Short Communication

Activity of carboxylesterases, glutathione-S-transferase and monooxygenase on *Rhipicephalus microplus* exposed to fluazuron

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The objective of this study was to assess the effect of the exposure to fluazuron on the activity of common pesticide detoxification enzyme groups in the cattle tick (*Rhipicephalus microplus*). Engorged females of a susceptible strain (POA) and a resistant strain (Jaguar) were exposed *in vitro* to fluazuron and their eggs and larvae were used to compare the activities of the general esterases, mixed-function oxidases (MFO) and glutathione-S-transferase (GST). The results showed significant elevation in MFO contents and esterases activity in the resistant strain when compared with the susceptible strain, in eggs and larvae respectively. In the POA strain, the MFO activity in eggs was down-regulated by fluazuron exposure. Based on these results, it can be concluded that different detoxification enzymes can act in distinct pathways depending on the tick’s development stage, and may be related to fluazuron detoxification in resistant strains.

1. Introduction

The tick *Rhipicephalus microplus* is one of the parasites with the greatest economic impact on cattle breeding [1]. According to Grisi et al. [2], the losses caused by this tick amount to US$ 3.24 billions per year in Brazil.

The acaricides belonging to the growth regulator class do not necessarily kill the ticks directly. Instead, they interfere in the parasites' growth and development process, interfering in molting and reproduction [3]. The benzoylphenyl ureas group is composed of molecules that inhibit the synthesis of chitin in arthropods [4]. One of the molecules belonging to this group is fluazuron. It inhibits the growth and development of arthropods by blocking the formation and deposition of chitin [5].

Many researchers have demonstrated the efficacy of fluazuron on larvae and nymphs of ticks, such as *R. microplus* [6] and *R. sanguineus* [7], besides other ectoparasites, like the mite *Sarcopes scabei* [8]. In *Rhipicephalus sanguineus* nymphs, it was demonstrated the effects of the arthropod growth regulator, fluazuron, in the formation of the integument and digestive processes [7]. However, the application of chemical products leads to the selection of resistance in these organisms, hampering their control. Resistance of cattle ticks to this substance was demonstrated an increase in the activities of these detoxification enzymes in resistant strains in comparison with susceptible strains [9,10], which indicate that drug resistance is the main obstacle to the efficient control of these arthropod populations.

In ticks, three enzyme systems are known that detoxify xenobiotic compounds: (i) esterases, (ii) mixed-function oxidases (MFO) and (iii) glutathione-S-transferase (GST) [11]. These enzyme complexes act making the drug more hydrophilic and thus less toxic to the organism. Biochemical assays with different detoxification enzymes can act in distinct pathways depending on the tick’s development stage, and may be related to fluazuron detoxification in resistant strains.

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In ticks, three enzyme systems are known that detoxify xenobiotic compounds: (i) esterases, (ii) mixed-function oxidases (MFO) and (iii) glutathione-S-transferase (GST) [11]. These enzyme complexes act making the drug more hydrophilic and thus less toxic to the organism. Biochemical assays with different populations of ticks and insects have demonstrated an increase in the activities of these detoxification enzymes in resistant strains in comparison with susceptible strains [12,13,14,15,16] within different active ingredients, but the exact detoxification mechanisms of benzoylphenyureas is still unclear.

Recently, Gaudêncio et al. [17] documented a series of alterations in...
the oxidative metabolism of *R. microplus* in response to exposure to fluazuron. The results indicate an inversion of the oxidative metabolisms in exposed ticks, characterized by an overlap of the enzyme related to fermentative pathways, besides variations of the hemolymph concentrations of some carboxylic acids, indicating that under these conditions, maintenance of the redox balance is carried out mainly by anaerobic pathways. Despite these results, information on detoxification enzymes in *R. microplus* after exposure to fluazuron is still scarce, limiting knowledge of the role of these enzymes in the detoxification step and their possible involvement in tick resistance. Therefore, to complement the data previously published, the aim of this study was to analyze the influence of exposure to fluazuron on the enzyme activity of complexes related to the detoxification of pesticides in *R. microplus* during its ontogenetic development (eggs and larvae), comparing the enzymes’ activities in resistant and sensitive strains.

2. Materials and methods

2.1. Ethical aspects

The present study was approved by the Bioethics Committee for Animal Experimentation of Rio de Janeiro Federal Rural University (CEUA-UFRRJ), under protocol number 023/2015.

2.2. In vitro exposure

The Guidelines for Resistance Management and Integrated Parasite Control in Ruminants, published by the Food and Agriculture Organization [10], establish a method to test the resistance to acaricides, based on the female immersion test [9].

The choice of the resistant strain (Jaguar strain) was based on the results obtained by Reck et al. [9], in which they observed traits denoting resistance of the Jaguar strain.

The in vitro tests were conducted with engorged females from a susceptible strain to fluazuron (POA strain, from Porto Alegre, RS) and a resistant strain (Jaguar strain, from a colony maintained at Instituto de Pesquisas Veterinárias Desidério Finamor, located in Eldorado do Sul, RS, Brazil), to compare the activities of the detoxification enzymes in eggs and larvae after laying. All the engorged females collected weighed between 200 and 300 micrograms.

The engorged females were separated into different experimental groups (control group and exposed group for each strain), each containing 10 ticks. The entire experiment was run in triplicate.

For the immersion tests, the active principle fluazuron, in powder form (Sigma Chemical Co., St. Louis, MO, USA), was diluted in a solution containing Triton X-100 at 2% diluted in sterile Milli-Q water and acetone (Merck, Darmstadt, Germany) to produce a stock solution of fluazuron at 1000 ppm. From this stock solution, a new dilution was carried out with sterile Milli-Q water to obtain a final fluazuron concentration of 50 ppm (0.02% Triton X-100 and 1% acetone). This concentration was chosen based on the results of [9], in which the authors observed that at the concentration of 50 ppm it was possible to easily observe the influence of fluazuron on the biological parameters evaluated. The control group did not contain the active principle fluazuron in the solution for the immersion test.

After one minute of immersion, the ticks were dried and stuck in jars enclosed with cotton balls to monitor larval hatching. The larvae from the biochemistry analyses were incubated in a climate chamber at 27°C ± 1°C at 80% relative humidity in Petri dishes, ventral side up, with double-sided adhesive tape and in water. Some of the larvae were immersed in a solution of fluazuron at 1000 ppm. From this stock solution, a new dilution was made of fluazuron at 50 ppm, which was used to perform the other enzyme assessments.

2.3. Preparing homogenates of eggs and larvae

Homogenates containing 50 mg of eggs from the fifth laying day were prepared by maceration in a microtube containing 300 μL of distilled water. The homogenates of larvae containing 50 mg from the pools of larvae were prepared by the same way. Then, the aliquots were removed from each sample to quantify the activity of mono-oxygenase (MFO). Subsequently, the samples were centrifuged at 10,000g for 1 min to perform the other enzyme assessments.

2.4. Determination of enzyme activities

The protocol to determine the enzyme activities followed the technical document from the Brazilian Ministry of Health, formulated by Oswaldo Cruz Foundation (2006) [22], containing the Method for Quantifying Activity Related to Pesticide Resistance of *Aedes aegypti*. This protocol is based on the method described by [18], with modifications.

A Biochrom EZ Reader 4000 was used for readings in these tests. The entire experiment was run in triplicate.

2.5. Determination of the activity of MFO

For the enzyme assay with MFO, each sample, containing a mixture of 20 μL of homogenate (before centrifuging), 60 μL of potassium phosphate buffer (90 mM, pH 7.2), 200 μL of a solution of 3,3′,5,5′-tetramethylbenzidine (TMBZ) (0.01 g TMBZ dissolved in 5 mL methanol plus 15 mL of 0.25 M sodium acetate buffer pH 5.0) and 25 μL of 3% hydrogen peroxide was placed in a well of a microplate (96-wells microplate). Then, the microplate was incubated while protected from light at room temperature for 2 h and the absorbance was read at 450 nm. The values were compared against a standard curve of purified cytochrome C. The results were expressed in equivalent units of cytochrome C/mg of protein.

2.6. Determination of the activities of carboxylesterases

The enzyme activities of the esterases were determined by addition of the substrates α and β-naphthyl. A mixture of 20 μL of the homogenate supernatant (for both α- and β-esterase), in 250 μL of a solution of α- or β-naphthyl acetate (120 μL of 30 mM α- or β-naphthyl acetate) dissolved in 24.75 mL of sodium phosphate buffer (20 mM, pH 7.2) was placed in each well of a 96-wells microplate. The reaction was incubated for 30 min at room temperature. Then 50 μL of a solution of Fast Blue B (0.045 g of Fast Blue B in 4.5 mL of distilled water added to a solution of 15 mL of 5% sodium dodecyl sulfate, SDS) was added. The reaction was incubated again at room temperature for 5 min, after which 200 μL of α- or β-naphthyl acetate/sodium phosphate and 50 μL of Fast Blue B were added. The absorbance readings were performed at 570 nm. The results were compared with a standard curve with known concentrations of α- or β-naphthyl and expressed in nmol of product formed/min/mg of protein.

2.7. Determination of the activity of glutathione-S-transferase

The enzyme activity of GST was measured using 10 μL of the homogenate supernatant added to 200 μL of glutathione and a solution of 1-chloro-2,4-dinitrobenzene (CDNB) (10 mM, pH 6.5, and 3 mM CDNB originally dissolved in methanol). The enzyme kinetics readings were performed at intervals of 5, 10, 15 and 20 min at 340 nm. The results were expressed in mM/min/mg of protein.

2.8. Protein assay

The protein contents of each well were measured by adding 300 μL of Bio-Rad reagent for protein quantification (1:4 diluted with dd H₂O...
from stock) to 10 μL of supernatant in each well, incubating for 5 min at room temperature, and then measuring absorbance at 570 nm. Absorbance was converted into protein concentration using a bovine serum albumin standard curve.

2.9. Statistical analyses

The results of the enzyme activities were expressed as mean ± standard deviation and submitted to analysis of variance (ANOVA) followed by the Tukey test for comparison of the means (InStat, GraphPad v.4.00 and v.3.02, Prism, Inc.).

3. Results and discussions

In the comparison between the susceptible (POA) and resistant (Jaguar) strains, the enzyme responses of exposure to fluazuron of eggs and larvae were different.

With respect to eggs, there was a significant (p < 0.05) increase in the activity of MFO in the exposed group of the Jaguar strain (7.62 ± 0.91 μg of cytochrome C/μg of protein) compared to the control group (4.17 ± 0.44 μg of cytochrome C/μg of protein). For the other enzymes evaluated in eggs (carboxylesterases and GST), no statistical differences were observed between the groups (Fig. 1). In the eggs from POA strain, MFO was significantly (p < 0.05) down-regulated by fluazuron exposure, decreasing in the treated group (4.68 ± 0.45 μg of cytochrome C/μg of protein) when compared to the control group (8.46 ± 0.73 μg of cytochrome C/μg of protein).

Considering the role of the monoxygenases in detoxification processes in ticks and insects, as reported by several researchers [13,15,19], the comparison between the activity of the enzyme in eggs from Jaguar strain and POA strain leads us to surmise the occurrence of an increase in the enzyme's activity within the resistant strain. This data was corroborated by the down-regulation suffered by MFO activity in the POA strain. According to [19], under normal conditions, the activity of monoxygenase in eggs is low, and it only increases in the molting period after the larvae hatch, a physiological effect caused by the metabolism of hormones related to ecdysis. Barros et al. [15] demonstrated that the horn fly, Haematobia irritans, resistance to cypermethrin is being primarily caused by an enhanced activity of P450 mono-oxygenases [15]. In the present study, the comparison of the enzyme activities in both strains showed that the activity of MFO rose only in the Jaguar strain, while in the POA strain this enzyme's activity declined after the treatment. That result suggests that in the eggs of the POA strain, the activity of MFO was suppressed. Therefore, it is possible to attribute the metabolism of fluazuron to the higher activity of MFO in eggs.

Although there was a significant difference in the comparison between the control group of the susceptible strain and the control group of the resistant strain for α-esterase activity, we do not attribute this result to the treatment effect, since they were only control groups. That finding can be related to the inherent phenotypic variations of different tick populations, with different genetic backgrounds.

With respect to the larvae, both general esterases (α and β esterases) had greater activity in the group exposed to fluazuron (α esterase: 129.63 ± 10.71 nM/μg of protein/min and β esterase: 137.58 ± 4.11 nM/μg of protein/min) of the Jaguar strain than the control group of the same strain (α esterase: 110.16 ± 8.83 nM/μg of protein/min and β esterase: 126.25 ± 5.95 nM/μg of protein/min) (p < 0.05) (Fig. 2). For the other enzymes evaluated in larvae (MFO and GST), no statistical differences were observed between the groups control and exposed in the same strain. The increase in the activity of carboxylesterases in the exposed group of larvae from resistant strain can be associated with their mechanisms of detoxification to fluazuron when compared to the exposed group of the susceptible strain. Many authors ever described that carboxylesterases are important enzymes present in ticks, involved in metabolizing active principles [14,18,19] and showed that significant elevation in esterase activities may indicate the involvement of esterases in the resistance processes [13,14]. A good correlation found between the results of bioassays and biochemical assays in our study was in accordance with the findings of other studies [20,21].

This study allows the conclusion that MFO, in the Jaguar strain, participate in the detoxification processes of fluazuron in eggs and draws the attention for the detoxifying mechanisms having a grand importance in resistance processes. Unexpectedly, after the larvae hatch, the carboxylesterases take over and enhance in activity, being the most important enzyme involved in the metabolism of the fluazuron. Based on these results, it can be concluded that these enzymes act in distinct ways depending on the tick's development stage, promoting fluazuron detoxification strategies that may contribute to the appearance of resistant strains. This study was important to warn the authorities in public health and veterinary departments to take appropriate actions to implement resistance management strategies.

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![Fig. 1. Comparison of the enzyme activities of carboxylesterases, glutathione-S-transferase and monoxygenase between the sensitive (POA) and resistant (Jaguar) strains in eggs from engorged females of Rhipicephalus microplus exposed to fluazuron.](image-url)
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References


