Effect of E-64 on reproductive parameters of Boophilus microplus

RESEARCH

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ABSTRACT

In this work we assayed the injection of E-64, and specific cysteine peptidases inhibitor, in vitellogenic females of the tick *Boophilus microplus*, with the aim of determining the effect caused on the ovaric development and oviposition. A significant reduction of the eggs lay efficiency, the ovary weight, the ovary vitellin content and the cysteine peptidase cathepsin L and cathepsin B-like activities was obtained in the injected group with the inhibitor. These results show the relevance of cysteine peptidases in the reproductive events of *B. microplus*.

Key words: *Boophilus microplus*, E-64, cysteine peptidases, ovary, vitellin, eggs, reproduction

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RESUMEN

Efectos del E-64 sobre parámetros reproductivos de *Boophilus microplus*. En este trabajo se ensayó la inyección de E-64, inhibidor específico de cisteíno peptidasas, en hembras vitelogénicas de la garrapata *Boophilus microplus*, con el objetivo de determinar su efecto sobre el desarrollo ovárico y la oviposición. En los grupos inyectados con el inhibidor, se obtuvo una reducción significativa de la eficiencia de la puesta de los huevos, del peso del ovario, del contenido de vitelina en el ovario y de las actividades cisteíno peptidasas semejantes a la catepsina L y a la catepsina B. Los resultados demostraron la importancia de las cisteíno peptidasas en los eventos reproductivos de *B. microplus*.

Palabras clave: *Boophilus microplus*, E-64, cisteíno peptidasas, ovario, vitelina, huevos, reproducción

Introduction

Ticks are obligated ectoparasites of terrestrial vertebrates. *Boophilus microplus* (*Acari: Ixodidae*) (Canestrini, 1887) is an ectoparasite of the bovines, really important in veterinary and widely distributed in the tropical and sub-tropical regions. The Bm 86 recombinant protein was used to produce the first commercial vaccine against *B. microplus* and the results have demonstrated the possibility of controlling tick populations by immunological resources. However, the vaccination with Bm 86 does not suppress the use of acaricides an efficient tick control [1], and, on the other hand, there is no similarity of amino acidic sequence between this antigen and any other protein of known function, which could make difficult to search efficacious antigens in other species [2].

Cysteine peptidases are involved in important functions in parasite organisms, among them the acquisition of nutrients, the invasion of cells of the host, the immune evasion, pathogenesis and virulence. Some of those enzymes have been considered as the potential targets towards which the antiparasitary chemical therapy must be directed, considering the powerful effects of the inhibitors of that class of peptidases on the parasites [3-5].

Studies performed in our laboratory demonstrated the presence of cysteine peptidases in the intestine, hemolymph, the ovary and the eggs of *B. microplus* [6-9].

The direct injection in ixodid ticks of anti-parasitic compounds and anti-antigen monoclonal antibodies of those species have been assayed in full females in order to assess their effect on the formation of eggs [10, 11].

Mainly considering the previously reported relationship between oogenesis and the presence of cysteine peptidases in the ovary of *B. microplus* [9], we decided to determine the effect of the injection of a specific inhibitor of this type of peptidases, E-64 on the ovaric development and the oviposition in full engorged female ticks (*B. microplus*).

Materials and methods

Reagents

The trans-epoxysuccinyl-L-leucylamide-(4-guanidine)-butane (E-64) inhibitor, the dymethylsulfoxide (DMSO) inhibitor, the N-carbobenzoxy-phenylalanyl-arginyl-4-methoxy-β-nafthylamide (Z-Phe-Arg-2NA) and N-carbobenzoxy-arginyl-arginyl-4-methoxy-β-nafthylamide (Z-Arg-Arg-2NA) and the reagents to develop the enzymatic activity were obtained from Sigma Chemical Co, USA.

Ticks

The *B. microplus* strain (*Babesia*, *sp.* Free) was kept in steps on Holstein cattle in the Biological and Pharmaceutical Laboratories (LABIOFAM). The full females were manually collected.

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Injection of E-64 in *Boophilus microplus* females

Thirty adult female ticks were processed before 24 hours after the collection in bovines. The E-64 inhibitor was solved in DMSO and diluted in physiological saline solution up to a concentration of 200 µM. A DMSO solution, with equal rate (1%) in physiological saline solution was used as control of the solvent. After individually weighed, the ticks were injected with 1.5 µL of each solution with a SGE micro-syringe, Australia, according to the method of Toro-Ortiz *et al.* (1997).

Effect of the injection of E-64 inhibitor on the efficiency of the oviposition of *B. microplus*

For determining the effect of the E-64 inhibitor on the eggs laid by *B. microplus*, the females were divided into 3 groups: control without injection (n=5), injected with DMSO (n=5) and with inhibitor (n=5). After injected, they were placed in individual vessels and incubated at 30 ºC y 90% HR for two weeks. The total eggs lay of each group was weighed and to perform the comparison among the treatments, the efficiency of the lay was calculated (EP=eggs weight/ticks weight).

Effect of the injection of the E-64 inhibitor on the ovaric development

For determining the effect of the injection of E-64 on the weight of the ovary, the content of vitellin and the cysteine peptidases activities of the ovary, 15 ticks were used divided into 3 groups, as in the former experiment. The ticks were incubated at 30 ºC and 90% relative humidity until the ovary dissection; 48 hours after the injection of the inhibitor (2 day of preoviposition). The ovaries were individually weighed and the size of three of their largest oocytes was measured with an ocular micrometer.

The measurement of the content of vitellin was performed through the calculation of the difference between the absorbance at 400 nm (near to the peak of absorption of the hemo group of the vitellin) and at 500 nm (wavelength for which there is no light absorption by the hemo group), of an extract prepared from the manual homogenization of each ovary and it was expressed in absorbance units [10].

The enzymatic activity of the extracts in front of Z-Phe-Arg-2NA and Z-Arg-Arg-2NA was determined in a reaction mixture containing by 50 µL of raw extract, 150 µL buffer (100 mmol/L citric acid, 200 mmol/L disodic phosphate, pH 4.0 and 6.5) and 5 µL of substrate (Z-Phe-Arg-2NA and Z-Arg-Arg-2NA, 0.5 mmol/L in the assay). L-cysteine 5 mmol/L was incorporated to each assay for a higher sensitivity. This mixture was incubated at 37 ºC by adjusting the temporal conditions to guarantee the work with starting velocities. The 2-naphtylamine released by the action of the enzyme was determined by an indirect method: the reaction is stopped by adding 200 µL coupling reagent that contents Fast Garnet GBC which react with the released 2 naphthylamine forming a red azo-compound. The amount of the product was measured at 520 nm in an Ultraspec III, Pharmacia, Sweden spectrophotometer. A curve was made by using 2-naphtylamine as a standard, whose co-tangent was 1.48×10^{-2} µmol. A unit (U) of enzymatic activity represents the production of 1 nmol 2-naphtylami-ne/min/mL enzyme in the assay [12].

Statistical analysis

The results were studied by a simple classification variant analysis (ANOVA), and later, when the differences were significant ($p<0.05$), the minimal squares test was used to compare the averages of each experiment. The results were expressed as an average value of each group ± the standard deviation.

The experiments described in this paper were twice performed in order to determine the effects of E-64 on the Efficiency of Lay and on the ovary development. The results of one of them are shown, because the same statistically significant differences were found among the groups when the experiments were repeated.

Results

To determine the effect of an inhibitor of cysteine peptidases on the parameters that represent the reproduction of B. *microplus*, E-64 was injected in full engorged female ticks (teleogines), stage where the vitellogenesis or synthesis of the main proteins of egg yolk is started.

Each experiment was carried out by using controls, without injection or injected with the E-64 solvent, DMSO, without showing significant differences between those groups in any case.

The efficiency of the lay of eggs was significantly affected ($p<0.05$) when injecting E-64 (Figure 1), reflected by a decrease in the relationship of the mass of eggs laid by the treated ticks, related to its body weight of 0.5316 ± 0.1082 in the group injected with DMSO, to 0.3016 ± 0.075 in the group injected with the inhibitor for a decrease of 43.3%.

In the group injected with E-64 the weight of the ovary significantly decreased ($p<0.05$) from 0.0227 ± 0.00416 g in the group injected with the solvent to 0.00835 ± 0.00335 g in the group injected with the inhibitor (Figure 2) for a 63.25% decrease. The measurement of the biggest oocytes did not show any significant difference among the groups (Table 1), what

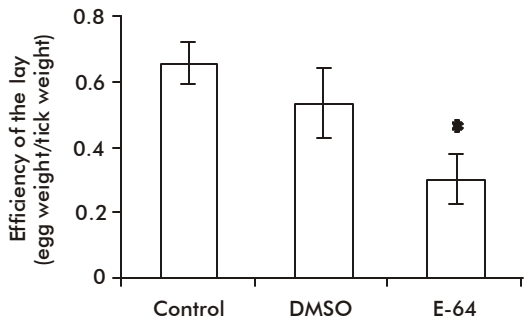

Figure 1. Effect of the injection of E-64 in teleogines of *B. microplus* on the efficiency of the lay of the eggs (n=5 for each group). (*) It indicates a statistically significant difference compared with the control group (minimal square test, $p<0.05$).

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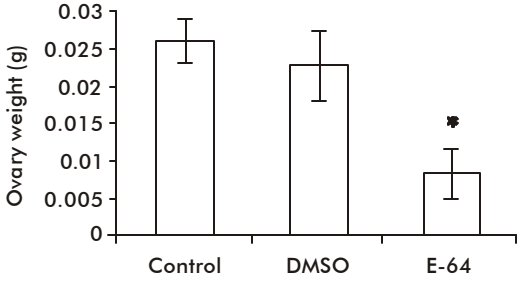

Figure 2. Effect of the injection of E-64 in teleogines of *B. microplus* on the weight of the ovary (n=5 for each group). (*) It indicates a statistically significant difference compared with the control group (minimal square test, $p<0.05$).

Table 1. Size of the biggest ovocytes present in the ovary of the *B. microplus* female as a result of the injection of the E-64 inhibitor. Three ovocytes were measured for each ovary of the 5 ticks studied in each group. The differences among the groups were not statistically significant (minimal square test).

Experimental group	Size of the biggest ovocytes (μm)	
	Mean average	Standard deviation
Control without injecting	52.0	± 1.549
DMSO	51.6	± 1.341
E-64	52.0	± 1.548

suggests that the decrease in the ovaric development and the eggs lay is own to the maturation of a lower number of oocytes in that period as a consequence of the presence of the inhibitor.

The vitellin content in this organ, expressed as the absorbance of the hemo group also decreased significantly, from 1.551 ± 0.395 U -to 0.587 ± 0.336 U (62.2% reduction) (Figure 3). It shows an effect of this compound on the vitellogenesis or the process of incorporation of vitellogenin in the oocyte.

Moreover, a statistically significant reduction ($p<0.05$) was observed in the cysteine peptidases activities detected in the ovary (Figure 4) in the group of ticks injected with the inhibitor. The hydrolytic activity of the Z-Phe-Arg-2NA substrate decreased

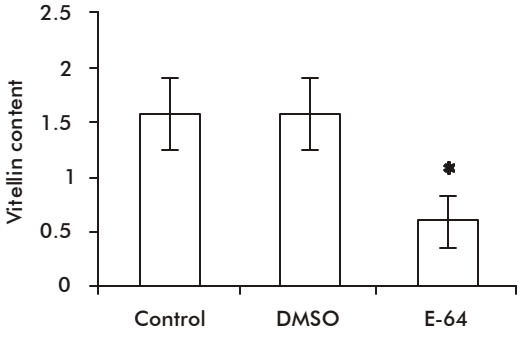

Figure 3. Effect of the injection of E-64 in teleogines of *B. microplus* on the content of vitellin reflected as the absorbance of its hemo group (Absorbance 400 nm- Absorbance 500 nm) in the extract of ovaries (n=5 for each group). (*) It indicates a statistically significant difference.

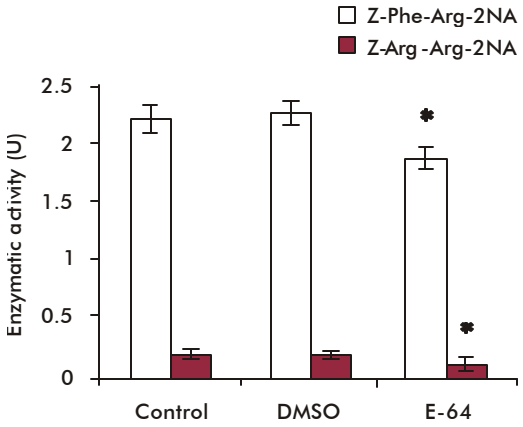

Figure 4. Effect of the injection of E-64 in teleogines of *B. microplus* on the Z-Phe-Arg-2NA and Z-Arg-Arg-2NA hydrolysis by the extract of ovaries (n=5 for each group). (*) It indicates a statistically significant difference compared with the control (minimal square test, $p<0.05$).

from de 0.9153 ± 0.0412 U in the group injected with DMSO to 0.7643 ± 0.0379 U, this is, a reduction of 16.5%,while the hydrolysis of the Z-Arg-Arg-2NA substrate decreased from 0.07843 ± 0.01845 U to 0.0415 ± 0.02172 U (47.1% reduction).

Discussion

In the *B. microplus* tick activities cathepsin L-like and cathepsin B-like, according to their specificity of substrate, were detected in the ovary of the adult female ticks until the end of oviposition [9], in the hemolymph of the females in the oviposition peak [7] and in the eggs of this species, where they perform an important function in the degradation of the proteins of egg yolk during embryogenesis [8]. The complete *in vitro* inhibition of the activity of those enzymes in the presence of E-64 made possible their classification as cysteine peptidases,

The significant reduction in the efficiency of the eggs lay obtained in this study as a consequence of the injection of E-64, without affecting eclosion and maturation of the larvas (not showed data), suggest a decrease in the number of eggs laid and not in their individual weight.

The ovary is the organ where *B. microplus* eggs formation takes place. In the ixodide ticks, the maturation of oocytes occurs asynchronically: simultaneously, in the ovary, oocytes with different degrees of development are found [13]. The decrease in the weight of the ovary in the ticks injected with E-64 could be the cause of the decrease of the efficiency of the eggs lay. The fact that there are no differences in the size of the largest oocytes suggests the occurrence of their complete maturation and strengthens the hypothesis of a decrease in the number of laid eggs and not in their individual weight.

The ability of producing great numbers of vitellogenin in a relatively short period seems the main evolutive strategy for the Ixodidae reproductive success. The synthesis of those hemolipoglycoproteins occurs in the fat body and in the midgut. Both in the ixodid ticks and in argasid, they are secreted to the hemolymph, where they are transported to be endocyted

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by the oocytes in the ovary to become vitellin, the main protein of the egg yolk [14] that constitutes 80% of the total content of proteins of the fresh egg. In the *B. microplus* egg, all the hemoproteins are derived from vitellin [15], so, the measurement of the content of hemo reflects a direct relationship with the presence of those proteins. As a result of the injection of E-64, a decrease of absorbance was produced, a characteristic of the hemo group in the ovary extract, what means a decrease of the amount of vitellin in that organ. This behavior can be given by the affection of the vitellogenin synthesis or its mechanism of transport and incorporation to the ovary. The direct participation of cysteine peptidases in those processes has not been shown. Nevertheless, the presence of that kind of peptidase [6, 16] in the intestine of full *B. microplus* female ticks suggests their participation in the digestion of the blood proteins ingested by the tick, and the vitellogenin synthesis depends of that process. However, from studies of artificial feeding with pepstatin, it is demonstrated that the peptidases aspartic perform a relevant function in the digestive events [17].

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The significant decrease in the cysteine peptidases activities of the ovary can be a consequence of the direct interaction with the inhibitor and/or the presence of those enzymes and the incorporation of vitellogenin. In fact, the studies in our laboratory suggest a possible hemolymphatic circulation of those cysteine peptidases before being incorporated to the ovary [7].

The presence of a lower enzymatic activity similar to catepsine B (able to hydrolyze the Z-Arg-Arg-2NA substrate) is well known and similar to catepsine L in the ovary of the group injected with the inhibitor. This result could be given by differences regarding the histolocation of both activities that would derivate in variations in the inhibitor accessibility. Later studies should be dedicated to make this finding clearer.

The decrease of the parameters that characterize the tick's reproductive process, in the presence of an inhibitor of cysteine peptidases, shows the importance of those enzymes in the life cycle of this ectoparasite and deserves a later study, because it is about a new possibility in the wide range of mechanisms proposed for the control of this arthropod.

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