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Deleterious effects of tributyltin on porcine vascular stem cells physiology

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ABSTRACT

The vascular functional and structural integrity is essential for the maintenance of the whole organism and it has been demonstrated that different types of vascular progenitor cells resident in the vessel wall play an important role in this process. The purpose of the present research was to observe the effect of tributyltin (TBT), a risk factor for vascular disorders, on porcine Aortic Vascular Precursor Cells (pAVPCs) in term of cytotoxicity, gene expression profile, functionality and differentiation potential. We have demonstrated that pAVPCs morphology deeply changed following TBT treatment. After 48 h a cytotoxic effect has been detected and Annexin binding assay demonstrated that TBT induced apoptosis.

The transcriptional profile of characteristic pericyte markers has been altered: TBT 10 nM substantially induced alpha-SMA, while, TBT 500 nM determined a significant reduction of all pericyte markers. IL-6 protein was detected in the medium of pAVPCs treated with TBT at both doses studied and with a dose response. TBT has interfered with normal pAVPC functionality preventing their ability to support a capillary-like network. In addition TBT has determined an increase of pAVPC adipogenic differentiation.

In conclusion in the present paper we have demonstrated that TBT alters the vascular stem cells in terms of structure, functionality and differentiating capability, therefore effects of TBT in blood should be deeply explored to understand the potential vascular risk associated with the alteration of vascular stem cell physiology.

1. Introduction

Tributyltin (TBT), an organotin compound (OTC), was widely used in the past as biocide in antifouling paints for ships and fishing nets (Sarkar et al., 2006). In spite of the fact that its use has been banned since 2008, due to its high environmental persistence, TBT is still detected in marine sediments up to 1–10 μg/L (Briant et al., 2013) and in samples of water collected next to harbors at concentrations up to 200–400 ng/L (Sousa et al., 2009; Radke et al., 2012).

Easily incorporated in cells and biological membranes, TBT triggered a wide range of toxic effects involving biochemical pathways, cell membranes, biomolecules depending on many factors including the animal species, the dose and the exposure time (Kotake, 2012; Pagliarani et al., 2013). Among its deleterious effects, the ability to disrupt endocrine system by binding specific hormone receptors is well documented (Nakanishi, 2008; Cho et al., 2012). TBT is a potent adipogenesis inducer in vertebrates (Grün et al., 2006). In stem cells TBT effects are mediated (Biemann et al., 2012) or not mediated (Biemann et al., 2014) by the activation of the proliferator-activated receptor (PPARγ).

Humans could be exposed to TBT by contaminated seafood and shellfish and the presence of TBT in human tissue was detected in the range of 3–100 nM (Kannan et al., 1999; Takahashi et al., 1999) as well as in human blood at concentrations ranging from 50–400 nM (Whalen et al., 1999). Two studies demonstrated that TBT may constitute a risk factor for cardiovascular disease impairing the coronary vascular reactivity to estradiol (dos Santos et al., 2012) and altering aorta morphology and functionality (Rodrigues et al., 2014) both using rodent models. In addition, we recently described in pig (Botelho et al., 2015) the effect of TBT on endothelial cells, demonstrating a wide range of effects all consistent with endothelial dysfunction.

The vessel wall has been reported as a source of different types of vascular progenitor cells (Zengin et al., 2006; Bautch, 2011; Lin and Lue, 2013; Psaltis and Simari, 2015) and increasing data suggest the possible involvement of these cells in vascular disease states, including atherogenesis and tumorigenesis (Tang et al., 2012; Zhang et al., 2013; Birbrair et al., 2015). We have recently isolated and characterized a population of Vascular Stem Cell-like cells from porcine aorta (porcine

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Aortic Vascular Precursor Cells pAVPCs) with mesenchymal property including adipogenic differentiation (Zaniboni et al., 2014, 2015). The biological similarities between pig and human at the genomic (Archibald et al., 2010; Bendixen et al., 2010), proteomic (de Almeida and Bendixen, 2012), anatomic and physiologic level (Niemann and Kues, 2003; Suzuki et al., 2011; Swindle, 2012), make the pig an excellent model for studying vascular and cardiovascular disorders (Forni et al., 2005; Bernardini et al., 2005; Zaragoza et al., 2011; Vilahur et al., 2011; Zannoni et al., 2012; Elmadhun et al., 2013a, 2013b).

Taking into account all these considerations, the aim of the present research has been to evaluate adverse effects of TBT on porcine vascular stem cells exploring the potential vascular risk associated with the occurrence of nanomolar concentrations of TBT in blood.

1.1. Chemicals and reagents

Heat inactivated FBS (fetal bovine serum), antibiotic–antimycotic, Dulbecco's phosphate buffered saline (DPBS), Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix were purchased from Gibco-Life technologies (Carlsbad CA,USA). Trypsin–EDTA solution 1X, Dimethyl sulphoxide (DMSO) and tributyltin chloride (TBT) were from Sigma-Aldrich (St. Louis, MO,USA). Pericyte Growth Medium was purchased from Promocell, (Heidelberg, Germany). Alexa Fluor 488 annexin/dead cell apoptosis kit (Molecular Probes, Eugene, USA) and CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega Corporation 2800 Woods Hollow Road Madison, WI 53711 USA) were used. RNA isolation was performed with NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Düren Germany), iScript cDNA synthesis kit and iTaq Universal SYBR Green Supermix were used for cDNA synthesis and qPCR analysis (Bio-Rad Laboratories Inc., Hercules, CA, USA).

1.2. Cell culture and treatments

Porcine Aortic Vascular Precursor cells (pAVPCs) were isolated and maintained as previously described (Zaniboni et al., 2014, 2015).

All experiments were performed with cells at the third passage. The first seeding after thawing was always performed in T-75 primary tissue culture flasks (Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA) and successive experiments were conducted in 24-wells or 96-well assay plates (Falcon Beckton-Dickinson). pAVPCs were cultured in Pericyte Growth Medium added with antimicrobial/antimycotic solution (1×) at 38.5 °C. TBT was diluted in DMSO until a 5 mM solution and then in culture medium to obtain desired concentrations for cell exposure. Control cells (TBT 0 nM) were treated with equivalent amount of DMSO used as vehicle (final concentration of DMSO <0.02%).

1.3. Effect of TBT on pAVPC viability

pAVPCs were seeded in a 96 well plates (tissue culture treated) and exposed to increasing doses of TBT (1, 10, 50, 500, 750, 1000 nM) for 24 or 48 h.

Cytotoxicity was evaluated by the CytoTox 96 Non-Radioactive Cytotoxicity Assay®. Briefly Supernatant was incubated with a reconstructed substrate mix for 30 min at room temperature, protected from light. Then, a stop solution was added and the absorbance was recorded at 490 nm. The maximum LDH activity was assessed by lysing cells, collecting the resulting medium and processing it as other samples. Cytotoxicity of TBT was calculated according to the equation:

% Cytotoxicity = Experimental LDH release (OD 490) / Maximum LDH release (OD 490).

To ascertain the mechanism of TBT-induced cellular death, the Annexin V/PI binding assay was used and the percentage of apoptotic and necrotic cell was evaluated through a flow-cytometric approach according to the manufacturers' instructions. Briefly pAVPCs cultured in 24 multiwell plates were incubated with 10 or 500 nM TBT. Cells were harvested, placed in Eppendorf tubes, centrifuged at 500 ×g for 10 min and resuspended in 100 μL of Annexin binding buffer. Annexin V-FITC (5 μL) and Propidium Iodide (1 μL) were added to cell suspension. After incubation, the cells were analyzed with a flow cytometer (FACSAria; BD Biosciences) by collecting 10^5 events.

1.4. Effect of TBT on gene expression of pericyte markers

pAVPCs were seeded in a 24 well plates and exposed to TBT 10 or 500 nM for 48 h. At the end of experimental time, treated or control cells were collected and stored until gene expression analysis.

Total RNA was isolated using the NucleoSpin® RNA Kit and high quality RNA, with A260/A280 ratio above 2.0 was used for cDNA synthesis. Total RNA (500 ng) was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit in a final volume of 20 μL. Quantitative real-time PCR was performed to evaluate gene expression profiles in CFX96 (Bio-Rad) thermal cycler using SYBR green detection system. A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 0.2 μM forward primer, 0.2 μM reverse primer, 1× iTaq Universal SYBR Green Supermix (Bio-Rad). One microliter of cDNA was added to 19 μl of the master mix. All samples were analyzed in duplicate. The qPCR protocol used was: 10 min at 95 °C, 40 cycles at 95 °C for 15 s and at 61 °C for 30 s, followed by a melting step from 55 °C to 95 °C (80 cycle of 0.5 °C increase/cycle).

The expression level of interest genes was calculated as fold of increase using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) in relation to pAVPCs cultured in standard condition (control). Primers sequence for α-SMA, PDGFR-β, Nestin, NG2 were reported in Zaniboni et al., 2015.

Since stability of reference genes is an essential requirement using primary cell culture (Bernardini et al., 2005, 2007, 2012) we decided to test multiple reference genes: RPL35, RPL4, GAPDH and HPRT. Data were normalized on the geometric mean of the most stable reference genes. Primers sequences, were previously reported by Alexander et al., 2012 and Zaniboni et al., 2015.

1.5. Effect of TBT on cytokine production

pAVPCs were exposed to TBT (10 and 500 nM) for 48 h then supernatants were collected from the transwell and 100 μl was used for quantitative determination of TNF-α and IL-6 by means of specific ELISA kits (Porcine TNF-alpha PTA00, Porcine Il-6 P6000B; R&D Systems, MN USA).

1.6. Effect of TBT on pAVPC functionality

To investigate TBT effects on the functionality of pAVPCs, *in vitro* angiogenesis assay was simulated by co-culturing pAVPCs pretreated with TBT (10 or 500 nM for 24 h) and Human Umbilical Vein Endothelial Cells (HUVECs) on extracellular matrix.

The co-culture experiments were carried out using 8-well (BD Falcon Bedford, MA USA) coated with undiluted Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix. Extracellular matrix coating was carried out for 3 h in a humidified incubator, at 38.5 °C, 5% CO_2.

Before co-culture, the HUVECs were stained with a green fluorescent dye (PKH67 Fluorescence cell linker kit -SIGMA) and pAVPCs with a red fluorescent dye (PKH26 Fluorescence cell linker kit -SIGMA) following the manufacturer's instructions.

After the staining procedure, the cells were mixed in a 1:10 ratio (pAVPCs: HUVECs). Images of the co-culture experiment were acquired using a digital camera installed on a Nikon epifluorescence microscope (Nikon, Yokohama, Japan).

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