the Bonferroni test as post ANOVA analysis using the GraphPad Instat 3.00 software (Graph Pad Software, Inc.). Differences with $P \leq 0.05$ are considered significant.

Results

Total GST activity (n=13), total Glutathione Peroxidase (GPx) activity (n=13) and total Glutathione Reductase (GSR) activity (n=7) were different in all the strains tested. In the TCBZ-resistant Sligo (1277 ± 32 nmol/min/mg protein) and TCBZ-resistant Oberon (1216 ± 16 nmol/min/mg protein) respectively higher than that in the TCBZ-susceptible Cullompton strain (800 ± 60 nmol/min/mg protein) total cGST activity was 59% and 52% respectively higher (P<0.001) than that in the TCBZ-susceptible (14). Regarding the GPx activity in the Sligo (83 ± 3.41 nmol/min/mg protein) and Oberon (81 ± 2.45 nmol/min/mg protein) and strains was, respectively higher than that in the TCBZ-susceptible Cullompton strain (49 ± 2.58 nmol/min/mg protein) was 69% and 65% respectively higher (P<0.001) than that in the TCBZ-susceptible (Figure 1and Table 1). And GSR activity in the Sligo (38 ± 2.07 nmol/min/mg protein) and Oberon (41 ± 1.25 nmol/min/mg protein) strains was, respectively higher than that in the TCBZ-susceptible Cullompton strain (29 ± 1.22 nmol/min/mg protein) was 31% and 41% respectively higher (P<0.001) than that in the TCBZ-susceptible (Figure 2 and Table 1), whereas CE activity in the Sligo (1.15 ± 0.27 umol/min/mg protein), Oberon (1.24 ± 0.31 umol/min/mg protein) and Cullompton strain (1.24 ± 0.32 umol/min/mg protein) strains did not differ between the different strains tested (Figure 3and Table 1).

Strain	TCBZ Resistance	Source	GST	GPX	GSR	CE
		(cita)	nMol/Min/mg Prot.	nMol/Min/mg Prot.	nMol/Min/mg Prot.	µMol/Min/mg Prot.
Cullompton		-7	800 ± 60	49 ± 2.5	29 ± 1.2	1.24 ± 0.32
Oberon	X	-7	1216 ± 16	81 ± 2.4	41 ± 1.25	1.24 ± 0.31
Sligo	X	-7	1277 ± 32	83 ± 3.4	38 ± 2.07	1.15 ± 0.27

Table 1: *F. hepatica* strains and enzymatic specific activity.

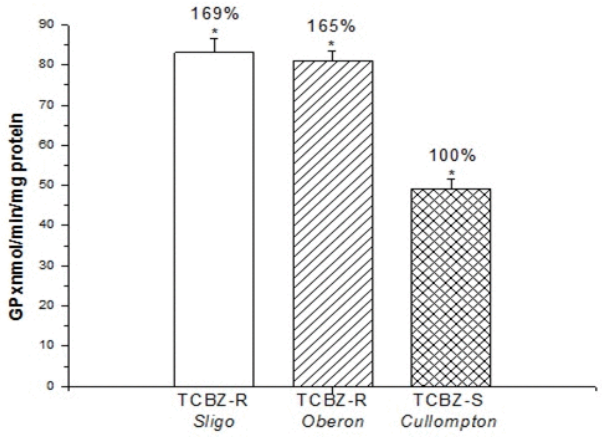

Figure 1: Citosolic enzymatic activity of total Glutathione Peroxidase (GPx) in *F. hepatica* TCBZ susceptible Cullompton strain and TCBZ resistant Sligo and Oberon strains to.

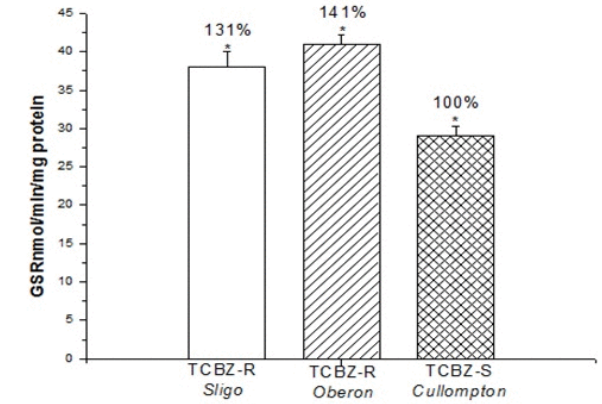

Figure 2: Cuantification the citosolic activity of total GlutathioneReductase (GSR) in *F. hepatica* susceptible (Cullompton strain) and resistant (Sligo and Oberon strains) to triclabendazole.

Discussion

Due to the lack of an effective vaccine, chemotherapy remains the main tool in the control of fascioliasis both in humans and livestock. Although other alternatives exist, current measures to control fascioliasis are based on the use of drugs such as Triclabendazole (TCBZ) [17]. Anthelmintic metabolic deactivation has been reported in the larvae and adults of helminth parasites. Fasciolicides interact with a number of different systems within the fluke and exert a variety of effects. So, it is difficult to determine whether flukicidal action is due to a single effect or a combination of effects [17]. Parasite defense mechanisms include detoxifying and anti-oxidant enzymes that would suppress anthelmintic oxidative toxicity [18].

Currently, there is a lack of knowledge about the mechanisms of detoxification and anthelmintic resistance in *F. hepatica*, our study suggests an active enzymatic role on TCBZ resistance [17]. The XMEs of helminth parasites may protect these organisms from the toxic effects of anthelmintics. It is well recognized that species-related differences in XME expression may affect not only the persistence of drugs and poisons in the body but also their susceptibility [17].

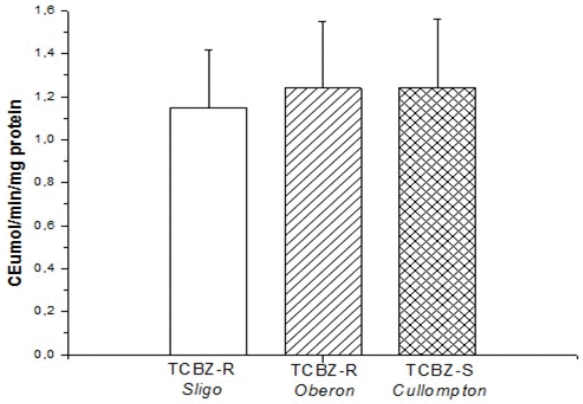

Figure 3: Cuantification the microsomal activity of total Carboxylesterases (CE) in *F. hepatica* susceptible (Cullompton strain) and resistant (Sligo and Oberon strains) to triclabendazole.

Glutathione (GSH) addition plays an important role in antioxidant defense in different tissues catalyze the reduction of oxidized GSH to reduced GSH which will be used by GST to reduce the peroxide and lipoperoxide, which they are reactive oxygen species. In this work is detected a multienzyme response involving at all the family of enzymes glutathione dependent. Carboxylesterase expressed no significant differences not being involved in the resistance phenomenon. These results contribute to the understanding of the mechanisms referred to the phenomenon of resistance to TCBZ.

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