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Research Report

The effects of okadaic acid on *Enchytraeus crypticus* (Annelida: Oligochaeta)

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Abstract

We describe the morpho-functional effects of different concentrations of okadaic acid (OA) on specimens of *Enchytraeus crypticus*. The results demonstrate that this experimental model is very sensitive to the treatment and presents time- and dose-related effects mainly involving an immune response associated with a reaction in the chloragogenous tissue. At the lower dose (100 nM), the main organs do not appear particularly affected except for a swelling of the coelomatic cavity and an increased number of circulating coelomocytes. At the higher dose (200 nM), the chloragogenous tissue extends in volume to occupy the body cavity almost completely, while the circulating amoebocytes and chloragocytic cells undergo conformational changes. At the highest OA dose (400 nM), there is a general cell suffering in the main animal organs. In control animals, the immunocytochemical reaction with anti-IL-6 antibody is positive in neuron cell bodies and fibres from the ventral nerve cord and in circulating amoebocytes. Following OA treatment, fewer immunoreactive cells are seen in the damaged nervous tissue, and the high number of recruited amoebocytes is also positive.

Key words: Annelid; *Enchytraeus crypticus*; okadaic acid; IL-6-like molecules

Introduction

Okadaic acid (OA) is produced by toxigenic dinoflagellates from the *Dinophysis* and *Prorocentrum* genera and is involved in fish death, diarrhetic shellfish poisoning and, consequently, human intoxication (Yasumoto *et al.,* 1978, 1985). The molecular mechanisms responsible of the various biological actions attributed to this toxin involve the specific inhibition of serine/threonine protein phosphatases 1 and 2A (Bialojan and Takai, 1988) which are critical components in signalling cascades regulating a variety of cellular processes in eukariotic cells. *In vivo* studies using mice as experimental models have described the distribution and excretion of OA following oral administration, as well as the morpho-functional modifications of toxin target organs (Edebo *et al.*, 1988; Ito and Terao, 1994; Ito *et al.*, 2002; Franchini *et al.*, 2005). The small intestine was particularly affected, with an edema in the lamina propria of villi and desquamation of degenerated epithelium, while the liver accumulated a significant amount of toxin, but was not damaged (Ito and Terao, 1994; Ito *et al.*, 2002). The mouse thymus was also particularly affected, with a severe alteration in structural architecture and a significant depletion in lymphoid elements. The ability of OA to provoke both immunostimulation and systemic immunotoxicity has also been highlighted (Franchini *et al.*, 2005). OA is a well known inducer of proinflammatory responses (Stanley *et al.*, 2001) and a stimulator of inflammatory cytokine gene transcription in murine macrophages (Tebo and Hamilton, 1994).

In the present investigation, the effects of OA on the structural organization of a species of enchytraeids are evaluated.

The enchytraeids have been widely used for many years in ecotoxicological laboratory tests, in view of their keystone ecological status and the fact that they can serve as indicator organisms for chemical stress (Didden and Rombke, 2001).

We have also examined the induced modifications for the presence and distribution of IL-6-like molecules. IL-6 is a multifunctional cytokine able to modulate a variety of physiological events that play a main role in the immune system and inflammation.

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Materials and Methods

Adult specimens of the worm *Enchytraeus crypticus* (Annelida, Oligochaeta, Enchytraeidae) were grown on agar medium for 1 month before the experimental procedure. The animals were cultured in 90 mm Petri dishes containing 1.1 % agar powder dissolved in distilled water and maintained at a temperature of 23 °C with a photoperiod of 8 h light and 16 h dark. The worms were fed with sterilized powdered rolled oats and water twice a week, checked daily and subcultured by transfer onto a new agar plate once every 10-14 days.

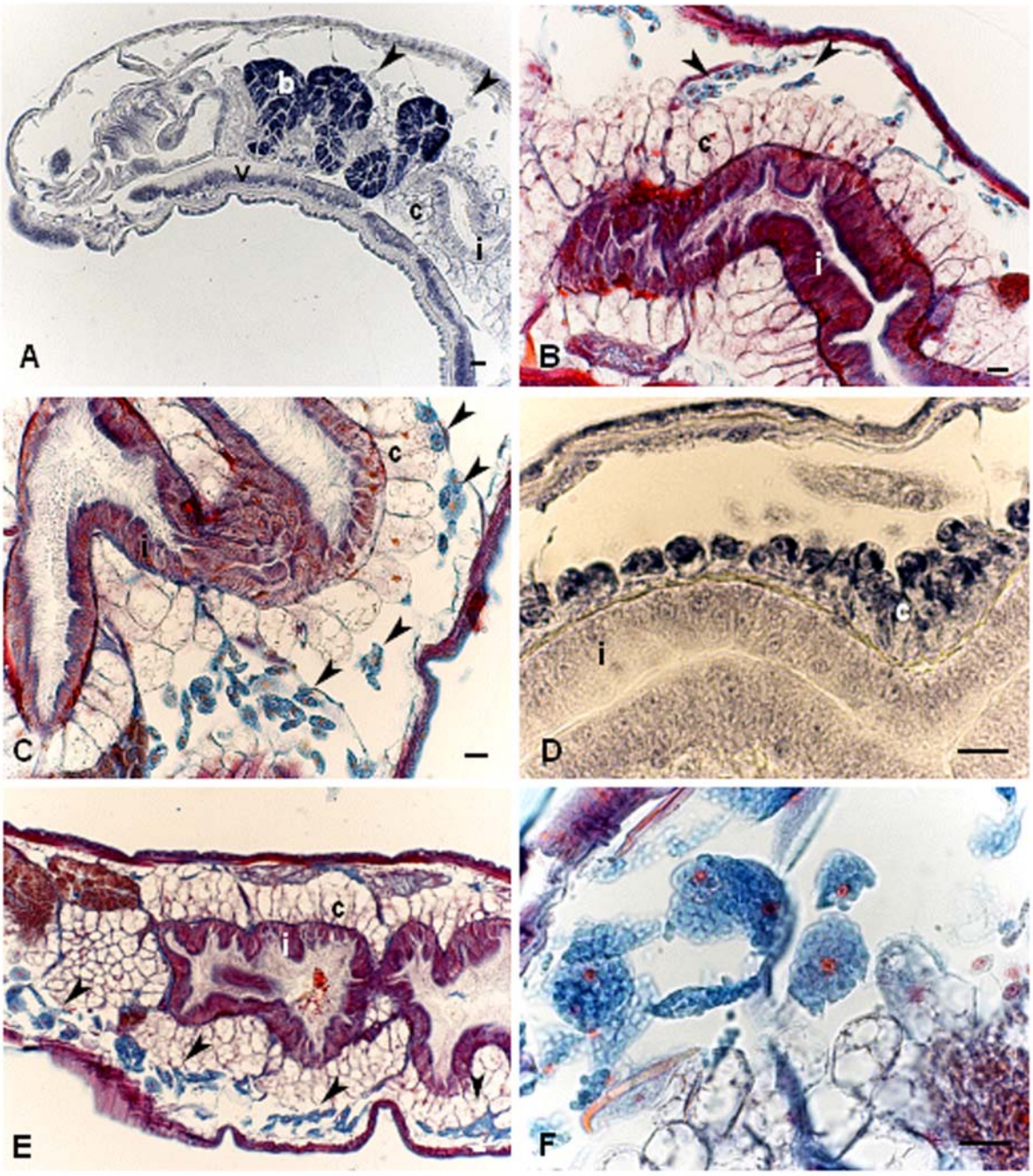

Fig. 1 Longitudinal sections from *E. crypticus* controls (A, B, D) and specimens treated with 100 nM OA for 48 h (C) and 200 nM OA for 12 h (E, F) stained with Mallory-Azan stain (B, C, E, F) and gallocyanin-chrome alum (A, D). The chloragogenous tissue (A, B) from controls formed one or two layers of round vacuolated and basophilic cells (D) surrounding the intestine. After OA treatment, the tissue showed a higher number of cell layers and expanded into the coelomatic cavity (E). The toxin also induced an increase in the number of circulating coelomocytes (C, E). Note the enlarged and rounded circulating chloragocytic cells (F). Brain, b; ventral nervous cord, v; chloragogenous tissue, c; coelomocytes, arrowheads; intestine, i. Bar = 10 μm

Thirty groups of 5 worms each were placed in 30 mm Petri dishes and treated as follows: 3 groups of control animals were fed normally at the beginning of the experiment, while 27 groups received powdered rolled oats mixed with a water solution of OA (Calbiochem, USA) at different final concentrations (100, 200 and 400 nM) for 12, 24 or 48 h.

Treated and control specimens were then collected, immediately fixed in Bouin's mixture and embedded in agar/paraffin, as previously described (Franchini *et al.*, 2003). The following histological and histochemical stains were performed on 7 µm transversal or longitudinal paraffin serial sections: hematoxylin-eosin and Mallory-Azan stains for general morphology, and gallocyanin-chrome alum reaction for nucleic acids (Bancroft and Gamble, 2002). The immunocytochemical procedure was carried out on controls and the variously treated *E. crypticus* using goat anti-IL-6 polyclonal antibody (R & D Systems, USA) diluted 1:500. Sections were incubated overnight at 4 °C in the primary antibody,

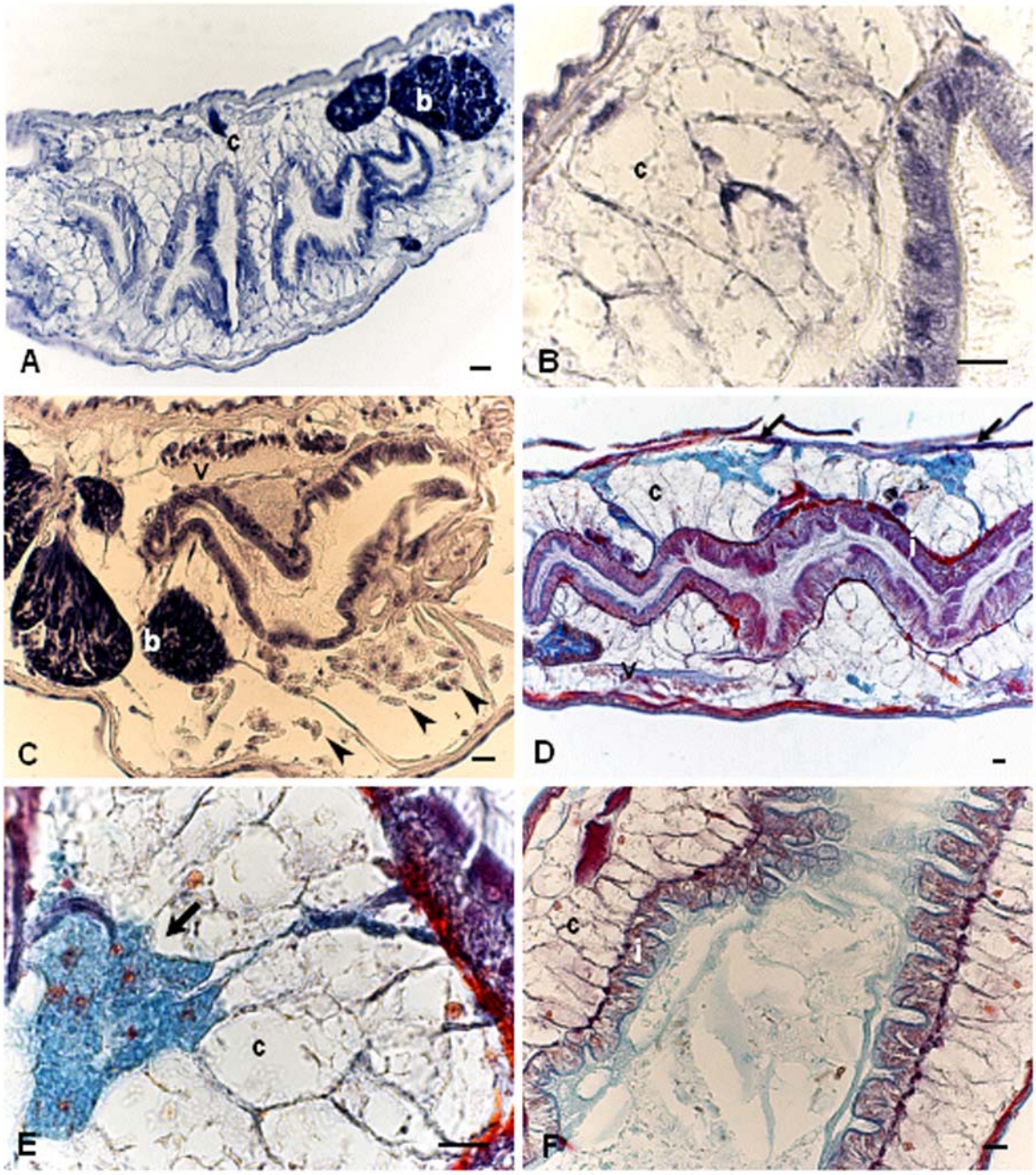

Fig. 2 Longitudinal sections from *E. crypticus* treated with 200 nM OA for 48 h (A-C) and 400 nM OA for 48 h (D-F) stained with Mallory-Azan stain (D-F) and gallocyanin-chrome alum (A-C). Brain, b; ventral nervous cord, v; chloragogenous tissue, c; clamped coelomocytes, arrows; intestine, i. Bar = 10 µm.

and immunoreactivity was visualised by an immunoperoxidase technique using avidin-biotin peroxidase complex (Hsu *et al.*, 1981). Controls of the immunocytochemical reactions were performed by substituting the primary antibodies with non-immune sera and/or absorbing the antibody with homologous antigen.

Results

Examination of the histological sections revealed that *E. crypticus* is sensitive to OA treatment in a time- and dose-related manner. At the lower dose (100 nM), the main organs did not appear particularly affected, except for a swelling of the coelomatic cavity, where, in comparison to controls, an increased number of circulating coelomocytes was observed after 48 h (Figs 1A-C). In *E. crypticus*, two main cell types were seen floating freely in the body cavity, a more numerous population of round or mostly elongated coelomocytes presenting a cytoplasm filled with large inclusions (Oligochaete designed chloragocytic cells), and smaller cells devoid of inclusions with an irregular cytoplasm (Oligochaete designed amoebocytes). In control worms the chloragogenous tissue presented one or two layers of large, vacuolated and basophilic cells surrounding the intestine (Fig. 1D). At the higher OA dose (200 nM) and after 12 h of treatment, this tissue differed in morphology, extended in volume, and an increased number of cell layers was observed (Fig. 1E). These cells were irregularly arranged and without cytoplasmic basophilia, while some were detached from the tissue and surrounded by amoebocytes that had undergone conformational changes into an active form. The circulating chloragocytic cells also changed morphology and became enlarged and rounded in shape (Fig. 1F). After 48 h, the cells in the chloragogenous tissue appeared highly vacuolated and tended to break. They occupied the body cavity almost completely, so that the coelomocytes were concentrated in the prostomium, where the tissue was not present (Figs 2A-C). The nervous system was also affected by a reduction in the nervous area, in particular in the ventral nerve cord extending through the entire animal length. At the highest OA dose (400 nM), a general cell suffering was found, i.e. the extended, irregularly shaped chloragogenous tissue cells embedded clumped coelomocytes (Figs 2D, E), the epithelial cell layer of the medio-posterior intestine increased in surface area (Fig. 2F), nephridia appeared disorganized in structure, and the ventral nerve cord comprising wrinkled nerve cells presented fewer neuronal cell bodies as a result of the invasion of expanding chloragogenous tissue (Figs 3A, D).

In control animals, the immunocytochemical reaction with anti-IL-6 antibody was positive in the nervous system. In particular, the antibody labelled neuron cell bodies and fibres located in the ventral nerve cord (Fig. 3A) and in the anterior ganglia near the mouth, but not brain nerve cells. One population of the circulating coelomocyte, i.e amoebocytes, was also positive (Figs 3B, C). After OA treatment, fewer immunoreactive cells were seen in nervous tissue (Fig. 3D) and the large number of recruited amoebocytes was positive, especially when seen in an active form with large and heterogeneous vacuola inside the cytoplasm (Figs 3E-G).

Discussion

The results from the present study demonstrate that the experimental model used is very sensitive to treatment with OA. The toxin induced time- and dose-related effects that mainly involved an immune response associated with a reaction in the chloragogenous tissue. The multi-functional cells in oligochaete chloragogenous tissue are known to be able to store glycogen, lipids and exogenous materials, such as pigments or metals, and have also been associated to immune defenses (Needham, 1966; Roots and Johnston, 1966; Prentø, 1979; Cooper 1981; Morgan 1981). In these cells, the chloragocytes, two cellular compartments have been implicated in metal trafficking: the cytoplasm organelles, called chloragosomes, undergo changes in autophagic derivatives, the debris vesicles. The subcellular modifications were found to be closely correlated with accumulated metal burdens in the tissue (Morgan and Turner, 2005). Following OA treatment, the tissue increases its volume considerably and shows morphological changes in the cells. The earthworm's chloragogenous tissue has some liver-like properties (Laverack, 1963; Prentø, 1987), and in *E. crypticus*, it may be involved in toxin accumulation and detoxification in a dose-related manner. The induction of a protein in response to toxin exposure, possibly related to detoxification reactions, has been demonstrated in the digestive gland of *Mytilus galloprovincialis* (Auriemma and Battistella, 2004). OA also induces an inflammatory cell response, and the number of circulating coelomocytes, known to be responsible for the animal's immune defence responses (Valembois *et al.*, 1982; Větvička and Šima, 1998), increases both in the chloragocytic cell population, which originates in the chloragogenous tissue surrounding the intestine (Fischer, 1993), and in the amoebocytes that change to an active form and show greater immunoreactivity to anti-IL-6 antibody. OA is a well-known specific inhibitor of serine/threonine protein phosphatase 1 and 2A. These molecules have been found to act as endogenous regulators of inflammatory cell signalling (Shanley *et al.*, 2001), and to play a role in the control and stimulation of proinflammatory cytokine gene expression by murine mononuclear phagocytes and other cell types (Falk *et al.*, 1994; Tebo and Hamilton, 1994; Wakiya and Shibuya, 1999; Feng *et al.*, 2006). The enhanced inflammatory reaction is associated, through a protein phosphatase 2A-mediated mechanism, with the regulation of the c-Jun NH_2-terminal kinase, one of the major mitogen-activated protein kinase signalling pathways (Shanley *et al.*, 2001; Avdi *et al.*, 2002). It has also been suggested that the dynamic interplay between kinases and phosphatases modulates the activity of several proteins that are crucial in neuronal functions. OA provokes effects in the structure of the nervous system, while the protein hyperphoshorylation due

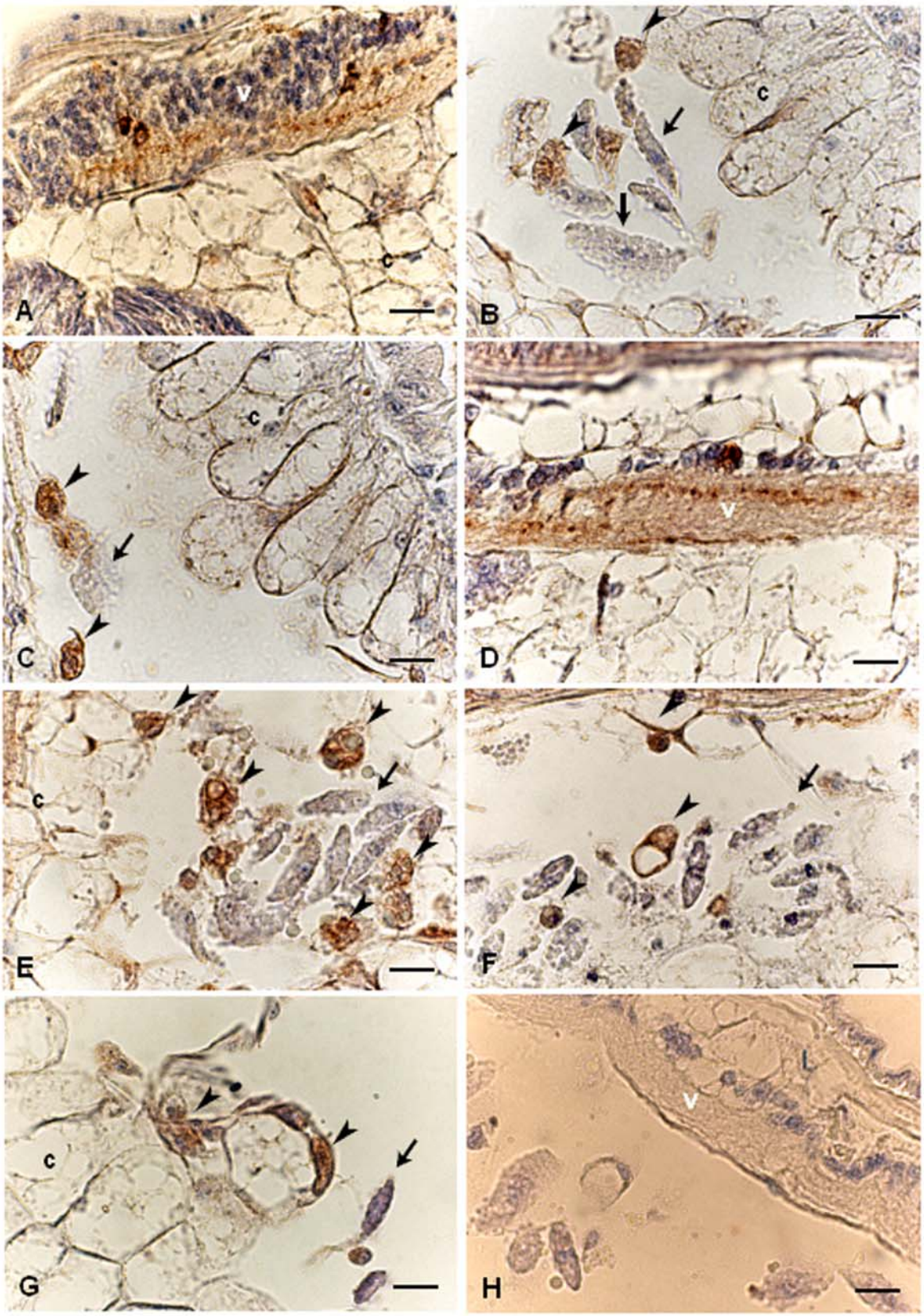

Fig. 3 Sections from *E. crypticus* controls (A-C) and specimens treated with 100 nM OA for 24 h (E), 200 nM OA for 24 h (F-H) and 400 nM OA for 48 h (D) immunostained with anti-IL-6 polyclonal antibody and haematoxylin nuclear counterstaining. Negative controls of the immunocytochemical reaction (H). Ventral nervous cord, v; chloragogenous tissue, c; negative chloragocytic cells, arrows; positive amoebocytes, arrowheads. Bar = 10 μm.

to the inhibition of phosphatases 1, 2A and possibly also calcineurin is known to induce neuronal stress and subsequent neurodegeneration (Tapia *et al.,* 1999; Arias *et al.,* 2002; Ramirez-Munguia *et al.,* 2003).

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