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Effects of tributyltin(IV) chloride exposure on early embryonic stages of *Ciona intestinalis*: *in vivo* and ultrastructural investigations

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The effects of tributyltin(IV) chloride (TBT chloride) solutions on ascidian embryos of *Ciona intestinalis* have been tested at different stages of development. It has been observed, *in vivo*, that TBT chloride inhibited cleavage of fertilized eggs and of embryo blastomeres giving rise to cellular masses that are not delimited by plasma membrane. Electron-dense precipitates of TBT chloride, probably as inorganic tin, have been observed by transmission electron microscopy in the egg cytoplasm of cellular masses.

The same type of precipitate was present also inside the mitochondria, whose structure appeared to be highly modified. These ultrastructural aspects are indicative of a degenerative process of the embryos after incubation in TBT chloride.

Keywords: Tributyltin(IV) chloride, early embryonic stages, Ascidiaceae

INTRODUCTION

The fertilization and embryonic development of several sea-animals could be used as biological processes applicable to water-quality monitoring. Among compounds detected in the aquatic environment, organotin(IV) compounds are present because of their biocidal and industrial use[1-3] and exert toxicity in marine plant and animal tissues.[4-6] Moreover, development of certain male sexual characteristics in females of dog whelks has been reported after TBT chloride contamination.[7]

The effects of organotin(IV) on embryonic development have been studied for some diorganotin(IV) derivatives on ascidians.[8,9] These animals are Urochordata which live by the shoreline, fixed on the rocks, etc., with a pelagic larva arising at the end of development. In this respect these animals may serve as useful indicators of the effects of pollutants on estuarine biota.

Considering that tributyltin(IV) compounds are much more toxic to aquatic fauna than dibutyltin(IV) compounds,[5] the aims of the present study have been:

(1) to determine the toxicity of tributyltin(IV) derivatives, and in particular of tributyltin-(IV) chloride (TBT chloride), to ascidian embryonic development with observations *in vivo*;
(2) to investigate the alterations of the cellular ultrastructure, during early embryonic stages, in order to ascertain the effects of the pollutants on marine embryos;
(3) to compare the effects of tributyltin(IV) chloride with those of previously employed dibutyltin(IV) compounds.[8,9]

EXPERIMENTAL

Adult specimens of *Ciona intestinalis* were collected from the Gulf of Palermo and Termini harbour (Palermo). Female and male gametes were removed from the gonoducts and transferred into agar-coated Syracuse dishes for fertilization.

In particular, observations have been carried out on the following groups.
(1) Controls: eggs fertilized in normal seawater and developed up to swimming larva stage.
(2) Unfertilized eggs dechorionated by hand with steel needles: some of these were fertilized in seawater, others were fertilized in 10^{-7} mol dm^{-3}

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TBT chloride solution in order to follow the event of fertilization.
(3) Embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the 4–8 cell stage until controls reached the swimming larva stage (Lot A). Other embryos, at the 4–8 cell stage, were treated with 10^{-9} and 10^{-11} mol dm^{-3} TBT chloride solutions.
(4) Embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the neurula stage until controls reached the swimming larva stage (Lot B).
(5) Embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the gastrula stage for 1 h and then maintained in normal seawater (Lot C).

The tributylin(IV) chloride (TBT chloride) was a kind gift from Schering (Bergkamen, Germany), which has been used without further purification. Concentrated stock solutions were obtained by dissolving stoichiometric amounts of the compound in Millipore-filtered seawater (MFSW). Working solutions (pH 7.25–8.50) were obtained by further dilution of the stocks in MFSW. Freshly prepared tributyltin(IV) chloride concentrations in the diluted solutions were used and were stable. Concentrations (10^{-7}, 10^{-9} and 10^{-11} mol dm^{-3}) and solution stability were checked as previously reported.[10]

Light and transmission electron microscopy

In vivo observations were made with a Leitz microscope.

Ciona Intestinalis control larvae, vitally stained for 5 min with Nile Blue sulphate (1 : 100 000 in MSFW) and maintained in culture at 18 °C, were observed and photographed with a Leitz Orthoplan microscope, using an Ilford FP4 plus film.

Embryos of Lots A, B and C were fixed with 2.5% glutaraldehyde in 0.2 mol dm^{-3} phosphate buffer (pH 7.5) solution and postfixed with 1% osmium tetroxide in the same buffer solution. The fixed material was dehydrated in graded alcohol and embedded in Epon 812.[11]

Semithin sections (1–2 μm thick) obtained with an Ultracut Reichert ultramicrotome, were stained with 1% Toluidine Blue at pH 2.5.[12] Sections were observed and photographed with a Leitz Orthoplan microscope, using an Ilford FP4 plus film.

For ultrastructural observation, thin sections obtained with an Ultracut Reichert ultramicrotome were contrasted with uranyl acetate and lead citrate,[13] and then photographed with a Philips EM 410 electron microscope, using Kodak electron microscope film (Estar thick base 4489).

RESULTS

In vivo observations

Effect of TBT chloride on fertilization

The dechorionated control eggs, after fertilization, presented the typical movements described by Ortolani.[14]

The dechorionated and fertilized eggs incubated in 10^{-7} mol dm^{-3} TBT chloride solution modified their shape in an abnormal way; they did not emit the second polar body and, in any case, did not cleave (Fig. 1).

Effects of TBT chloride on the 4–8 cell stage

The embryos incubated in 10^{-7} mol dm^{-3} TBT chloride solution were blocked and presented fusion of some blastomeres. Those incubated in 10^{-9} mol dm^{-3} TBT chloride solution were abnormal embryos which presented blastomeres of different sizes, indicating fusion of some cells. Those incubated in 10^{-11} mol dm^{-3} TBT chloride solution gave rise to 50% of blocked anomalous embryos and 50% of anomalous larvae which presented short and twisted tails and were deprived of adhesive and sensorial organs.

When the embryos at the four-cell stage were incubated for 15 min and then transferred into normal seawater, the embryos blocked at anomalous 4–8 cells in 10^{-7} mol dm^{-3} TBT chloride solution.

In 10^{-9} and 10^{-11} mol dm^{-3} TBT chloride solutions, only 60% of normal larvae were obtained. The 4–8 cell stage embryos which were incubated 1 h in 10^{-7} mol dm^{-3} TBT chloride solution and then transferred into normal seawater blocked as anomalous embryos.

Effects of TBT chloride on gastrula stage

The embryos incubated in 10^{-7} mol dm^{-3} TBT chloride solution at the beginning of gastrulation

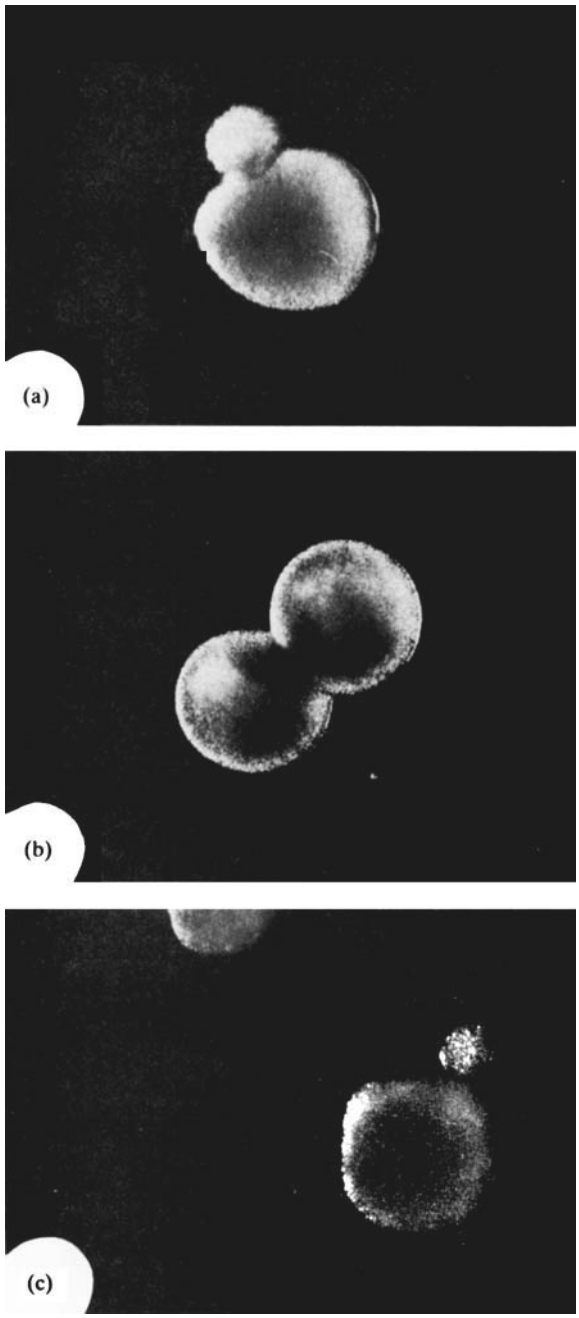

Figure 1 Dechorionated and fertilized eggs of *Ciona intestinalis* incubated in 10^{-7} mol dm^{-3} TBT chloride solution modify their shape in an abnormal way; they do not emit the second polar body and not cleave. Magnification ×150.

for 1 h gave rise to anomalous embryos without differentiation, with a dorsal blastoporal lip which did not close, thus indicating an alteration of the gastrulation process.

Light- and electron-microscope observations

Controls

Observation with the light microscope, carried out on control embryos, showed development up to swimming larvae.

The swimming larva of *Ciona intestinalis* was about 1.5 mm long and completely covered by a coat ('tunic' or 'test') (Fig. 2). The larva was constituted of a cephalic region (cephalenteron) containing sensorial organs and of a tail, for swimming in seawater.[15] Several test cells were noted adhering to the test (Fig. 2).

Effects of TBT chloride on the 4–8 cell stage (Lot A)

The observations carried out on Lot A showed embryos with anomalous structure.

The light-microscope observations of semi-thin sections evidenced a globe-shaped embryo not covered by the test. The embryo consisted of irregular cellular masses, externally surrounded by a layer of test cells. These cellular masses, with no nuclei inside, were detached from each other, separated by large spaces (Fig. 3a). Ultrastructural observations with the electron microscope showed the occurrence of electron-dense precipitates of TBT chloride in the cell masses (fig. 3b), not delimited with a plasma membrane.

Several organelles were present in the cell masses which, at higher magnification, showed the typical structure of peroxisomes (Fig. 3d). In fact their diameter was about 0.5 μm and they contained an electron-dense nucleoid, dispersed in a homogeneous matrix, with a typical eccentric position (Fig. 3d).

In the cell masses several globe-shaped mitochondria were also present. Their ultrastructure was highly modified in comparison with orthodox mitochondria. The inner membrane was continuous and separated from the outer one by a well delimited space. The mitochondrial cristae did not arise from the inner membrane and, in the section, they appeared as tubular vesicles dispersed in the mitochondrial matrix (Fig. 3c).

Moreover, TBT chloride precipitates were present in the mitochondria as strongly electron-dense granules of different dimensions (Fig. 3c).

Effects of TBT chloride on the gastrula stage (Lot B)

The observations carried out on Lot B showed the anomalous structure of embryos which stopped

developing as a consequence of treatment with 10^{-7} mol dm^{-3} TBT chloride solution. Globe-shaped embryos, without test (Fig. 4a), have been evidenced by semi-thin section observation with the light microscope. The embryos were ringed by test cells, and consisted of cell masses, separated from one another by large spaces and with one or more nuclei (Fig. 4a). The ultrastructural observations showed TBT chloride electron-dense precipitates in the cell masses (Fig. 4b, c) not delimited by outer cytoplasmatic membrane, evidencing one or more nuclei. As many as three nuclei could be found in some of the masses (Fig. 5b). As to the ultrastructural appearance of the nucleus, some TBT chloride precipitates have been found in the two-layered nuclear membrane, while precipitates are absent in the nucleoplasma. TBT chloride precipitates were present, in particular, in some widened regions of the two-layered nuclear membranes (Fig. 5a).

Several mitochondria showing TBT chloride electron-dense granules inside were present in the cell masses. The mitochondrial ultrastructure, highly modified in comparison with orthodox mitochondria, was identical to that found in Lot B mitochondria (Fig. 5c).

One-hour effects of TBT chloride on the gastrula stage (Lot C)

The observations obtained on Lot C, with both light and electron microscopy, were identical to those obtained for Lot B.

DISCUSSION

This paper reports data on toxic effects of tributyltin(IV) chloride (TBT chloride) during the early stages of ascidian embryonic development. In particular, studies have been carried out with both the light and the electron microscope on *Ciona intestinalis* embryos incubated in TBT chloride solutions immediately after fertilization, during segmentation and during gastrulation.

The *in vivo* observations show that the processes which generally occur in the egg after fertilization are inhibited as a consequence of TBT chloride incubation.

In particular, TBT chloride incubation affects ooplasmic segregation and prevents emission of the second polar body and egg cleavage. Observations have been carried out also on embryos incubated in TBT chloride during the early developmental stages (4–8 cell stage) and during gastrulation. These embryos present strong anomalies and their development is blocked. These anomalies resemble those previously obtained after treatment with some dibutyltin(IV) compounds.[8,9] In these reports, the stoppage of embryonic development was related to the toxic damage caused by organotin(IV) derivatives to the plasma membrane, to the cytoskeleton and to the chromosomal structure.[8,9]

The effects of TBT chloride incubation seem to be proportional both to concentration and to exposure time. After one hour of incubation with TBT chloride, these effects are already evident and irreversible. In fact, there are no differences between the embryos treated with TBT chloride solution at the gastrula stage for one hour and then transferred into normal seawater and the embryos treated with TBT chloride solution at the gastrula stage until controls reached the swimming larva stage. The results obtained show mitosis block of blastomeres that, on the contrary, form cell masses which can be either devoid of a nucleus or polynucleated.

In order to ascertain which cellular structures are modified by TBT chloride, ultrastructural investigations have been carried out by trans-

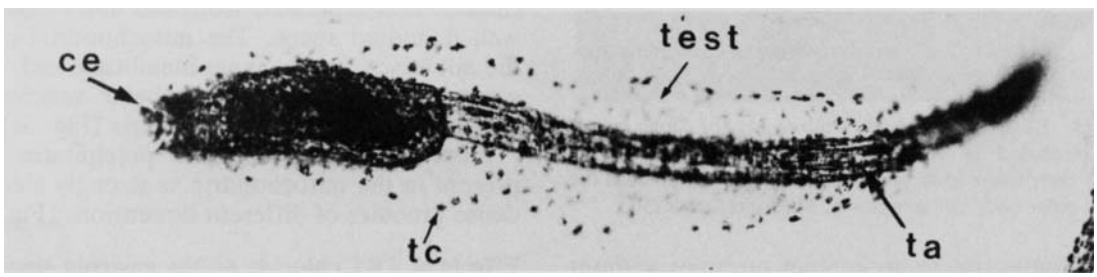

Figure 2. Control: *Ciona intestinalis* swimming larva vitally stained for 5 min with Nile Blue sulphate (1:100 000 in MSFW). The larva is completely covered by a coat (test) and is constituted of a cephalic region or cephalenteron (ce) and by a tail (ta) for swimming in seawater. Several test cells (tc) adhere to the test. Magnification ×120.

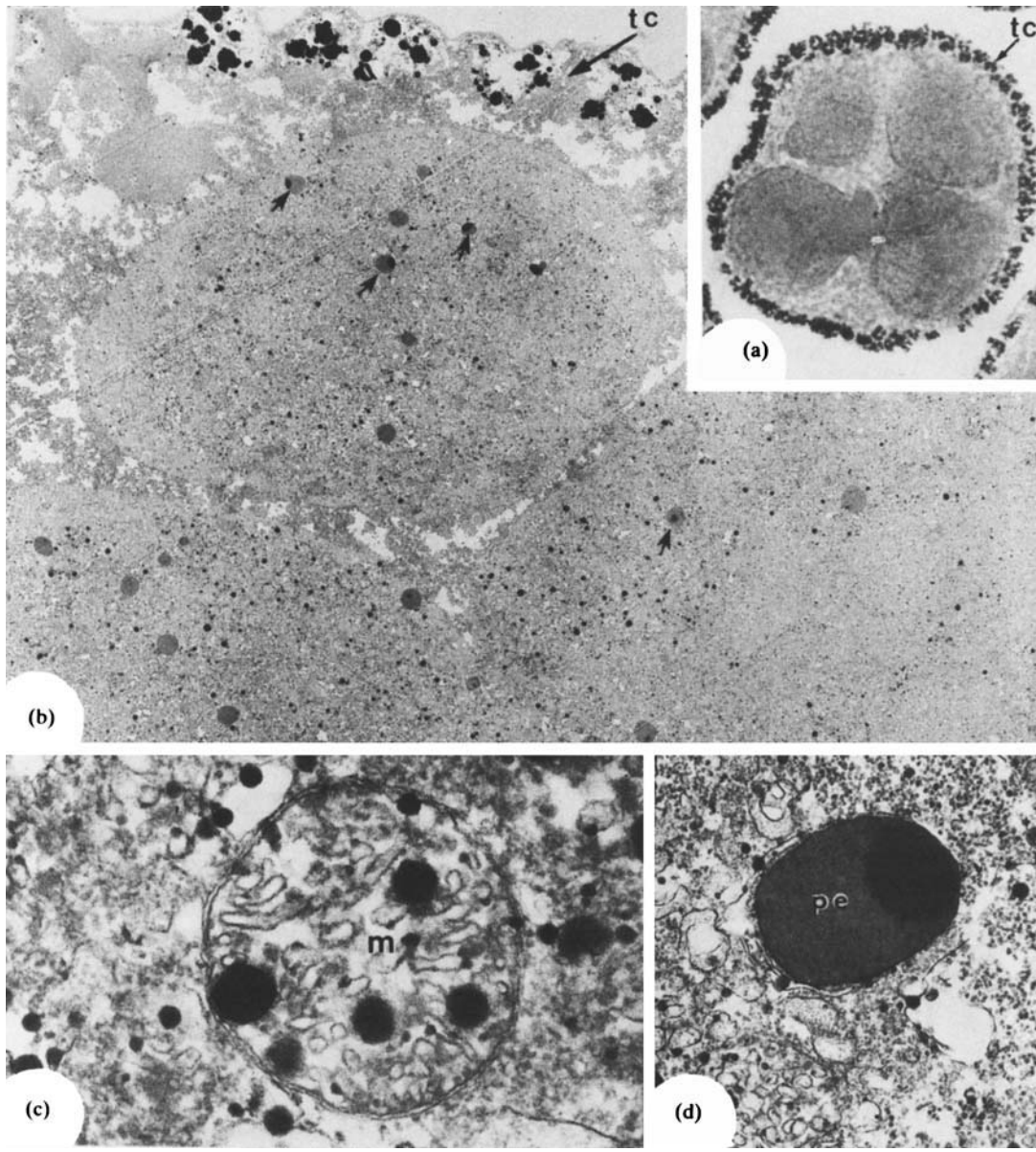

Figure 3 Lot A: *Ciona intestinalis* anomalous embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the 4–8 cell stage until controls reached the swimming larva stage.

(a) Semithin section stained with 1% toluidine Blue (pH 2.5). The anomalous embryo is globe-shaped, surrounded by a layer of test cells (tc). Inside the embryo cell there are anucleated masses, separated from each other. There is no test on the embryo. Magnification ×312.

(b)–(d) Electron-dense precipitates of TBT chloride in the cell masses (b) together with, at high magnification, the typical ultrastructure of peroxisomes (pe) (d). The mitochondria ultrastructure (m) is highly modified. Electron-dense precipitates of TBT chloride are present in the mitochondria, whose cristae, at the section, appear as vesicles dispersed in the matrix (c).

Magnification: (b) ×2500; (c) ×26 350; (d) ×52 000.

mission electron microscopy (TEM). TBT chloride effects on cellular structures are evident. Ultrastructural observations show that granular electron-dense precipitates of TBT chloride are accumulated in the cell masses originated by the fusion of blastomeres. The same type of precipitate is found in the mitochondria whose structure is highly modified. Cell masses are devoid of

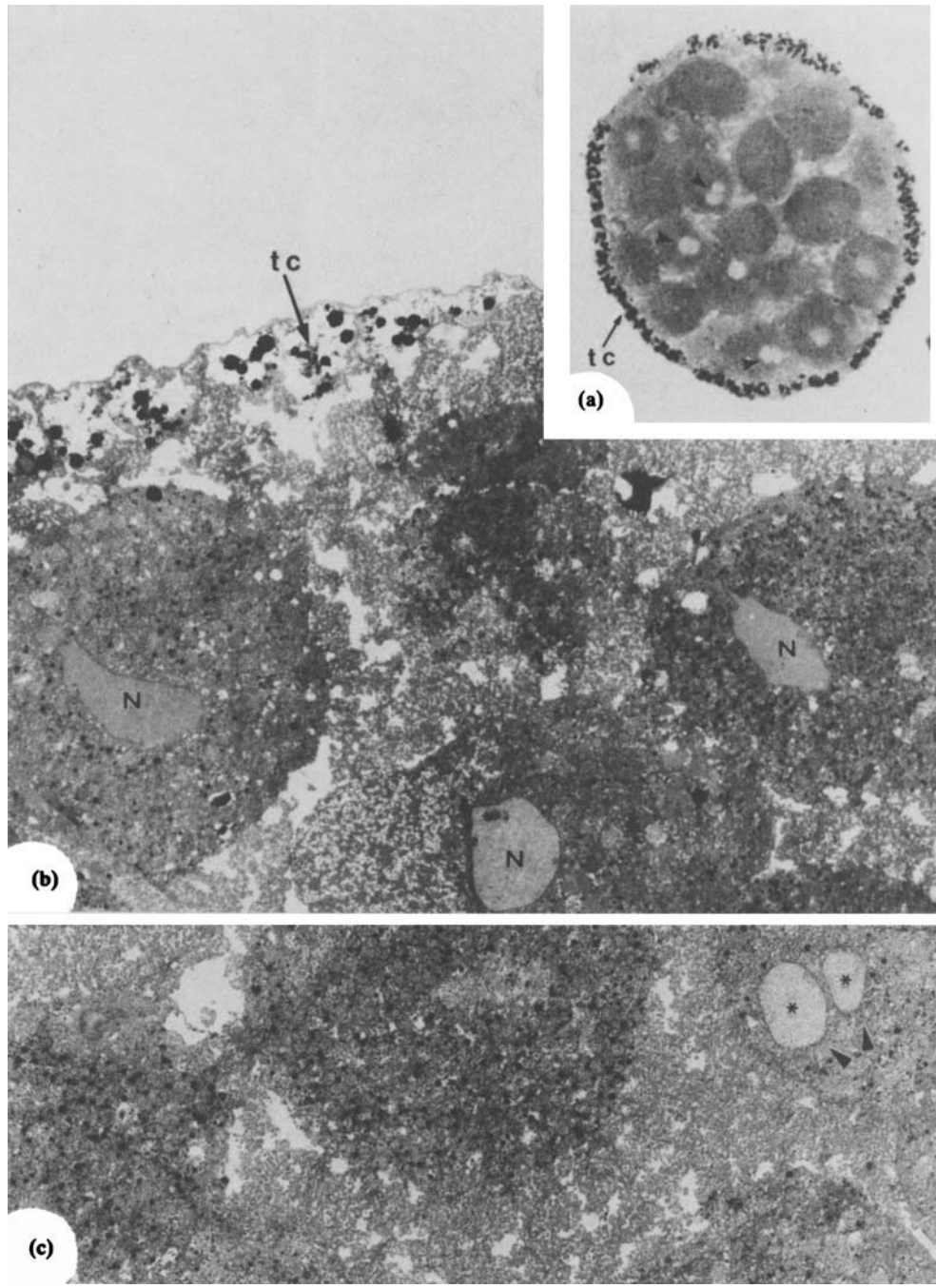

Figure 4. Lot B: *Ciona intestinalis* embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the gastrula stage until controls reached the swimming larva stage.

(a) Semithin section stained with 1% Toluidine Blue (pH 2.5). The annomalous embryo is globe-shaped, surrounded by a layer of test cells (tc). Inside the embryo cell there are mono- and bi-nucleated masses, separated from each other (arrows). There is no test on the embryo.

(b), (c) Embryo is surrounded by a layer of test cells (tc). It consists of cellular masses, not delimited by plasma membrane, containing electron-dense precipitates of TBT chloride. One or more nuclei are present in these masses (d, arrows); N, nucleus.

Magnification: (a) ×380; (b) ×2100; (c) ×2700.

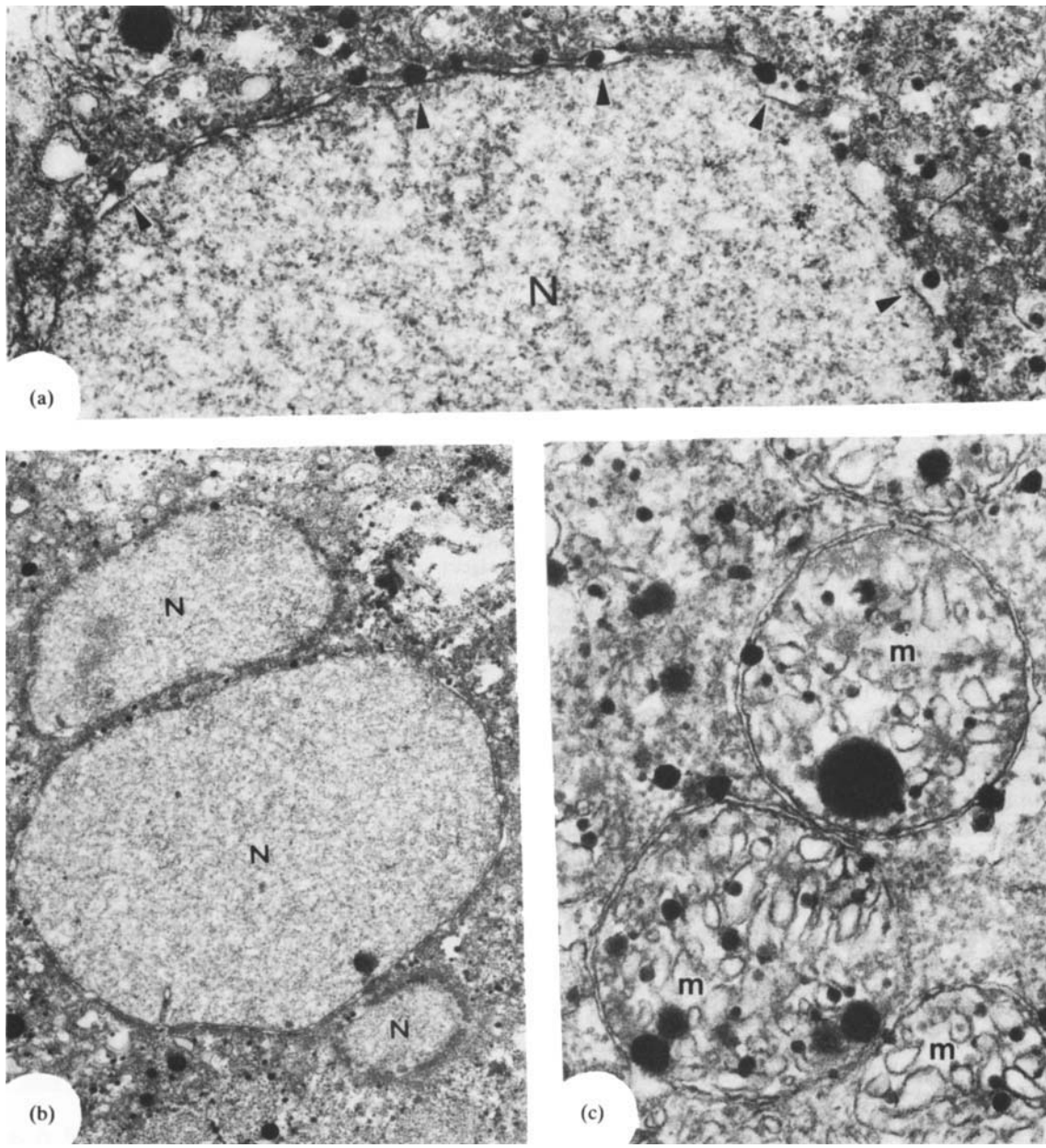

Figure 5. Lot B: *Ciona intestinalis* anomalous embryos treated with 10^{-7} mol dm^{-3} TBT chloride solution at the gastrula stage until controls reached the swimming larva stage.

(a), (b) Ultrastructural aspects of the nucleus. The two layers of nuclear membrane are separated by a wide space inside which, at some enlarged points, TBT chloride precipitates are present (a, arrows). Some cellular masses also show three nuclei (b). No TBT chloride precipitates occur in the nucleoplasm. N, nucleus.

(c) The mitochondria (m) are globe-shaped and their ultrastructure is highly modified. The mitochondrial cristae do not arise from the inner membrane and, in the section, appear as tubular vesicles dispersed in the matrix. TBT chloride precipitates are present in the mitochondria.

Magnification: (a) ×28 750; (b)×9900; (c) ×58 500.

cytoplasmatic membrane. The embryos incubated at the gastrula stage in TBT chloride present polynucleated cell masses. TBT chloride precipitates are also found in the nuclear membrane space which, at some points, is enlarged.

Major ultrastructural modifications of cytoplasmic and nuclear membranes and of mitochondria are evidence of a degenerative process occurring in the embryo and can be related to the stoppage of normal development.

Further data are reported in the literature on damage to the mitochondria caused by pollutants. Engel and Fowler[16] observed, by TEM, ultrastructural modifications caused by copper and cadmium in the mitochondria of mulluscan gill tissue. Alterations at chromosomal levels have been demonstrated by Vitturi *et al.*[10] in *Truncatella subcylindrica* treated with dibutyl(IV) derivatives. The presence of several peroxisomes in the embryos incubated at the 4–8 cell stage represents an interesting piece of information in this report. It is quite difficult to evaluate the influence of the occurrence of these organelles due to the damage caused to embryonic development by TBT chloride. That the larger number of peroxsomes could be associated with the cell damage occurring in the embryos is a clear possibility.

In conclusion, the toxic damage of TBT chloride on ascidian embryonic development is due to ultrastructural modifications of both cytomembranes and mitochondria. In agreement with the hypothesis of Mansueto *et al.*[8,9] organotin(IV) toxicity probably involves the activity of proteins with a basic role in embryonic development. This hypothesis could explain the occurrence of some events brought about by TBT chloride incubation, such as egg cleavage inhibition, stoppage of blastomere mitosis during segmentation and their fusion into cell masses devoid of cytoplasmic membranes.

Also, the anomalous blastomere arrangement during gastrulation could be related to the stoppage of cellular migration and to the absence of cellular recognition, or to other interactions.

The hypothesis of a possible relation between the lack of protein functionality and the blockage of embryonic development is in agreement with Longwell and Hughes' investigation of *Scomber scombrus*, according to which mitosis stopped after dibutyltin(IV) dichloride treatment.[17] According to the above-mentioned Authors, the organotin (IV) derivative inhibits the polymerization of tubulin.[18]

Further research on the late development stage and on larvae, which could clarify the effects of TBT chloride, is in progress.

The importance of the present investigation is correlated with the fact that the hydrolysed antifouling organotin(IV) derivatives, such as TBTO, in seawater, at the pH values at which our investigation has been carried out (pH 7.5), decompose giving tributyltin(IV) chloride and tributyltin(IV) carbonate as main subproducts.[18]

These latter are less toxic than the parent compounds, but are responsible for citotoxic activity. On the other hand, the toxic effects observed by our ultrastructural investigations on the early stages of development of ascidians for tributyltin(IV) chloride are in agreement with previous reports.[8,9,19]

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