

KINETICS OF POLYCARION MACROPHAGE FORMATION IN GRANULOMATOUS INFLAMMATORY RESPONSE OF *Piaractus mesopotamicus* HOLMBERG, 1887 (OSTEICHTHYES: CHARACIDAE). EXPERIMENTAL MODEL

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ABSTRACT

The aim of this essay was the evaluation of the inflammatory giant cells formation in a granulomatous inflammatory response by the implant of a glass cover slip in the subcutaneous tissue in fishes. Young *Piaractus mesopotamicus* Holmberg, 1887 were anesthetized with benzocaine and the glass cover slips were implanted in their subcutaneous tissue. The results showed some macrophages and some foreign body giant cells after three days. After seven days, a larger number of macrophages were present and more foreign body giant cells were formed. At this time a little number of Langhans giant cells was observed. After 14 days, the Langhans giant cells were the predominant type and the number of foreign body giant cell decreased. At this time the number of macrophages had also increased. After 30 days, the giant cells were not individualized and a fibrous hyaline capsule was present. In the 45th day after the implant, a thick hyaline connective capsule was observed. These results indicate that the implant of glass cover slips in fishes induced the multinucleated giant cell formation, similarly to what had been observed in mice, despite the difference in the kinetics of macrophage accumulation -in rats and mice, the accumulation of these cells was more intense in the first three days after the implant, while in *P. mesopotamicus*, the number of these cells increased seven days after the implant.. This model proved to be appropriate for studies about the kinetic of polykaria formation in the granulomatous inflammatory response in vivo.

Key words: *Piaractus mesopotamicus*, multinucleated giant cells

CINETICA DA FORMAÇÃO DE MACRÓFAGOS POLICARIONTES NA RESPOSTA INFLAMATÓRIA GRANULOMATOSA EM *Piaractus mesopotamicus* HOLMBERG, 1887 (OSTEICHTHYES: CHARACIDAE). MODELO EXPERIMENTAL

RESUMO

O objetivo deste ensaio foi o de avaliar a formação de gigantócitos policariontes na inflamação crônica granulomatosa induzida pelo implante de lâminulas de vidro no tecido subcutâneo de pacu, *Piaractus mesopotamicus*. Para tanto, foram utilizados pacus jovens, anestesiados com benzocaína, que receberam implantes de lâminulas de vidro circulares com 11 mm de diâmetro no tecido subcutâneo. Os resultados demonstraram que após três dias havia pequeno número de macrófagos e os primeiros gigantócitos binucleados do tipo corpo estranho presentes na lâminula. Aos sete dias, os macrófagos e células gigantes tornaram-se mais numerosos e os primeiros gigantócitos tipo Langhans foram observados. Aos 14 dias predominavam os gigantócitos tipo Langhans sobre os do tipo corpo-estranho. Aos trinta dias após o implante, havia se formado uma cápsula de tecido conectivo, observada macroscopicamente, ao redor da lâminula, coincidindo com a dificuldade de individualização de gigantócitos. Aos 45 dias, todas as lâminulas apresentaram-se encapsuladas e ao exame microscópico já não era possível observar as células. Esses resultados demonstram que até 14 dias após o implante, o modelo é adequado ao estudo da cinética da formação de macrófagos policariontes na resposta inflamatória crônica granulomatosa in vivo.

Palavras-chave: *Piaractus mesopotamicus*, macrófagos policariontes

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INTRODUCTION

Fish culture is at a moment of great expansion and consolidation in Brazil, needing the production of new knowledge, which depends mostly on the creation or adaptation of experimental models in fish. The pathology of aquatic organisms, in particular, has developed and brought interest, not only in the point of view of the comparative study to mammals, but also for the practical application of its results. The creation or adaptation of experimental models in animals makes it possible to investigate some morbid phenomena in controlled environmental conditions, reducing its interference in the results.

The formation of multinucleated giant cells or polycarion macrophages is recognized since the beginning of the 1960 (GILLMAN and WRIGHT, 1966). In order to study the formation mechanism of such cells, RYAN and SPECTOR (1970) developed an experimental model which consisted in sterilized round glass cover slips inserted in the subcutaneous tissue of mice. These authors noticed that macrophage adhere onto this surface and that after some time they turn into giant cells. These macrophages are formed by the morphophysiological transformation of monocytes from bone marrow, and depend on their turnover, since the mitosis allows the formation of only two generations of these cells. About five days after the glass cover slips insertion, macrophages begin to fuse their membranes, forming the so-called polycarion macrophages. Initially, their nuclei are distributed randomly in the cytoplasm, and this giant cell is recognized as a "foreign body giant cell". Seven to ten days after cover slips insertion, the nuclei begin to be disposed asymmetrically in one edge of the cell cytoplasm, forming a crown-like figure, recognized as Langhans giant cell, whose lifespan is of approximately seven to ten days. (RYAN and SPECTOR, 1970; MARIANO and SPECTOR, 1974).

In birds, *Gallus gallus domesticus*, differently from what happens to mice, the formation of giant cells in glass cover slips inserted in the subcutaneous tissue is more premature, beginning to form 12 hours after insertion, and becoming more expressive from 18 hours on (GRECCHI *et al.*, 1980).

In fishes, the presence of giant cells is associated with different etiological agents, such as the parasite *Jauela glandicephalus* in *Paulicea lutkeni* Steindachner, 1877 (EIRAS and REGO, 1989); the encapsulated larvae L3 of *Pseudoterranova decipiens* in rainbow trout (*Oncorhynchus mykiss*, WALBAUM, 1792), experimentally infected (RAMAKRISHNA and BURT,

1991) and the chronic inflammatory reaction induced by the intramuscular inoculation of BCG *Oreochromis niloticus* Linnaeus, 1758 (MATUSHIMA, 1994). In this last case, after three days, giant cells are rare but become more frequent after seven days, acquire more organization after 21 days and within 33 days these cells are able to phagocytose BCG; thrombocytes, lymphocytes, eosinophils and plasma cells were also seen but the epithelioid cells were the main component of the granulomatous reaction. Melanomacrophages could also be seen.

Fish macrophages are easily isolated from lymphohaemopoietic organs and the peritoneal cavity, due to their capacity of adherence (SECOMBES, 1996) and can be kept in culture for a long time (SECOMBES, 1990; MUÑOZ *et al.*, 1999). In some fish species macrophages fused spontaneously and formed giant cells (MCG) after few days in culture (SECOMBES, 1985; 1990; MORIMOTO *et al.*, 1996).

There is not, in the consulted literature, an experimental model which could allow the evaluation of the quantitative or qualitative evolution kinetics of polycarion macrophages in the granulomatous response of fishes. Therefore, the objective of this paper is to evaluate the model of round glass cover slip insertion in the subcutaneous tissue of pacu, *Piaractus mesopotamicus*, with regard to the foreign-body granulomatous response and polycarion macrophages kinetics.

MATERIAL AND METHOD

Fish and water quality

In order to reach the proposed objectives, 50 outbreed young pacus (*Piaractus mesopotamicus*, HOLMBERG, 1887), males or females acquired in Tabatinga, SP, Brazil, were used. The fishes had initial average length of 11.08 (0.41cm and initial average weight of 32.06 (3.56 g. Animals were maintained in five tanks (250 L capacity 10 fishes/tank), with dechlorinated water. Each tank had its supplementary aeration. Every day, in the morning, temperature was measured in every tank with an Hg bulb thermometer. Every week pH, electric conductivity and dissolved oxygen were measured (GOLTERMAN *et al.*, 1978). During the experimental period, all the tanks remained under appropriate conditions of temperature ($30.0 \pm 0.63^\circ\text{C}$); conductivity (186.85 ± 2.78 S/cm), pH (8.14 ± 0.31) and dissolved oxygen (5.17 ± 0.82 mg/L) (SIPAÚBA-TAVARES, 1995).

Cover slip implant

Round 11 cm diameter glass cover slips (Glasstécnica®) were inserted in fish subcutaneous tissue on the lateral-dorsal region, behind left operculum. In order to do so, fishes were previously anesthetized with benzocaine (1g/10L). After asepsis, the scales were gently taken out and a small incision was done in the skin. The cover slips - previously sterilized in a flame - were inserted and the skin was stitched with simple nylon knots. Animals were then taken back to their tanks and a salt bath (10 g NaCl/ L/15 minutes) was prepared, and on the two following days a new salt bath (1 g NaCl/ L/15 minutes) was prepared, also for each tank.

The glass cover slips were taken out at different times: 3, 7, 14, 30 and 45 days after they had been inserted. For each one of these moments, 10 fishes were used.

Fish sacrifice, cover slip removal and preparation

Animals were sacrificed by prolonged benzocaine immersion (1g/L water). Cover slips were carefully taken out, washed with 0.65% buffered saline solution, fixed in Bouin's fixative for 10 minutes, stained in

hematoxilin-eosin and mounted for citological examination. The morphology of the reactive connective tissue capsule that surrounded the glass coverslips after 45 days was evaluated in histological 6 mm thickness preparations stained with hematoxilin-eosin.

RESULTS

The data presented here show that the fish tanks remained under controlled temperature, pH, conductivity and dissolved oxygen conditions throughout the experiment. The little variations that occurred were within adequate limits for fishes. The cleaning of the tanks on alternate days avoided organic matter accumulation and ensured the maintenance of water quality.

Three days after cover slip insertion, the first glass cover slips were taken out. The washing process removed the non-adherent glass material and made it possible to observe many accumulated macrophage and rare binuclear giant cells (Figure 1). After seven days, the number of isolated macrophages had increased; its greater organization in macrophage clumps was noticeable, as well as the predominance

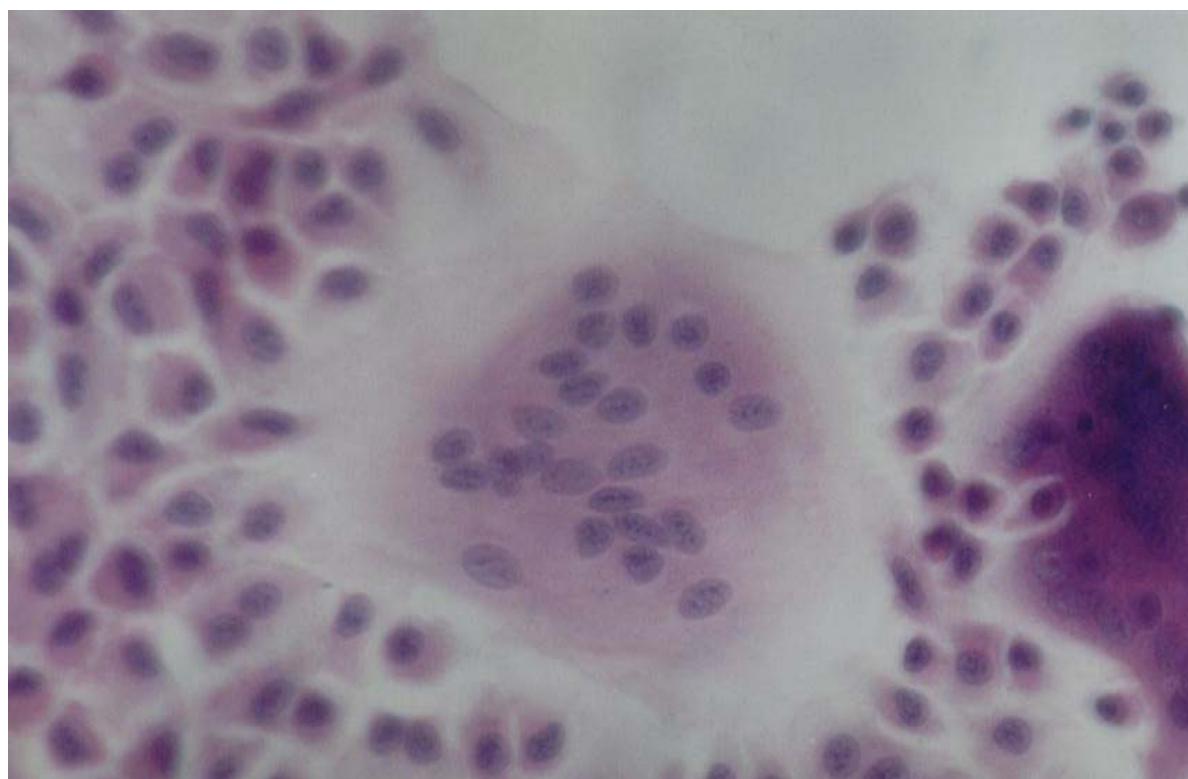


Figure 1. Isolated macrofages and a foreign body giant cell type with randomly distributed nuclei in the cytoplasm seven days after the cover slip implant. HE x 1000

of foreign body giant cells (cells with randomly distributed nuclei in the cytoplasm, as it can be seen in Figure 1. At this moment, besides the presence of foreign body giant cells, other giant cells were present and their nuclei were asymmetrically distributed at one edge of the cells, forming a "crown-like" figure, which is the characteristic morphology of Langhans giant cells (Figure 2). The observations made 14 days after glass cover slips implants showed that the number of Langhans giant cells was greater than that of foreign body giant cells and that the number

of macrophage had stabilized.

After 30 days, it was not possible to individualize giant cells anymore, due to the intense connective proliferation forming a capsule constituted by fibroblasts, fibrocytes, collagen fibers, mononuclear cells and neo-vessels.

Forty-five days after the cover slip implants, giant cells were not present anymore, but a hyaline connective capsule with the predominance of collagen components, fewer neo-formed vessels and less cellularity was present.

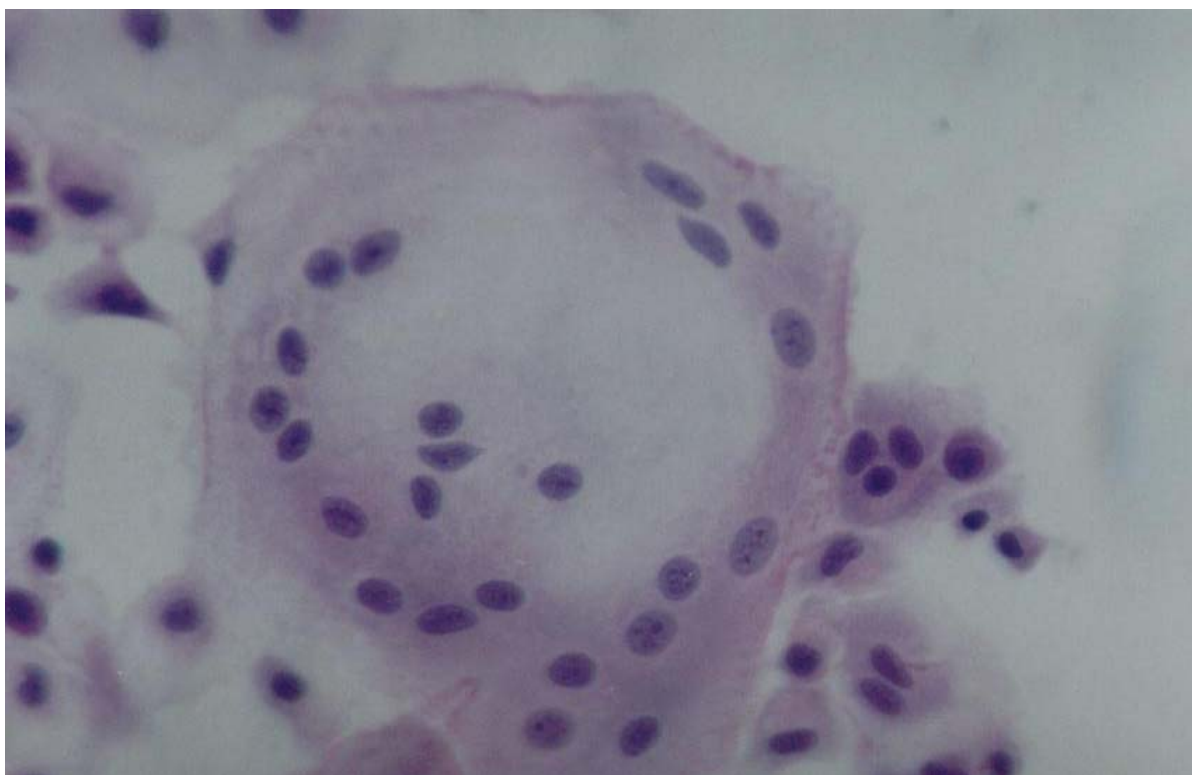


Figure 2. Polycarion macrophages with nuclei forming a crown-like figure recognized as Langhans giant cell seven days after the cover slip implant. HE x 1000

DISCUSSION

The results demonstrated that the round glass cover slip implant model in subcutaneous tissue of young pacu induced the formation of foreign-body granuloma and polycarion macrophages. These cells are foreign body type and are present in small number three days after implants. The Langhans giant cells were seen after seven days, and after 30 and 45 days a connective tissue capsule surrounded the glass cover slips. Thrombocytes, lymphocytes, plasma cells and

epithelioid cells were not observed. These results are similar to the ones observed in rats/mice in the pioneer studies carried out by RYAN and SPECTOR (1970) and MARIANO and SPECTOR (1974). These authors noticed that giant cells began to form four days after the cover slip implants and that their permanency depends on daily macrophage turnover. This turnover is more intense on the first days after inserting the cover slips and the giant cell lifespan is of approximately six to seven days. In a different way,

in the present research, the macrophage accumulation was less intense on the first three days after cover slip insertion and became more intense after seven days. Despite this difference in macrophage accumulation kinetics between *P. mesopotamicus* and mice/rats (RYAN and SPECTOR, 1970; MARIANO and SPECTOR, 1974), the formation of giant cells and their morphological evolution in *P. mesopotamicus* have similar patterns to those species throughout time. In other words, in both cases foreign body giant cells are formed at first after macrophage migration, and four days later they "grow" forming the first Langhans type giant cells that become the predominant cell type 14 days after cover slip implants were done. The Langhans cell type probably derives from foreign-body type cell and represents a more advanced/mature cell stage (MARIANO and SPECTOR, 1974). The significance and the determining factor(s) for cell fusion is not known (MARIANO, 1995) but it is dependent on the nature of the causal agent (BIRMAN and MARIANO, 1985).

In this study, it was observed the beginning of the formation of a connective tissue capsule surrounding the glass cover slips, presenting fibroblastic proliferation and rare mononuclear cells 30 days after they had been implanted. Forty five days after cover slip insertion, this structure presented fibrous and hyaline aspect, with collagen compound predominating. In mice, such structure began to form seven days after cover slip implant, and was observed as a light fibroblast proliferation and diffuse mononuclear cells proliferation (BIRMAN and MARIANO, 1985).

The glass cover slip insertion in birds *Gallus gallus domesticus* demonstrated that, differently from the observed in mice/rats and the observed in this study with *P. mesopotamicus*, polycarion macrophage form earlier. Giant cells are already observed 12 hours after cover slip insertion, and their formation is intensified after 18 hours, for birds have more facility to chronify lesions owing to the quick transformation of the macrophages into epithelioid cells (GRECCHI *et al.*, 1980).

When crustaceans are invaded by living or inert foreign bodies, the hemocytes react against them by means of phagocytosis and multicellular reaction, resulting in "encapsulation" or "nodule formation", which is structurally similar to the granulomas observed in mammals RATNER and VISON (1985).

Fishes take an intermediary filogenetic position if

compared with *Gallus gallus domesticus* and crustaceans. For this reason, we expected polycarion macrophage formation also from 12 hours after cover slip insertion. Otherwise, in times inferior to three days (12 and 24 hour) only fibrin, cellular debris and few neutrophils in a fibrin net were observed. There is possibly some evolutionary / physiological explanation for these characteristics of inflammatory reaction in *P. mesopotamicus*, and it brings new perspectives to the area of comparative pathology.

The polycarion macrophage individualization in glass cover slips is very clear and well noticed up to 14 days after it had been inserted. So, in studies where the count of such cells is necessary, periods of more than 14 days may represent technical impossibility.

Many studies of mammals and fish macrophages have focused on the isolation and culture of these cells *in vitro* including the giant cell formation. At this model the authors evaluated killing, phagocytosis, respiratory burst and enzymatic activities under different conditions and stimuli (COUSO *et al.*, 2002). However, it is inadequate when the evaluation of granulomatous response and/or giant cell formation *in vivo* is concerned because a lot of influences were blocked or stimulated. The fact that the macrophages fused spontaneously and formed giant cells (MCG) after a few days in culture (SECOMBES, 1985; 1990; MORIMOTO *et al.*, 1996) is inappropriate for a comparison with the *in vivo* situations. Little is known about the mechanisms that induce or prevent MCG formation *in vitro* (COUSO *et al.*, 2002).

The results of this research indicate that this experimental model is adequate and may be useful to study foreign-body granuloma and polycarion macrophage in fishes in different physiopathological situations *in vivo*. Different agents can be injected or inoculated in the subcutaneous pocket where the glass cover slip will be inserting and can induce different granulomatous responses (BIRMAN and MARIANO, 1985) and the fish organism can be submitted to different conditions like stress and others. In some situations, this model and the isolation *in vitro* of macrophages can be used together and complementarily.

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REFERENCES

- BIRMAN, E.G. and MARIANO, M. 1985 The influence of inflammatory agents on giant cell formation. *Braz. J. Med. Biol. Res.*, 18: 507-512.
- EIRAS, J.C. and REGO, J. 1989 Giant cell reaction associated with *Paulicea lutkeni* (Osteichyces, Pimelodidae) infection with *Jauela glandicephalus* (Cestoda, Protocephalidae). *Rev. Ibér. Parasitol.*, 49: 217-218.
- GILLMAN, T. and WRIGHT, L.J. 1966 Probable *in vivo* origin of multinucleate giant cells from circulating mononuclear. *Nature*, 209: 263-265.
- GOLTERMANN, H.L., CLYNO, R.S., OHNSTAD, M.A. 1978 *Methods for Physical and Chemical Analysis of Freshwater*. 2^o ED., Oxford: Blackwell Scientific, (JNP Handbook,8) 213 pp.
- GRECCHI, R., A. M SALIBA; MARIANO, M. 1980 Morphological changes, surface receptors and phagocytic potentials of fowls mono-nuclear phagocytes and trombocytes *in vivo* and *in vitro*. *J. Pathol.*, 130: 23-31.
- HOLMBERG, E.L. 1887 Viaje a Misiones. *Bol. Acad. Nac. Ci.*, 10: 22-387.
- MARIANO, M. and SPECTOR, W. G. 1974 The formation and properties of macrophage polycarions (inflammatory giant cells). *J. Pathol.*, 113:1-19.
- MATUSHIMA, E.R. 1994 *Avaliação do processo inflamatório crônico granulomatoso induzido experimentalmente através da inoculação de BCG em peixes da espécie Oreochromis niloticus – Tilápia do Nilo*. São Paulo, 1994. (Tese Doutorado. Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo).
- RAMAKRISHNA, N.R.; BURT, M.D. 1991 Tissue response of fish by larval *Pseudoterranova decipiens* (Nematoda; Ascaridoidea). *Can. J. Fish. Aquacult. Sci.*, 48: 1623-1628.
- RYAN, G.B. & SPECTOR, W.G. 1970 Macrophage turnover in inflamed connective tissues. *Proc. R. Soc.*, 175: 269-92.
- SIPAÚBA-TAVARES, L.H. 1995 *Limnologia Aplicada à Aqüicultura*. Jaboticabal: Funep, 70p.