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Organometallic complexes with biological molecules. XV. Effects of tributyltin(IV)chloride on enzyme activity, Ca^{2+}, and biomolecule and synthesis in *Ciona intestinalis* (Urochordata) ovary

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Considerable attention has been given in recent years to the possibility that xenobiotics in the environment may affect reproduction in animals. In this study, the relative impact of tributyltin(IV) (TBT) chloride, one of the most toxic environmental pollutants, was investigated using *Ciona intestinalis* ovary as a model system. The pleiotropic effects of TBT exposure are concentration dependent and include a decrease of ATP levels, lipid content and nucleic acid content and synthesis. In contrast, a marked increase in calcium (Ca^{2+}) and glucose content is observed. Furthermore, TBT alters enzymatic activity, inhibiting creatine kinase and stimulating alkaline phosphatase and cholinesterase (at concentrations higher than 10^{-5}M in sterile sea water solution). The implications of these effects on reproduction and embryonal development are discussed, along with the possibility that they reflect an extreme cellular defence mechanism triggered to avoid deleterious consequences for the survival of the species. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

There is evidence that many estuarine and coastal waters, in particular within the Mediterranean Sea, are heavily polluted by organotin compounds. In a number of reports, the effects of organotin compounds on animals and mammalian cells have been investigated. Some organotin compounds are neurotoxic and immunotoxic.[1] In tunicates, they affect phagocytic activity of haemocytes,[2–4] apoptosis[5] and cytoskeletal alteration during the first cell cleavage[6,7] and in phagocytes.[8,9]

Organotin compounds also inhibit phagocytosis and exocytosis in the rabbit.[10] It has been demonstrated that tributyltin(IV) (TBT) derivatives affect chromosome structure in molluscs and fish.[11,12] Moreover, a number of biochemical systems have been shown to be sensitive to organotin compounds, e.g. oxidative phosphorylation and ATPase activity are inhibited in calf heart mitochondria.[13,14] Reduced levels of nucleic acids, lipids, proteins, glucose and ATP content have been observed in ascidian embryos after treatment with TBT porphinate derivatives.[15] At the ultrastructural level, the plasma membrane, mitochondria and myofibril structure of ascidian embryos exposed to organotins are damaged.[16,17]

Recently, it has been demonstrated that many substances can compromise the reproductive system. They can impair the production of gametes and alter genotype, structure and functionality, with the risk of severe damage to the fertilization process and the embryo.[18–20] In many marine prosobranch snails, TBT compounds induce abnormalities in the female sexual apparatus,[21,22] leading to reproductive failure and to population decline.[23] Other

reports indicate that fish, birds, reptiles, mammals and other species inhabiting environments polluted with synthetic compounds also suffer reproductive problems.[19,20] Previous research on ascidian gametes has shown that exposure to organotin compounds leads to reduced sperm motility and loss of the fertilization power of eggs.[6,7] In this study, the ovary of *Ciona intestinalis* was chosen as a model system in order to understand better the biological mechanisms underlying the TBT chloride (TBTCl) toxicity on the reproductive system. During ascidian oogenesis it is possible to demonstrate three periods of synthetic activity: the first period is characterized by mitotic activity of the germ cells and DNA synthesis is predominant; in the second period, RNA (particularly rRNA) and proteins are intensively synthesized; the third period is mainly characterized by synthesis of yolk proteins and lipids.[24,25] It also seems that the test cells which surround the oocytes contribute to this intense synthetic activity by furnishing nutritive substances to the cytoplasm of the oocytes. A high incorporation of proteins and nucleic acid precursors is observed in these cells.[24,26] In particular, in ascidians the determination of cell fate during embryogenesis appears to be mediated by cytoplasmic factors or determinants. These are thought to originate during oogenesis, localize in the egg, segregate into different cell lineages during cleavage and eventually regulate gene expression.[27–29]

This study investigates the effects of TBT exposure of the *C. intestinalis* ovary on nucleic acid, protein and lipid metabolism, cellular ATP and Ca^{2+} levels and enzymatic activity.

EXPERIMENTAL

Chemicals

Sterile sea water (SSW) was obtained by filtering and pasteurizing at 80 °C normal sea water, containing 100 μg of chloromycetin/ml. TBTCl was a gift from Witco GmbH (Bergkamen, Germany). A 0.1 mM TBTCl solution was prepared by dissolving the compound in 0.07% dimethylsulfoxide (DMSO) containing SSW. Then 10^{-5} and 10^{-7} M solutions were obtained by dilution and their total tin contents were checked using a Perkin Elmer model 3100 atomic absorption spectrometer (equipped with a Perkin Elmer model 100 flow injection analysis system for atomic spectroscopy) according to standard procedures. The solvent DMSO, used because of the low solubility of the compound in non-coordinating solvents, was a Merck (Darmstadt, Germany) reagent. ^{3}H-thymidine (25–30 Ci mmol^{-1}, TRK 120), ^{3}H-uridine (25–30 Ci mmol^{-1}, TRK 178) and, ^{3}H-leucine (25–30 Ci mmol^{-1}, TRK 178) were from Amersham chemicals (Buckinghamshire, UK).

Ca^{2+} content (Kit N. 587 A) and the enzyme activity of creatine kinase (Kit N. 45.1), cholinesterase (Kit N. 420 MC) and alkaline phosphatase (Kit N. 104-LS) were determined by using appropriate reagents from Sigma Chemie GmbH (Steinheim, Germany).

Cultures

Ovaries of *C. intestinalis* were removed from a number of animals and, after washing in SSW, were divided into three batches: the first was cultured in SSW and used as a control; the other two were cultured in TBTCl solutions at concentrations of 10^{-5} M and 10^{-7} M (all in SSW), and used as tests to study biochemical TBT effects. After 24 h incubation at room temperature, ovaries of the three batches were washed several times in SSW and frozen at −80 °C until appropriate extraction and analysis. To investigate the effects of TBT exposure on the synthesis of nucleic acids and proteins, ovaries were incubated for 24 h at 25 °C in 10^{-5} and 10^{-7} M TBT-containing media, in the presence of labeled radioactive precursors to DNA, RNA and proteins: ^{3}H-thymidine, for DNA synthesis; ^{3}H-uridine, for incubation in control ovaries and for RNA synthesis; ^{3}H-leucine, for protein synthesis. Control ovaries were incubated for 24 h in SSW, where the appropriate labelled precursors were dissolved. 10 μCi ml^{-1} of appropriate precursor was used for the incubation and the incorporation of the label was stopped after 24 h by adding 0.1 vols of 10^{-2} M of the same, but non-labeled, precursor in SSW.

Ovaries of each batch were washed several times in SSW and kept at −80 °C until appropriate extraction and analysis.

Extractions and analysis

DNA, RNA, proteins, lipids, glucose and ATP were extracted and analysed as previously described.[15] To study the TBT effects on nucleic acid and protein synthesis, ovaries, previously incubated in each of the radioactive precursors, were homogenized separately, and each homogenate was divided into three parts. From each fraction, the

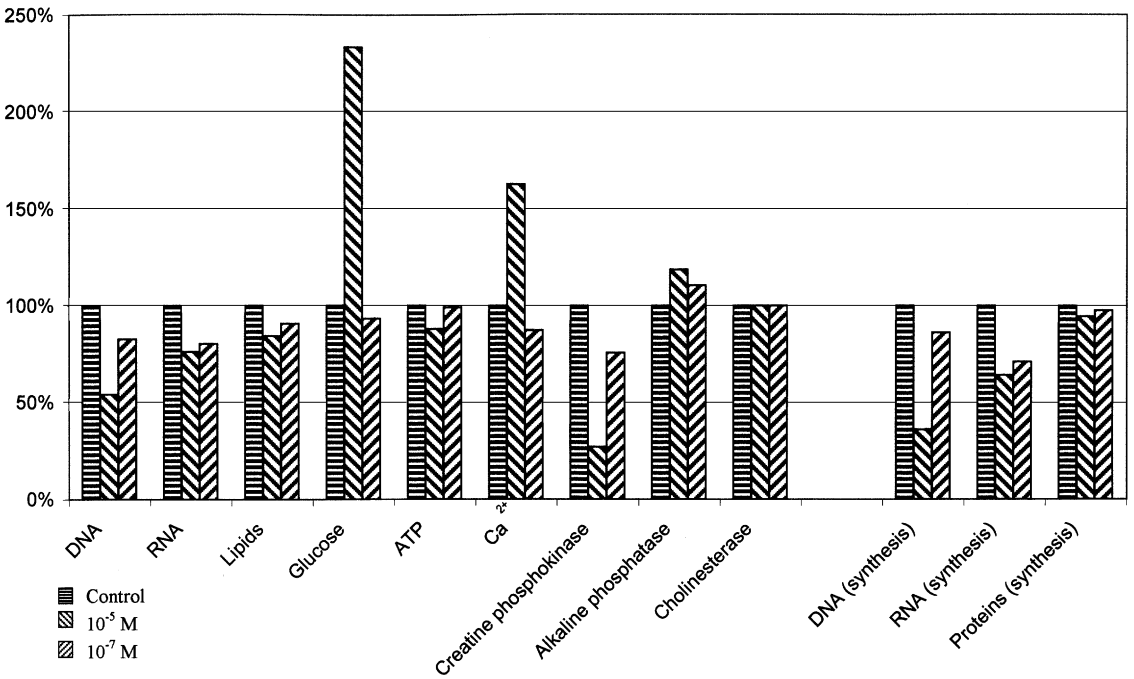

Figure 1 Effects of TBT exposure of *C. intestinalis* ovary, on creatine kinase, alkaline phosphatase ativity, ATP, Ca^{2+}, glucose, lipids, nucleic acids and protein content and synthesis. All data are plotted as a percentage of control values (100%). The concentration of each variable measured, at the two different TBT levels (control $\rightarrow 10^{-5}$ M TBT level and control $\rightarrow 10^{-7}$ M TBT level), were analysed with a *U* test.[32]

extraction and analysis of DNA, RNA and protein were performed as usual.[15]

Radioactivity was measured as cpm (counts per minute) with a Beckman LS1800 liquid scintillation counter. The scintillation solution was an aqueous counting scintillant, ACS (Amersham, Buckinghamshire, UK). Aliquots were also withdrawn from the initial homogenates to determine Ca^{2+} content and enzymatic activity, (in International Units) of creatine kinase, cholinesterase and alkaline phosphatase. All values were the average of three determinations ±S.D. and were normalized to protein mass as in *C. intestinalis*; a constant protein amount corresponds to a prefixed egg-number.[30,31] The data were plotted as percentages of the control value, which was considered 100%.

Table 1 Relative amount of nucleic acids (micrograms of nucleic acids per milligram of proteins; average of three determinations). The percentage with respect to the control is reported in parentheses

Culture	DNA	RNA
Control	9.9 ± 0.2	117 ± 2
TBT, 10^{-5} M	5.4 ± 0.2 (54.5%)	89 ± 3 (76.1%)
TBT, 10^{-7} M	8.2 ± 0.2 (82.8%)	94 ± 9 (80.3%)

RESULTS

Figure 1 summarizes the effects of TBT exposure of *C. intestinalis* ovaries on creatine kinase and alkaline phosphatase activity, ATP, Ca^{2+}, glucose, lipids, nucleic acids, protein content and synthesis. As all data are plotted as a percentage of control values, the effect of TBT on cholinesterase activity could not be plotted in Fig. 1, as the enzyme is not detectable in control ovaries (*vide infra*).

The total nucleic acid contents in control and TBT-exposed ovaries are shown in Table 1.

The DNA content decreases to almost half of the control value in the ovaries exposed to 10^{-5} M TBT. A substantial reduction of the RNA content is also observed under the same conditions. In Table 2, newly synthesized proteins and nucleic acids are analysed by measuring the specific activity of the

Table 2 Specific activity (cpm/μg) of the protein, DNA and RNA 'ex novo' synthesized after exposure of the ovaries to TBTCl. The percentage of each specific activity with respect to the control is reported in parentheses

Culture	Proteins	DNA	RNA
Control	39 ± 2	25 ± 2	18 ± 1
TBT, 10^{-5} M	37 ± 2 (94.9%)	9 ± 0.3 (36%)	12 ± 1 (66.7%)
TBT, 10^{-7} M	38 ± 2 (97.4%)	22 ± 1 (88%)	12.8 ± 0.4 (71.2%)

Table 3 Lipids, glucose, ATP and Ca^{2+} concentrations, expressed as micrograms of the compound per milligram of proteins (average of three determinations), in the ovaries after exposure to TBTCl. The percentage of each concentration with respect to the control is reported in parentheses

Culture	Lipids	Glucose	ATP	Ca^{2+}
Control	463 ± 10	0.15 ± 0.01	17.69 ± 0.65	3.8 ± 0.4
TBT, 10^{-5} M	390 ± 12 (84.2%)	0.35 ± 0.03 (233.3%)	15.5 ± 0.5 (87.6%)	6.3 ± 0.5 (165.8%)
TBT, 10^{-7} M	420 ± 15 (90.7%)	0.14 ± 0.01 (93.3%)	17.53 ± 0.48 (99.10%)	3.4 ± 0.4 (89.5%)

biomolecules extracted from the ovaries exposed to TBT in the presence of the appropriate radioactive precursor. Values are expressed as specific activity of ^{3}H-DNA (cpm of incorporated ^{3}H-thymidine over micrograms of total DNA extracted), ^{3}H-RNA (cpm of incorporated ^{3}H-uridine over micrograms of total RNA extracted), and ^{3}H-proteins (cpm of incorporated ^{3}H-leucine over micrograms of total proteins extracted).

The most drastic effect appears to be on nucleic acid synthesis. A sharp decrease of DNA specific activity to 36.0% of the control value is observed in the ovaries exposed to 10^{-5} M TBT solution. A much less drastic decrease, 88.0% of the control value, is also observed in ovaries exposed to 10^{-7} M TBT solution.

A noticeable effect on RNA synthesis is also found. Ovaries exposed to 10^{-5} M and 10^{-7} M TBT are characterized by decreases of 66.7% and 71.2% in RNA specific activity respectively. Interestingly, there is very little difference in protein specific activity with respect to the control value. Overall, TBT seems to alter preferentially the nucleic acid metabolism.

From Table 3, 15.8% and 9.3% reductions of lipid content (with respect to the control value) are observed in ovaries exposed to 10^{-5} M and 10^{-7} M TBT solutions respectively. ATP content basically remains at control values in ovaries exposed to 10^{-7} M TBT, whereas a 12.4% reduction of ATP is observed upon exposure to 10^{-5} M of pollutant. TBT induces a greater than twofold increase of glucose content when ovaries are exposed to a 10^{-5} M solution of pollutant, and causes a slight decrease when present at a lower concentration (10^{-7} M). A similar pattern is also observed when investigating Ca^{2+} content after TBT exposure. The pollutant, in fact, induces a 65.8% increase of Ca^{2+} in ovaries exposed to 10^{-5} M TBT, and causes a 10.5% decrease at lower concentrations (10^{-7} M). Table 4 shows specific activities of the enzymes investigated with values expressed in International Units over milligrams of protein. When assaying for creatine kinase activity, a dramatic 73.0% decrease, with respect to the control value, is found in ovaries exposed to 10^{-5} M TBT, whereas a 24.3% decrease is induced by 10^{-7} M TBT. In contrast, alkaline phosphatase activity increases at

Table 4 Enzyme activity, expressed in International Units per milligram of proteins (average of three determinations), after exposure of the ovaries to TBTCl. The percentage of each concentration with respect to the control is reported in parentheses

Culture	Creatine kinase	Alkaline phosphatase	Cholinesterase
Control	0.37 ± 0.07	0.25 ± 0.06	0
TBT, 10^{-5} M	0.10 ± 0.04 (27.0%)	0.29 ± 0.04 (116.0%)	0.6 ± 0.2
TBT, 10^{-7} M	0.28 ± 0.08 (75.7%)	0.27 ± 0.05 (108.0%)	0

both TBT concentrations tested, with the highest increase of activity, being detected in ovaries exposed to the highest concentration of TBT used in this study. Interestingly, cholinesterase activity was found only in the ovaries exposed to 10^{-5} M TBT, being undetectable in the control ovaries, and in the ovaries exposed to 10^{-7} M TBT.

DISCUSSION

The dose-concentration-dependent behaviour of TBT toxicity on *C. intestinalis* ovary is consistent with previous data obtained *in vivo* at the ultrastructural level, in eggs and embryos.[7,16] Moreover, it has been noticed that the effects are also incubation-time-dependent.[7,11,16,17] TBT already leads to a decrease of nucleic acids upon exposure to the lowest concentration (10^{-7} M) used in this study, causing a dramatic reduction of nucleic acid content and synthesis at the highest concentration (10^{-5} M) used. Moreover, when compared with control values, TBT causes a greater relative reduction of DNA than of RNA content. The DNA decrease in the ovary is indicative of reduction of cellular reproduction, the first step of oogenesis, suggesting TBT inhibition of the production of germinal cells, which give rise to eggs, with drastic consequences for the survival of the species. The test cells will, most likely, also be affected, as in oogenesis their role is to reproduce actively; it is suggested that in this period their function is to nourish and protect the egg.[24,26] In this study, we did not address how TBT decreases DNA content. However, one possibility is that it could do so by triggering apoptosis, an innate cellular suicidal defence program, known to be well conserved through evolution.[33] A molecular hallmark of apoptosis is a characteristic degradation of cellular DNA, and various *in vitro* studies have shown that exposure to TBT, rather than being directly cytotoxic, actually triggers programmed cell death. TBT is, indeed, well known to induce apoptosis in mammals and has also been reported to trigger apoptosis in fish,[34] marine sponges[35] and tunicates.[5] The available evidence strongly indicates that the intracellular Ca^{2+} increase observed upon TBT exposure plays a pivotal role in this mode of cell death. Interestingly enough, our data show that 10^{-5} M TBT also induces a marked increase of Ca^{2+} content in *C. intestinalis* ovaries. Organotin-induced apoptosis has been thoroughly investigated using rat thymocytes as a model. In this system, TBT promotes cellular suicide by activating cysteine proteases (called caspases), which selectively cleave vital cellular substrates and this results in internucleosomal fragmentation of DNA by selectively activated DNases.[36,37]

TBT is also known to induce a rapid increase of intracellular Ca^{2+} levels (*vide supra*) and Ca^{2+} chelation by EGTA [ethyleneglycol-bis-(*β*-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid] and/or BAPTA, [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid], and can block caspase activation and TBT-induced apoptosis. In this pathway, the rise in Ca^{2+} content is a prerequisite for postmitochondrial events involved in caspase activation leading to induction of apoptosis, events which TBT-exposed cells grown in a Ca^{2+}-free medium are able to evade, dying by necrosis.[38]

Another feature observed during TBT-induced apoptosis is mRNA degradation.[39] mRNA is the primary product of the gene information contained in the DNA molecule: it is the template used for translation, the synthesis of proteins. An intense synthesis of RNA occurs in the nucleus (a major portion being synthesized in the nucleolus: rRNA) of germinal cells during ascidian oogenesis.[25] This RNA will be used during the first steps of embryonal development with synthetic activity resuming only later, that gastrula stage.[30]

Reduction of RNA content was observed in the ovaries exposed to TBT-containing media and can be interpreted as a direct effect due to decrease of RNA stability, or as an indirect effect linked to alteration of gene activity. With regard to protein synthesis, at both concentrations used in this study, only a small reduction with respect to control values was observed in ovaries exposed to TBT. Taken at face value, the data suggest that TBT does not have a remarkable effect on translation in *C. intestinalis* ovaries. However, one must keep in mind that a strong increase in the synthesis of a specific subset of proteins could render detection of a more general decrease of protein synthesis more difficult. In this regard, it is worth noting that TBT is a potent inducer of the heat-shock response,[38–40] promoting neo-synthesis of stress proteins from mRNA already present in the oocytes as well as by inducing the synthesis of new mRNA encoding such proteins.

The synthesis of stress proteins represents a fundamental universal protective mechanism necessary for cell survival under a variety of unfavourable conditions. Considering marine invertebrates, induction of stress protein synthesis has been observed in crayfish.[41,42] Moreover, TBT has

been also shown to promote synthesis of such polypeptides in the rotifer *Brachionus plicatilis*.[43] Furthermore, 10^{-5} M TBT has been reported to decrease protein synthesis significantly in other cell-types;[44] in that study, it was a dramatic reduction of the ATP levels that seemed to be responsible for the effect. In this respect, it is remarkable that in *C. intestinalis* ovaries exposed to 10^{-5} M TBT, ATP content decreases only by 12.4%. Considering that TBT exposure causes severe damage to mitochondria,[16,17] the major cellular sites of ATP production, one would expect massive ATP depletion, unless cellular ATP levels were maintained through an alternative pathway. Indeed, it is well known that triorganotins disturb mitochondrial activity by binding to a component of the ATP synthase complex, inhibiting mitochondrial ATP synthesis.[44,45]

Interestingly, it has recently been reported that intracellular ATP levels can modulate the mode of cell death after exposure to TBT, with necrosis following ATP depletion and apoptosis following glucose-dependent maintenance of ATP levels.[46] Furthermore, the same study provides evidence for the requirement of cellular ATP for caspase activation. In order to gain insight into the apparent maintenance of ATP levels, despite the TBT perturbation of mitochondrial activity, we decided to quantify cellular glucose content in our system.

Indeed, we observed a dramatic increase of glucose in 10^{-5} M TBT-exposed ovaries, with respect to the control ovaries, making it very tempting to speculate a possible induction of a compensatory mechanism aimed at maintaining cellular ATP levels by mobilizing glucose to increase glycolitic ATP production. In further investigating the effects of TBT exposure on energetic metabolism, creatine kinase activity was assayed.

This enzyme catalyses the transfer of inorganic phosphate from ATP to creatine, producing phosphocreatine (energetic reserve) and ADP. Our data show decreased enzyme activity following TBT exposure. In particular, the 73.0% reduction in creatine kinase activity observed at the highest TBT concentration (10^{-5} M) used in this study can only be partially explained by the mild ATP depletion observed after TBT treatment. It is possible that TBT disturbance of the mitochondrial proton gradient could divert electrons from the respiratory chain, leading to the formation of reactive oxygen species, which have been reported to inactivate creatine kinase by oxidating critical SH groups.[47] Alternatively, the reduced activity of creatine kinase could be due to a direct interaction of the organotin with the protein. This inhibitory effect of TBT on enzymatic activity seems to be specific to creatine kinase, in that alkaline phosphatase increases and cholinesterase activity is present only in ovaries exposed to TBT 10^{-5} M, being absent both in the control ovaries and in those exposed to TBT 10^{-7} M. Alkaline phosphatase is synthesized in the endodermic cells during embryonal development and is segregated in the cells that become the branchial and digestive tissues of the post-metamorphic juvenile and adult.[48,49] It has been demonstrated that actinomycin D (inhibitor of RNA synthesis) does not affect the synthesis of this enzyme, whereas translation inhibitors reduce its expression. Therefore, the mRNA coding for alkaline phosphatase must already be present in the cytoplasm of oocytes and segregated in the endodermic cells during development.[50] Our observations indicate a moderate increase of alkaline phosphatase activity after exposure of ovary cells to TBT. It is interesting that the same result has been observed in unfertilized eggs of *C. intestinalis* treated with calcium ionofore A 23187,[51] which, like TBT, is known to trigger cell death through a mechanism in which an increase of Ca^{2+} content seems to play a major role.[45]

The effect of TBT exposure on enzyme activity is more dramatic for cholinesterase. In our experiments the enzyme is not detectable in control ovaries,[52] nor in ovaries incubated in 10^{-7} M solution, but it becomes clearly detectable in ovaries incubated in 10^{-5} M solution. Previous reports indicate that cholinesterase is a major contributor to total enzymatic activity in *C. intestinalis*.[53] However, this enzyme appears at the neurula stage, localizing in muscle cells of the swimming larva. Perhaps the anticipated detection of cholinesterase in TBT-exposed ovaries is due to a positive regulation of the enzyme's expression, mediated by TBT. Indeed, there is evidence of changes in gene activity observed in response to physical and chemical stress.[54,55] Furthermore, TBT has been shown to induce gene regulatory pathways through activation of NF-κB, a transcription factor that controls the inducible expression of various genes involved in cellular defence mechanisms. Interestingly, TBT-induced NF-κB activation is preceded by an increase in intracellular Ca^{2+} and is almost completely abrogated by BAPTA.[56] Finally, we observed a slight decrease of lipid content. This could reflect molecular disorder at the level of the plasma membrane and other cytomembranes (mitochondria, nuclear envelopes, etc.)

induced by TBT.[16] Indeed, TBT is a well known membrane-active molecule[57,58] and its effect on biomembranes is a fundamental aspect of TBT toxicity. By disturbing membrane structure, it affects cell function, as cellular interactions with the surrounding environment are mediated by cell membrane components. TBT is also a well known anion carrier in membranes[59,60] and could sever important cytoskeletal interactions by sequestering anionic poshatidylinositol-4,5-diphosphate.[61] In addition, one can imagine a potential production of tri-*n*-butylstannylperoxy free radicals[59] which could lead to lipid peroxidation, extending the lipotoxic effects to non-membrane components, such as yolk lipids, critical during ascidian oogenesis.

We conclude that TBT strongly affects Urochordata oogenesis by interfering with normal cellular metabolism and enzyme activity, possibly by triggering extreme cellular defence mechanisms. Changes in germinal cells caused by TBT could cause deleterious alterations, such as changes in gene expression, which could lead to altered production and, consequently, an anomalous segregation of cytoplasmic determinants into different lineages during early cleavage, thus causing anomalous embryo development. The likely possibility that, upon TBT exposure, these cells choose to activate cellular suicide to avoid aberrant embryonal development is currently being tested. In conclusion, this study shows that the female reproductive system in Urochordata is heavily affected, at a biochemical level, by the chemical pollutant TBT.

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