



Mitochondrial toxicity of triclosan on mammalian cells



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ABSTRACT

Effects of triclosan (5-chloro-2'-(2,4-dichlorophenoxy)phenol) on mammalian cells were investigated using human peripheral blood mono nuclear cells (PBMC), keratinocytes (HaCaT), porcine spermatozoa and kidney tubular epithelial cells (PK-15), murine pancreatic islets (MIN-6) and neuroblastoma cells (MNA) as targets. We show that triclosan (1–10 $\mu\text{g ml}^{-1}$) depolarised the mitochondria, upshifted the rate of glucose consumption in PMBC, HaCaT, PK-15 and MNA, and subsequently induced metabolic acidosis. Triclosan induced a regression of insulin producing pancreatic islets into tiny pycnotic cells and necrotic death. Short exposure to low concentrations of triclosan (30 min, $\leq 1 \mu\text{g/ml}$) paralyzed the high amplitude tail beating and progressive motility of spermatozoa, within 30 min exposure, depolarized the spermatozoan mitochondria and hyperpolarised the acrosome region of the sperm head and the flagellar fibrous sheath (distal part of the flagellum). Experiments with isolated rat liver mitochondria showed that triclosan impaired oxidative phosphorylation, downshifted ATP synthesis, uncoupled respiration and provoked excessive oxygen uptake. These exposure concentrations are 100–1000 fold lower than those permitted in consumer goods. The mitochondrial mechanism of triclosan differs from that of valinomycin, cereulide and the enniatins by not involving potassium ionophoric activity.

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Abbreviations: EC₅₀, concentration that diminishes the respective vitality parameter by $\geq 50\%$; $\Delta\Psi$, electric transmembrane potential; $\Delta\Psi_p$, membrane potential of the plasma membrane; $\Delta\Psi_m$, membrane potential of the mitochondrial membrane; BCF, bioconcentration factor; PBMC, monocyte-enriched peripheral blood mononuclear cells; HaCaT, a spontaneously immortalized (non-neoplastic) keratinocyte cell line; PK-15, a porcine kidney tubular epithelial cell line; MNA, a murine neuroblastoma cells; MIN-6, a murine pancreatic beta cell line; TPP⁺, tetraphenylphosphonium; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; MIC, minimal inhibitory concentration; PI, propidium iodide; RLM, rat liver mitochondria; PN, pyridine nucleotides.

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1. Introduction

The antibacterial preservative 5-chloro-2'-(2,4-dichlorophenoxy) phenol (mol. wt. 289.5), trivial names triclosan and irgasan, was introduced to commerce in 1964, in products of health care industry in 1972 and is since then in wide spread world-wide use in personal care products, textiles, food contact materials [81,82,36]. Triclosan is poorly biodegradable, lipophilic (log K_{ow} 4.76) [29], bioaccumulates in biota (BCF=2.5) and found ubiquitous almost everywhere in the indoor and outdoor environment, including drinking water resources [12,33,23,13,20,93,68,86].

The antibacterial effect of triclosan is believed to be through its potent inhibitory effect on the bacterial type enzyme of fatty acid biosynthesis, enoyl-acyl carrier protein reductase (ENR), the Fab I step [38]. Triclosan inhibits the purified enzyme with an IC_{50} of 120 nM [57] and inhibits the growth of bacteria with MIC of 0.25 $\mu\text{g ml}^{-1}$ (*E. coli*) to $>1000 \mu\text{g ml}^{-1}$ (*Pseudomonas aeruginosa*) [39]. Since this enzyme is believed to occur in prokaryotic organisms only, triclosan has been considered harmless to humans [16,80,34] and approved at high concentrations (up to 0.3 wt%) in direct human contact, such as tooth paste, mouth washes, creams, wet wipes, diapers and other personal and occupational hygiene products [11,36,50,55,59,60].

However, *in vitro* studies have revealed that triclosan is a xenoestrogen [56,42,52], potent inhibitor of the proinflammatory TLR-signaling pathway in epithelial cells and plasminogen activation in human oral fibroblasts [9,96] and, at low concentration, adversely affects the motility of human sperm [7,78]. In addition, epidemiological studies strongly indicate that exposure to triclosan causes allergic sensitization in children, retards fetal development in utero, affects the thyroid function and alters the inflammatory responses of epithelial cells [24,67,15,51,69]. Triclosan also induces formation of antibiotic resistant nasal biofilms of *Staphylococcus aureus* and is a possible promoter of antibiotic resistance in pathogenic bacteria [97,88].

Human exposure to triclosan has been documented through the skin, orally and respiratory system, due to its widespread use. The oral use of care products may be the most significant route of exposure to triclosan for adults [1,5,60]. Triclosan in human body fluids (plasma, urine, breast milk) and tissues, including fetuses, was first reported from Sweden and subsequently in numerous countries on all continents [1,5,75,21,8,90,58,11,50,71,73].

Considering the large scale of human exposure, the environmental persistence and the reported adverse health effects of triclosan there is a need for understanding the biochemical mechanisms of its toxicity [35]. In this paper we report on the metabolic targets of triclosan toxicity at concentrations relevant for human exposure in primary and cultured human, porcine and murine cells and describe the mitochondriotoxic properties of this chemical.

As indicator cells for observing toxic effects we used human primary blood cells, monocyte-enriched peripheral blood mononuclear cells (PBMC) freshly isolated from healthy human blood and keratinocytes non-neoplastic. These two cell types represent the major

organs responsible for human innate immunity [3]. Porcine spermatozoa resemble more closely than any other spermatozoa their human counterparts [85,95] and porcine kidney tubular epithelial cells representing the susceptibility of a major elimination route of chemicals. To represent the nervous system and the cells responsible for the glucose homeostasis murine neuroblastoma cells (MNA) and insulin producing pancreatic β -cells were chosen [41].

2. Materials and methods

2.1. Target cells used for *in vitro* toxicity assessments.

Human cells. Monocyte-enriched peripheral blood mononuclear cells (PBMC) were retrieved from buffy coats of healthy individual donors from the Finnish Red Cross Blood Service (with ethical permission), Helsinki, Finland, by the Ficoll gradient method of Bøyum [18]. The cells were used on the day of isolation. Each lot of isolated PBMC was checked for viability (trypan blue exclusion) and used when viability was $>80\%$. HaCaT is a spontaneously immortalized (non-neoplastic) keratinocyte cell line, from adult human epidermis and exhibits normal differentiation [19]. The cells were handled as previously described [41].

Porcine cells. Spermatozoa were delivered by commercial suppliers (Figen, Ltd., Tuomikylä, Finland, and Véghfarm Kft, Pakod, Hungary). All disposables used in sperm contact, were pretested for sperm motility affecting effects as described elsewhere [7]. The semen was diluted to 27×10^6 cells ml^{-1} using commercial extender (MR-A Kudus S.A. Madrid Spain or Androstar Plus, Minitube, Tiefenbach, Germany), and used within 48 h as described by [6,7]. The spermatozoa were exposed as suspension in seminal plasma diluted to 27×10^6 sperm cells ml^{-1} , with the commercial extender, containing 2–5 mM K^+ and 150 to 200 mM Na^+ for all exposures, excepting the measurements of glucose consumption. Since the glucose concentration in the commercial sperm extenders is extremely high (150 mM), the sperms were prewashed with TALP-HEPES medium (Bavister and Yanagimachi 1977, as cited by [79]). The sperm cells and the pelleted cells were resuspended into TALP-HEPES medium to 75×10^6 cells ml^{-1} , with glucose set at 15 ± 0.5 mM. Exposures were done for 24 h at 22 ± 2 °C. For motility readings the sperm cells were warmed to 37 °C (5 min).

PK-15 is a non-neoplastic porcine kidney tubular epithelial cell line [28], cultured as described earlier [74].

Murine cells. Neuroblastoma cells (MNA) were from the Department of Veterinary Virology, The Finnish Food Safety Authority EVIRA (Helsinki). MIN-6 cells, kindly donated by J. Miyazaki, is a murine pancreatic beta cell line that grows as islets and retains glucose-inducible insulin secretion [63,41] and was supplied by the Department of Virology, National Institute of Health and Welfare, Helsinki.

All cells, except the spermatozoa, were maintained in RPMI 1460 (10% fetal bovine serum complete medium), cultivations and exposures were performed in tissue culture cabinet (Heracell 150i, Thermo Scientific) in a humidified (RH $>90\%$) atmosphere, at 37 °C, with 5 vol% CO_2 in air.

2.2. Toxicity endpoint measurements with intact cells

The toxicity endpoints were determined by exposing the test cells to serial dilutions (dilution step of 2) of triclosan (dissolved in methanol or ethanol), ≥ 9 steps, exposure concentrations from 100 μg to 0.1 $\mu\text{g ml}^{-1}$. The assays were executed with ≥ 3 replicates, resulting into a standard deviation of $<40\%$. Vehicle controls with the same amount of the respective solvent were run in parallel to each exposure. MNA, PK-15 and MIN-6 were dispersed by trypsin treatment and dispensed in RPMI 1640 (complete) medium. The cells were exposed to triclosan as suspension (PBMC, sperm cells), monolayers (HaCaT, PK-15, MNA) or islets (MIN-6). Triclosan, dissolved in methanol, was dispensed into the wells to concentrations indicated and incubated for the indicated exposure time.

Measurement of glucose consumption, acidification and resazurin reduction. Endpoints were measured in the 24 well microplates, 1 ml of the cell suspension (in the maintenance medium) per well, after 24 and 48 h exposure to triclosan. Triclosan (six dilutions; 20–0.5 μl per well, dissolved in methanol) was dispensed into the six wells. Vehicle controls contained the same amount of solvent (methanol). Glucose was measured from 0.5 μl samples aseptically drawn from the wells, with a glucose meter (Precision Xceed, Abbott Diabetes Care Ltd, Berkshire, UK). At the end of exposure, the presence of reducing equivalents was measured fluorometrically (excitation 544 nm, emission 590 nm; [4]) and pH colorimetrically (phenolphthalein), after ventilation (lid removed, 1 h, to evaporate CO_2) (Fluoroskan Ascent reader, ThermoFischer Helsinki, Finland).

Sperm motility. Motility was microscopically measured as described by [7] except that endpoints for the two kinds of sperm motility were separately recorded, progressive motility [14,78,27] and the shivering (i.e. whiplash flagellar beating in absence of progressive motility). The rapid progressive motility of the sperms (prewarmed to 37 °C) was assessed in phase contrast microscope with a heated stage (Olympus CKX41 and software Cellsense standard version 11.0.06 (Olympus Soft Imaging Solutions GmbH, Muenster, Germany) by differentiating the proportion of spermatozoa exhibiting high amplitude in tail beating from those expressing shivering motility only. For the human eye, rapid and progressively motile sperm cells looks as if each sperm cell possesses two tails, the angle between the tails being $\geq 40^\circ$ [79]. The amount of sperm cells exhibiting “two tails”, was calculated using the CellSense standard software. The assays were performed in triplicate.

Fluorescence microscopic methods. For assessing mitochondrial and cellular membrane potentials, the cells were single or double stained (see figure captions) using the fluorogenic dyes 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, the membrane potential $\Delta\Psi$ responsive dye) or propidium iodide (PI, 2.4 mg ml^{-1} in water) plus calcein AM (acetoxymethylester) for viability, as described previously [40].

Flow cytometry analyses. Viability (plasma membrane integrity) was assessed using the Sperm Viability Kit (L-7011, containing SYBR 14 and PI) following the instructions

of the manufacturer. Mitochondrial transmembrane potential was assessed with JC-1 (T3168) following the protocol of Garner and Thomas [32]. Plasma membrane phospholipid asymmetry within the viable sperm subpopulation was assessed with merocyanine 540 according to [37], with modification: dead cell counterstain was To-Pro3. Beckman Coulter FC 500 dual laser flow cytometer was used, with a 488 nm argon laser (20 mW) for excitation of SYBR 14, PI, JC-1 and merocyanine, and a 635 nm HeNe laser (25 mW), for To-Pro3. The cells were identified based on forward and side scatter properties and non-sperm events were gated out from the analyses. List mode files were analyzed with Flowing free flow cytometry data analysis software (version 2.5.1., www.flowing.com).

Potassium efflux assay. Assay of potassium ions (K^+) efflux from sperm cells and PBMC were performed and described previously [41]. Briefly, the cells were pelleted by centrifugation, resuspended (54×10^6 cells ml^{-1}) in isotonic, sodium phosphate buffered K^+ free medium and placed in a measurement cuvette provided with magnetic stirring, temperature control (24 °C), and a potassium selective electrode (NIKO-ANALIT, Moscow, Russia) linked to PC recording software (Record 4, IBC, Pushchino, Russia). The concentration of K^+ in the extracellular medium was recorded once per second. The electrode signal was calibrated by adding 100 μM of KCl into the cuvette at the end of each run, as done in a previous study [74]. The isotonic (300 m Osm kg^{-1}) K^+ free medium contained 150 mM NaCl, 5 mM NaH_2PO_4 , 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM glucose and 10 mM HEPES (pH adjusted to 7.2 with Trizma base). Alamethicin was used as a positive reference.

2.3. Toxicity endpoints measured with isolated rat liver mitochondria (RLM)

RLM were isolated from male Wistar rats by a standard method as described previously [89]. The mitochondria were washed twice in mannitol buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES-Tris, pH 7.4, potassium-free), resuspended in the same buffer to 60–80 mg protein ml^{-1} and kept on ice for analyses. Mitochondrial functions were determined as described earlier [89]. Oxygen uptake was measured with a Clark electrode and the mitochondrial membrane potential ($\Delta\Psi_m$) with the aid of tetraphenylphosphonium (TPP^+) [46] using a TPP^+ -selective electrode (NIKO, Moscow, Russia). Mitochondrial functions were measured in a 1 ml closed chamber at 25 °C with magnetic stirring in the Standard Medium containing 120 mM KCl, 2 mM KH_2PO_4 , and 10 mM HEPES (pH adjusted to 7.3 with a few grains of TRIZMA base), with 5 mM glutamate plus 5 mM malate, or succinate in the presence of rotenone as the respiring substrates. Details are given in the respective figure legends.

2.4. Media and reagents

Triclosan (CAS 3380-34-5), analytical grade, $\geq 97.0\%$ (HPLC), Dulbecco's phosphate buffered saline (PBS; Mg and Ca free), resazurin sodium salt (CAS 62758-13-8), carbonyl cyanide-(trifluoromethoxy) phenyl-hydrazone (FCCP, CAS 370-86-5), TPP^+ , malate and glutamate, MgATP, rotenone

$\geq 95\%$ (CAS 83-79-4), alamethicin (CAS 27061-78-5) and HEPES (CAS 7365-45-9) analytical grade $\geq 99.0\%$ were from Sigma, 2,4 (α)-dinitrophenol (CAS 51-28-5) from Merck. The tissue culture media, RPMI 1460 glucose containing (15.8 mM) and RPMI1640 glucose free, trypsin 10 \times with EDTA, for cell detachment, were purchased from Lonza (Verviers, Belgium). For Complete medium, L-glutamine (Gibco 21875), heat inactivated 10% fetal bovine serum (FBS), PenStrep (penicillin 10,000 units and streptomycin 10,000 $\mu\text{g ml}^{-1}$), all from Gibco (Invitrogen, Carlsbad CA) were added. Glucose strips were from Lifescan (Johnson & Johnson, Espoo Finland). The fluorogenic dyes – JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; dissolved in dimethyl sulfoxide DMSO), propidium iodide and calcein AM—were all obtained from Invitrogen (Carlsbad, CA, USA). The other chemicals were of analytical grade and purchased from local suppliers. Cultivation chamber slides # 154534 were from Labtek (Thermo Fisher Scientific, Vantaa Finland).

3. Results

3.1. Low concentration of triclosan impairs oxidative metabolism of human, porcine and murine cells

Peripheral blood mononuclear cells (PBMC, enriched with monocytes) freshly retrieved from individual human healthy donors, were exposed to 0.1–100 $\mu\text{g ml}^{-1}$ triclosan. As seen in Fig. 1A, triclosan (2.5 μg per ml, 8.6 μM , final conc.) four-folded the consumption rate of glucose (Δ mM glucose vs. exposure time) by the PBMC (12×10^6 cells per ml in RPMI medium). Exposure to 5 or 10 $\mu\text{g ml}^{-1}$ (17–35 μM) accelerated the PBMC glucose consumption rate so much that after 30 h the medium became exhausted of glucose (residual <1 mM). Higher dosages of triclosan (>25 $\mu\text{g ml}^{-1}$) inhibited or blocked consumption of glucose, indicating loss of cell viability (Fig. 1A). Concomitant to accelerated glucose consumption, triclosan exposure induced metabolic acidification, visible as a pH drop of ≥ 1 units in the medium (from 7.3 to 6.0, Fig. 1B). Exposure to 50 $\mu\text{g ml}^{-1}$ of triclosan practically zeroed glucose consumption (Fig. 1A) and inhibited the generation of reducing equivalents (Fig. 1B).

The human keratinocytes (HaCaT) and porcine kidney tubular epithelial (PK-15) cell lines were exposed to triclosan similarly as PBMC. These cells responded to low exposures (<5 $\mu\text{g ml}^{-1}$) of triclosan by accelerated glucose consumption, followed by acidification of the extracellular medium and, finally, by inhibition of generation of metabolites measurable by the resazurin reduction test (exposure to 10–20 $\mu\text{g ml}^{-1}$), as summarized in Table 1. PK-15, HaCaT and PBMC were exposed also with an initial concentration of 6–7 mM. It was found that 2.5–10 $\mu\text{g ml}^{-1}$ triclosan induced acceleration of glucose consumption and acidification.

The murine neuroblastoma (MNA) cells responded to triclosan by acceleration of glucose consumption and metabolic acidification. The MNA cells were more sensitive to triclosan than the PBMC or the epithelial cells: the EC₅₀ values for acceleration of glucose uptake and

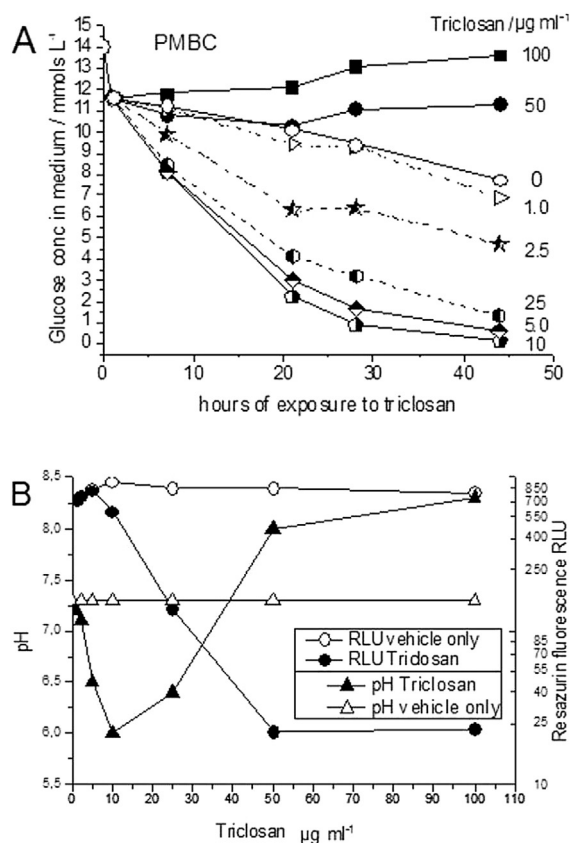


Fig. 1. Effects of triclosan on glucose metabolism by human primary PBMC. PBMC freshly isolated from healthy human donor blood were exposed to 1–100 $\mu\text{g/ml}$ of triclosan for 44 h. Concentration of glucose in the medium was measured at indicated time points during the 44 h exposure (Panel A). The resazurin fluorescence as an indicator of the presence of NADH and pH in the medium an indicator of anaerobic metabolism were measured at the end of the exposure (at 44 h) (Panel B).

induction of acidosis were low, 1 $\mu\text{g ml}^{-1}$. Exposure to 2 $\mu\text{g ml}^{-1}$ triclosan effectively blocked glucose uptake by the MNA (Table 1).

The rate of glucose consumption of porcine sperm cells (washed cells tested in TALP-HEPES medium, 75×10^6 cells ml^{-1} , 24 h) was low (<0.5 mM). The production of resazurin reducing equivalents was blocked when exposed to 5–13 $\mu\text{g ml}^{-1}$ triclosan. There was no measurable drop of pH of the medium (<0.5 pH units) at any of the tested exposure concentrations (1–50 $\mu\text{g ml}^{-1}$ triclosan).

With murine pancreatic islets (MIN-6) the rate of glucose consumption, was low, 0.5–1 mM per 24 h. Exposure to triclosan neither accelerated glucose uptake by the islets nor was there any measurable acid production. Production of resazurin reducing equivalents by these cells was inhibited by exposure $\leq 5 \mu\text{g ml}^{-1}$ triclosan, indicating metabolic death of the islet cells.

The above results, summarized in Table 1, show that low concentrations of triclosan seriously interfered with the energy metabolism of the primary cells (human PBMC, porcine sperm), of the non-neoplastic continuous human and porcine epithelial cells (HaCaT, PK-15) and the two murine cell lines (MNA, MIN-6).

Table 1

Toxicity endpoints of triclosan toward differentiated human, porcine and murine cells. EC₅₀ indicates the lowest exposure concentrations where the indicated cell damage was microscopically observed for >50% of the exposed cells or observed as a 50% increase or decrease in the metabolic reaction (medium pH, resazurin reactivity; glucose consumption) compared to the exposure to vehicle only, at the same time point. The tested range of triclosan concentrations was 0.1–100 µg ml⁻¹. The endpoints were measured after 24 h exposure, at 20 °C (sperm cells) and 37 °C (other cells).

Exposure cell type	Triclosan exposure concentration – EC ₅₀ µg ml ⁻¹						
	ΔΨ _m loss ^a	Acceleration of		Inhibition of		Cellular damage	
		glycolysis ^b	acidosis ^c	glycolysis ^d	resazurin reduction ^e	motility loss ^f	PI uptake ^g
Human							
PBMC	5	2.5–5.0	5	≤50	20	–	20
Keratinocyte HaCaT	5	5	5	50	20	–	20
Porcine							
Sperm cell ^h	1	No	No	No	13	≤1 ⁱ	5–12
Kidney PK-15	5	5–10	10	50	20	≥2.5 ^j	25
Murine							
MNA neuro-blastoma	2–5	1	1	2	5	–	2
MIN-6 insulinoma	5–10	no ^k	No acidosis	no ^k	≤5	–	5–10

^a Observed by fluorescence microscopy, EC₅₀ here means the exposure concentration which causes the yellow–orange fluorescent emission of the mitochondria inside the JC-1 stained cells to turn into green fluorescence.

^b The rate of glucose consumption (µmol ml⁻¹ h⁻¹) by the cells doubled compared to the vehicle control at the same time point.

^c Indicates that pH of the medium acidified by >0.5 unit compared to the vehicle control at the same time point.

^d Indicates the exposure concentration where the glucose consumption during 24 h was ≤50% of that in the culture spiked with vehicle only.

^e Resazurin fluorescence lower compared to the vehicle control at the same time point. This is measured at the end of the experiment, i.e. after 44–48 h exposure.

^f Measured (microscopy) for the sperm cells only: proportion of motile cells decreased to <50% compared to the vehicle control at the same time point.

^g >50% the cells fluoresce red (microscopy) when stained with propidium iodide (PI).

^h For assessing triclosan effects on glucose consumption, acidosis and of resazurin reduction, the sperm cells were washed twice in TALP-HEPES medium [79] and resuspended to 75 × 10⁶ cells ml⁻¹ in the same medium, glucose concentration set at 15 ± 0.5 mM. These endpoints were measured after 24 h exposure at 22 °C.

ⁱ Progressive motility.

^j Shivering motility.

^k Glucose consumption of the MIN-6 cell culture was <0.5 mM in 24 h (exposed and nonexposed).

3.2. Mitochondria in human, porcine and murine cells were depolarised by exposure to low micromolar concentrations of triclosan

Since triclosan induced metabolic acidosis in the tested mammalian cells, its effect on mitochondrial and cellular membrane potentials (ΔΨ) was investigated. The membrane permeant, fluorogenic dye JC-1 shifts its fluorescent emission from orange (energized membrane) via yellow to green, when ΔΨ depolarises from >150 mV to ≤100 mV, Fig. 2. Additionally, live/dead staining, calcein AM (viable cells fluoresce green) combined with propidium iodide (PI, dead cells fluoresce red), were used to assess the effect of triclosan on the viability of the cells, Figs. 2 and 3.

The lowest concentrations of triclosan that accelerated glucose consumption by PBMC (1–2.5 µg ml⁻¹, Fig. 1A), depolarised the mitochondrial transmembrane potential (ΔΨ_m) of the PBMC, visible as a change in JC-1 fluorescent emission from intensely orange to yellow and green (1.5 µg ml⁻¹ triclosan, Fig. 2 top row). PI positive, swollen cells became prevalent in cells exposed (8 h) to ≥6 µg ml⁻¹.

HaCaT and PK-15, lost their ΔΨ_m when exposed to ≥6 µg ml⁻¹ triclosan (24 h, Fig. 2, 3rd row from top). PI positivity (indicator of necrotic death) in these cells was observed at exposures to 20–25 µg ml⁻¹ triclosan (Fig. 3).

Sperm cells mitochondria are wrapped around the axoneme of the sperm tail, proximal to the head [77]. The sperm cell mitochondria were highly responsive to

triclosan exposure: the bright orange–yellow fluorescence of the spermatozoan mitochondria faded within 30 min of exposure to ≤1 µg ml⁻¹ triclosan. The same exposure converted the progressive, rapidly swimming sperms into cells that expressed whiplash motility only (Fig. 2, row 4). Higher exposure (≥2.5 µg ml⁻¹) terminated also the shivering, whiplash motility.

Coincident to the motility changes, novel yellow–orange fluorescence emerged in the acrosomal hemisphere of the sperm head (Fig. 2, 30 min exposures to 1 and 2.5 µg ml⁻¹ triclosan) indicating a local upshift of ΔΨ in this part of the cytoplasmic membrane. This was followed (24 h of exposure), by emerging of yellow–orange fluorescing spots in the distal parts of the sperm tail, where no mitochondria are located (Fig. 2, row 5 from top). To answer the question, whether an efflux of K⁺ down the concentration gradient (intracellular >100 mM K⁺, sperm extender 2–5 mM) could increase the negative charge on the cytoplasmic side of the cell membrane, we tested, whether exposure to triclosan would induce leakage of K⁺ from the intracytoplasmic space. The concentration of K⁺ ions in the extracellular medium was measured, using a K⁺ specific electrode, while exposing the sperm cells to triclosan. Similar test was done with PBMC. The results (not shown) were that K⁺ efflux from boar spermatozoa and PBMC occurred at exposure to ≥15 µg ml⁻¹ and >10 µg ml⁻¹ triclosan, respectively. At these concentrations the cell membrane permeability barrier toward PI was damaged (Fig. 2, top row and Fig. 3, 2nd row from top) and motility was lost (sperm cells).

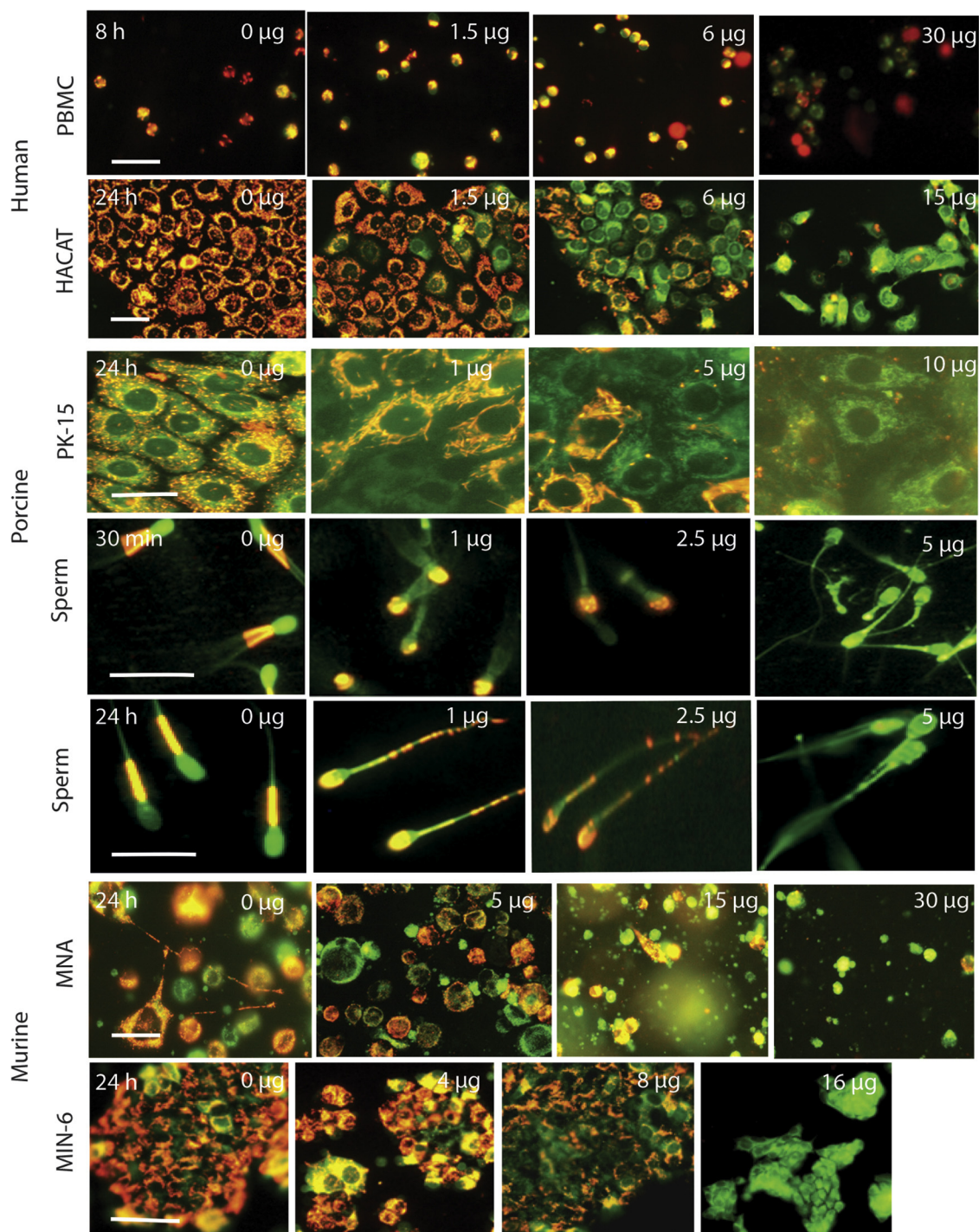


Fig. 2. Exposure to triclosan dissipated mitochondrial membrane potential in human PBMC and keratinocytes (HaCaT), porcine kidney tubular epithelial cells (PK-15) and spermatozoa, murine neuroblastoma cells (MNA) and pancreatic islets (MIN-6, insulin producing). After exposure to the indicated concentrations ($\mu\text{g/ml}$) and times (minutes, hours) of triclosan, the cells were stained with the membrane potential responsive fluorogenic dye JC-1 ($\mu\text{g/ml}$) or double stained with (JC-1 and propidium iodide, PI; PBMC and HaCaT). The fluorescent emission of JC-1 shifts from red-orange via yellow to green when the membrane potential ($\Delta\Psi$) diminishes from ≥ 150 mV toward ≤ 100 mV. Necrotic cells (PI positive) are visible in PBMC ($30 \mu\text{g}$) as swollen, intensely red cells. The images are representative of three independent microscopic views. Scale bar, $30 \mu\text{m}$.

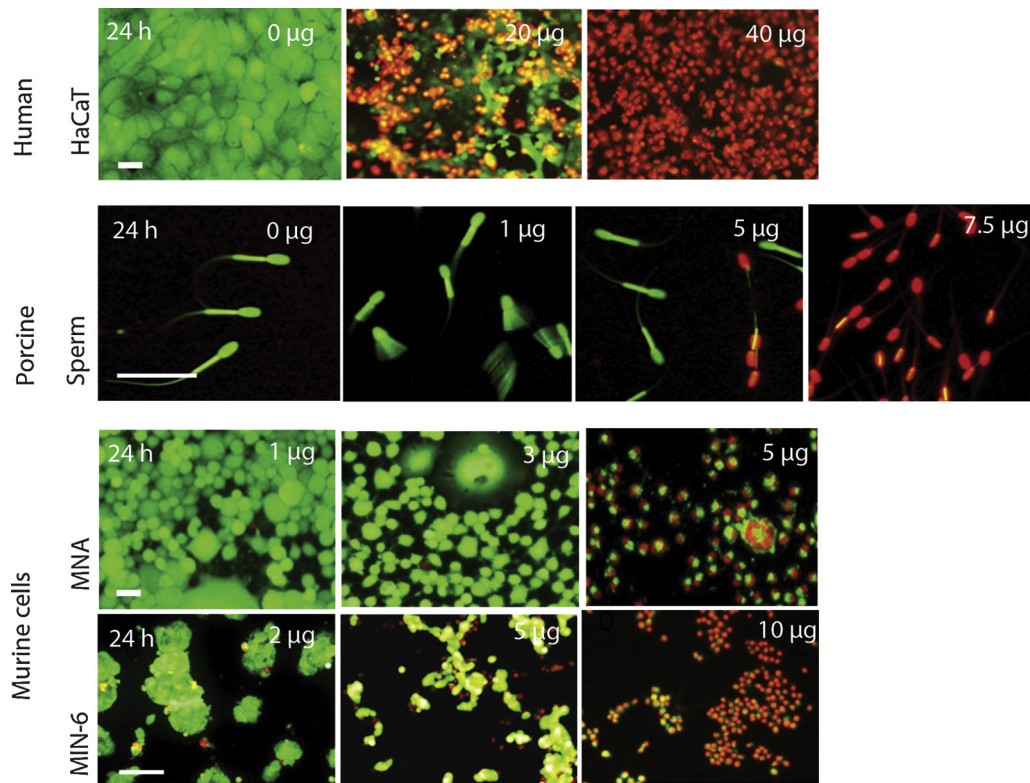


Fig. 3. Microscopic assessment of death of human, porcine and murine cells upon exposure to triclosan. The concentration of triclosan exposure (24 h) is shown for each panel. After exposure, the cells were double stained with acetoxyethyl calcein (live stain, green fluorescence) and propidium iodide (PI, death stain, red fluorescence indicates permeabilisation of the cell). The cells exposed to vehicle only (methanol) show no permeability to PI. The cells exposed to triclosan show dose-dependent increase of PI-permeabilized (red) cells. At the highest exposures, the cytoplasmic content (green) has leaked out, leaving the red stained cell nuclei visible. Measure bar, 30 μm .

To obtain a quantitative view on the sperm damaging effects of triclosan flow cytometric measurements were done on cells stained with merocyanine 540 and SYBR 14 in addition to PI and JC-1. The original flow cytometric dot plots are shown in Fig. 4A, quantitative results summarized in Fig. 4B. Fig. 4B shows that loss of viability (SYBR14/PI), disorganization of membrane lipids (M540/To-Pro3) and loss of $\Delta\Psi$ occurred in response to exposure 9 $\mu\text{g ml}^{-1}$ ($\pm 40\%$) triclosan. This matches well with microscopic observations of the sperm cells, PI positive at 7.5 $\mu\text{g ml}^{-1}$ triclosan (Fig. 3) and fading of yellow and orange fluorescence at 5 $\mu\text{g ml}^{-1}$ triclosan. Mitochondria ($\Delta\Psi_m$) of the sperm cells were depolarised already in response to exposure of 1 $\mu\text{g ml}^{-1}$ triclosan (Fig. 2, sperm cells). The flow cytometric observation shows that part of the JC-1 aggregates (yellow, orange) were retained until higher exposures ($> 5 \mu\text{g ml}^{-1}$ triclosan) is due to the triclosan-induced emerging elevation of membrane potential in the acrosomal area of the sperm head and the fibrous sheath of the flagellum (=distal part of the tail), Fig. 2, 3rd row from bottom).

The murine neuroblastoma cells, MNA, responded to triclosan exposure by morphology: the axon-like sprouts and the large sized cells, typical of MNA, regressed and became increasingly positive for PI-staining (Figs. 2 and 3, 2nd row from bottom). The JC-1 staining indicates that the MNA

cells lost the $\Delta\Psi_m$ when exposed to 5 $\mu\text{g ml}^{-1}$ when the cells permeated PI (red fluorescing nuclei).

For the murine pancreatic β -cell islets (MIN-6, insulin producing) microscopy showed that exposure to triclosan led to disintegration of the islet structure and induced conversion of the fragmented islets into tiny, pycnotic cells (Fig. 3, bottom row) with no energy metabolism. Resazurin reduction ceased at exposure to 2 $\mu\text{g ml}^{-1}$ triclosan, Table 1.

3.3. Combined toxic responses to triclosan of primary and cultivated mammalian cells

The experiments with PBMC were conducted separately from cells of five different individuals, with similar results indicating that differences between individuals was within one step (dilution step of 2; $\leq 40\%$ standard deviation of three replicates) of dilution. Experiments with sperm cells were executed separately for each individual ejaculate. Differences between individual boars for motility also were within one step of dilution. The values in Table 1 are representative for 20 individual ejaculates standardized to cell density of 27×10^6 spermatozoa ml^{-1} in sperm extender.

Spermatozoa (porcine), neuronal cells (murine, MNA) showed EC_{50} values for the different toxicity endpoints of 1 and 2 $\mu\text{g ml}^{-1}$ triclosan whereas for the PBMC (human)

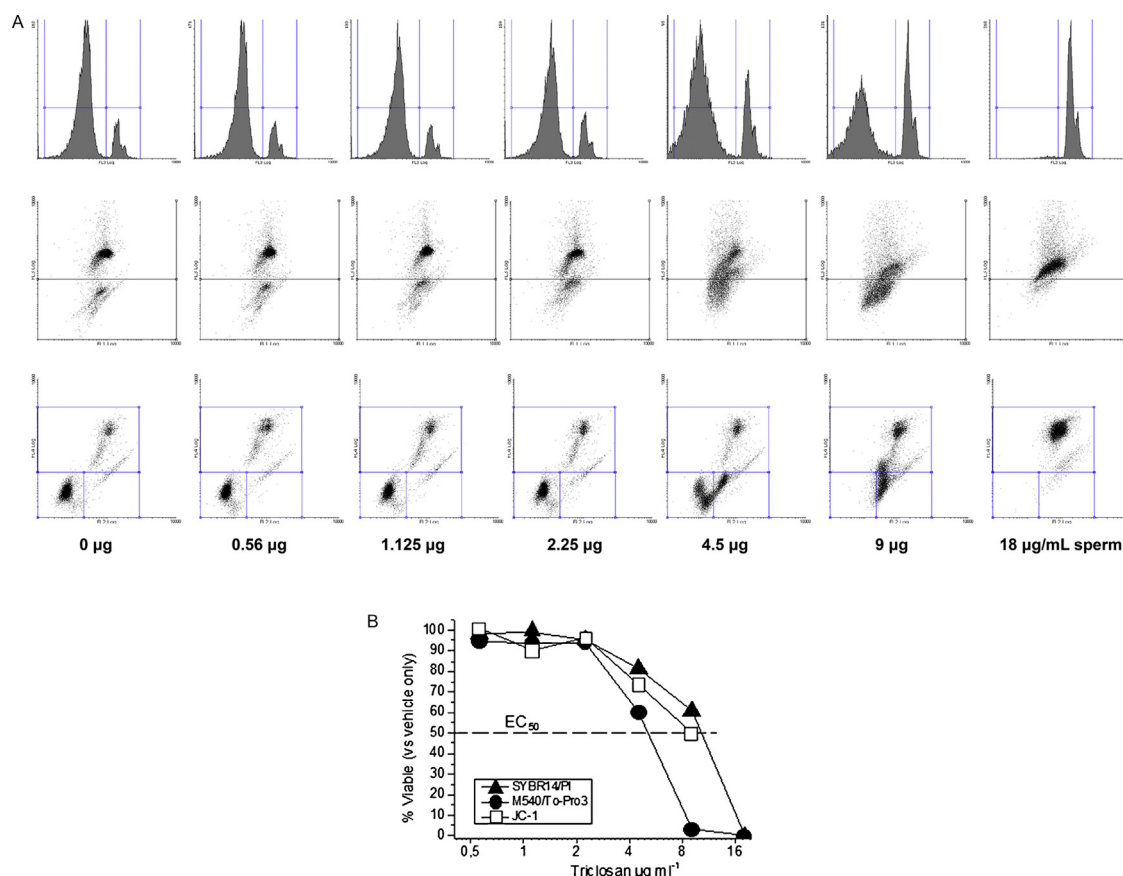


Fig. 4. (A) Original flow cytometric dot plots of spermatozoa exposed to 0.5–18 $\mu\text{g ml}^{-1}$ triclosan (dilution step = 2). Top row: SYBR14 (left panel – viable)/PI (right panel – dead) stained. Middle row: top panel JC-1 aggregates ($\Delta\Psi$ high), bottom panel JC-1 monomers ($\Delta\Psi$ lost). Bottom row: M540 (bottom panels)/To-Pro3 (top panel – dead). M540 bottom left panel: viable intact. M540 bottom right panel: membrane disordered. (B) Quantitative effects of triclosan on selected viability parameters of boar spermatozoa measured by flowcytometry. SYBR14/PI, M540/To-Pro3, JC-1 aggregation value measured after 24 h exposure at 20 °C.

and the epithelial cells (human HaCaT, porcine PK-15) the EC_{50} values of different endpoints ranged from 5 to 50 $\mu\text{g ml}^{-1}$. The MNA, growing as 20–30 μm diameter cells with >50 μm axon-like appendices (Fig. 2, bottom 2nd row), and MIN-6, typically growing as large islets (>50 μm , Fig. 3, bottom row), lost their infrastructure in response to low exposure 1 and 2 $\mu\text{g ml}^{-1}$ triclosan (Fig. 3, two bottom rows). Thus the spermatozoa and neural cells (MNA) were more sensitive to triclosan induced toxicity than were the PBMC or the epithelial cells.

3.4. The effect of triclosan on mitochondrial functions

We used purified rat liver mitochondria (RLM) as a tool to investigate whether a triclosan driven mitochondrial dysfunction was involved in the toxic outcomes presented in Figs. 1–4 and Table 1.

The effect of triclosan on the membrane potential, $\Delta\Psi_m$, of purified mitochondria was assessed using a TPP-selective electrode (Fig. 5). It showed that triclosan induced a concentration-dependent decrease of the $\Delta\Psi$ of the mitochondria, observed within 1 min of exposure to 0.36–0.73 $\mu\text{g ml}^{-1}$ triclosan (Fig. 5A). The observed pattern is similar to the action of the classical uncoupler FCCP,

used as reference (Fig. 5B). The relative efficacy of 1 μM triclosan in dissipating the mitochondrial $\Delta\Psi$ approximated that provoked by 25 nM of FCCP. Subsequent measurements of the redox state of pyridine nucleotides (PN), and of oxidative phosphorylation by the mitochondria, showed that triclosan induced a decrease of the pyridine nucleotide (PN) fluorescence of RLM (Fig. 5C). This effect was dose-dependent. Triclosan caused not only a decrease in PN fluorescence but also inhibited oxidative phosphorylation.

Added FCCP (1 μM) caused complete oxidation of PN (Fig. 5C). The time of phosphorylation of added ADP increased in a dose dependent response to triclosan, and after addition of 2.9 $\mu\text{g ml}^{-1}$ triclosan the PN was almost completely oxidized and hardly any response to the addition of ADP was observed (Fig. 5C). Thus, triclosan acted like an uncoupler: it not only enhanced PN oxidation but also inhibited oxidative phosphorylation.

To find out if triclosan acted on the mitochondrion by uncoupling ATP synthase from the terminal oxidase (cyt aa3), triclosan was dispensed into cuvettes with RLM respiring on glutamate plus malate (Fig. 6A), or succinate with rotenone. Rotenone was added as a known non-competitive inhibitor of the respiratory chain Complex I. 2,4-dinitrophenol was used as the reference uncoupler

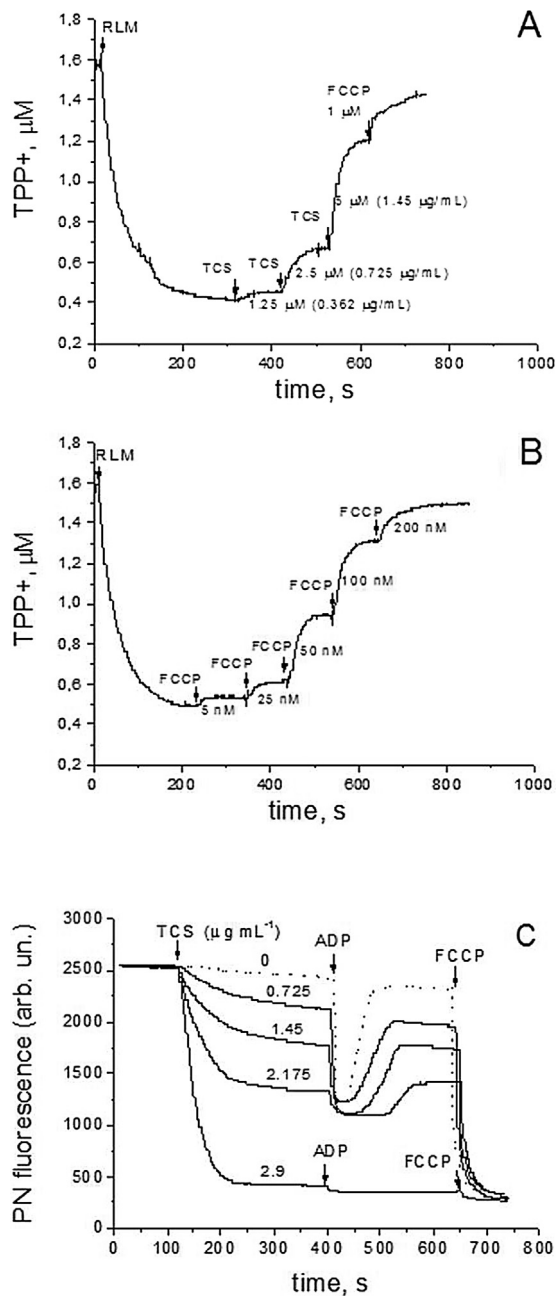


Fig. 5. Effects of triclosan on the membrane potential, redox state of pyridine nucleotides and on oxidative phosphorylation in isolated rat liver mitochondria. $\Delta\Psi_m$ was measured with TPP⁺-selective electrode in rat liver mitochondria (RLM) (1 mg protein ml⁻¹) in standard medium with 5 mM glutamate plus 5 mM malate as the respiring substrates. Triclosan (TCS) (A) or FCCP (B) was added at concentration and time points indicated. Panel A shows TCS induced a concentration-dependent decrease of the mitochondrial membrane potential ($\Delta\Psi_m$, measured with TPP⁺-selective electrode) of isolated RLM. Panel B shows FCCP induced a concentration-dependent decrease of the mitochondrial membrane potential. Panel C shows TCS induced a concentration-dependent decrease of pyridine nucleotides (PN) fluorescence and uncoupling of oxidative phosphorylation in RLM. Trace 0 shows the vehicle (methanol) control. The final concentrations of ADP and FCCP were 200 μM and 1 μM, respectively. The traces shown are representative for three separate experiments.

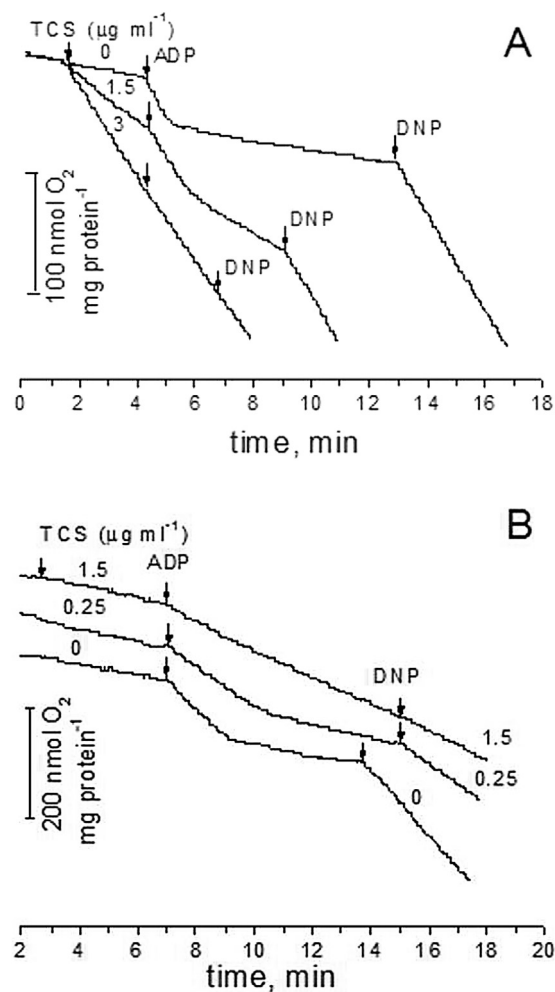


Fig. 6. The effect of triclosan (TCS) and 2,4-dinitrophenol (DNP) on rates of oxygen consumption and oxidative phosphorylation of RLM (1 mg protein ml⁻¹ in standard medium) respiring on 5 mM glutamate plus 5 mM malate (A) or succinate in the presence of rotenone (B). Trace 0 shows the vehicle (methanol) control. The final concentrations of ADP and DNP were 200 μM and 50 μM, respectively. The traces shown are representative for three separate experiments.

(Complex V). The responses of oxygen consumption to added ADP were measured.

As seen in Fig. 6A, the upshift of oxygen consumption in response to added ADP, faded away (in a dose-responsive manner) when triclosan was dispensed into the cuvette prior to the ADP. Adding 50 μM (9.2 μg ml⁻¹) 2,4-dinitrophenol, a known uncoupler of the complex V, caused a dose-dependent upshift of oxygen consumption in response to added ADP. When exposed to 3 μg ml⁻¹ (10 μM) triclosan the mitochondria ceased to respond to added ADP or to the reference uncoupler 2,4-dinitrophenol (50 μM or 9.2 μg ml⁻¹) (Fig. 6A), indicating 100% of uncoupling of the oxidative phosphorylation had already taken place. It thus appears that the uncoupling power of 10 μM triclosan equals that of 50 μM 2,4-dinitrophenol.

Fig. 6B shows the responses of mitochondria to triclosan oxidizing succinate in the presence of rotenone. As seen in Fig. 6B the addition of triclosan caused only a slight

increase of the respiration rate in State 2, and decreased the rates of respiration in State 3 and of uncoupled respiration. The result means that triclosan-enhanced oxygen consumption was prevented because the required feed of reducing power from Complex I was blocked by rotenone. At a concentration of $1.5 \mu\text{g ml}^{-1}$ triclosan induced a significant inhibition of the respiration rate in State 3 that did not respond when the classical uncoupler 2,4-dinitrophenol was added. The results in Figs. 6A and B confirm that triclosan inhibited oxidative phosphorylation and caused loss of the respiratory control and that triclosan impaired oxidative phosphorylation by acting as an uncoupler. This confirms that the site of action role of triclosan is downstream in the respiration chain, as an uncoupler of the ATP synthetase complex (V) of the mitochondrion, thereby causing dysfunction of the mitochondrial oxidative phosphorylation.

4. Discussion

The results shown in this paper represent a new dimension on the mechanisms of action of triclosan on intact, mammalian cells of six different types, expanding its toxicity targets beyond those described so far. Several of the toxic endpoints were acute, measurable after short exposure times, 30 min (sperm motility, $\Delta\Psi_m$) or hours (excessive glucose consumption, metabolic acidification in PBMC, HaCaT, PK-15, MNA; cessation of resazurin reduction followed by necrotic cell death in MIN-6). The effects occurred at exposure concentrations encountered in human body fluids [50,55,64,69,71].

Exposure to low micromolar ($\leq 5 \mu\text{g ml}^{-1}$) concentrations of triclosan down shifted oxidative phosphorylation (aerobic ATP generating machinery) and caused loss of $\Delta\Psi_m$ in intact, non-neoplastic human and porcine cells, as well as in isolated RLM. Concomitant to this, and a novel finding for triclosan, upshifting of glucose consumption was observed in the exposed cells, followed by metabolic acidification, similar to that described for cells where mitochondria were damaged by microbial toxin, cereulide. Cereulide is known as highly mitochondrio toxic in vitro [74] as well as for humans in vivo [61,70,84,65], where mitochondrial dysfunction caused pH of blood to decrease from 7.3 to ≤ 6.8 leading to mortality.

Cells that are exclusively dependent on mitochondrial energy production may be expected to be sensitive to defects in their energy retrieval. Our results showed that Triclosan reduced the ability of cells representing the innate immunity system, PBMC and keratinocytes (HaCaT), to produce ATP by oxidative phosphorylation. Cells of the myeloid lineages derive their energy exclusively from glycolysis, whereas T and B cells use amino acids, glucose and lipids as energy sources for oxidative phosphorylation. As lymphocytes proliferate, they become increasingly dependent on glucose uptake (reviewed by [53]). Extracellular acidity stimulates production of IL-1 β by human monocytes and can be recognized as a danger signal stimulating the innate and adaptive immune responses [44]. Bone resorption by macrophage-like osteoclasts also is stimulated by protons, being maximal at pH 7.0 [10]. Thus

substances that cause metabolic acidification by human cells may adversely affect health.

MIN-6 are pancreatic islet cells, used in numerous studies as a model for studies of dysfunction of insulin producing β -cells [87,26]. The pancreatic β -cells are known to be unable of aerobic glycolysis and do not exhibit Crabtree effect [25,43] in response to mitochondrial inhibition [83,22,45]. This explains why these cells did not respond by increased glucose consumption and acidosis to triclosan exposure. This inability leads to shortage of energy and necrotic cell death, and not apoptosis, when MIN-6 cells are exposed to mitochondrial toxins [54,41]. Necrotic cell death has been suggested to lead to autoimmune destruction of the β -cells [49,17].

Triclosan has been shown to inhibit boar sperm motility and recently it was shown that human spermatozoa are similarly inhibited [14,78]. A novel effect of triclosan in the present paper is visualized in the response of sperm cells (boar) by hyperpolarisation of the cytoplasmic membranes in the acrosomal area (the forehead) and of the fibrous sheath. The fibrous sheath is the principal piece of the boar sperm flagellum (Fig. 2), where there are no mitochondria [62,47]. The flagellar and acrosomal hyperpolarizations occurred when the mitochondria already were depolarized ($\Delta\Psi_m$ lost) by 1 or $2.5 \mu\text{g ml}^{-1}$ triclosan (Fig. 2). The sperm cells showed hyperactive, shivering motility (Fig. 3) although the progressive mobility had ceased (Table 1). It is known that boar spermatozoa possess two isomorphic sets of glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12) and pyruvate kinase (PK, E.C. 2.7.1.40) [31]. The sperm-specific forms of GAPDH-S and PK-S were shown by immunofluorescence microscopy to be located in the distal (principal) part of the sperm flagellum and the acrosomal region of the sperm head, whereas the somatic isomorphs, GAPDH and PK, of these enzymes are localized in the midpiece of the flagellum, in the mitochondria [30,31]. It thus appears that when the ATP synthesis by mitochondrial oxidative phosphorylation was dysfunctionalized by triclosan (which acts as uncoupler), ADPH-S and PK-S produced reducing power (NAD(P)H, visible as hyperpolarization) and ATP (visible as induced shivering flagellar motion). The results indicate that triclosan exposure, at concentration where the mitochondria are dysfunctionalised, does not affect the glycolytic ATP generated by the sperm-specific GAPDH-S and PK-S. Due to the hyperpolarisation of the non-mitochondrial parts of the sperm cell, when the mitochondria in the midpiece are already depolarized, flowcytometric methods alone could lead to an erroneous interpretation of the energy status of the sperm cell hence such methods cannot be used to reliably assess the effect of triclosan on sperm cells.

It was recently shown that triclosan (and several other endocrine disrupting chemicals, EDCs) induces hyperactivation and rheotaxis of human sperms by activating the spermatozoan, progesterone and prostaglandin inducible Ca^{2+} channel, CatSper [78]. The resulting Ca^{2+} influx controls sperm capacitation, chemotaxis and acrosome exocytosis [2,76,72,78]. Since boar spermatozoa possesses [94] CatSper Ca^{2+} channel similar to the human, we suggest that the shivering motility (Fig. 3), visible in boar sperms exposed to triclosan ($1\text{--}2.5 \mu\text{g ml}^{-1}$), and the

simultaneously visible exocytosis/hyperpolarization of the acrosome (Fig. 2) are consequence of triclosan action on the CatSper channel. Hence, triclosan may contribute to the subfertility and decline in sperm quality occurring particularly in urban environments where the population is more heavily exposed to triclosan-containing products than in rural areas.

Hyperpolarisation of the sperm head, concomitant to loss of $\Delta\Psi_m$ has earlier been described to occur in boar spermatozoa in response to microbial toxins, cereulide (1 ng ml^{-1}), valinomycin (2.5 ng ml^{-1}) and enniatin B ($5\text{ }\mu\text{g ml}^{-1}$) [40,89,91]. Cytoplasmic hyperpolarization by these substances appeared to depend on toxin induced efflux of K^+ ions from the cells, increasing the negative charge inside. We tested whether exposure to triclosan caused K^+ leakage from the sperm cells, and found K^+ efflux only at concentrations where the permeability barrier against PI of the sperm cell membrane collapsed.

The MNA, representing neural cells showed in this study the highest sensitivity toward triclosan and responded by necrotic cell death. Since necrotic cell death is considered detrimental and it is believed that it is linked to autoimmune disorders, the current findings suggest that the potential role of triclosan in the development of neurological disorders should be studied.

We used RLM to verify the effects observed by exposure to triclosan on intact cells or islets. For this aim we used RLM in a medium similar to that of live mitochondria in intact cells. These results showed that triclosan uncoupled oxidative phosphorylation similar to classical uncouplers, FCCP and 2,4-dinitrophenol. This supports those presented by [66], even though we used a different medium and more sensitive method (TPP⁺ electrode) for the measurement of membrane potential.

Triclosan is known to specifically inhibit bacterial fatty acid synthesis [38,39,57] and therefore its effects were considered to be limited to prokaryotes. However, in this paper we show that spermatozoa and somatic mammalian cells were much more sensitive toward triclosan than some bacteria, e.g. *Pseudomonas aeruginosa*, with MIC concentration $>1000\text{ }\mu\text{g ml}^{-1}$ for inhibiting the biosynthesis of FAS type II fatty acid synthesis [39].

Effects resembling those of triclosan have been shown in literature for other phenolic compounds. Loss of sperm motility at same low micro molar range was reported on human sperm for 4-octylphenol and triclosan [78]; decrease of the lytic function of human natural killer cells was shown to be more effective for triclosan compared to nonylphenol [92] and induction of mitochondrial dysfunction caused by bisphenol A was also reported in human lymphoblasts [48].

5. Conclusion

Triclosan exerted adverse effects toward different somatic and reproductive cells at extracellular concentrations of $<1\text{--}5\text{ }\mu\text{g ml}^{-1}$. Considering a $\log K_{ow}$ of 4.76 and BCF of 2.5, low but repeated external exposures may result into adverse effects especially in cells that are non-renewable (pancreatic β -cells) or where there is no elimination route,

e.g. testis or neuronal cells. Triclosan as a mitochondrial toxic chemical, depending on its tissue distribution and elimination, may lead to unexpected long-term toxic effects similar to what has resulted into withdrawal of numerous mitochondrial toxic pharmaceuticals.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found in the online version.

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