CARRAGEENIN INDUCED INFLAMMATION IN *Piaractus mesopotamicus* (OSTEICHTHYES: CHARACIDAE) CULTURED IN BRAZIL

Maurício Laterça MARTINS [1,5]*; Flávio Ruas de MORAES [2]*; Rodrigo Yudi FUJIMOTO [3]; Eduardo Makoto ONAKA [4]; Fabiana Rizzi BOZZO [2]; Julieta Rodini Engrácia de MORAES [2]

ABSTRACT

The present work evaluated the acute inflammatory response induced by carrageenin (500 µg) injection into the swim bladder of pacu (*Piaractus mesopotamicus*) after consecutive stress of capture. Fish were subjected to air exposition of 30 seconds repeatedly at 60 minutes intervals over six hours. The cellular inflammatory component was evaluated six hours after either the carrageenin or the control (saline injection), by total and differential counts. Cortisol and glucose were analyzed each hour. In the inflammatory exudate, thrombocytes were the most frequently observed cells followed by macrophages. A significant reduction of cortisol levels was observed after the third stress of capture, as well as an increase in glucose after the second stimulus of stress. Differential counts of circulating leucocytes and thrombocytes show increased percentage of special granulocytic cells and a significant neutrophilia in stressed fish. Non-stressed fish injected with carrageenin presented neutrophilia and lymphopenia. In the swim bladder, carrageenin provoked congestion, interstitial haemorrhage, dissociation of the collagen sheaf and inflammatory infiltrate.

Key words: *Piaractus mesopotamicus;* inflammation; carrageenin; consecutive stress; cortisol; glucose; haematology

INFLAMAÇÃO AGUDA PROVOCADA POR CARRAGENINA EM PACU, *Piaractus mesopotamicus* (OSTEICHTHYES: CHARACIDAE), CULTIVADO NO BRASIL

RESUMO

Este trabalho avaliou a resposta inflamatória aguda induzida por injeção de carragenina (500 µg) na bexiga natatória de pacu (*Piaractus mesopotamicus*), após estresse consecutivo de captura. Os animais foram submetidos a exposição fora da água por 30 segundos, repetindo-se o processo a cada 60 minutos num período de seis horas. Seis horas depois das injeções de solução salina (controle) ou carragenina, o componente da resposta inflamatória foi analisado pela contagem total e diferencial de células. O cortisol e a glicemia foram analisados a cada hora. A análise do exsudato inflamatório mostra predominância de trombócitos, seguidos por macrófagos. Significativa redução dos níveis de cortisol observa-se nos animais após o terceiro estímulo estressante, bem como aumento da glicemia após o segundo estímulo. A contagem diferencial de leucócitos e trombócitos circulantes mostra maior porcentagem de células granulocíticas especiais e significativa neutrofilia após o estresse. Os animais que receberam injeção de carragenina apresentaram neutrofilia e linfocitopenia. Na bexiga natatória, a injeção de carragenina provocou congestão, hemorragia intersticial, dissociação dos feixes de colágeno e infiltrado inflamatório.

Palavras-chave: *Piaractus mesopotamicus*; inflamação; carragenina; estresse consecutivo; cortisol; glicemia; hematologia

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[1] *Departamento de Aqüicultura, CCA, Universidade Federal de Santa Catarina (UFSC) - SC, Brasil*
[2] *Departamento de Patologia Veterinária, Universidade Estadual Paulista (UNESP) - Jaboticabal, SP, Brasil*
[3] *Núcleo de Estudos Costeiros, Universidade Federal do Pará (UFPA), Campus de Bragança - Belém, PA, Brasil*
[4] *Instituto de Pesca, Centro Avançado de Pesquisa Tecnológica do Agronegócio do Pescado Continental - São José do Rio Preto, SP, Brasil*
[5] *Endereço/Address: Rod. SC 404, km 3, Itacorubi, C.P.: 476, CEP: 88040-900 - Florianópolis, SC, Brasil*
e-mail: mlaterca@cca.ufsc.br
** Bolsista de Produvidade em Pesquisa/CNPq*

INTRODUCTION

The first observations on inflammation in fish were made by METCHINIKOFF (1905), who studied phagocytosis by injection of guinea pig erythrocytes into the visceral cavity of *Carassius auratus*. Prior to this, MESNILL (1895) recorded *Bacillus anthracis* phagocytosed by mononuclear leucocytes of fish, and very much later, WEINREB (1958) described histological changes induced by intraperitoneal injection of turpentine. In fish, turpentine injection (REZNIKOFF and REZNIKOFF, 1934); inoculation of mycobacteria (JAKOWSKA and NIGRELLI, 1953); *Aeromonas hydrophila* (POST, 1963); flavobacterium (KLUGE, 1965); complete Freund's adjuvant, *Staphylococcus aureus* and squalene (FINN and NIELSEN, 1971a, b; JENKINS and KLESIUS, 1998); carrageenin (TIMUR *et al*., 1977); lipopolysaccharide (LPS) of *Salmonella typhimurium* (PAULSEN *et al*., 2001); myxozoan parasites (KOVÁCS-GAYER, 1983) have all been studied. The swim bladder has been used for inflammation studies with carrageenin (MATUSHIMA and MARIANO, 1996; MARTINS *et al*., 2004) and for studies on the non-specific immune response (ENDO *et al*., 1995; CHEN *et al*., 1998).

Aquaculture may cause stress to fish due to methods of capture and transport, as well as high stocking density, reduction of dissolved oxygen, pH and temperature changes and high content of ammonia or pollutants (SMART, 1981). The result is hyperactivity of interrenal tissue with an increase in cortisol and catecholamine levels (CECH *et al*., 1996). The inhibition of the inflammatory and immune response (ELLSAESSER and CLEM, 1986; MAULE *et al*., 1989) is a consequence of an increase in corticosteroid levels, favouring the development of infectious diseases (FEVOLDEN *et al*., 1992) and parasitic diseases (YOKOYAMA *et al*., 1996) that are influenced by the fish species and intensity and duration of stress.

Carrageenin is a sulphated polysaccharide derived from Irish moss and used in studies on inflammation. When injected in the posterior foot pad of the rat it induces acute inflammation and an increase in the corticosteroid level after two hours (MORAES and GARCIA LEME, 1982). In addition, in Nile tilapia (*Oreochromis niloticus*) carrageenin induces non-specific acute inflammation with the presence of thrombocyte and macrophage (MATUSHIMA and MARIANO, 1996). The same fact was observed by MARTINS *et al*. (2004), using LPS, also in Nile tilapia.

In this paper, the cellular exudate of the swim bladder, glucose and cortisol levels and the differential leucocyte and thrombocyte counts in the blood of an important freshwater fish, *Piaractus mesopotamicus* Holmberg, 1887, following administration of 500 mg carrageenin or saline solution in association with consecutive stress of capture were examined.

MATERIAL AND METHODS

Maintenance of fishes

A total of 72 fish with 369.2±153.1 g body weight and 22.0±2.9 cm length were randomly distributed in 12 flow-through aquaria of 250 L, receiving 1 L/min of water. During the experiment, the values of some physical and chemical water characteristics were maintained as follows: temperature, 27.8±0.8 °C; pH, 7.3±0.8; electrical conductivity, 186.0±1.2 µS/cm; dissolved oxygen, 3.7±0.4 mg/L; and alkalinity, 92.2±0.8 mg/liter. The animals remained in the aquaria during a period of one week for acclimatization and fed *ad libitum* a commercial diet.

Induction and evaluation of the inflammatory process

After anesthetizing with a solution of 1 g benzocaine to 10 L of water, fish of six aquaria (n=36) received into the swim bladder an injection of 500 mg carrageenin (marine colloids) dissolved in 0.5 mL sterile saline solution 0.65%, in the anterior-medial region (1.0 cm to the right direction of operculum), at the same point of lateral line. The fish of the other six aquaria (n=36) were injected in the same location with the same volume of sterile saline solution (control group). This procedure was adopted for fish submitted (n=18) or not (n=18) to the consecutive stress of capture. Six hours after the injection, fishes of all groups were killed by deepening of the anesthetized state and their swim bladder was washed with 0.5 mL PBS containing 0.001 ml EDTA 5%. With the aid of a Pasteur pipette, the content was carefully collected into centrifuge tubes maintained in ice and diluted to 1:4 in order to determine the total number of leucocytes (number/mL) in Neubauer chamber. Afterwards, the content was centrifuged at 150 G for 10 minutes, and the supernatant discarded. A drop of serum obtained from the same fish species was added to the precipitate, and smears of the exudate cells were made for the differential counts of macrophages, lymphocytes, granulocytes and thrombocytes. After drying, the smears were fixed with methyl alcohol (three minutes) and stained with Giemsa (two drops

of Giemsa for one mililiter of boiled distilled water) for 15 minutes.

Induction of repeated stress of capture

Fish were subjected to air exposition in net during 30 seconds, applied repeatedly at 60 minutes intervals over 6 hours, as detailed by MARTINS *et al*. (2000). Blood samples were collected as follows: time 0 - basal blood sample and first stress; time 1 - injection with carrageenin or saline solution and sample after 5 min; time 2 - stress and sample after 5 min; time 3 - stress and sample after 5 min; time 4 - stress and sample after 5 min; time 5 - blood sample 90 min after the time 4. With this procedure three stresses of capture were made.

Circulating levels of blood cortisol and glucose and the differential blood cell count of leucocytes and thrombocytes

Blood samples were withdrawn (2.0 ml) from the caudal vein into a syringe containing a drop of 10% EDTA solution, to determine the circulating levels of cortisol by radioimmunoassay (Coat-a-Count DPC) and glucose (KING and GARNER, 1947). For the differential count of leucocytes and thrombocytes, air-dried blood smears were prepared using the ROSENFELD (1947) method, in which 100 cells were counted. In order to observe the evolution of cortisol and glucose levels five fishes were killed at the given times: 'zero` to 'five`, as anteriorly explained.

Histopathological evaluation of the swim bladder

After collection of exudates from the swim bladder, sections 6 mm thick, stained with haematoxilin-eosin or Masson's trichrome, were examined and fixed in 10% buffered formalin, dehydrated and embedded in paraffiny wax.

Statistical analyses

The animals were randomly distributed between the two groups (stressed and non-stressed) and the two treatments (injection of carrageenin and saline solution). The averages were compared by the Tukey test, at 5% probability (STEEL and TORREY, 1980) and the percentage from the differential count of the blood and exudate cells were transformed in arc sin ($\sqrt{P+0.5}$).

RESULTS

Cellular composition of the swim bladder exudate

The results demonstrate significant ($P < 0.05$) accumulation of total leucocytes six hours after injection of carrageenin in the swim bladder of *Piaractus mesopotamicus*. The induction of stress did not affect the total leucocyte counts in the saline solution injected fish, but in those stressed and injected with carrageenin there was a significant increase ($P < 0.05$) in total leucocytes, when compared with the non-stressed fish (Table 1). The mean percentage values of granulocytes, lymphocytes and macrophages recovered from the exudate did not differ ($P < 0.05$) between treatments. In saline solution injected and non-stressed fish, the predominant cells in the exudate were thrombocytes and macrophages. When the fishes were submitted to stress, there was a significant increase ($P < 0.05$) in thrombocytes in those injected with saline solution. Nevertheless, in carrageenin injected fish no difference was observed in the percentage of macrophages (Table 1).

Table 1. Mean values and statistics of the differential counts of mononuclear and polymorphonuclear cells (%) and the total leucocyte count (number/μL) in the exudate in the swim bladder of *Piaractus mesopotamicus*, six hours after injection with saline solution (control) or carrageenin. Capital letters: for comparison between treatments, and lower case letters: for comparison between groups. C.V.: variant coefficient; *P < 0.05; **P < 0.01

Parameter	*Treatment*	*Stressed Group*	*Non-stressed group*	*F test for group*	*F test for treatment*	*C.V.*
Granulocytes	Control	1.00 aA	2.44 aA	0.30	0.79	66.80
	Carrageenin	3.90 aA	2.33 aA			
Lymphocytes	Control	0.44 aA	1.00 aA	3.24	2.85**	58.08
	Carrageenin	7.10 aA	1.90 aA			
Macrophages	Control	5.44 aA	2.80 bA	0.00	8.22**	48.47
	Carrageenin	9.50 aA	31.09 aA			
Thrombocytes	Control	38.33 bA	8.56 bB	7.32**	60.75**	32.91
	Carrageenin	79.50 aA	63.86 aA			
Total leucocytes	Control	281.80 bA	424.00 bA	14.23	23.29**	17.59
	Carrageenin	7,745.80 aA	3,767.50 aB			

Histopathological analysis of the swim bladder

The injection of carrageenin provoked congestion associated with a discrete and focal interstitial haemorrhage, a dissociation of the collagen sheaf indicating oedema, and the presence of a cellular inflammatory infiltrate. In the latter, thrombocytes were the predominant cells followed by macrophages and desquamated cells. The deposition of fibrin and the displacement of the mucosa with the associated dissociation of the collagenous sheaf were observed. In saline solution injected fish the alterations were similar but less severe.

Circulating levels of cortisol and glucose and differential count of leucocyte and thrombocyte

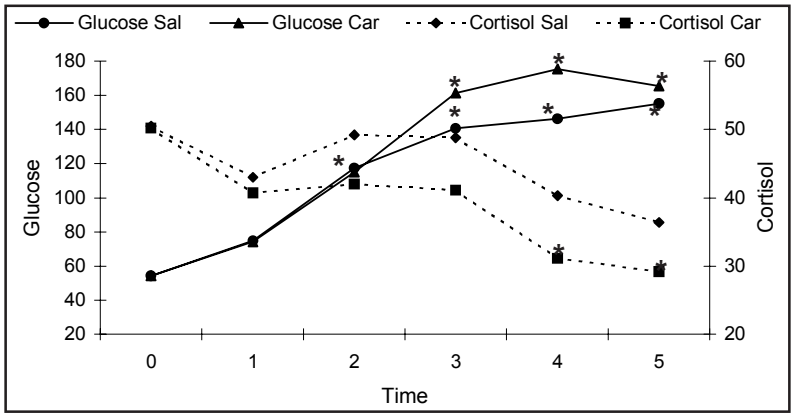
There was no significant difference between saline solution and carrageenin injected fish submitted to consecutive stress of air exposition. Nevertheless, a decrease in the cortisol levels ($P < 0.05$) was observed at times four and five in relation to the basal values (Figure 1). In both groups, a graded increase in the blood glucose concentration was recorded two hours later in the saline solution group and three hours later in the carrageenin one, when compared with the non-stressed fish.

The differential counts of circulating leucocytes and thrombocytes showed increased number of special granulocytic cells and significant neutrophilia in stressed fish. In non-stressed fish, carrageenin injection provoked neutrophilia. On the other hand, lymphopenia ($P < 0.05$) occurred in carrageenin injected and non-stressed fish. The monocyte percentage did not change in this situation (Table 2).

Table 2. Mean values and statistics of the differential counts of leucocytes and thrombocytes (%) in the blood of *Piaractus mesopotamicus* injected with saline solution (control) or carrageenin. Capital letters: for comparison between the times, and lower case letters: for comparison in vertical line. S.G.C.: special granulocytic cell; C.V.: variant coefficient; * $P < 0.05$; ** $P < 0.01$

Parameter	*Treatment*	*Stressed group*	*Non-stressed group*	*F test for group*	*F test for treatment*	*C.V.*
S.G.C.	Control	8.50 aA	3.10 aB	42.58**	3.34**	39.79
	Carrageenin	5.80 aA	2.20 aB			
Neutrophils	Control	32.50 aA	7.90 aB	194.85**	13.24**	21.69
	Carrageenin	23.30 bA	12.90 bB			
Lymphocytes	Control	4.40 aA	8.20 aA	0.04	3.33**	35.07
	Carrageenin	4.50 aA	4.20 bA			
Monocytes	Control	11.10 aA	10.80 aA	3.54**	0.31	33.65
	Carrageenin	10.80 aA	8.60 aA			
Thrombocytes	Control	45.20 aB	68.90 aA	120.19**	8.49**	11.44
	Carrageenin	56.00 bB	71.90 aA			
Eosinophils	Control	0.00 aA	0.00 aB	3.914*	3.12*	6.09
	Carrageenin	0.00 aA	0.00 aB			

Figure 1. Mean values of glucose (mg/100 mL) and cortisol (µg/dL) in Piaractus mesopotamicus after injection with 0.5 mL saline solution (Sal) and 500 µg carrageenin (Car) at different sample times. * Indicates significant difference between the sample times within each parameter ($P < 0.05$).



DISCUSSION

The data demonstrate that the injection of 500 mg carrageenin provoked an inflammatory response that was made up of 79.50% thrombocytes, 31.09% macrophages and lower numbers of granulocytes and lymphocytes. These results are similar to those observed in *Oreochromis niloticus*, three hours after carrageenin injection in the same cavity in which it was provoked accumulation of 63% thrombocytes and 17% macrophages in the exudate (MATUSHIMA and MARIANO, 1996). Thrombocytes are found and well described in birds, reptiles, amphibians and fishes. Although they do not belong to the leucocyte group, the presence of thrombocytes in the inflammatory reaction and the phagocytic activity are discussed. The defensive function of thrombocytes has been reported for several animals (GRECCHI *et al*., 1980; KAJIGAYA *et al*., 1985; SUZUKI 1986; DIAS and SINHORINI, 1991; MATUSHIMA and MARIANO, 1996). Because of these characteristics, many authors have incorrectly included thrombocytes in the differential count of leucocytes (CHONDAR, 1982; MURRAY, 1984; LEA-MASTER *et al*., 1990; HOUSTON *et al*., 1996). In order to correct that error, unpublished results were obtained at the laboratory at which the present work was developed, using the designation "blood cells of organic defense", when the objective is the organism defense. The present data indicate thrombocytes as the principal component in the acute inflammatory reaction induced by carrageenin in *P. mesopotamicus*, in spite of their origin and participation, at least in part, in the organic defense mechanisms.

According to the literature, the composition of the inflammatory exudate depends on the substance, site of lesion and fish species. Then, *Salmo gairdneri* injected with turpentine revealed accumulation of neutrophils six hours after injection (WEINREB, 1958); the same was observed in tilapia four days after paraffin injection (SUZUKI, 1986); intramuscular injection of 2% silica in *Carassius auratus* provoked migration of lymphocytes and macrophages (Janssen and Waaler, 1967, *apud* FINN, 1970); the presence of lymphocytes and granulocytes in rainbow trout inoculated with *Yersinia ruckeri* was observed by GRIFFIN (1983); migration of neutrophils and macrophages in *Pleuronectes platessa* two days after the intraperitoneal injection of oyster glycogen or *Vibrio algynoliticus* was registered (MACARTHUR *et al*., 1984); presence of macrophages three days after the intraperitoneal administration of squalene, incomplete Freund adjuvant, goat serum, tioglicolate or saline solution was observed in *Ictalurus punctatus* (JENKINS and KLESIUS, 1998). Within 24 hours, the carrageenin granuloma into the myotomal muscle of *P. platessa* showed inflammatory response containing neutrophils, macrophages and lymphocytes (TIMUR *et al*., 1977). NARNAWARE and BAKER (1996) have observed 78% reduction in lymphocyte/thrombocyte number in circulating blood of trout injected with saline solution. In this work such observations were confirmed in tropical fish injected with carrageenin and corroborated the findings of MARTINS *et al*. (2004). Increased number of neutrophils in the exudates confirms the observations of MARTINS *et al*. (2004) in stressed tilapia.

Contrarily to the expected, the total count of cells recovered from the exudate was significantly higher in the stressed fish than in the non-stressed ones. In fact, this increase was probably due to thrombocytes presence. However, this phenomenon can be explained by the reduction in cortisol levels of stressed fish. According to MARTINS *et al*. (2000), six hours after handling stress in *P. mesopotamicus*, reduction of the cortisol was observed. MCCORMICK *et al*. (1998) also registered a decrease in cortisol three hours after stress in the Atlantic salmon, in oposition to the observed by FEVOLDEN *et al*. (1992) and MCDONALD and MILLIGAN (1997). Increased cortisol levels in *P. mesopotamicus* (KRIEGER-AZZOLINI *et al*., 1989) and in *Rhamdia quelen* (BARCELLOS *et al*., 2004) occurred one hour after stress of capture. Contrarily to the results obtained in the present work, MARTINS *et al*. (2004) did not observe alteration of the cortisol levels in tilapia injected with carrageenin. Following this thought, the interval of time utilized in this work was able to detect the presence of cortisol. In this assay, the cortisol level was evaluated before and five minutes after each stress and each hour. Thus, the increase and the subsequent decrease of cortisol levels are little probable and are reinforced by the increase in the number of cells in the inflammatory focus. In fact, it is possible that the fish sensitivity to stress has diminished like a block of hypothalamic-hypophisis-interrenal axis (MCCORMICK *et al*., 1998).

In rats, factors produced in the inflammatory focus in a carrageenin-induced reaction and the release of corticosteroids from the hypothalamus-pituitary-adrenal axis inhibit the inflammatory response

(GARCIA LEME and SHAPOVAL, 1975; MORAES and GARCIA LEME, 1982). In this situation, a single application of carrageenin could induce elevation of corticosterone concentration, when compared to control animals (MORAES and GARCIA LEME, 1982). Rats with hypothalamic lesion, hypophisectomized, with lack of medular layer of adrenal or adrenalectomized, showed improvement of oedematous and leucocytic response to carrageenin (MORAES *et al*., 1987). In mammals, corticosteroids reduce the adesion of leucocytes to vascular endothelium, diapedesis and chemotaxis, limiting the migration of white cells to the lesioned site (FARSKY *et al*., 1995). On this point of view, it is possible the occurrence of the same inflammatory behavior in fish because of the presence of inflammatory cells in response to decreased cortisol levels, as related by MARTINS *et al*. (2000).

In this work, the consecutive stress of capture associated with carrageenin or saline solution injection was not sufficient to provoke an elevation of plasma cortisol concentration. Although the cause of cortisol failure was not found, the increased inflammatory response and the hormone values showed coherence, by the fact that cortisol is an anti-inflammatory substance.

In tropical fish, glucose may increase depending on the stress to which they are submitted, as related by KRIEGER-AZZOLINI *et al*. (1989), BARCELLOS *et al*. (2001) and TAVARES-DIAS *et al*. (2001). This work shows an increase in the glucose levels from time 2 through time 5. This means that these levels were kept high during the whole period. These findings are according to those of MARTINS *et al*. (2004) in stressed fish. By the way, there was no difference between saline solution and carrageenin injected fish. In this case, the injection of substances did not alter glucose levels that have been confirmed by MARTINS *et al*. (2004). Contrarily to that found with cortisol, glucose increased not only in saline solution but also in carrageenin injected fish after stress.

MATUSHIMA and MARIANO (1996), utilizing the same model for studies on inflammation, obtained similar results, observing congestion, oedema and an inflammatory infiltrate with a predominance of cells similar to thrombocytes. On the other hand, when TIMUR *et al*. (1977) studied chronic inflammatory response of carrageenin in plaice, necrosis of muscle fibers was evident after three days, with the presence of macrophages and lymphocytes. The results of the present study show thrombocytes as the most observed cells followed by macrophages and mucosa displacement with dissociation of collagen sheaf.

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