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Effects of tributyltin(IV) chloride on the gametes and fertilization of *Ascidia malaca* (Ascidiacea: Tunicata)

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***Ascidia malaca* gametes before fertilization incubated in 10^{-5} or 10^{-7} M solutions of tributyltin(IV) chloride, TBTCl, for 3 h appear highly damaged under transmission electron microscopy observation. Also, the fertilization process is affected by the compound: the damaged spermatozoa are present in the vitelline coat and the egg does not cleave. An increase of microbodies, structurally similar to peroxisomes, have been detected in the egg peripheral cytoplasm, probably in relation to their role in alleviating damage to some cellular components. The results have shown that the reproduction of ascidians under unfavourable environmental conditions is prevented. Copyright © 2003 John Wiley & Sons, Ltd.**

KEYWORDS: gametes and reproduction; ascidians; tributyltin(IV) chloride; peroxisomes proliferation

INTRODUCTION

Over the past several years there has been much interest on the effects of pollutants on organisms. Recently, there has been more emphasis on toxic components that exert deleterious effects on reproduction, producing a variety of consequences on communities such as an increased mortality or reduced fertility. The increase of infertility in the industrialized regions has focused the attention of researchers on the factors that can compromise the endocrine system of animals living in highly polluted environments. Reproductive disorders in animals include altered fertility, impaired hormone secretion and modified reproductive anatomy.[1,2] The sperm motility and its fertilizing ability are affected by different compounds in some species.[3–9] Imposex, the development of male primary sexual characteristics in female gastropods, is described for the dogwhelk *Nucella lapillus*, an estuarine snail,[10,11] in *Ilyanassa obsoleta*,[12,13] and in *Hexaplex trunculus*.[14] It appears that development of imposex is a highly specific response to organotin(IV) compounds and it occurs in intertidal gastropods in relationship to intense traffic and marinas.[15] Imposex caused by tributyltin (TBT) results in a decline of reproduction potential and the eventual disappearance of populations.[16,17]

In the hermaphroditic *Ascidia malaca* (Ascidiacea: Tunicata), gametes are released into the seawater, where fertilization occurs. Spermatozoa, unfertilized and fertilized eggs may consequently be subjected to the effects of many man-made environmental pollutants. As the tunicates, from a phylogenetic point of view, occupy an important position between invertebrates and vertebrates, at present, they appear to be a useful model for studying various aspects of reproduction in invertebrates; but much information, especially on fertilization, is of general significance.[18] Moreover, according to Fukumoto,[19] fertilization in ascidians has characteristics of both mammals and marine invertebrates; acrosome reaction in ascidians, particularly in some recently examined species,[20–24] occurs in fundamentally the same way that has been observed in mammalian spermatozoa.

Recent studies[25,26] showed that the female reproductive system in urochordata is heavily affected, at a biochemical level, by TBTCl. As there is no information at an ultrastructural level regarding the impact of TBT on gametes and on fertilization process, the aim of this research is to examine

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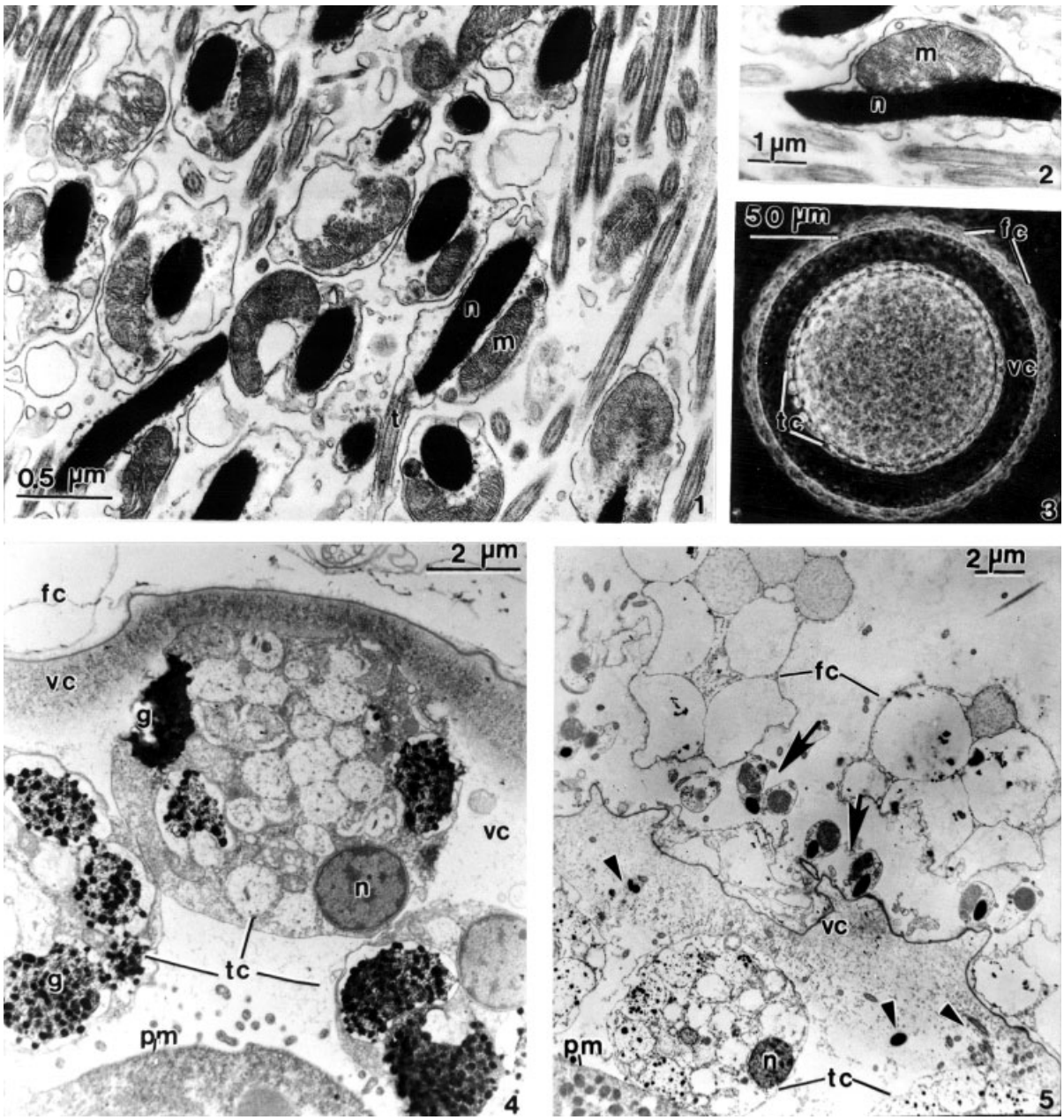

Figure 1. TEM micrograph of fully differentiated *A. malaca* spermatozoa (control) from the sperm duct: m = mitochondrion, n = nucleus, t = tail.

Figure 2. TEM micrograph of a longitudinally sectioned head of a control *A. malaca* spermatozoon.

Figure 3. Phase-contrast micrograph of a living unfertilized *A. malaca* egg (control): fc = follicle cell; tc = test cell; vc = vitelline coat.

Figure 4. TEM micrograph of envelopes of an unfertilized *A. malaca* egg (control): n = test cell nucleus; g = test cell granule; pm = egg plasma membrane.

Figure 5. TEM micrograph of envelopes of a fertilized *A. malaca* egg (control) showing many sperm nuclei and tails in the vitelline coat (arrowheads); intact spermatozoa are also present between the vitelline coat and the follicle cells (arrows).

the gametes structure and fertilization after TBTCl treatment by means of light and electron microscopies in order to obtain meaningful data on toxicological studies regarding reproduction of ascidians. This research could also be useful for comparative ecotoxicological studies in other taxa, including humans.

MATERIALS AND METHODS

Adult specimens of *A. malaca*, an ascidian self-fertile species, were collected in the Gulf of Palermo and its vicinity. Female and male gametes were removed from the gonoducts of 40 dissected animals. Before insemination, dry sperm of 20 individuals was diluted in Millipore-filtered sea water (MFSW, pore size 0.45 μm) to a final concentration of approximately 0.1 v/v (pH 7.8, salinity 37‰, $T = 22\,^{\circ}C$). After insemination, the eggs of 20 individuals were washed twice in the same medium. Part of the living egg was examined under a Leitz Diaplan light microscope and part was fixed for transmission electron microscopy (TEM). The samples were prefixed with 3% glutaraldehyde in 0.1 M cacodylate buffer in sea water (pH 7.2) containing 4% sucrose for 30 min at room temperature (22 °C) and post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C. Then they were dehydrated in an ethanol–propylene oxide series and embedded in Dow epoxy resin.[27] Sections were stained with uranyl acetate and lead citrate[28] and examined with a Philips EM 410.

TBTCl was a kind gift from Witco GmbH (Bergkamen, Germany). Concentrated stock solutions were obtained by dissolving stoichiometric amounts of the compound in 0.07% dimethylsulfoxide (DMSO) containing MFSW. The total tin content was reported by Puccia *et al.*[26] Working solutions (pH 7.25–8.5) were obtained by further dilution of the stocks in MFSW. Freshly prepared 10^{-5} and 10^{-7} M TBTCl solutions were used.

Observations have been carried out on the following samples:

1. Controls: spermatozoa, unfertilized and fertilized eggs incubated in MFSW with and without 0.07% DMSO.
2. Spermatozoa treated with TBTCl solutions for 3 h.
3. Unfertilized eggs treated with TBTCl solutions for 3 h and afterwards fertilized with untreated sperm.
4. Eggs fertilized in TBTCl solutions and processed after 3 h.

All experiments were repeated in triplicate ($n = 3$). Control fertilized eggs, both in MFSW and MSFW + 0.07% DMSO, were observed to develop up to the swimming larval stage.

RESULTS

Controls

The control *A. malaca* spermatozoa (Figs 1 and 2) are characterized by a compact tapered nucleus with a closely applied single large mitochondrion and a flagellum with the typical 9 + 2 microtubule pattern.[29] The acrosome, because of its extremely reduced size, can be recognized only in an appropriate plane of section. The control spawned eggs of *A. malaca* are endowed with a very complex vestment composed of a relatively thick vitelline coat layer with follicle cells on their outer surface and test cells on their inner surface, overlying the egg plasma membrane (Fig. 3).

The follicle cells constitute a single layer and appear highly vacuolated; at their basal region they are separated by narrow clefts, which are the only means by which the sperm can reach the subjacent vitelline coat (see Fig. 5). The test cells form a single but discontinuous layer of vacuolated cells, which move freely within the texture of the vitelline coat (Figs 4 and 5). This encloses the test cells and consists of three different fibrous layers; its thickness, about 20 μm *in vivo*, decreases during TEM preparations. Fertilization in ascidians is a multistep process leading to spermatozoa meeting and fusing with eggs; many spermatozoa penetrate through the clefts of the follicle cells (Fig. 5, arrows) and cross the vitelline coat (Fig. 5, arrowheads); only one of them enters the egg. After 3 h, the eggs incubated in MFSW or in MFSW + 0.07% DMSO develop up to the early gastrula stage.

Effects of TBTCl on spermatozoa

Spermatozoa incubated for 3 h in 10^{-5} or 10^{-7} M solutions of TBTCl showed similar anomalies for the two different concentrations, strongly injuring all sperm components (Fig. 6); in particular, the nucleus shows various degrees of degeneration with a modified shape, a homogeneous and weakly osmophilic content and a highly damaged membrane.

Effects of TBTCl on eggs before and after fertilization

A. malaca unfertilized eggs incubated in 10^{-5} and 10^{-7} M solutions of TBTCl for 3 h and observed under a light microscope show the loss of many follicle cells and a change in the morphology of the vitelline coat, which appears thicker and more translucid than in controls. TEM observations reveal some modifications in the ultrastructural morphology of the test cells and of the egg cortical cytoplasm (Figs 7 and 8). Follicle cells, rarely found in incubated eggs, do not show significant signs of alterations. In the test cells, the highest modifications concern their nucleus and the granules. These latter appear very different from those of controls (Fig. 4): they are elongated, peripherically located and have an almost homogeneous very electron-dense content (Figs 8 and 9). The nucleus is very irregularly shaped; the nuclear membrane also has an irregular outline and the nuclear content is less electron-dense and finely granular. Damaged mitochondria are scattered in the egg periphery and peroxisome-like organules are seen in the

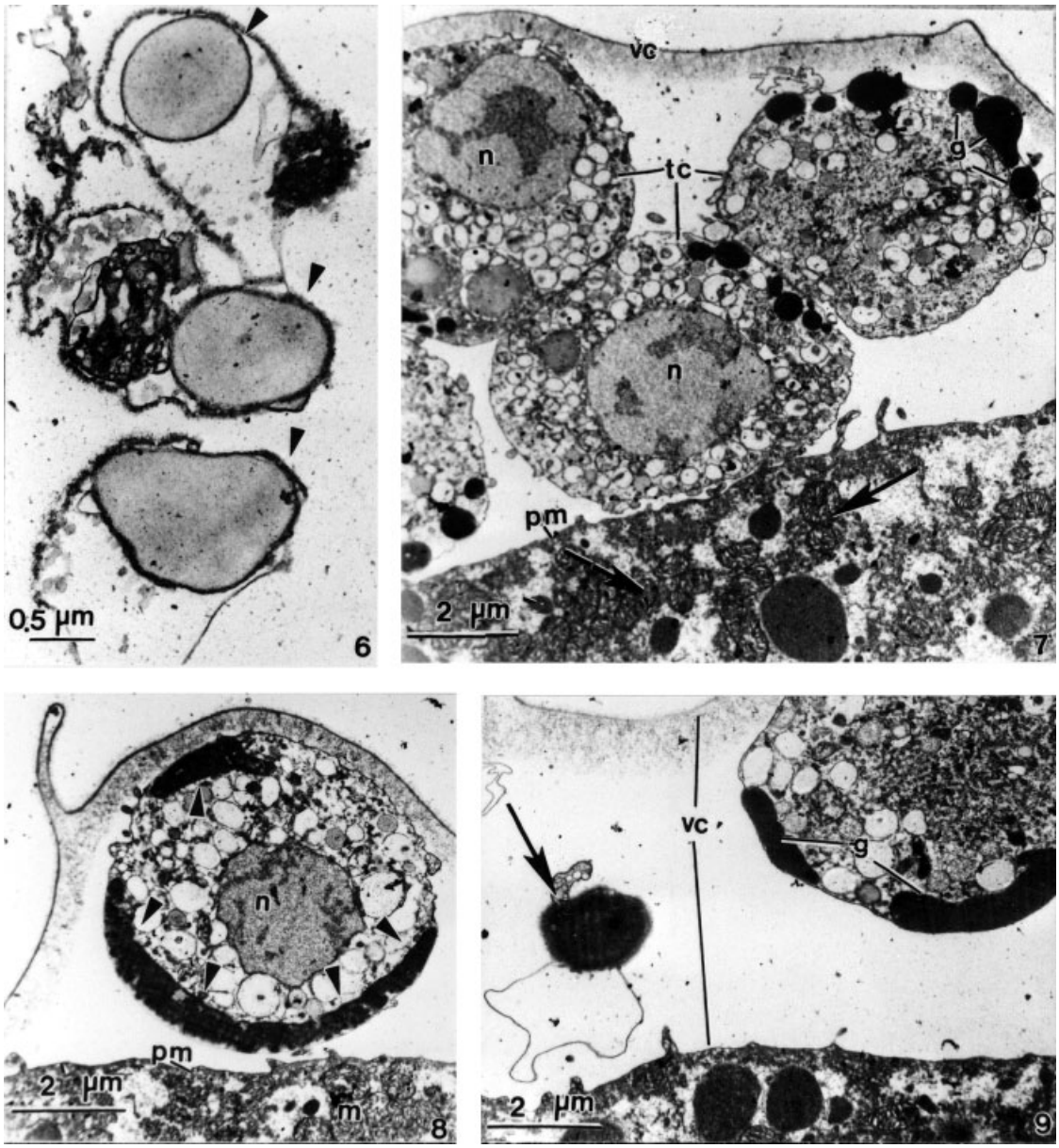

Figure 6. TEM micrograph of *A. malaca* spermatozoa incubated for 3 h in 10^{-5} M TBTCl solution. All the sperm components appear highly damaged and the nuclei of the spermatozoa are greatly modified (arrowheads).

Figure 7. TEM micrograph of *A. malaca* unfertilized egg incubated for 3 h in 10^{-7} M TBTCl solution showing highly modified test cells nuclei; a group of damaged mitochondria are present in the cortical egg cytoplasm (arrows): n = test cell nucleus; g = test cell granule; pm = egg plasma membrane; tc = test cell; vc = vitelline coat.

Figure 8. TEM micrograph of *A. malaca* unfertilized egg treated for 3 h in 10^{-7} M TBTCl solution; the test cell granules appear highly modified (arrowheads): m = mitochondria.

Figure 9. TEM micrograph of *A. malaca* fertilized egg incubated for 3 h in 10^{-7} M TBTCl solution showing a nucleus of a damaged spermatozoon in the vitelline coat (arrow).

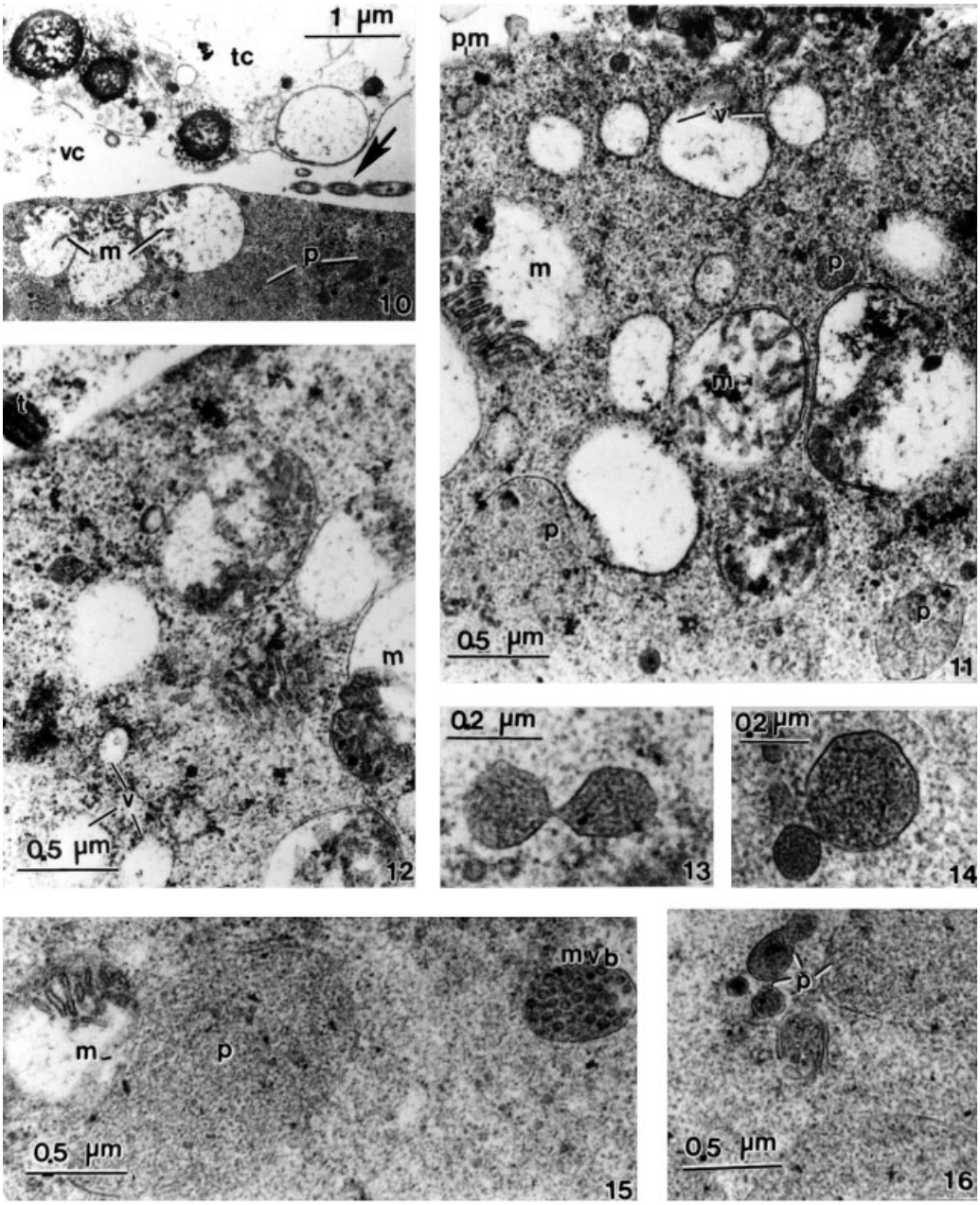

Figure 10. TEM micrograph of *A. malaca* fertilized egg treated for 3 h in 10^{-5} M TBTCl solution showing sperm tail in the vc (arrow); damaged mitochondria and peroxisomes are present in the cortical cytoplasm: m = mitochondria; p = peroxisomes; tc = test cell; vc = vitelline coat.

Figure 11. TEM micrographs of the cortical cytoplasm of a fertilized *A. malaca* egg exposed for 3 h in 10^{-5} M TBTCl solution showing damaged mitochondria, peroxisomes and vesicles: pm = egg plasma membrane; t = sperm tail; v = vesicles.

Figure 12. TEM micrographs of the cortical cytoplasm of a fertilized *A. malaca* egg exposed for 3 h in 10^{-5} M TBTCl solution showing damaged mitochondria, peroxisomes and vesicles: pm = egg plasma membrane; t = sperm tail; v = vesicles.

Figure 13. High magnification of peroxisomes in division.

Figure 14. High magnification of peroxisomes in fusion.

Figure 15. TEM micrographs of particulars of a fertilized *A. malaca* egg exposed for 3 h in 10^{-5} M TBTCl solution showing a damaged mitochondrion, a multivesicular body, vesicles, peroxisomes and other organelles in the cortical cytoplasm: mvb = multivesicular body.

Figure 16. TEM micrographs of particulars of a fertilized *A. malaca* egg exposed for 3 h in 10^{-5} M TBTCl solution showing a damaged mitochondrion, a multivesicular body, vesicles, peroxisomes and other organelles in the cortical cytoplasm: mvb = multivesicular body.

cytoplasm. These eggs, if fertilized in normal sea water, do not cleave.

In eggs fertilized in TBTCl medium, TEM observations reveal some modifications in the ultrastructural morphology of the test cells and of the cortical egg cytoplasm (Figs 9–16), similar to those previously described in unfertilized eggs incubated in TBTCl solutions. Many spermatozoa, even if highly damaged, are observed at the vitelline coat level, but never into the cortical cytoplasm of the eggs (Figs 9 and 10; arrow). In fact, the eggs after 3 h of incubation in TBTCl do not cleave.

The mitochondria of the eggs show a serious degree of damage; many vesicles, multivesicular bodies and other organelles of varying shape and granular content are also scattered in the cortical cytoplasm (Figs 10–12 and 15). The latter consist of a single membrane and contain material that appears slightly denser than the cytoplasm; from their characteristics they could be interpreted as peroxisomes in various degrees of genesis (Figs 13, 14 and 16).

DISCUSSION

The results described above constitute evidence of some deleterious effects of TBTCl on gametes and on the fertilization process. Spermatozoa are strongly damaged; these changes are indicative of possible implications for fertility potential. Unfertilized eggs incubated for 3 h in TBTCl solutions are seriously damaged: the mitochondria are anomalous and microbodies are present in great numbers compared with the controls. The treated eggs, if fertilized with normal sperm, do not cleave, as Mansueto *et al.*[8] have also demonstrated in *Ciona intestinalis*.

The same anomalies are found in the fertilized eggs, where the vitelline coat is different from that of the controls and it contains many damaged spermatozoa. In all cases, the eggs after 3 h of incubation in TBTCl solutions do not cleave: no sperm nuclei have been detected in the periphery of eggs. Following Franchet *et al.*,[30] TBT impedes sperm–egg interactions at the egg coat level in *Phallusia mammillata*, but it does not prevent sperm–egg fusion in naked eggs even if it has a deleterious effect on the sperm-activated channels of the egg membrane. *In vivo* studies have shown that dechorionated eggs of *C. intestinalis*, fertilized in toxic compound solutions and incubated for 10 h, generally eject the first polar body and present delayed and abnormal fertilization movements, and in no case did they cleave.[8]

The follicular cells detach very soon from the eggs; those rarely found in incubated eggs do not show significant signs of alteration. Fundamentally, they greatly influence the sperm behaviour in sperm–egg interaction.[31] Instead, the test cells show several alterations in their nucleus, nuclear envelope and granules. Many functions have been attributed to these cells in the events that occur between oogenesis and hatching: nutritive contribution to the oocytes during oogenesis;[32–35] production of hatching enzymes;[36] synthesis of pigments;[37] incorporation of vanadium and iron into the oocyte;[38,39] deposition of ornaments on the tunic in the tailbud stage;[40] and a role in development and metamorphosis in *C. intestinalis*.[41] The granules, bigger than those of controls, peripherically located and electron-dense, could be complex lipids in different degrees of oxidation of a lipofuscin nature.[42] A big granule positive to the lipofuscin reaction has been shown in the periphery of the test cells in *Ascidiella aspersa*.[34] A correlation between age and lipofuscin accumulation using histological techniques has been clearly established in crustaceans and other marine animals.[43,44] Enesco *et al.*[45] and Guarino *et al.*[46] have demonstrated that pollutants affect lipofuscin in cells, as probably occurs in test cells of ascidians.

TBTCl exposure causes several types of damage to mitochondria of gametes, either on the external membrane or on cristae. TBT is known for its effects on structure and metabolism of the mitochondria, inhibiting ATP synthesis by binding to a component of the ATP synthase complex.[25,26,47–50] Consequently, the sperm are unable of moving to fertilize the female gamete.

The anomalous nuclei of test cells, in particular the finely granular chromatin, could be related to alterations of chromosomes, as demonstrated in *Truncatella subcylindrica* treated with dibutyltin(IV) derivatives and in *Aphanius phasciatus* with diorganotin(IV) chloride and triorganotin(IV) chloride.[51,52] There is evidence that contaminant exposure often leads to changes in chromosome and gene structure.[53]

Spherical organelles, bounded by a single membrane, are abundant in unfertilized and fertilized eggs incubated for 3 h in TBTCl. In a previous study,[47] an abundance of organules that had the structure of peroxisomes were in evidence in the first embryonic stages of *C. intestinalis* treated with TBTCl.[47] In the digestive gland cells of molluscs, peroxisomes have been observed to proliferate in response to organic pollutants.[54,55] In our research, the abundance of organules bounded by a single membrane could be interpreted as being peroxisome-like organules that proliferate in the presence of TBTCl, indicating a possible role in the metabolic removal of toxic agents and in alleviating damage to egg components. We have initiated research to identify whether these organules are real peroxisomes, because they are the focus of interest among cellular and medical biologists for their involvement in genetic and degeneration diseases:[56] some inherited diseases have been linked to peroxisomal deficiencies in humans.[57] Actually, the problem is how new peroxisomes can form; that is, how can an organelle with no genome propagate itself, under exposure to some environmental pollutants. At present, 23 different genes (PEX genes) have been identified as being involved in different aspects of their biogenesis.[58] It may be that TBTCl can cause alterations in the expression of various genes involved in the cellular defence mechanism, by priming the various steps for the proliferation of these organelles. This hypothesis is supported by a change of gene expression by TBTCl stimulating

alkaline phosphatase and cholinesterase activity in the ovary of *C. intestinalis*.[26] In our study, many vesicles of different size are seen in the egg cytoplasm; some of them are in close contact with each other: the fusion of small peroxisomal vesicles for the formation of larger ones in *Yarrowia lipolytica* has been reported.[59] We suppose that the small vesicles observed in the egg cytoplasm could be preforms of peroxisome biogenesis; moreover, the association of peroxisomes with mitochondria could be significant for the transport of small molecules of lipids as substrates for cellular respiration.

In conclusion, this research has demonstrated that TBTCl strongly affects reproduction in ascidians and suggests that it can produce, as with many other pollutants, a reduction not only in their populations, but also in other species of marine biota.

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