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India ink induces apoptosis in the yellow clam *Mesodesma mactroides* (Deshayes, 1854). Optical and ultrastructural study

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ABSTRACT

This paper reports on the acute inflammatory and cellular process in the yellow clam, *Mesodesma mactroides*, induced by injection of India ink into the muscular foot. Histological observations with optical and electronic microscopy were made at 24 and 48 h after injection. The induced cellular inflammatory response consisted of a general hemocyte infiltration without necrosis and apoptotic activity. Migration of ink-laden phagocytes across the intestinal epithelium was recorded. It appeared that the yellow clam "excreted" ink particles through the gill and kidney. The positive staining for apoptosis was observed in the digestive gland. Electronic microscopy revealed ultrastructural changes of endoplasmic reticulum stress and apoptotic bodies in the digestive gland. The mechanism by which the India ink particles induce apoptosis remains unknown, but might possibly be associated with the endoplasmic reticulum stress. This work has highlighted features that require further discussion in the restricted field the inflammatory responses of mollusks.

Key words: India ink, inflammation process, endoplasmic reticulum stress, apoptosis, *Mesodesma mactroides*.

INTRODUCTION

Interest in the immunity of bivalves has been increasing continuously over last decades due to the catastrophic mortalitiy in aquaculture-produced species and due to the decline of natural stocks of economic value (Song et al. 2010).

For decades, the yellow clam *Mesodesma mactroides* (Deshayes 1854) (Mesodesmatidae) was the major shellfish resource from the sandy beaches of the Atlantic coast of Argentina, Uruguay and the southern part of Brazil (Castilla and Defeo 2001). However, a combination of overexploitation and high mortality rates along the entire distribution range of the species culminated in the collapse of these natural stocks (Fiori and Cazzaniga 1999).

In recent years, there has been great emphasis on the study of parasites of the yellow clam and the resulting pathologies (Cremonte and Figueras 2004, Carvalho et al. 2013a, b). However, basic pathological processes, such as inflammation, have been generally overlooked.

Classic studies using injected dyes have greatly helped investigators describe the phagocytic cells and their role in local and general inflammatory processes in oysters (Pauley and Sparks 1966).

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Essentially all animal cells have the ability to undergo apoptosis by activating an intrinsic cell suicide program, when they are no longer needed, or have become seriously damaged. The execution of this program leads to a morphologically distinct form of cell death, termed apoptosis (Kerr et al. 1972, Wyllie et al. 1980). It is now generally accepted that apoptosis is of crucial importance for the development and homeostasis of metazoan animals (Bergmann et al. 1998).

Apoptosis in mollusks is involved in the larval development process (Gifondorwa and Leise 2006) and most likely constitutes an important immune response that can be initiated by different inducers (Terahara and Takahashi 2008).

The endoplasmic reticulum (ER) is a centrally located, multifunctional, and multiprocess intracellular organelle supporting many mechanisms required by virtually every cell (Groenendyk et al. 2010). The membrane performs a remarkable number of diverse functions, including protein synthesis, translocation across the membrane, integration into the membrane, folding, posttranslational modification (including *N*-linked glycosylation, and synthesis of phospholipids and steroids on the cytoplasmic side of the ER membrane, and regulation of Ca^{2+} homeostasis). Development and maintenance of optimally functioning ER membrane is essential for virtually all cellular activities, from intracellular signaling to control of transcriptional pathways; from ion fluxes to control of energy metabolism; from protein synthesis to multisubunit assembly; and from lipid synthesis to transcriptional regulation of steroid metabolism. One of the major advantages of the centrally located ER network for the cell is the ability to control the composition and the dynamics of the ER luminal environment in an extracellular environment- independent way. Furthermore, the ER is not an isolated organelle because it has developed sophisticated mechanisms of communication with many other cellular compartments, especially the mitochondria, the plasma membrane, and the nucleus (Anelli and Sitia 2008, Frischauf et al. 2008).

ER stress conditions in mammals and humans have been observed in numerous diseases including Alzheimer disease, Creutzfeldt–Jakob disease, Huntington disease as well as cardiovascular diseases, indicating that ER stress- induced apoptosis is an important factor in pathophysiological conditions (Boya et al. 2002).

The purpose of the present study was to describe the inflammatory and celular responses experimentally induced after injection of a foreign body (India ink) into the muscular foot of the yellow clam *M. mactroides*

MATERIALS AND METHODS

All experimental protocols were approved by the Federal University of Rio Grande (FURG) Animal Care Committee.

Clams Husbandry

Specimens of yellow clam (n = 60) were collected in December 2012 at the intertidal zone of the Cassino Beach, (32˚19'54.2" S 052˚17'20.4" W), Brazil, by excavating the sand with a shovel. Specimens measured 40-60 mm in shell height (mean = 47 mm). Once in the laboratory, the clams were maintained for one day in tanks filled with 50 L of seawater, which was continuously aerated (temperature = 23 °C, salinity = 33 ppt), and were fed with the microalgae *Nannochloropsis occulata*.

Experimental Design

Prior to injection, the clams were anesthetized with a benzocaine solution (250 mg/L). Using a 25 gauge needle fitted to a syringe, approximately 0.05 ml of a suspension of Indian ink (Royal Talens, Holland) or filtered seawater (30 clams for each group) was injected into the muscular foot of the *M. mactroides*,

Following the injections, the clams of each group were placed back in separeted tanks with 50 L of aerated seawater until the appropriate period of time had elapsed. Water exchange, tank cleaning and microalgae were provided daily. Ten clams were analyzed at 24 and 48 h after the injection.

Sample Processing

Optical microscopy

Clams were shucked and the meat fixed in 20% buffered formalin for 24 h. Sections that were approximately 5 mm thick, including the mantle, gills, gonad, digestive gland, kidney, and foot were then taken from each specimen. Tissue samples were embedded in Paraplast ® (Sigma, St. Louis, MO, USA) and 5 μm sections were stained with hematoxylin and eosin (H&E).

Ultrastructural analysis

For electron microscopy small fragments of the mantle, gills, gonad, digestive gland, kidney, and foot were cut into 1 mm blocks and immediately fixed in phosphate buffered glutaraldehyde (pH 6.9 at 4 °C), washed in Millonig's solution and post-fixed in 1% osmium tetroxide; the tissue blocks were then dehydrated in a graded series of ethanol-acetone, immersed in propylene oxide and embedded in Durcupan ACNI (Fluka Chemie A.G., Switzerland). Ultrathin sections were cut with a LKH ultramicrotome and doublestained with uranyl acetate and lead citrate before examination in a Jeol JEM-8T electron microscope (Jeol, 32, Tokyo, Japan).

Apoptosis Analysis

Detection of apoptosis was performed in several tissues of both animals injected with Indian ink and control group animals injected with physiological solution. These samples were fixed in 20% buffered formalin and embedded in Paraplast®. For apoptosis detection, terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labelling (TUNEL) was performed according to the manufacturer's recommendations by using the ApogTag plus Peroxidase *in situ* Apoptosis Detection Kit (S7101; Chemicon, International). Anti-cleaved caspase-3 immunostaining was assessed using a rabbit anti-cleaved caspase-3 polyclonal antibody (Asp175; Cell Signalling Technology, Danvers, MA) as previously described (Schoner et al. 2010)

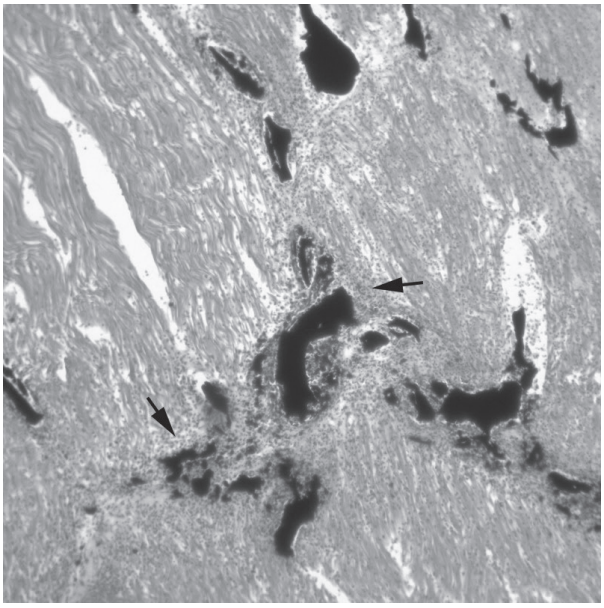

Figure 1 - Muscle tissue, 24 hours after the ink injection, with clumps of India ink granules surrounded by massive infiltration of hemocytes (arrow). H&E staining. (See the colors in the online version).

RESULTS

All of the experimental clams survived until the end of the trial.

Optical Microscopy

Within 24 h after the India ink injection, a massive migration of hemocytes to the location of the injection at the muscular foot was observed. Clumps of India ink granules were seen to be encapsulated, and small granules were phagocytosed and surrounded by inflammatory infiltrate (Fig. 1).

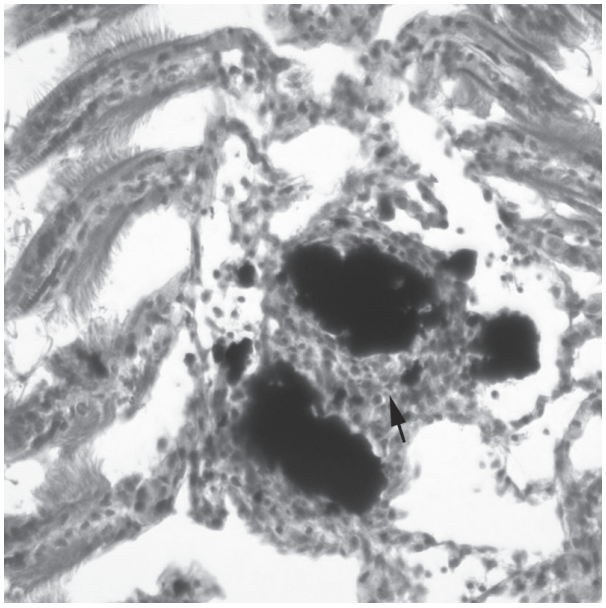

Figure 2 - Gill, 48 hours after the ink injection, with India ink encapsulated and surrounded by inflammatory infiltrate (arrow). H&E staining. (See the colors in the online version).

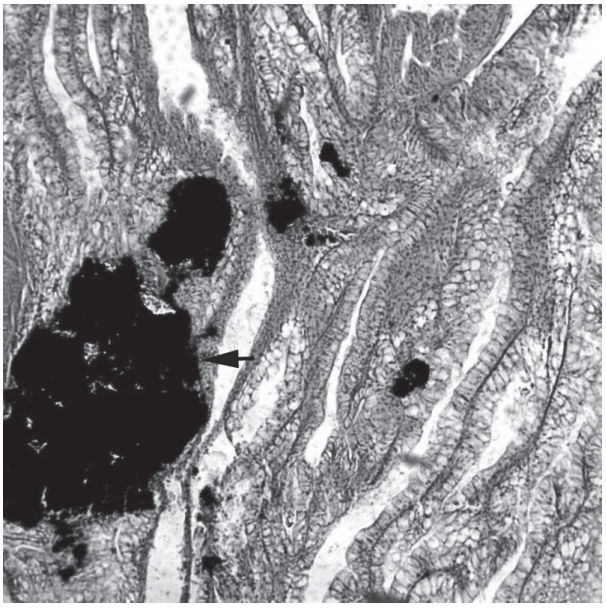

Figure 3 - Kidney, 48 hours after the ink injection, with India ink between the tubular structures (arrow). H&E staining. (See the colors in the online version).

Within 48 h after the India ink injection, gross examination of the experimental clams revealed that the India ink had spread to all parts of the body. Clumps of India ink accumulated in the gills, where they caused severe inflammation with massive infiltration of hemocytes, obstruction of the vessels and hemorrhage (Fig. 2). Other organs in which the ink particles accumulated were the kidney and the digestive tract (Fig. 3).

Evaluation of the Apoptotic Process by Optical Microscopy

Forty-eight hours after injection of Indian ink into the muscular foot of *M. mactroides*, it was possible to observe the presence of apoptotic cells in digestive gland tubules and muscle cells. Apoptosis was not observed in animals that were not injected with India ink. Tissues were counterstained with hematoxylin and eosin to aid in the morphologic evaluation of normal and apoptotic cells (Fig. 4).

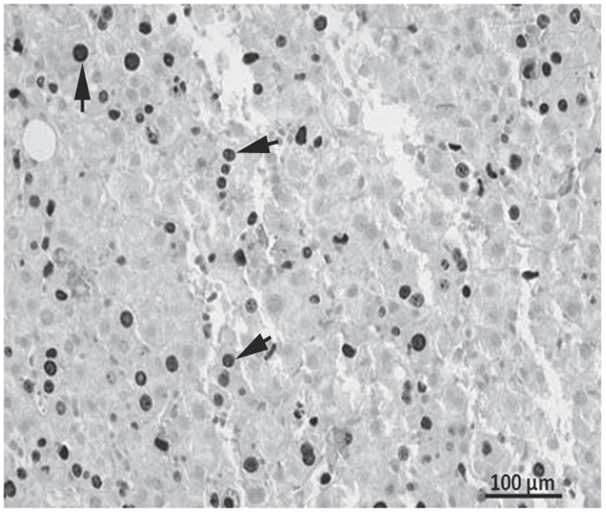

Figure 4 - Presence of apoptotic cells (arrow) in digestive gland tubules, 24 hours after the ink injection. (See the colors in the online version).

Electronic Microscopy

Within 24 h after the injection of the India ink, gill and kidney cells displayed various abnormalities, with the digestive gland being the most affected. The shape of the nucleus was often irregular. In some cells the mitochondria were swollen, these

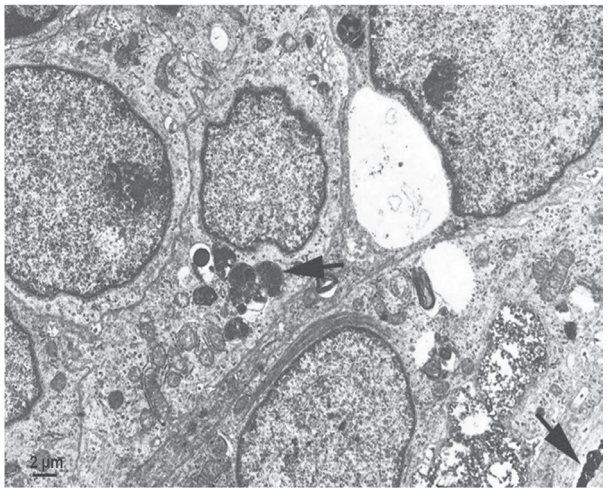

Figure 5 - Low magnification of kidney tissue, 24 hours after the ink injection. Cells display numerous cytoplasmic osmiophilic lamellar structures. (arrows).

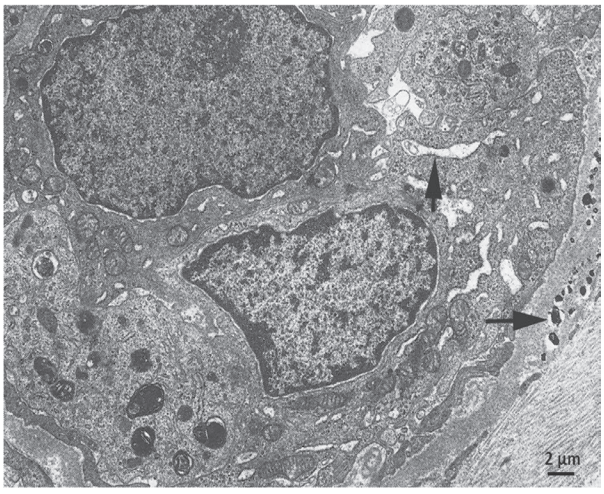

Figure 6 - Gill cell, 48 hours after the ink injection, showing dilated endoplasmic reticulum (ER) (short arrow) and osmiophilic and electrodense lamellar structures (long arrow), which may correspond to India ink. M: mitochondria.

cells were empty or contained osmiophilic lamellar structures that were suggestive of india ink with a variable density. Forty-eight h after the India ink injection, similar condensed cell fragments containing fragmented nuclear masses were found in the voluminous membrane bound vacuoles in the cytoplasm of cells. The basement membrane appeared fragmented and had disappeared in some areas. When present, it was not closely apposed to the cytoplasmic membrane of the kidney cells, which is the normal configuration. Granular hemocytes were most frequently detected in contact with epithelial cells and presented a rather regular, round or oval nucleus. Their cytoplasm was usually pale and contained scarce isolated ribosomes and a few mitochondria. Furthermore, these cells usually lacked a well developed endoplasmic reticulum and a Golgi apparatus, and one or several lysosomal vesicles of variable size were frequently observed. The abundance of inflammatory cells in the gill and kidney tissue, which was represented by osmiophilic lamellar or granular electron dense material, was often associated with india ink. Fibroblasts predominated in the fibrotic areas, and a basement membrane-like substance was frequently in contact with their cell surfaces. Some fibroblasts showed morphologic signs of great synthetic activity, as evidenced by cytoplasm containing numerous cisternae of rough ER that was dilated and filled with material of medium electron density. Within 24 h after the India ink injection, apoptotic bodies were found in the muscle cells (Figs. 5, 6, 7, 8, 9).

DISCUSSION

Bivalve hemocytes are primarily responsible for the defense against pathogens and foreign particles (Ottaviani et al. 2010). Phagocytosis and encapsulation are two major mechanisms used by hemocytes to eliminate nonself substances and dead cells (De Vico and Carela 2012). Phagocytosis is a process by which nonself molecules and cell debris are recognized and ingested, while encapsulation is the cellular immune defense reaction against foreign bodies that are too large to be phagocytosed (De Vico and Carela 2012).

The inflammatory response that occurs after pathogen invasion and/or cellular injury is a local defense reaction from the host tissue (Cone 2001).

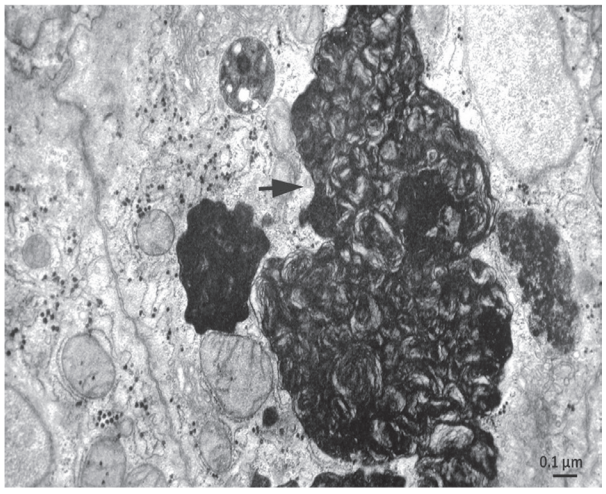

Figure 7 - Higher magnification of figure 5, osmiophilic and electrodense lamellar structures (arrows), which may correspond to India ink.

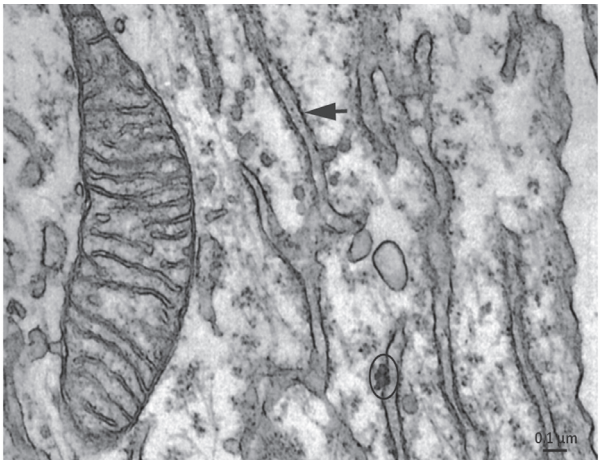

Figure 8 - Higher magnification of digestive gland cell which thickening of the membrane of the endoplasmic reticulum (arrow) , dilated cisterns, ribosome dissociation and electron-subjects grouping the outside of the membrane (0).

The functional basis of the inflammatory response has not been substantially modified during evolution, and the basic pattern is quite similar, regardless of the nature of the injurious agent, the site of its occurrence, or the injured organism (Ottaviani et al. 2010).

According to the manufacturer, Royal Talens, the carbon black pigment in Indian Ink is extremely fine, less than 2 µm. These particles are in constant motion in the ink, continually bumping into one another. The special preparation method of the ink ensures that they do not form lumps. If a large amount of water is added too quickly, however, the binder (shellac) that surrounds the particles dissolves. The particles then clot to form larger particles and sink to the bottom.

The strong hemocyte infiltration around the site of India ink was probably due to chemotaxis on the part of these cells in response to a release of chemicals from the necrotic muscle and connective tissue cells in the area. Phagocytosis of India ink by individual phagocytic cells in *M. mactroides* is similar to the response observed in other mollusks (Pauley and Krassner 1972).

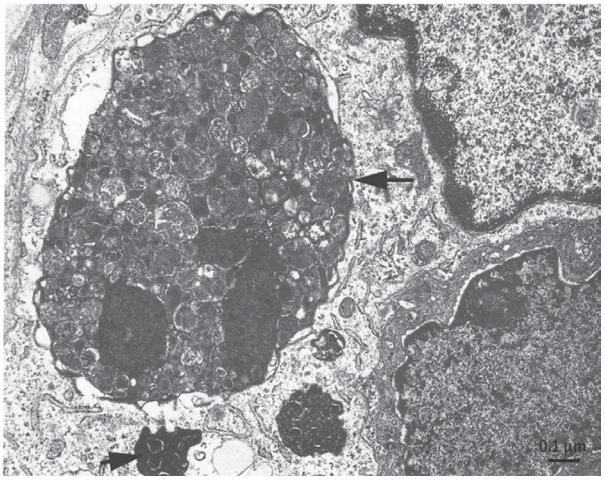

Figure 9 - Digestive gland cell, 48 hours after the ink injection, with a voluminous cytoplasmic membrane bound vacuole containing an apoptotic body (long arrow) and electrodense structures (short arrow), which may correspond to India ink.

Soluble substances are eliminated primarily through the gill, kidney, and intestine. In vertebrates, insoluble particulate material, such as carbon, is sequestered in the liver, spleen, lungs, and lymph nodes, all of which are part of the mononuclear phagocyte system (MPS) (Hirsch and Fedorko 1970). Disposal of particulate material

is very slow, except in the lung MPS cells, and may persist for many years. The main pathway of elimination in invertebrates is either the intestine or the renal-pericardial complex. According to Pauley and Krassner (1972), the intestine and the kidney play an important role in the elimination of ink particles from the sea hare *Aplysia californica*. The fact that ink was injected intramuscularly in the foot muscles may account for this result.

The electron microscopic study focused on the abnormalities in the gill, kidney and muscle tissue. A constant feature was the presence of conspicuous necrosis of gill cells and several electrolucent areas of irregular size that did not have apparent osmiophilic material and were not limited by a membrane, which seem to correspond to India ink in the gill, kidney, muscle, and intestinal cells.

Abnormalities in the gill and in the kidney epitelial basement membrane were also observed. These included the presence of areas of rarefaction containing osmiophilic inclusions in a basement membrane that was otherwise thickened or multilayered. The significance of these basement membrane abnormalities is unknown. However, the suggestion has been made that they result from the deposition of India ink (Cotran 1965).

This is the first electron microscopy study of invertebrates treated with India ink. In vertebrates, however, a few studies have been performed that were based on electron microscopy. Cotran (1965) studied endothelial cells of rats and mice "overloaded" with India ink, and the vascular endothelium was examined by electron microscopy for evidence of phagocytosis. Phagocytosis of carbon was demonstrated in the endothelium of small myocardial vessels, the endocardium, the pulmonary capillary endothelium, the aorta, and the glomerular and peritubular capillary endothelia. Some carbon remained in the endothelium of the heart vessels for at least 7 days after overloading. Carbon particles were also present in circulating mononuclear cells and in perivascular phagocytes.

Apoptosis is a process with typical morphological signatures, including plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation (Kerr et al. 1972, Wyllie et al. 1980). A family of cystein-dependent aspartate-directed proteases, called caspases, is responsible for the proteolytic cleavage of cellular proteins that contribute to the characteristic apoptotic features, such as the cleavage of caspase-activated DNase that results in internucleosomal DNA fragmentation. Two pathways for activating caspases have been studied in detail. One pathway starts with ligation of a death ligand to its transmembrane death receptor, followed by recruitment and activation of caspases in the death-inducing signalling complex. The other pathway involves the participation of mitochondria, which release caspase-activating proteins into the cytosol, thereby forming the apoptosome, where caspases will bind and become activated.

It is well established that prolonged ER stress can lead to cell apoptosis. Several novel pathways have been identified that can offer explanations on how cells trigger programmed cell death when faced with irreparable damages that cannot be rescued by the unfolded protein response. Despite these important discoveries, the *in vivo* molecular mechanisms underlying ER stress induced apoptosis are just emerging. Furthermore, it is unclear whether observations derived from specialized cell lines reflect tissue-specific or general mechanisms and whether results from *in vitro* reconstitution assay systems apply to endogenous cellular mechanisms (Li et al. 2006).

The efficient functioning of the ER is essential for most cellular activities and for survival. Conditions that interfere with ER function lead to the accumulation and aggregation of unfolded proteins. If the stress is prolonged, or the adaptive response fails, apoptotic cell death ensues. In mammals and humans, many studies have focused on how this failure initiates apoptosis, as ER stress-induced

apoptosis is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases (Szegezdi et al. 2006). In this study, mollusks treated with India ink showed apoptosis, whereas those treated with a physiological solution did not. The cause of apoptosis in this context is still unknown; however, India ink accumulation within the cells may be related to ER stress.

Bordem (2012) showed ultrastructural changes of ER stress, such as thickening of the membrane of the endoplasmic reticulum, dilated cisterns, ribosome dissociation and electron-subjects grouping the outside of the membrane. Some of these findings were observed herein, in cells studied with electron microscopy.

Previous studies have shown that bivalve hemocytes synthesize and secrete catecholamines including noradrenaline and dopamine (Ottaviani and Franceschi 1996). However, noradrenaline has the capacity of inducing apoptosis of oyster hemocytes (Lacoste et al. 2002) and norepinephrine induces endoplasmic ER stress in PC12 cells. These three factors (apoptosis, norepinephrine and ER stress) could be related with a foreign body in mollusks.

CONCLUSION

In conclusion, India ink proved to be a useful tool for experimental model for inflammation in mollusks. Lastly, note that India ink iduced apoptosis, and the cause of this phenomenon is still unknown and the association of apoptosis and ER in mollusks requires further studies.

ACKNOWLEDGMENTS

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RESUMO

Este artigo reporta o processo inflamatório agudo e celular no marisco branco *Mesodesma mactroides*, induzido através de injeção de tinta nanquim no pé muscular. Obervações histológicas com microscopia óptica e eletrônica foram realizadas 24 e 48 h após a injeção. A resposta celular inflamatória induzida consistiu de infiltração hemocitária geral sem ocorrência de necrose e atividade apoptótica. Migração de fagócitos carregados de tinta nanquim através do epitélio intestinal foi registrada. Parece que o marisco branco excretou partículas de nanquim pelas brânquias e rim. A tinção positiva para apoptose foi observada na glândula digestiva. Microscopia eletrônica da glândula digestiva revelou mudanças ultraestruturais de estresse do retículo endoplasmático e corpos apoptóticos. O mecanismo pelo qual as partículas de tinta nanquim induzem apoptose continua desconhecido, possivelmente associado ao estresse do retículo endoplasmático. Este trabalho destacou características que exigem discussões na área restrita das respostas inflamatórias de moluscos.

Palavras-chave: tinta nanquim, processo inflamatório, estresse do retículo endoplasmático, apoptose, *Mesodesma mactroides*.

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