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Influence of low environmental temperature on the phagocytic activity of bullfrog (*Rana catesbeiana*) thrombocytes*

Influência da baixa temperatura ambiental sobre a atividade fagocítica de trombócitos de rã touro gigante (*Rana catesbeiana*)

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SUMMARY

The influence of low environmental temperature on phagocytic activity of bullfrog (*Rana catesbeiana*) thrombocytes induced by the injection of colloidal carbon in the dorsal lymphatic sac was investigated. Results showed that low environmental temperature decreased the thrombocyte phagocytic activity. Thrombocytes of animals treated and kept at 6°C showed a slow initial activity at 1h (16.3 ± 4.3; results expressed as mean ± SEM of positive thrombocytes in 400 cell analysed/animal; n=6), which increased slightly at 6h and 12h (45.8 ± 12.2; 55.5 ± 9.6), reaching a maximum reaction at 3d and 7d (80.3 ± 27.5; 78.3 ± 29.5). In contrast, bullfrogs maintained at 24°C presented an initial high reaction at 1h (90.0 ± 16.7), increasing markedly until 1d (196.0 ± 49.8), and then decreasing until 7d (56.0 ± 10.6). These data corroborate previous studies which demonstrated the effects of environmental temperature on multiple factors related to the host's protective mechanisms.

UNITERMS: Frogs; *Rana catesbeiana*; Platelets; Phagocytosis; Temperature.

INTRODUCTION

Thrombocytes are blood cells found in birds, reptiles, amphibians and fishes, whose role in blood coagulation has previously been determined. Therefore, thrombocytes are considered as analagous to the platelets [11].

Morphologically, thrombocytes are characterized as small to medium size, round, oval or spindle-shaped cells, having a strongly basophilic, round to oval shaped and central nuclei. The cytoplasm usually has a pale, homogenous, eosinophilic appearance. A distinct feature observed in amphibians thrombocytes is the presence of uniform, spherical azurophilic granules within the cytoplasm, which can assume either a polar or a diffuse distribution [4,15].

At ultrastructure level, the presence of an open canallicular system, similar to the one seen in platelet, composed of long invaginated cell membranes, could be seen in the cells. The function of these systems has been suggested to be the exchange between plasmatic and endogenous substances. Yet, the occurrence of a cytoskeleton composed of microtubules, responsible for conformation changes, has already been described [5].

However, the role of thrombocytes in inflammatory reactions in non-mammal vertebrates has been subject to some discussion. Their phagocytic activity has been described in amphibians and chickens [1,4,6,9,12], and a possible participation as active components of the inflammatory cellular exudate has already been discussed [7,8,13,16,20,21,24].

Environmental temperature has fundamental influence on ectothermic vertebrates (reptiles, amphibians and fish), since these animals are incapable of generating endogenous heat to maintain their physiologic activities [18,22,23]. On a similar basis, multiple factors related to the host's defense mechanisms, including antibody formation, seasonal immunologic variation and locomotor performance are modulated by environmental temperature [2,14,17,19].

Regarding the inflammatory response, modulation of thrombocytes by environmental temperature was clearly demonstrated by Finn & Nielsen 10 (1971) for teleost fish and by Dias & Sinhorini [7,8] (1991, 1992) for tadpoles and adult bullfrogs (*Rana catesbeiana*). Those authors demonstrated that low temperatures were able to delay the cellular component of the experimentally induced inflammmatory reaction.

The objective of this study was to investigate the influence of low environmental temperature on the phagocytic activity induced by the injection of colloidal carbon in the dorsal lymphatic sac of bullfrogs (*Rana catesbeiana*).

MATERIAL AND METHOD

Animals

Eighty-four specimens of *Rana catesbeiana*, obtained at the

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Setor de Ranicultura, Instituto de Pesca do Estado de São Paulo, were employed. The animals measured 7cm long and weighed 30g on average, at the time of the experiment. All animals were adapted to the laboratory conditions for at least 30 days before the experiments.

They were kept in standardized 25 x 12 x 20cm glass aquaria, at maximum population density of 8 animals/aquarium. The animals were fed mice/rat twice a week. The light cycle established for all groups was 12h light/12h dark.

Experimental Protocol

Groups and environmental temperature: The animals were randomly divided in two groups of 42 specimens each and maintained at selected temperatures of 6°C and 24°C. For both temperatures, an interval of confidence of ± 1°C was considered. Bullfrogs were adapted to the selected temperatures for, at least, one week prior to the experimental procedure. At pre-set times of 1h; 6h; 12h; 1d; 2d; 3d and 7d, six individuals per group were killed and samples were collected.

Anesthesia: The animals kept at 24°C were anesthetized in a glass jar with diethyl ether, followed by section of spinal cord and encephalic rupture. The specimens kept at 6°C were submitted to section of the spinal cord and encephalic rupture without previous anesthesia.

Induction of phagocytosis by colloidal carbon: A volume of 0.1ml of colloidal carbon was injected into the lumen of dorsal lyphatic sac, at its mid-section.

Collecting of samples and evaluation of the phagocytosis: At pre-set times the animals were anesthetized and their coelomic cavity opened. Samples of 1.5ml of peripheral blood were collected directly from the heart with the aid of siliconised glass pipettes, previously treated with 8% EDTA solution. For each sample, multiple peripheral blood film slides were obtained and stained with modified May-Grunwald-Giemsa technique [1].

Analysis of the phagocytic activity was carried out by examining 400 thrombocytes from each specimen with a Zeiss-Jenamed 2 photomicroscopy. The results obtained in the differential analysis were expressed as mean ± SEM of positive thrombocytes for the phagocytosis of colloidal carbon.

Statistical analysis: Quantitative evaluation of the phagocytic activity between the groups were confronted using the T test and the level of significance set at $P<0.05$.

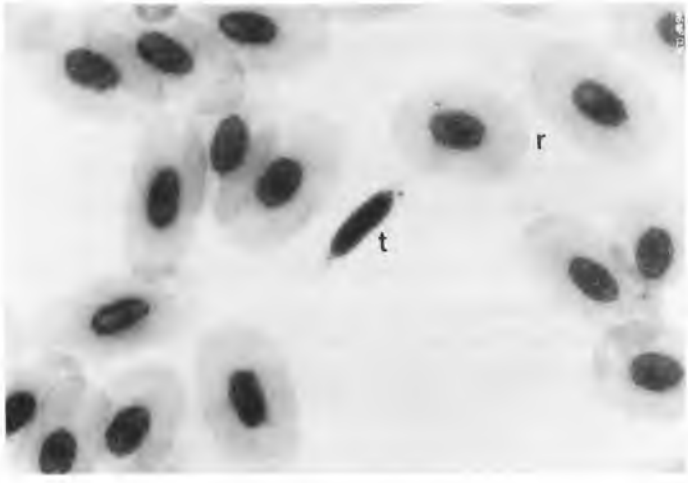

Figura 1
Photomicrograph of a thrombocyte (t) showing the presence of dark brown to black, variably sized granules within the cytoplasm. r = red blood cell. Modified May-Grunwald-Giemsa, 800x.

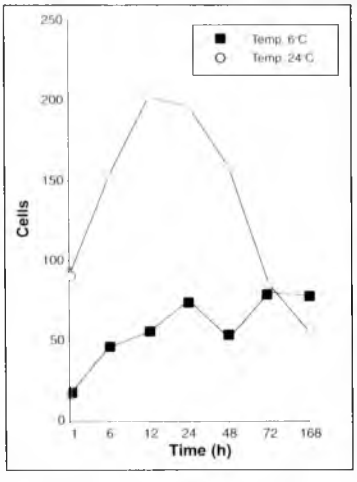

Figura 2
Influence of environmental temperature on the thrombocyte phagocytic activity induced by injection of colloidal carbon in bullfrog. The results express the number of thrombocytes considered positive for phagocytosis in 400 cell/ animal. São Paulo, July, 1993.

RESULTS

The data obtained are expressed on Tab.1 and Fig.1. Phagocytosis was observed as early as 1h post injection of colloidal carbon (90.0 ± 16.7), in animals kept at 24°C, increased markedly until 1d post injection (196.0 ± 49.8) and then decreased until the 7d (56.0 ± 10.6).

On the other hand, phagocytic activity of bullfrogs thrombocytes kept at 6°C was significantly lower than the ones described at 24°C for all times, but 3d and 7d. After 1h, the number of positive thrombocytes was 16.3 ± 4.3, increased slightly at 6h and 12h (45.8 ± 12.2; 55.5 ± 9.6), reaching maximum reaction on 3d and 7d (80.3 ± 27.5; 78.3 ± 29.5).

Morphologically, thrombocytes were characterized as oval to spindle-shaped cells, with a pale, homogenous, eosinophilic cytoplasm, displaying a central, oval-shaped and basophilic rough nuclei. Positive cells revealed the presence of dark brown to black, variably sized granules within the cytoplasm; otherwise, the remaining common features were preserved (Fig. 2).

DISCUSSION

The methodology applied was successful in achieving the

Table 1

Effect of environmental temperature on the thrombocyte phagocytic activity induced by injection of colloidal carbon in the dorsal lymphatic sac of bullfrog (*Rana catesbeiana*). São Paulo, July, 1993.

TIME	TEMPERATURE 6°C	TEMPERATURE 24°C
1h	16.3 ± 4.3*	90.0 ± 16.7
6h	45.8 ± 12.2*	152.5 ± 28.8
12h	55,5 ± 9.6*	201.6 ± 27.4
1d	75.3 ± 17.9*	196.0 ± 49.8
2d	51.5 ± 13.2*	158.5 ± 48.9
3d	80.3 ± 27.5	85.3 ± 23.0
7d	78.3 ± 29.5	56.0 ± 10.6

proposed objectives. The injection of colloidal carbon via dorsal lymphatic sac proved to be a reliable method to induce phagocytosis. The dorsal lymphatic sac is an ample chamber limited by lymphatic endothelium and surrounding skin. It occupies most of dorsal aspects of frogs and toads and is readily accessible. Once injected, the introduced substance quickly reaches the blood stream. Colloidal carbon proved an appropriate material, because its granules are promptly visualized within phagocytes, and, as inert substance, does not seem to interfere with physiologic thrombocyte activity.

The role played by thrombocytes in the inflammatory response of non-mammal vertebrates has been discussed. In fish, thrombocytes have already been described as the predominant component of inflammatory cellular exudate induced by carrageenin in the natatory bladder of *Oreochromis niloticus* [20]. In reptiles, the presence of thrombocytes has already been reported in the inflammatory site in turtles *Trachemis dorbignyi* [21].

In amphibians, Jordan [18] (1925) and Dawson [6] (1933) suggested a possible role played by these cells in the phagocytosis of foreign body in salamander and leopard frog, respectively [15,23]. Dias; Sinhorini [7] (1991) suggested that thrombocytes could be a major inflammatory cell in tadpoles of *Rana catesbeiana* [8]. However, the same authors, using an ultrastructural approach, did not observe thrombocytes in the inflammatory reaction induced by foreign body in the same animals [8]. In avian species, the thrombocytes phagocytic activity has been clearly proposed by several authors [1,4,9,12]. In contrast, Ishida et al. [13] (1985) and Kajigaya et al. [16] (1985) did not obtain similar results while investigating the inflammatory response induced by foreign body in chickens [13,16].

In this study, particles of colloidal carbon were seen within thrombocytes cytoplasm in animals kept at 24°C as soon as 1h post injection, suggesting a dynamic action in purifying the blood stream of foreign bodies. Due to the high number of circulating thrombocytes found in most non-mammal vertebrates, this potential effect could imply significant value in the host defense mechanisms.

Environmental temperature showed a statistically significant effect on the thrombocyte phagocytic activity. Animals kept at 24°C presented a much higher number of positive thrombocytes in all experimental times, but 3d and 7d, when contrasted with the results of bullfrogs kept at 6°C. These results corroborate the data of previous studies, which demonstrated the effects of environmental temperature on multiple factors related to the host's protective mechanisms, such as antibody formation, immunological response [14,17] and inflammatory reaction [7,8,10].

The presence of granules of colloidal carbon within the thrombocytes cytoplasm may also be partially explained due to occurrence of a canallicular open system in these cells. This system allows a quick and substantial exchange of substance between the thrombocytes and the surrounding plasmatic fluid. Consequently, granules of colloidal carbon could be trapped within those open canallicular systems and be mistaken as phagocytized particles, as has already been suggested for mammal platelets [3]. However, it is unlikely that this passive phenomenon could be modulated by environmental temperature. Further studies, investigating the ultrastructure characteristics of this phenomenon, as well as the role played by thrombocytes facing biological agents, might offer answers to these and other arising questions.

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RESUMO

O objetivo do presente trabalho foi o de investigar a influência da baixa temperatura ambiental sobre a atividade fagocítica de trombócitos de rã touro gigante (*Rana catesbeiana*). O modelo indutor de fagocitose utilizado foi a injeção de carvão coloidal no saco linfático dorsal. Os resultados alcançados mostraram que o frio foi capaz de modular significativamente a capacidade fagocítica dos trombócitos. Animais tratados e mantidos a 6°C exibiram uma lenta atividade inicial à 1h (16,3 ± 4,3; resultados expressos como média desvio padrão de trombócitos positivos em 400 células analisadas/animal; n=6), que aumentou discretamente às 6h e 12h (45,8 ± 12,2; 55,5 ± 9,6), alcançando o máximo de reação aos 3d e 7d (80,3 ± 27,5; 78,3 ± 29,5). Por outro lado, rãs mantidas a 24°C apresentaram uma forte resposta inicial à 1h (90,0 ± 16,7), aumentando marcadamente até 1d (196,0 ± 49,8), e então diminuindo até 7d (56,0 ± 10,6). Os resultados obtidos suportam estudos prévios que demonstram a importância da temperatura ambiental sobre múltiplos processos relativos aos mecanismos de defesa desses animais.

UNITERMOS: Rãs; *Rana catesbeiana*; Plaquetas; Fagocitose; Temperatura.

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