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Toxicology of yessotoxin: an update

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Effects of yessotoxin (YTX) on the skeletal muscle: an update

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Abstract

Yessotoxins (YTXs) are algal toxins originally included in the diarrheic toxins. After oral intake, YTXs induce only ultra-structural changes (packages of swollen mitochondria) in cardiac cells. The aim of this study was to investigate the possible effects of YTX on the other contractile striated tissue, the skeletal muscle, *in vitro* and *in vivo*. *In vitro*, in skeletal mouse myotubes, YTX (0.01-1.0 μM) influenced cell excitability in a concentration- and time-dependent way. In the *in vivo* study, transmission electron microscopy analysis did not reveal any ultra-structural alteration of skeletal muscle after acute (1 mg kg^{-1}) or repeated (1 and 2 mg kg^{-1} day^{-1}, for 7 days)

oral administration of YTX to mice. The observation that effects were detected *in vitro* but not *in vivo* supports the hypothesis of a low YTX bioavailability to skeletal muscle after *per os* intake. Therefore, our results seem to exclude a toxic effect in skeletal muscle when YTX is consumed as a food contaminant.

Keywords: yessotoxin; skeletal muscle cells; calcium; videomicroscopy; transmission electron microscopy.

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Introduction

Yessotoxin (Figure 1) and analogues (YTXs) are ladder-shaped lipophilic polycyclic ether compounds of algal origin, initially included in the Diarrheic Shellfish Poisoning (DSP) toxins group, and often detected in mussels together with the primary DSP toxin okadaic acid (OA). YTXs are produced by *Protoceratium reticulatum* (= *Gonyaulax grindley*) (Satake *et al.* 1997a), *Lingulodinium polyedrum* (= *Gonyaulax polyedra*) (Paz *et al.* 2004) or *Gonyaulax spinifera* (Rhodes *et al.* 2006), organisms that are distributed. worldwide. So far, no human poisoning induced by YTXs has been reported (Toyofuku 2006). Moreover, since YTXs do not provoke diarrhoea, they have been recently reclassified and separately regulated from DSP toxins (CEE 2002).

"[Insert Figure 1 about here]"

The oral toxicity of YTXs appears to be low. After acute oral administration of YTX (1-10 mg kg^{-1}) in mice, only ultrastructural changes in cardiomyocytes were observed: the morphological alterations were detectable only in restricted areas of the heart close to the capillaries (Terao *et al.* 1990; Aune *et al.* 2002; Tubaro *et al.* 2003). In a more recent study consisting of 7 oral doses administered over a 3 weeks, a No Observable Adverse Effect Level (NOAEL) of 5 mg YTX kg^{-1} was established (Espenes *et al.* 2004). In the absence of human data and applying a safety factor of 100, a provisional acute reference dose of 50 μg kg^{-1}, corresponding to a Regulatory Limit (RL) of 12 mg YTXs kg^{-1} shellfish meat, was recently proposed (Toyofuku 2006). However, repeated daily oral administration of YTX (1 or 2 mg kg^{-1} day^{-1}, for 7 days) in mice induced ultrastructural changes such as alterations of fibrils and packages of swollen

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mitochondria in cardiomyocytes near capillaries, similar to those observed after acute oral intake (Tubaro *et al.* 2004; Callegari *et al.* 2006).

In vitro studies showed that YTX causes apoptosis in skeletal muscle cell lines *via* a mitochondrial pathway (Korsnes *et al.* 2006), suggesting that also the skeletal muscle could represent a target tissue for the toxin. The aim of the present study was to further investigate the effects of YTX on the skeletal muscle both *in vitro* and *in vivo*. *In vitro* experiments were performed to investigate if YTX affected the intracellular calcium transients generated by the action potentials. *In vivo* experiments were performed to analyze the possible ultrastructural effects of YTX on skeletal muscle cells of mice because no effect was previously observed by light microscopic examinations after acute or repeated oral administration of YTX (Tubaro *et al.* 2003 and 2004).

Materials and methods

Materials

YTX was isolated from *P. reticulatum* collected in Mutsu Bay (Japan) (Murata *et al.* 1987). The purity of the final preparation was confirmed by mass spectrometry and liquid chromatographic methods (Satake *et al.* 1997b; Yasumoto and Takizawa 1997). YTX was dissolved in 95 % aqueous ethanol and, for oral administration, aliquots of the stock solutions were properly diluted with saline solution (0.9 % NaCl), in order to administer the appropriate dose of the toxin and reduce the concentration of ethanol to 1.8 % (v/v). For *in vitro* studies, aliquots of the stock solution of YTX were properly diluted, in order to reach a concentration of ethanol in the cells medium lower than 1 % (v/v).

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Dulbecco's modified Eagle's medium (DMEM), horse serum, penicillin, streptomycin and glutamine were purchased from ICN Biomedicals (Irvine, USA). Bovine fetal calf serum was purchased from PAA Laboratories (Pasching, Austria), 3 % glutaraldehyde from Serva (Heidelberg, Germany) and Dow Epoxy Resin (DER 332; Unione Chimica Europea; Milan, Italy). All other chemicals were Carlo Erba products of analytical grade (Milan, Italy).

Cell cultures

Skeletal muscle cell cultures (i28) were established from mouse satellite cells (Irintchev *et al.* 1997). Briefly, satellite cells were isolated from the hind leg muscles of 7-day-old male Balb/c mice killed by cervical dislocation. Muscle tissue was minced and then treated with collagenase and trypsin. Expansion and enrichment of myogenic cells were achieved by repeated plating and cultivation in DMEM with D-valine containing 20 % fetal bovine serum. Muscle cells could be maintained as exponentially growing myoblasts over 40 passages in the presence of a medium consisting of HAM'S F-10 containing 20 % fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). To induce cell differentiation and myotube fusion the medium was replaced, 1 day after plating, with DMEM supplemented with 2 % horse serum and L-glutamine, penicillin, and streptomycin as above. Cells were maintained at 37 °C in CO_2 (5 %)-enriched air. Experiments were performed on myoblasts maintained in culture from the 5th to the 40th passage and cultured up to 10 days in differentiation medium.

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Video-imaging

Video-imaging was used to measure the intracellular calcium concentration ($[Ca^{2+}]_i$). Experiments were carried out at 37 °C on Fura-2 loaded myotubes attached to a coverslip treated with matrigel. All the experiments were performed on myotubes bathed in an external solution containing 2 mM $CaCl_2$. Briefly, the cells were alternatively excited at 340 and 380 nm and the fluorescence images were acquired with an intensified CCD camera [Hamamatsu Photonics, Hamamatsu, Japan]. The acquisition rate was 4 frames/s. The calculation of ratio images (Grynkiewicz *et al.* 1985) and the temporal plots of the fluorescence signals were performed off-line.

Animals and experimental conditions

Experiments were carried out in conformity with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC), concerning animal welfare.

Female CD-1 mice (18–20 g) were purchased from Harlan Italy (S. Pietro al Natisone, Italy). Groups of three animals were acclimated for 1 week under the conditions of controlled room temperature (21 ± 1°C), humidity (60–70 %), and a fixed artificial light cycle (7.00 – 19.00 h). Dust-free poplar chips were used as bedding (Harlan Italy; S. Pietro al Natisone, Italy). Mice were fed a global diet for rodents containing 18.5 % protein (Harlan Italy; S. Pietro al Natisone, Italy), and had free access to food and water.

YTX, dissolved in saline solution (0.9 % NaCl) containing 1.8 % ethanol (v/v), was administered to mice by gavage in a volume of 10 ml kg^{-1} body weight. The toxin was administered as a single dose (1 mg kg^{-1}) or as repeated daily doses for 7 days (1 mg

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kg^{-1} day^{-1}). Control animals were given 10 ml kg^{-1} day^{-1} of the vehicle (saline solution containing 1.8 % ethanol v/v). The toxin or vehicle was administered in the morning (9.00–10.00 h). Twenty-four hours after the last administration, the mice were killed by ketamine hydrochloride (350 mg kg^{-1}, administered intraperitoneally). Skeletal muscle (right hind leg) was removed and fixed for examination by transmission electron microscopy (TEM).

Transmission electron microscopy analysis

Blocks of skeletal muscle smaller than 1 mm^3 were promptly fixed in a solution of 3 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h at room temperature and 2 h at 4 °C, rinsed three times (10 min each wash) in the same buffer and post fixed in 1 % osmium tetroxide for 1 h at 4 °C. The samples were then dehydrated in graded ethanols and embedded in Dow Epoxy Resin 332 (Lockwood 1964). Ultrathin sections were cut by an ultratome Leica Ultracut UCT8 (Leica, Mikrosysteme Aktiengesellschaft, Wirn, Austria), double stained with uranyl acetate and lead citrate (Venable and Coggeshall 1965) and examined with a Philips EM 208 transmission electron microscope.

Statistical analysis

In vitro data are expressed as means ± standard error. The Student's *t* test was used to examine statistical significance, assumed for *p* values lower than 0.05.

Results and discussion

Mouse myotubes differentiated *in vitro* generate spontaneous action potentials, accompanied by $[Ca^{2+}]_i$ transients and contractions. The mean event frequency is 0.16

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Hz. The spontaneous action potentials, $[Ca^{2+}]_i$ transients and contractions resemble those triggered by the nerve in skeletal muscle fibers being inhibited by tetrodotoxin and verapamil (Lorenzon *et al.* 2002). The video-imaging technique was used to study the effect of YTX on the skeletal muscle cell excitability, measuring the spontaneous $[Ca^{2+}]_i$ transients.

Incubation of myotubes with YTX (1.0 µM for 20 min) did not affect the frequency of $[Ca^{2+}]_i$ transients (Figure 2). However, after 1 hour, an almost complete inhibition of spontaneous activity was observed: the percentage of active cells was reduced by 96 % with respect to the control cells (Figure 3). No effect was observed at the lower concentrations used (0.1 and 0.01 µM).

"[Insert Figure 2 about here]"

When the incubation time was increased to 24 hours, 0.1 µM YTX significantly reduced the spontaneous activity (77 %). After 48 hours of incubation, the percentage of active cells was reduced by 97 % at all the YTX concentrations tested (Figure 3). It has to be pointed out that the inhibition of the spontaneous activity after 48 hours incubation was associated with a dramatic reduction in the number of viable cells. Thus, YTX affected both the excitability and the viability of skeletal myotubes *in vitro* and its effect was concentration- and time-dependent.

In silent myotubes incubated for 24 hours with 0.1 or 1.0 µM YTX, we tested the responsiveness to physiological stimuli, such as depolarization induced by perfusion

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with 40 mM KCl, a classic membrane depolarizing agent, or 20 mM caffeine, a well known agonist of the intracellular Ca^{2+} channels present on the surface of the sarcoplasmic reticulum.

"[Insert Figure 3 about here]"

The percentage of cells responsive to KCl and caffeine was similar to that observed under control conditions (Figure 4). Therefore, 24 hours incubation with YTX had no effects on the mechanism controlling Ca^{2+} homeostasis in skeletal muscle cells.

"[insert Figure 4 about here]"

The effects of YTX on skeletal muscle were also evaluated *in vivo* after single (1 and 2 mg kg^{-1}) and repeated daily oral administration in mice (1 or 2 mg kg^{-1} day^{-1}, for 7 days). The mice were sacrificed 24 hours after the single or last dose and morphological changes in skeletal muscle were analyzed by TEM. No morphological differences in the skeletal muscle sections were noted between control and YTX-treated mice, neither after acute administration nor at the end of the repeated YTX treatment. Particular attention was given to the mitochondria and myofibrils. The myofibrils presented a normal fine structure similar to the controls and unaltered mitochondria were observed between the fibrils (Figure 5).

"[Insert Figure 5 about here]"

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In conclusion, we observed significant *in vitro* effects on cell excitability and viability when skeletal muscle cells were incubated with YTX. However the toxin did not cause tissue damage or ultrastructural changes in the skeletal muscle *in vivo*. These results support the hypothesis that YTX is poorly absorbed after *per os* intake and that its bioavailability to skeletal muscle is too low to provoke significant morphological changes. On the contrary, under the same experimental conditions, *in vivo* YTX provokes ultrastructural myocardium changes. Thus, cardiac tissue seems to be the specific target for YTX. Further studies are in progress to further clarify the differing responses of cardiac and skeletal muscle, an aspect that could be an important determinant in view of the proposed deregulation for YTX . The present EU Regulatory Limit for YTX is 1 mg of YTX equivalents per kg of shellfish meat (CEE 2002). However, this tolerance level was established considering the estimated No Observable Adverse Effect Level of 1 mg YTX kg^{-1} body weight, based on the results obtained from acute oral exposure to mice, as well as assuming an average shellfish consumption of 100 g and applying a safety factor of 600 due to the lack of data and indications of effects on the cardiac muscle (Espenes *et al.* 2004).

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Figure captions:

Figure 1. Chemical structure of yessotoxin.

Figure 2. Representative trace showing the $[Ca^{2+}]_i$ transients generated by myotubes under control conditions. YTX does not affect the frequency of the cell activity within 20 min of incubation (number of observed cells = 15).

Figure 3. Effect of YTX (0.01, 0.1 and 1.0 μM) on the number of myotubes exhibiting Ca^{2+} spiking activity after 1, 24 or 48 hours incubation. For each experimental point, 10 optical fields were randomly chosen and at least 70 cells were analyzed. * significantly different from the control, $p<0.05$, Student-s t-test.

Figure 4. Responsiveness of myotubes to depolarisation by KCl (40 mM) or caffeine (20 mM) after 24 hours incubation with YTX (0.1 and 1.0 μM). The two traces show representative $[Ca^{2+}]_i$ transients elicited by KCl and caffeine perfusion. The histograms show the percentage of responsive cells detected in the presence of the two stimuli. For each experimental point 3 optical fields were randomly chosen and at least 16 cells were analysed.

Figure 5. Electron micrographs of longitudinal sections of skeletal muscle from control (upper image) and YTX-treated mice (2 mg kg^{-1} day^{-1}, for 7 days; lower image). Similarly, no ultrastructural alterations consequent to YTX administration were observed after acute treatment.

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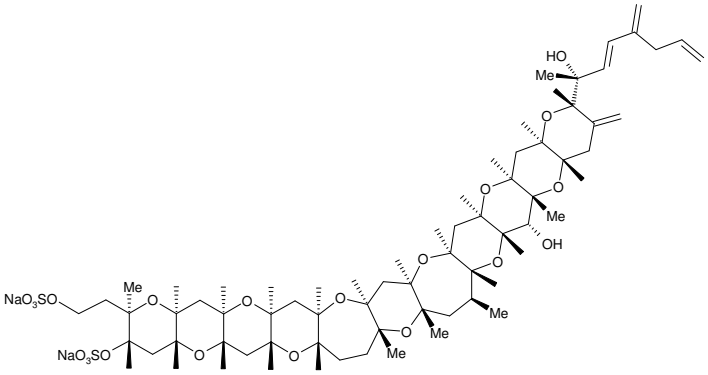

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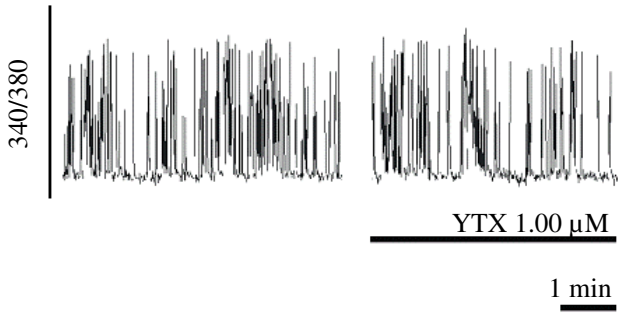

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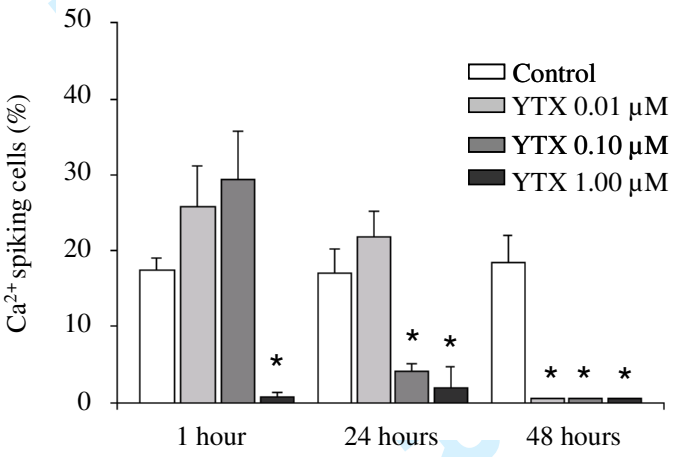

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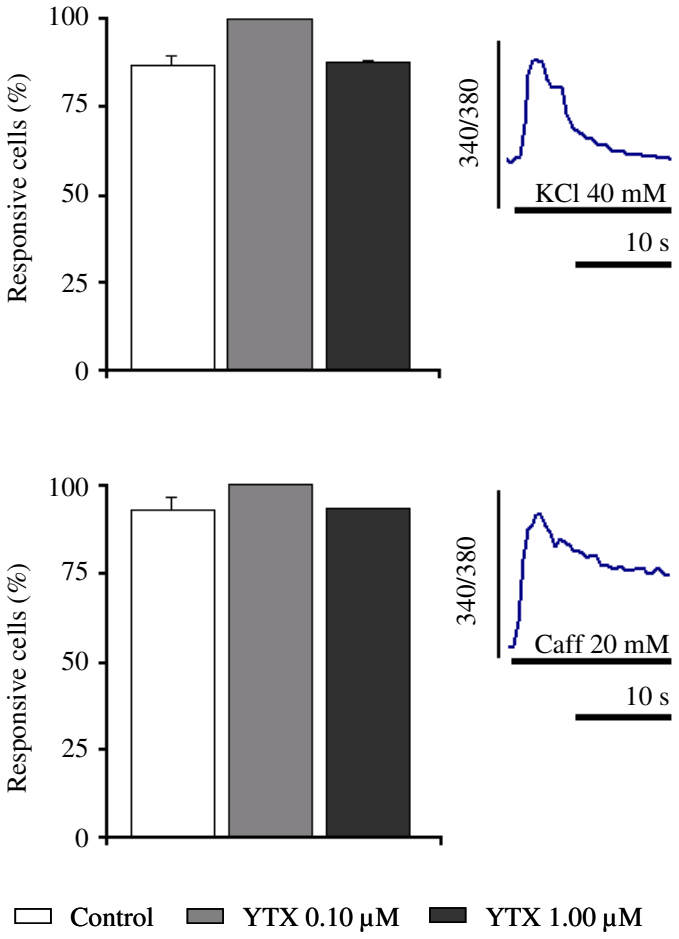

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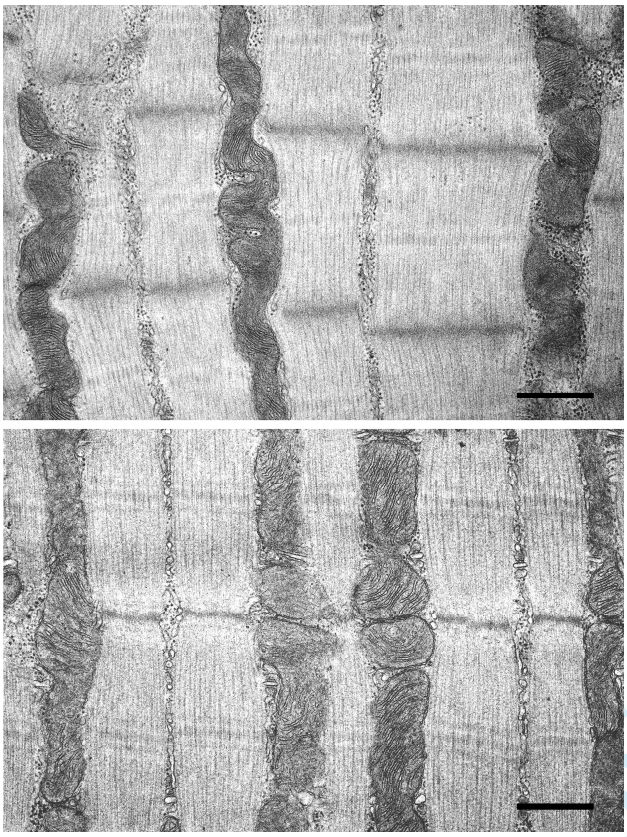

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