Kinetics of the inflammatory reaction induced by carrageenin in the swimbladder of *Oreochromis niloticus* (Nile tilapia)

Cinética da reação inflamatória induzida pela carragenina na bexiga natatória de *Oreochromis niloticus* (Tilápia-do-Nilo)

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SUMMARY

The swimbladder, natural cavity of *Oreochromis niloticus* (Nile tilapia), weighing 100 to 150g, was used to study the carrageenin induced cell kinetics (n=42). Inoculation of 0.1ml of the irritant (0.5%) into the swimbladder determined an inflammatory reaction characterized by local congestion and edema of its wall and cell migration, mainly mononuclear, into the cavity. These phenomena began 3 hours following injection of the irritant and reached a peak as early as 24 hours. Light and electronic microscopies of the blood cells were undertaken to characterize the inflammatory cells that might migrate to the swimbladder. Thrombocytes, predominantly, migrated into the cavity, at different time intervals, after the inoculation of the irritant. Few macrophages, lymphocytes, granulocytes and other morphofunctionally undefined cells also took part in the exudate composition.

UNITERMS: Inflammation; Carrageenin; Fish.

INTRODUCTION

The most complete comparative study of the inflammatory process was written by Metchinikoff [20] (1968). This author, centering the inflammatory process on the phagocytic activity of macrophages and microphages, was the first to demonstrate that mononuclear cells of fish have phagocytic activity.

The study of the inflammatory process in fish did not advance much during this century [6,11,25,29,30]. Cellular elements of this process are not yet determined and many other doubts still exist. Little is known about the chemical mediation of process in this animal class, nor about the interference of the immune response and, maybe most important of all, the relationship between infectious agents and inflammation in the pathogeny of many infectious diseases waits for elucidation [7,29].

Facing the lack of knowledge in this research field, we decided to study the kinetics of the inflammatory reaction in fish using the swimbladder's natural cavity of *Oreochromis niloticus* as site for the noxious stimulus of the carrageenin.

MATERIAL AND METHOD

Animals

Male and female fish (n=100) of the species *Oreochromis niloticus* (Nile tilapia), weighing 80-100g, were used. The animals were kept in 50 l water tanks. Water was constantly aerated by compressor pumps connected to internal filtres, and maintained at 22°C ± 1°C of temperature. Fishes were fed with commercial balanced feed "ad libitum" during the whole experimental period.

Anesthesia

Animals were anesthetized by immersion in a benzocain solution (1:10.000) [9,32].

Induction of the inflammatory response

A solution of carrageenin (0.5%) (Marine Colloids, Illinois, USA) in buffered phosphate saline solution (PBS) containing calcium and magnesium was prepared immediately before use. With a tuberculin type needle (27G x 1.2"), 0.1ml of this solution was injected into the swimbladder of the anesthetized fish. To inject correctly into this natural cavity, the needle was introduced just below the lateral line of the animal.

Collecting of inflammatory cells

Animals were anesthetized and their spinal cord were sectioned. The inflammatory cells were harvested by the washing swimbladder cavity with 2.0ml PBS plus 0.03ml EDTA (5%) at 03, 06, 09, 12, 24, 48 and 72 hours after the carrageenin injection. The cells were maintained in the same solution and conserved in an ice bath until cytologic analysis.

Inflammatory cell cytology

The cell suspension was centrifuged at 800 rpm for 10 minutes in a clinical centrifuge, and 0.1ml of fish serum was then added to the sediment. The cells were resuspended, smeared and fixed
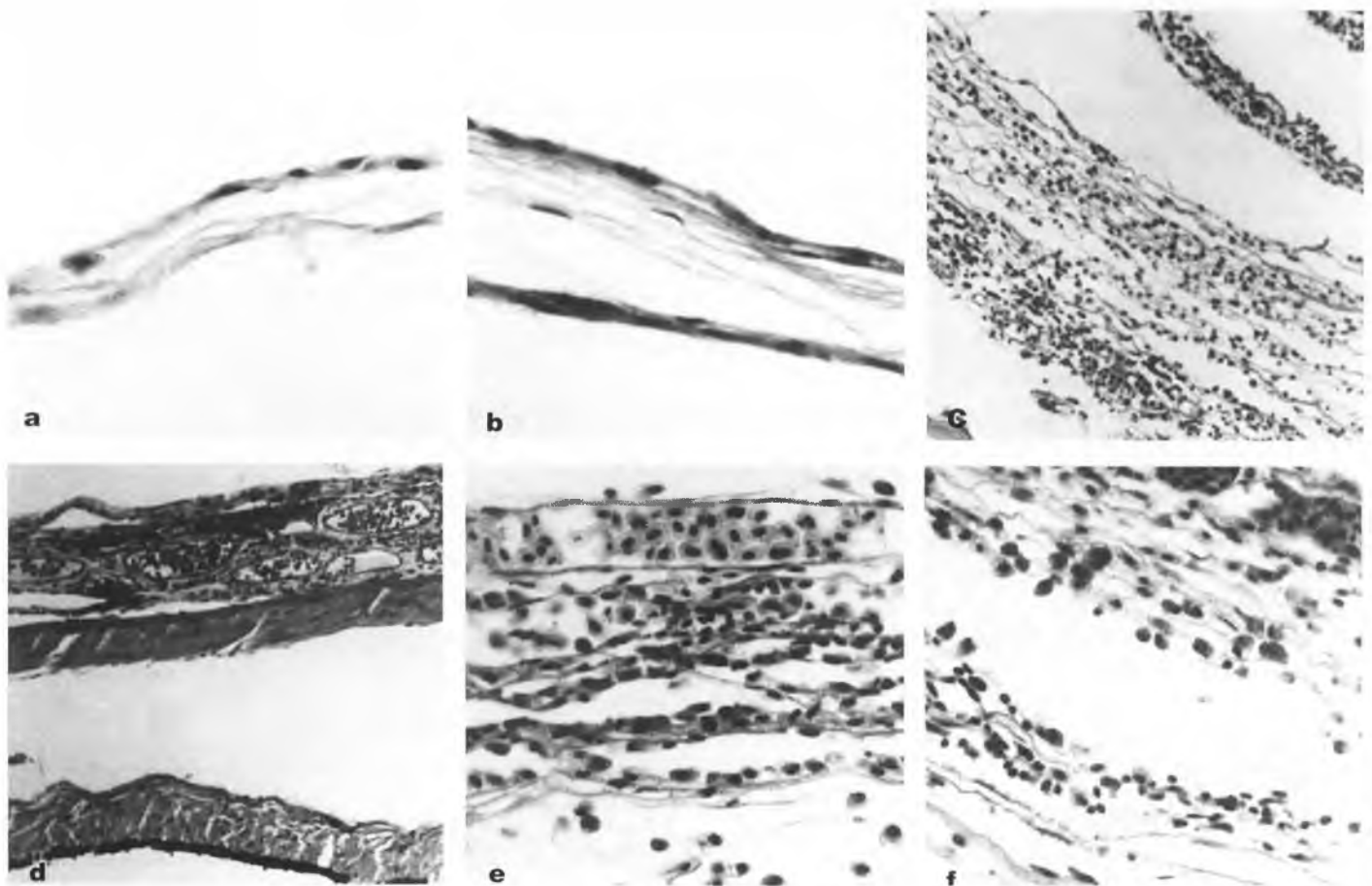
Figure 1

Photomicrograph of normal carrageenin inflammed swimbladder *O. niloticus*. Normal swimbladder wall showing the single layer of cells of the internal lining, and the subjacent delicate weakly bound connective tissue (a) and (b) H.E. stain, x500. Swimbladder wall 24 hours after injection of carrageenin, with intense diffuse inflammatory infiltrate (c); vascular congestion, (d); migration of mononuclear cells to the connective tissue (e) and (f) H.E. stain, x500.

in methanol. They were then stained with Giemsa and a differential count was done.

Morphological pattern of the inflammatory cells was obtained comparing them to the morphology of fish blood cells observed in blood smears stained similarly with Giemsa. Blood was obtained by puncture of caudal veins.

Electron microscopy

The swimbladders were washed with 1.0ml of a 2% glutaraldehyde solution diluted in Milloning buffer solution [22]. Cells were centrifuged and post-fixed in a 2% solution of osmium tetroxide. The pellets formed were embedded in Araldite [18]. Ultrafine sections were obtained and stained with uranil acetate [30] and lead citrate [33]. Sections were examined under an EM 201 Philips-Holland transmission electron microscope.

Normal cell morphology for comparison was obtained from blood collected from caudal vein and mixed with an equal volume of 2% glutaraldehyde solution in Milloning solution. The white portion of the clot was removed after centrifugation for 10 minutes

at 800 rpm and processed as described above.

Swimbladder histology

At various times after carrageenin injection into the swimbladder, the animals were anesthetized, bled, sectioned transversally, and sections of the anterior, medial and posterior region of the swimbladder were obtained. These fragments were fixed in Bouin's liquid 12 hours and then processed for inclusion in paraffin. Five urn sections, showing the whole circumference of the organ, were stained by the hematoxilin-eosin method (H.E.) and picrosirius red [16].

Adherence and spreading of inflammatory cells on glass surfaces

Cells were harvested from swimbladder 24 and 48 hours after the injection of the irritant. Cellular sediment obtained by centrifugation was then resuspended in PBS. The cellular suspension was placed on glass slides and kept inside plastic chambers for 1 hour at room temperature. The slides were then
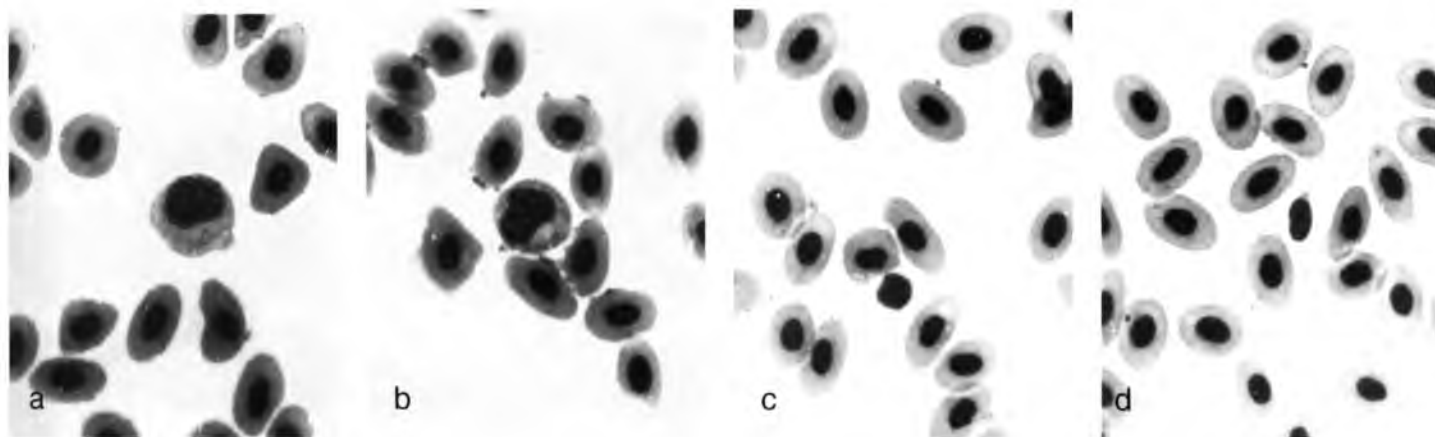
Figure 2

Photomicrograph of blood smears, showing nucleated erythrocytes, cells resembling monocytes (a; b), lymphocytes (c), and thrombocytes (d). Giemsa stain, x1250.

fixed with glutaraldehyde (2%) in Milloning buffer solution, and examined by phase-microscopy.

RESULTS

Swimbladder morphology

Gross aspects:

The Nile Tilapia's swimbladder is a hollow organ, extending from the cranial region to the base of the tail, in the animal sub-vertebral region.

Histology:

The organ is delimited internally by a single layer of flat cells resting upon a delicate basal membrane as made evident by the picrosirius red method. Beneath this membrane, a vasculature morphologically comparable to capillaries can be seen infiltrating a sparse and weakly bound connective tissue (Fig. 1a, 1b).

In sections from anterior and medial, but not from posterior portions of the swimbladder, projections of cells from the internal lining of the organ can be seen. These projections surround capillaries, venules and arterioles, resulting in a typical fish vascular network.

Histology of inflammed swimbladder:

Histological examination of the swimbladder 6 hours after the carrageenin injection evidenced a mild congestion of the capillary network located bellow the internal cellular lining of the organ, as well as of the vascular tissue similar to venules of slightly larger caliber. A discrete edema of the connective tissue that surrounded the microvasculative was also observed.

Twelve and twenty-four hours after the injection of the irritant, a marked inflammatory infiltrate with discrete areas of hemorrhage beneath the intern cellular lining of the organ, or in the intraluminal vascular projections, was observed. In this cellular infiltrate mononuclear cells, with small nuclei, condensed chromatin and sparse cytoplasm predominated. But cells with broad cytoplasm and replete with delicate eosinophilic granules
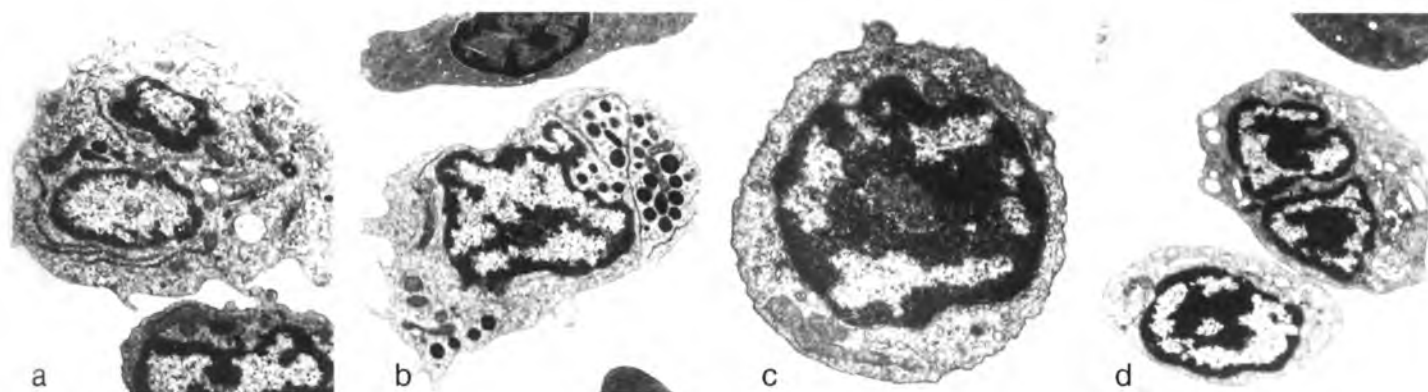

Figure 3

Electron micrograph of blood cells showing typical monocyte (a), x17,136; granulocyte (b), x15,936; lymphocyte (c), x17,472; and thrombocytes (d), x17,136.
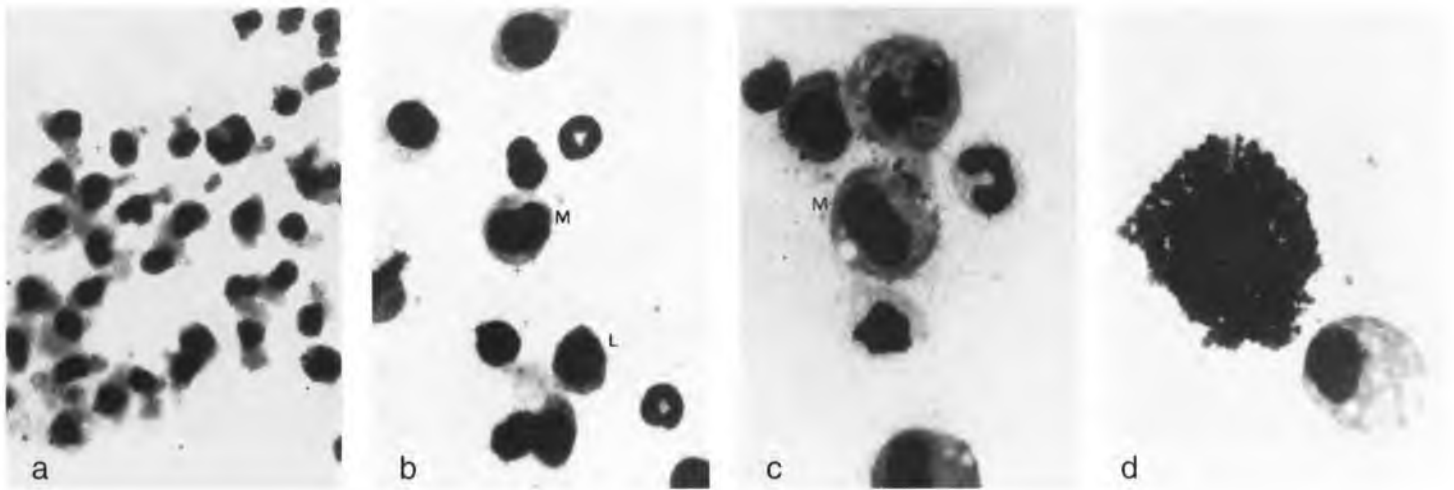
Figure 4

Photomicrograph of swimbladder inflammatory exudate smears. In (a), cells resembling thrombocytes can be seen. In (b) cells resembling macrophages (M), lymphocytes (L) and cells with ring nuclei can be seen. In (c) macrophages (M) and unidentified cells can be seen. Photomicrograph (d) shows a typical granulocyte and macrophage. Giemsa stain, x1250.

were also observed. At this time, perivascular connective tissue showed intense edema (Fig. 1c, 1d, 1e, 1f).

Blood and inflammatory cell morphology

Blood cells:

Based on the hypothesis that the inflammatory cell infiltrate of fish, like that of mammals, originates mainly from blood cells, a brief analysis of leukocytes of the animals used in this study was performed.

Optical microscopy observation of the blood smears evidence nucleated erythrocytes and cells resembling monocytes, granulocytes, lymphocytes and thrombocytes (Fig. 2a, 2b, 2c, 2d). These same cellular elements were identified by electron microscopy as well (Fig. 3a, 3b, 3c, 3d).

Inflammatory cells:

No cells were harvested when PBS was used to wash the swimbladder of normal fish. Cells of predominantly mononuclear type were observed 3 hours after injection of carrageenin.They were characterized as thrombocytes and macrophages based on comparisons with blood (Fig. 4a, 4b, 4c). These cells were present in the exudation up to 72 hours after injection. Cells resembling lymphocytes were rarely observed. Typical granulocytes were observed 12 hours after injection of the irritating (Fig. 4d). In relation to macrophages, ultrastructural analysis confirmed optical microscopy observations (Fig. 5a, 5b). Although phagocytic vacuoles were present, carrageenin was not seen inside them.

Other mononuclear cells showed a cytoplasmatic outline with few projections, some electron-dense granules in the cytoplasm, rare phagocytic vacuoles and chromatin similar in all respects to that of circulating thrombocytes; they did not, however, show the typical canalicular system of the latter. In most of these cells two

cytoplasmic areas of lighter coloration, without a clear membrane outline (Fig. 5c, 5d), were observed. No cells with typical granulocyte morphology were observed by electron microscopy.

Differential analysis of cells in the inflammatory exudate:

Differential analysis of cells that migrated into the swimbladder's lumen after the carrageenin injection is expressed in Tab. 1. A total count of 100 cells was attempted for each smear.

Results presented in Tab. 1 show that cells similar to thrombocytes predominate in the exudate. From the 3rd hour on, this was statistically significant in relation to other cell types. The proportion of cells similar to macrophages was small but constant throughout the experiment. Other cells, resembling lymphocytes and granulocytes, only appeared occasionally.

TABLE 1

Kinetics of inflammatory cells in the swimbladder's exudate of Nile tilapia. The results are expressed by means of differential count of inflammatory cells (n=42), São Paulo, 1988.

Time (hours) after carrageenin	Cell type		
	Thrombocytes	Macrophages	Others
Control	0	0	0
03	63	11	2
06	71	17	3
09	92	07	1
24	84	13	0
48	85	15	0
72	90	10	0

Inflammatory cell adherence and spreading on glass surfaces:

Inflammatory cells obtained 24 hours after injection of carrageenin adhere to glass slide surfaces at room temperature. Most of these cells, after 1 hour adherence, had a fusiform

shape with a single cytoplasmatic projection. Other ones had various numbers of these projections. Cells with small, ovoid nuclei, surrounded by cytoplasmatic projections that spread over the glass surface were also observed.

DISCUSSION

Initial studies of the inflammatory response in teleost fish date from the end of last century and beginning of this one [19,20,21]. Neither these studies, nor more recent ones [8,10,15,17,24,27,28,30], however, clearly established the nature of the cells involved in the inflammatory process, nor their kinetics when faced with different stimuli. The lack of this information is partly due to the inadequacy of the methods used to identify the inflammatory cells, and partly to the difficulty in establishing an experimental model that would permit the isolation of cells from the inflammatory exudate.

As results demonstrated here, the swimbladder proved to be adequate for this goal. The ease with which an irritant could be injected and inflammatory cells could be washed out of the organ, the lack of free resident cells in the organ's cavity, the lack of communication of its lumen with that of other compartments, and the possibility of precise histological examinations of its structures, offer ample opportunities for the study of the inflammatory reaction in this species.

Histological analysis, 6 hours after the injection of carrageenin into the swimbladder, revealed discrete edema and congestion of the subjacent microcirculation and basal membrane that lines the organ. As time went by, these manifestations increased in intensity. Twelve hours after the injection of the irritant, the extravascular connective tissue was invaded by cells similar to thrombocytes, but granulocytes were scarce. The same response could be seen in the vascular network that projects into the organ. The method did not permit the characterization of the route used by cells to migrate from the tissues to the organ's lumen. Probably this phenomenon

occurred mainly via the vascular network projecting into the lumen of the bladder.

Washing the swimbladder's cavity enabled to harvest inflammatory cells at different times after injection of the irritant. Ultrastructural analysis of these cells revealed based on morphological standards of blood cells, the predominance of thrombocytes and, in smaller quantities, macrophages and granulocytes. It was interesting to observe that thrombocytes detected in the exudate showed a greater quantity of electron-dense granules and a less evident canalicular network. A similar phenomenon was observed by Suzuki [27] (1984), who observed that thrombocytes lose their canalicular system after phagocytosis of *E. coli*. Morphological plasticity of thrombocytes was clearly shown by Greecchi et al.[14] (1980), who demonstrated marked morphofunctional changes in chicken thrombocytes cultivated "in vitro". The most evident change was the acquisition of receptors for Ig Y (Ig G), when cells were kept in culture for different periods. Another feature that characterizes thrombocytes in the exudate is the fact that, similarly to what occurs with chicken thrombocytes [14], fish thrombocytes adhere and spread over glass surfaces. Further studies are needed for the evaluation of other functions of these cells, such as phagocytic and secretory capacity.

Comparing the morphology of mononuclear cells from the blood, it was possible to characterize and quantify the cells in the exudate induced by carrageenin in the swimbladder using optical microscopy. The differential analysis of the exudate showed that by the 3rd hour after injection, about 60% of cells could be characterized as thrombocytes; this proportion remained throughout the experiment. Macrophages and other cell numbers (granulocytes and lymphocytes) remained low, close to 10%. The predominance of thrombocytes in the inflammatory exudate induced by carrageenin in the Nile tilapia resembles to that which occurs in the chicken [14] and in the amphibian.

Thrombocytes present in the blood of chicken [1,2,3,13,14,26], fish [3,8,11,27] and amphibian[5] are considered cells with functions similar to
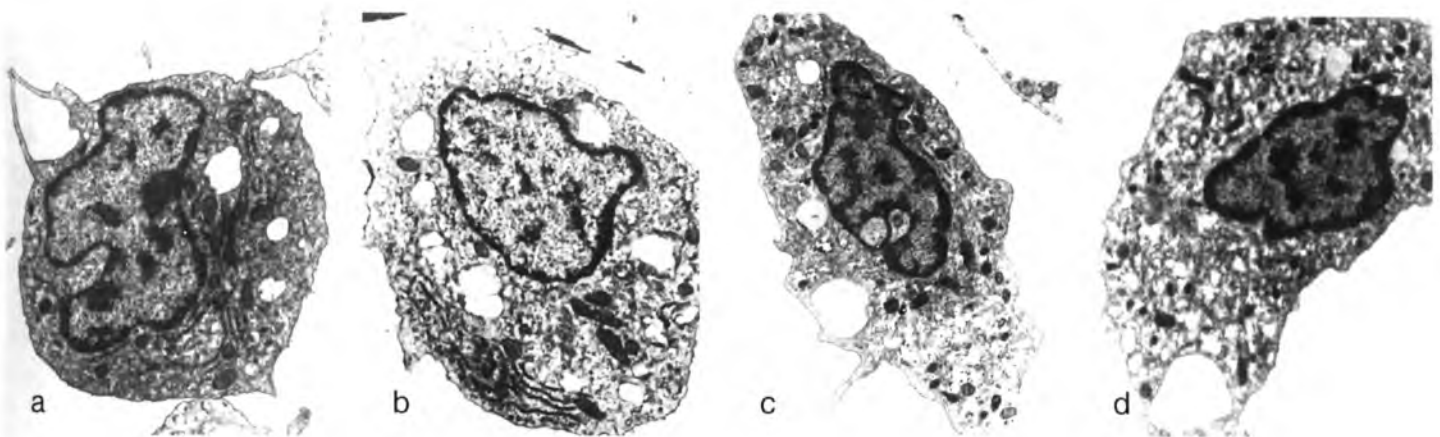

Figure 5

Electron micrograph of mononuclear cells from the inflammatory exudate. Typical macrophages are observed in (a), x16,608, and (b), x15,792. In (c), x16,608, and (d), x16,128, cells with morphology similar to that of thrombocytes can be

those of platelets in mammals[12]. The strong participation of these cells in the inflammatory exudate of chicken[14] and as demonstrated here, of fish, opens up questions concerning the phylogenetics of these cells, mainly because in mammals the participation of platelets in the inflammatory process is not yet totally clarified[23,29]. Contrary to what occurs in mammals, granulocytes and macrophages have a reduced numerical participation in acute inflammatory exudate in fish, as showed here.

RESUMO

A cavidade natural da bexiga natatória de *Oreochromis niloticus* (Tilápia-do-Nilo), pesando entre 100 e 150g, foi utilizada para o estudo da cinética celular inflamatória induzida pela carragenina (n=42). A injeção de 0,1ml do irritante (0,5%) na luz da bexiga natatória determinou um processo inflamatório caracterizado por congestão e edema de sua parede e migração de células, predominantemente mononucleares, para a cavidade. Este fenômeno teve início 3 horas após a injeção do irritante, atingindo um máximo às 24 horas. Para caracterizar as células inflamatórias que migraram para a cavidade do órgão, foi realizada uma análise das células sanguíneas desses animais em nível de microscopia de luz comum e eletrônica. Pode-se demonstrar que as células que migraram para a luz da bexiga natatória após diferentes tempos da injeção do irritante eram predominantemente trombócitos. Poucos macrófagos, linfócitos, granulócitos e outras células não caracterizadas morfofuncionalmente também faziam parte do exsudato.

UNITERMOS: Inflamação; Carragenina; Peixes.

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