Effect of tributyltin on mammalian endothelial cell integrity

REVIEW

EFFECT OF TRIBUTYLTIN ON MAMMALIAN ENDOTHELIAL CELL INTEGRITY

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Abstract

Tributyltin (TBT), is a man-made pollutants, known to accumulate along the food chain, acting as an endocrine disruptor in marine organisms, with toxic and adverse effects in many tissues including vascular system. Based on the absence of specific studies of TBT effects on endothelial cells, we aimed to evaluate the toxicity of TBT on primary culture of porcine aortic endothelial cells (pAECs), being pig an excellent model to study human cardiovascular disease. pAECs were exposed for 24h to TBT (100, 250, 500, 750 and 1000nM) showing a dose dependent decrease in cell viability through both apoptosis and necrosis. Moreover the ability of TBT (100 and 500nM) to influence endothelial gene expression was investigated at 1, 7 and 15h of treatment. Gene expression of tight junction molecules, occludin (OCLN) and tight junction protein-1 (ZO-1) was reduced while monocyte adhesion and adhesion molecules ICAM-1 and VCAM-1 (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) levels increased significantly.at 1 hour. IL-6 and estrogen receptors 1 and 2 (ESR-1 and ESR-2) mRNAs, after a transient decrease, reached the maximum levels after 15h of exposure. These findings indicate that TBT deeply alter endothelial profile, disrupting their structure and interfering with their ability to interact with molecules and other cells.

Keywords: adhesion molecules, endothelial cells, estrogen receptors, inflammation, tight junctions, Tributyltin

Abbreviations: porcine Aortic endothelial Cells (pAECs), tributyltin (TBT), occludin (OCLN), tight junction protein-1 (ZO-1) intercellular adhesion molecule-1 (ICAM-1) vascular cell adhesion molecule-1 (VCAM-1), interleukin-6 (IL-6) , estrogen receptors 1 and 2 (ESR-1 and ESR-2)

1 Introduction

Among man-made pollutants, known to accumulate along the food chain, organotins, and mainly trisubstituted tin compounds, are especially dangerous, due to their wide industrial exploitation as polyvinyl chloride stabilizers, catalysts, pesticides and biocides in antifouling paints.

Tributyltin (TBT) interact by both covalent and non-covalent bonds with biomolecules and membrane structures and is considered among the most toxic substances ever deliberately introduced into environment (Pagliarani et al. 2013).

In spite of bans of TBT use (IMO 2001), the residue in marine environment is still an important concern (Horiguchi, 2012) due to its environmental persistence (Hoch, 2001; Fent et al, 2004). The contamination of aquatic environments is especially harmful. Bioaccumulation in tissues of exposed species (Frouin et al., 2010) leads to contamination of seafoods (Ma et al. 2011) and in turn, mainly through the food chain, of terrestrial species including human (Kannan et al., 1999; Takahashi et al., 1999).

TBT exerts a toxic effect acting as a classical endocrine disruptor for marine organisms causing imposex in gastropod mollusks (Gallo and Tosti, 2013). TBT shows also toxic and adverse effects in many kinds of cells and tissues of a variety of species, including mammalian (Ohshima et al., 2005) in which affects endocrine system through different pathway. Organotins are potent inhibitors of 11β-hydroxysteroid dehydrogenase type-2 (11β-HSD2) (Atanasov et al., 2005) and shows proadipogenic activity in some cell lineages like human and mouse multipotent stromal stem cells (Kirchner et al. 2010; Li et al., 2011; Penza et al., 2011).

Different cell types have depicted dissimilar levels of tolerance to TBT, resulting in a diversity of effects and in specific toxic concentrations for every cell lineage. In a cultured human granulosa-like tumor cell line, Saitoh et al. (2001) found a toxic TBT concentration of 1000 ng/mL, causing cell death within 24h, while 200 ng/mL induced apoptosis of the cells. In neurons continuously exposed to TBT for 3 days, Yamada et al. (2010) observed a TBT-induced death at 30 nM in 4-6 days cultures and at 50 nM in 14-16 days cultures, which means that older neurons

80 are more resistant to TBT toxicity. Significant loss of viability was observed in neuroblastoma cells
81 incubated for 24h with doses of TBT ranging from 250 nM onward, with a linearity found between
82 250 nM and 2 μM (Ferreira et al., 2013) and in a Sertoli-germ cell co-culture incubated for 6h with
83 a minimum dose of 300 nM (Mitra et al., 2013a).

84 The information on human exposure to butyltin compounds is limited; some studies found
85 TBT, DBT(Dibutyltin), and MBT (Monobutyltin) levels in human tissues in the range of 3-100 nM
86 (Kannan et al., 1999; Takahashi et al., 1999). Butyltin compounds were already found in human
87 blood in concentrations ranging between 64 and 155 ng/mL (Whalen et al., 1999), in particular TBT
88 have been found up to 261 nM. This variability could be related to human diet, food habits, gender
89 and physiological stage which should be taken into account; controlled trial in animal models could
90 overcome this problem.

91 Recently, very interesting studies, using rodent model, correlated TBT to cardiovascular
92 disorders impairing the coronary vascular reactivity response to estradiol and producing endothelial
93 denudation in isolated rat heart (Dos Santos et al., 2012) and demonstrated TBT ability to reduce
94 vasoconstrictor response in isolated aortic rings of female rats (Rodrigues et al., 2014).

95 Among cellular components of vasculature, the endothelium is especially susceptive to
96 plasma toxicants because it is structurally arranged in a single layer of cells that first come into
97 contact with blood vessel contents. Further, injuries to endothelial cells are implicated in the
98 pathophysiology of several diseases (Yamada et al., 2011) and in particular in the cardiovascular
99 ones (Mordi and Tzemos, 2014).

100 Based on the recent demonstration that TBT can influence vascular system and on the
101 absence of specific studies of TBT effects on endothelial cells, we aimed to evaluate the toxicity of
102 TBT on mammalian endothelial cells, using primary cultures of porcine Aortic Endothelial Cells
103 (pAECs), being swine an excellent animal model in the field of cardiovascular research (Forni et
104 al., 2005; Vilahur et al., 2011; Zaragoza et al., 2011; Zannoni et al., 2012; Gessaroli et al., 2012).
105 In addition, we aimed to investigate the ability of TBT to influence gene expression of markers
106 involved in structure and function maintenance and in response to injuries of endothelial cells., in

and in estrogen sensitivity. Finally we evaluate the alteration of endothelial function induced by TBT through monocytes adhesion assay.

2 Materials and methods

2.1 Chemicals and reagents

Human endothelial SFM medium, Heat inactivated FBS (fetal bovine serum) and Fungizone were purchased from Gibco-Life technologies. Trypsin-EDTA solution 1X, Dimethyl sulphoxide (DMSO) and tributyltin chloride (TBT) were from Sigma-Aldrich and Dulbecco's phosphate buffered saline (DPBS) from EuroClone. AlexaFluor 488 annexin/dead cell apoptosis kit (Molecular Probes, Eugene, USA Invitrogen) and CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega. Promega Corporation 2800 Woods Hollow Road Madison, WI 53711 USA) were used. NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG Postfach 10 13 52 D-52313 Düren Germany) was used for RNA isolation and IScript cDNA synthesis kit, IQ Supermix and IQ SyBR Green Supermix.(Bio-Rad Laboratories Inc., Hercules, CA, USA) were used for cDNA synthesis and RT-PCR analysis.

2.2 Cell culture

pAECs were isolated and maintained as previously described by Bernardini and colleagues (2005) and used from the third to the sixth passage. The first seeding after thawing was always performed in T-25 tissue culture flasks ($3x10^5$ cells/flask) (T25-Falcon, Beckton-Dickinson, Franklin Lakes, NJ, USA) and successive experiments were conducted in 24-well (qPCR analysis and monocyte adhesion assay) or 96-well assay plates (cell viability) (Falcon Beckton-Dickinson) with confluent cultures. Cells were cultured in Human endothelial SFM medium, added with FBS (5%) and antimicrobial/antimycotic solution (1x Gibco-Life technologies code 15240-062) at 38.5°C. The tributyltin chloride was diluted in DMSO until a 5mM solution and therefore in culture medium to obtain desired concentrations for cell exposure.

2.3 Cell Viability

The ability of TBT to induce cytotoxicity was evaluated by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega BioSciences LLC San Luis Obispo, CA, USA) that quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis.

Confluent pAECs were incubated for 24h in a 96 well plate with TBT (0, 100, 250, 500, 750, 1000 nM) and the supernatant was collected to be analysed. The maximum LDH activity was assessed by lysing cells, collecting the resulting medium and processing it as other samples. 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. Cytotoxicity of TBT was calculated according to the equation:

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

To determine the ability of TBT to induce apoptosis or necrosis the Annexin V binding assay was employed. This assay detect phosphatidilserine (PS) externalization on the plasma membrane.

In viable cells PS is located on the cytoplasmic surface of the cell membrane. However during the early stage of apoptosis, PS is translocated from the inner to the outer leaflet of the plasma membrane.

Confluent pAECs cultures were incubated in a 24 well plate with increasing doses TBT and the Annexin V / PI binding assay (Alexa Fluor® 488, Life Technologies) was used.

Cells were harvested, placed in eppendorf tubes, centrifuged at 500 x g for 10 min and resuspended in 100 µL of Annexin binding buffer. Annexin V-FITC (5µL) 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 at least 10^4 events.

2.4 RNA isolation and quantitative real time PCR (qPCR)

To determine the ability of TBT to influence endothelial gene expression, confluent pAECs were incubated for different time (1, 7, 15 hours) with different doses of TBT (0, 100 or 500nM).

Total RNA was isolated using the NucleoSpin®RNA Kit (Macherey-Nagel GmbH & Co. KG, Germany), and one µgof total high quality RNA(A_{260}/A_{280} ratio above 2.0) was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD Laboratories Inc., California, USA) in a final volume of 20 µL. Swine primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA) for each studied gene: estrogen receptor 1 and 2 (ER-1; ER-2); tight junction proteins: occludin (OCLN) and tight junction protein-1 (ZO-1); adhesion molecules: vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1); cytokine: interleukine-6 (IL-6). Moreover, primers for the following reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and Hypoxanthine-guanine phosphoribosyltransferase (HPRT-), were designed in order to evaluate the most suitable using BestKeeper Software (Pfaffl et al., 2004) for qPCR normalization. Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Table 1. Quantitative real-time PCR was performed to evaluate gene expression profiles in iCycler (Bio-RAD) using SYBR green I detection system. The amplification reaction (25 µL) contained 12.5 µL of IQ SYBER Green Bio-RAD Supermix (Bio-RAD), 1 µL of each forward and reverse primer (5 µM), 2.5 µL cDNA and 8 µL of water. All samples were performed in duplicate and controls lacking cDNA template were included to determine the specificity of target amplification. The real-time program included an initial denaturation for 1min 30s at 95°C, 40 cycles of 95°C for 15s, and 60°C for 30s, followed by a melting step with ramping from 55°C to 95°C at a rate of 0.5°C/10s. Specificity of the amplified PCR products was confirmed by melting curve analyses and agarose gel electrophoresis. The expression level of interest genes was calculated as fold of increase using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) in relation to pAECs cultured under standard conditions (control).

2.5 Monocytes adhesion assay

Separation of peripheral blood mononuclear cells (PBMC) was performed by Lympholyte-H (Cedarlane, Burlington, NC, USA) following the instruction. PBMC were washed twice with PBS, suspended in DMEM supplemented with 10% FBS and placed on a Petri dish for 1h at 38.5°C in a humidified 5% CO_2 atmosphere to allow cells adhesion. Then, the culture medium containing non-adherent cells was removed and medium was replaced. After 48h of culture, cells were detached using a scraper and suspended in DMEM supplemented with 10% FBS (Cutini et al., 2012).

Monocytes adhesion assay was performed according to Lim et al. (2011). pAECs were treated with TBT 0, 100 or 500 nM for 1h. Fresh culture medium containing $1.5x10^4$ PBMC was added in each well and incubated for 2h at 38.5°C in a humidified 5% CO_2. Unbound cells were removed by washing with PBS. PBMC adhering to pAEC layer were counted in 5 randomly selected fields for each well.

2.6 Statistical analysis

All the data were analysed with the Shapiro-Wilk test, to assess whether they are modelled by a normal distribution, and with the Levene test, to assess whether the variances are comparable.

Normal distribution of data and homoscedasticity were assumed, with p value > 0.05. Data were analyzed through Students *t*-test comparing treatments in relation to the control. Statistical analysis was carried out by using R software (http://www.R-project.org)

3 Results

3.1 Effect of TBT on cell viability

pAECs exposed to TBT lost gradually their typical morphology of continuous monolayer and an increasing number of detached cells appeared related to the increase of TBT dose (Fig. 1A). The loss of cells viability is demonstrated by LDH release: TBT induced significant decrease of cell viability from the dose of 250nM (Fig 1B). The flow cytometric data showed that TBT is capable to

induce both apoptosis and necrosis (Fig 1C), even if apoptosis was prevalent at lower doses (data not shown).

3.2 Effect of TBT on gene expression

The mRNA expression stability of the commonly used reference gene (GAPDH, HPRT, β-Actin) revealed that β-actin expression was reduced after TBT exposure (data not shown). Therefore, GAPDH and HPRT geometric mean value of Ct was used for normalization of qPCR data.

Both tight junction molecules OCLN and ZO-1 were lower in pAECs treated respect to pAECs under standard culture conditions starting from 7h of exposure at 500 nM TBT (Figure 2 B; D); moreover ZO-1 expression showed significant decrease also at low dose (Fig. 2 C). The adhesion molecules, VCAM-1 and ICAM-1 showed a significant transient increase at 1 hour at both doses, followed by a decrease (Fig. 3).

IL-6 mRNA was transiently reduced at early times for both TBT doses. After 15h of exposure at TBT 500 nM the IL-6 expression was significantly increased (Figure 4).

The transcript of both estrogen receptors was significantly decreased at both doses after 1 and 7 hours, then, at 15 hours, both receptors increased even if only ER-1 was significantly increased with TBT 500 nM (Fig. 5).

3.3 Effect of TBT on monocyte adhesion

Increased monocyte adhesion was observed after TBT treatment the monocyte adhesion assay (Fig 6), As shown in Fig 6C the TBT treatment significantly increased monocyte adhesion

4 Discussion

Among different cellular types, the endothelium, uniquely positioned at the interface between the vessel wall and flowing blood, can be a relevant target for TBT toxic action. The endothelial layer, in fact, regulates multiple functions such as maintenance of normal vascular

240 tone, modulation of coagulation, and immune responses (Maney et al., 2011) and may contribute
241 to vascular disorders (Aki et al., 2008). Therefore, being endothelial cells directly exposed to the
242 TBT accumulated in the bloodstream as a result of biomagnification, it is important to investigate in
243 which extent they are affected in relation to its principal features.

244 Our study demonstrated that TBT significantly reduced the viability of pAECs, displaying a
245 consistent dose-response relation. Accordingly, the cell death mechanism, namely apoptosis or
246 necrosis, is known to depend on the TBT dose and exposure time (Pagliarani et al.,2013). The
247 cytotoxic effect of TBT on pAECs was observed at 250nM dose, comparable to that one exhibited
248 in neuroblastoma cell (Ferreira et al., 2013). A great variability in cell susceptibility to TBT is
249 reported in the literature: mouse neurons exhibit signs of toxicity and death from concentrations of
250 30 nM (Yamada et al., 2010) while rat or trout hepatocytes begin to die from 2 µM (Reader et al.
251 1999, Jurkiewicz et al., 2004).

252 The lowest dose utilized had no significant effects on cell viability, however, it had an
253 relevant effect on gene expression, this result is to be considered taking into account that in human
254 blood TBT was found till 155 ng/mL (Whalen et al., 1999).

255 Expression of genes related to tight junctions (OCLN and ZO-1) was reduced by TBT
256 exposure confirming the morphological alterations observed and consistently with an increased
257 number of round and detached cells. Therefore, from a functional point of view, TBT alters the
258 typical architecture of endothelial cell-cell junctions, consistently with previous reports in various
259 mammalian cell types such as epithelial cell *in vitro* (Tsukazaki et al., 2004) prostate cells *in vivo*
260 (Barthelemy et al., 2007) and isolated heart cells *ex vivo*(Dos Santos et al. 2012)

261 Adhesion molecules, responsible for mediating the aggregation of cells to endothelium, are
262 considered markers of endothelial dysfunction and are used to predict potential vascular risk
263 (Wiseman et al., 2014). VCAM-1 is constitutively expressed on endothelial cells and mediates
264 tethering and rolling of lymphocytes and monocytes (Tu el al., 2013). ICAM-1 is constitutively
265 expressed on the cell surface of endothelial cells and leukocytes and functionally activates
266 leukocyte-endothelial adhesion and migration. The effect of TBT on ICAM-1 and VCAM-1 gene

267 expression, in our model, followed the same trend with an initial increase and a subsequent
268 decrease, in accordance with the modification that occurs at the beginning of an endothelium
269 dysfunction. (Burger et al., 2012). Consistently, as adhesion molecules mediate the adherence of
270 circulating leukocytes to the vascular endothelium, TBT treatment strongly increased monocytes
271 adhesion to pAECs.

272 The response of endothelial cells to a toxic injury often involves several changes in
273 inflammatory mediators. IL-6 is a pleiotropic cytokine, it regulate B cell differentiation,
274 immunoglobulin production, T cell proliferation and participate to the regulation of inflammatory
275 responses driving adhesion molecules synthesis in endothelial cells (Fogam 2011). IL-6 is also a
276 potent proangiogenic cytokine regulating vascular endothelial growth factor (VEGF) secretion
277 (Nilsson et al., 2005; Pober et al., 2009); different cell types produce IL-6 among these endothelial
278 cells are one of the major sources of plasma IL-6 (Tanaka et al., 2011).

279 In our model, TBT was able to reduce IL-6 mRNA expression at early times for both doses.
280 However TBT 500nM strongly increased the IL-6 mRNA expression after 15h of treatment, in
281 accordance with data observed by Mitra et al. (2013) in neural dissociated cortical cells and likely
282 in an effort to induce cell survival through VEGF.

283 The interaction of organotin with nuclear receptors is well known, they augment
284 adipogenesis via the interaction with PPARγ/RXRα (Grün and Blumberg, 2006) and inhibit
285 estrogen with a systemic action and/or through repression of ER synthesis (McAllister and Kime,
286 2003, Delfosse et al., 2014). The expression of the two estrogen genes receptors was altered by
287 TBT: the expression decreased till 7h at both doses, whilst a strong increase was observed after
288 15h only with the highest dose. Similarly, Zhang et al. (2013) shown that TBT affected the ERs
289 gene expression in males rockfish (*Sebastiscus marmoratus*) leading to an increase or a decrease
290 depending on the applied concentrations.

291 Further investigations will be necessary to clarify the functional impact of this estrogen
292 receptor alteration. The multiple effect of estrogens mediated by ERs includes endothelial
293 proliferation, endothelial apoptosis inhibition, modulation of adhesion molecules with an overall

protective effect against cardiovascular diseases (Mendelsohn and Karas 1999; Mosca et al., 2011). In fact it is well know that the incidence of cardiovascular disease differs significantly between man and woman being lower in man and in post-menopausal women.

Overall the exposure of endothelial cells to TBT induced a wide range of effects all consistent with endothelial dysfunction that represents the first stage of many vascular diseases. in In our model TBT induced a shifts from an anti- to a pro-adhesive endothelial phenotype that is the first step of inflammatory process and an important risk factor for cardiovascular diseases (Aird, 2008). Moreover we demonstrate the alteration of cell-cell junctions that is known to be responsible in vivo of alteration of permeability exiting in edema and vascular fragility (Dejana et al., 2009).

The role of ER in triggering the TBT effects on endothelial cells and, on turn, on cardiovascular diseases, must be deeply investigated due to the clear protective effect that estrogens exert on cardiovascular system and the power of TBT to alter steroidogenesis.
Therefore, further *in vivo* investigation using pig model will be necessary to understand the pathological implication of TBT contamination in the development of cardiovascular alterations.

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Figure 1. A) Representative images of pAECs morphology under standard culture conditions or in the presence of increasing TBT doses; cultures exposed to TBT demonstrated a higher number of detached cells. Effect of TBT on cell viability. B) Cytotoxicity assessed by LDH release assay on pAECs exposed to increasing TBT concentrations. Data represents mean ± S.E. of four independent experiments **P < 0.01. C) Annexin V / PI binding assay, representative cytograms showing control (left) and pAECs exposed to TBT 1000 nM (right). TBT treatment induced both apoptotic and necrotic cells.

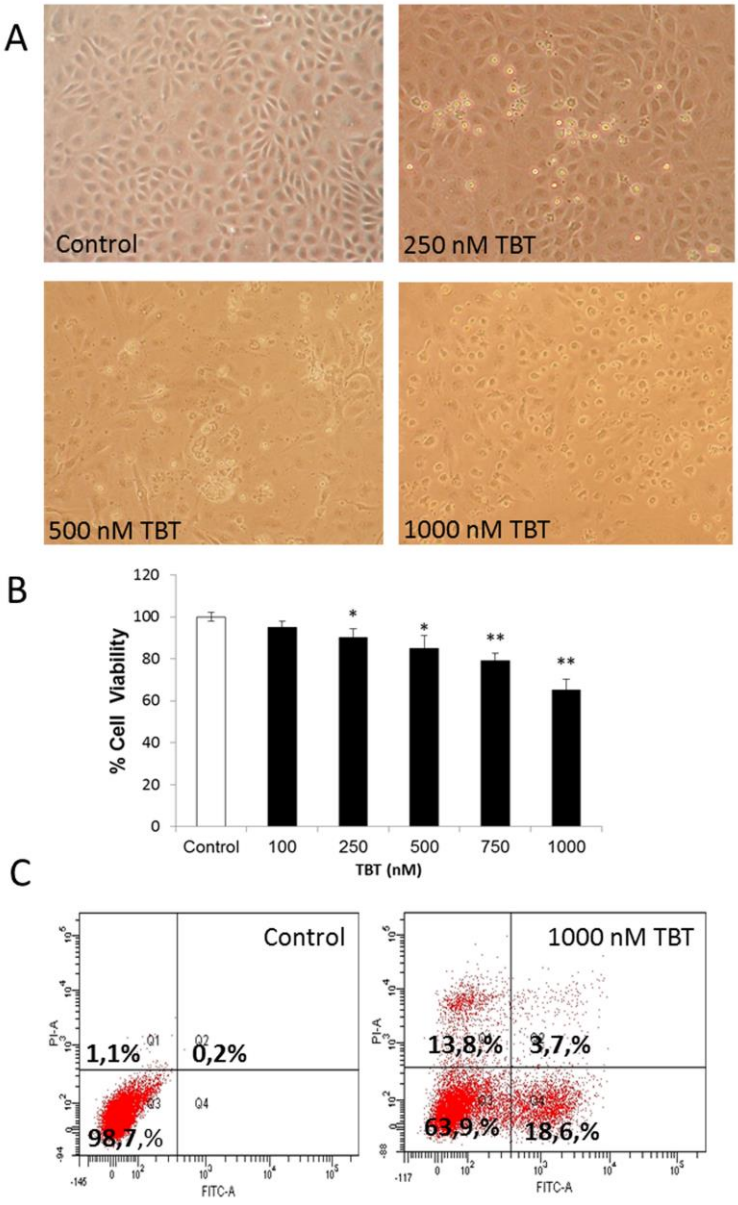


Figure 2. Relative gene expression of tight junction: OCLN (A,B) and ZO-1(C,D) in pAECs treated with TBT (100 and 500 nM) at different period (1, 7 and 15 hours). Relative expression was calculated as fold of change in respect to the control cells ($2^{-\Delta\Delta Ct}$ method). Error bar represents the range of relative expression.

**P < 0.01 and *P < 0.05 when compared to control.

Occludin (OCLN); Zonula Occludens-1 (ZO-1)

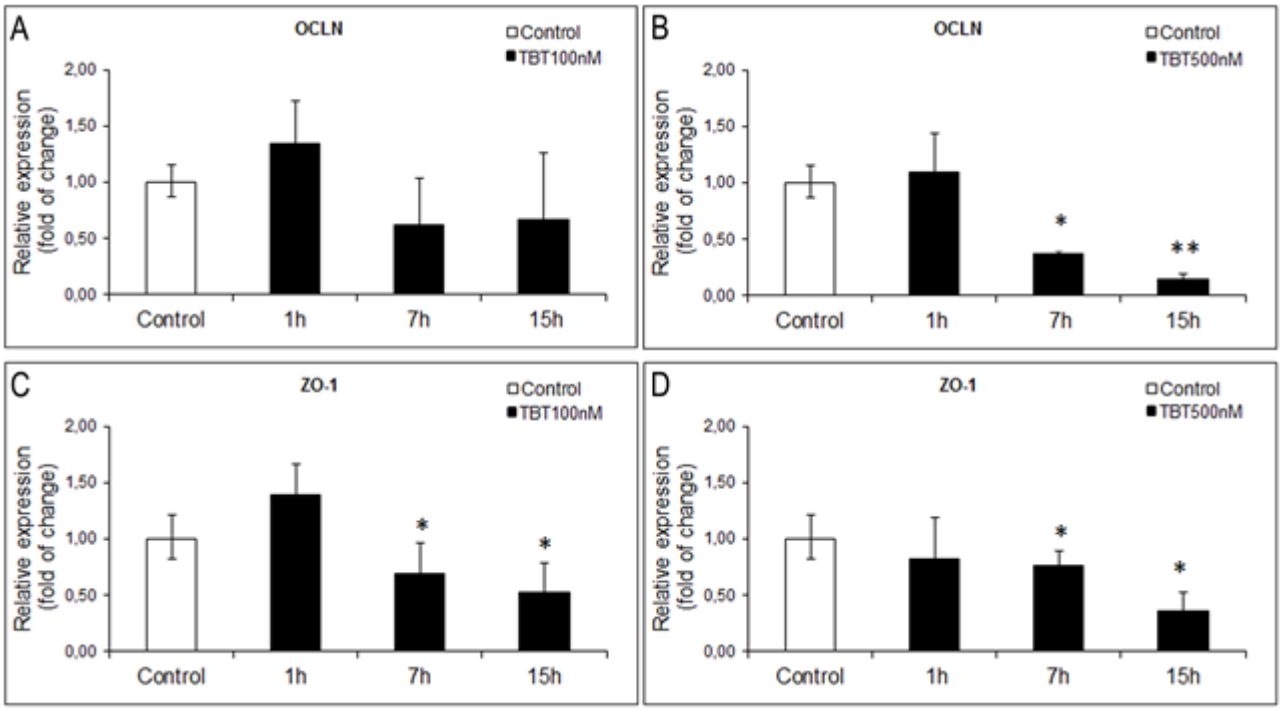


Figure 3. Effect of TBT exposure on expression levels of genes related to adhesion molecules:VCAM-1 (A,B), ICAM-1 (C,D) in pAECs treated with TBT (100 and 500 nM) at different period (1, 7 and 15 hours). Relative expression was calculated as fold of change in respect to the control cells ($2^{-\Delta\Delta Ct}$ method). Error bar represents the range of relative expression.

**P< 0.01 and *P < 0.05

Intercellular Adhesion Molecule-1 (ICAM-1); Vascular Cell Adhesion Molecule-1 (VCAM-1)

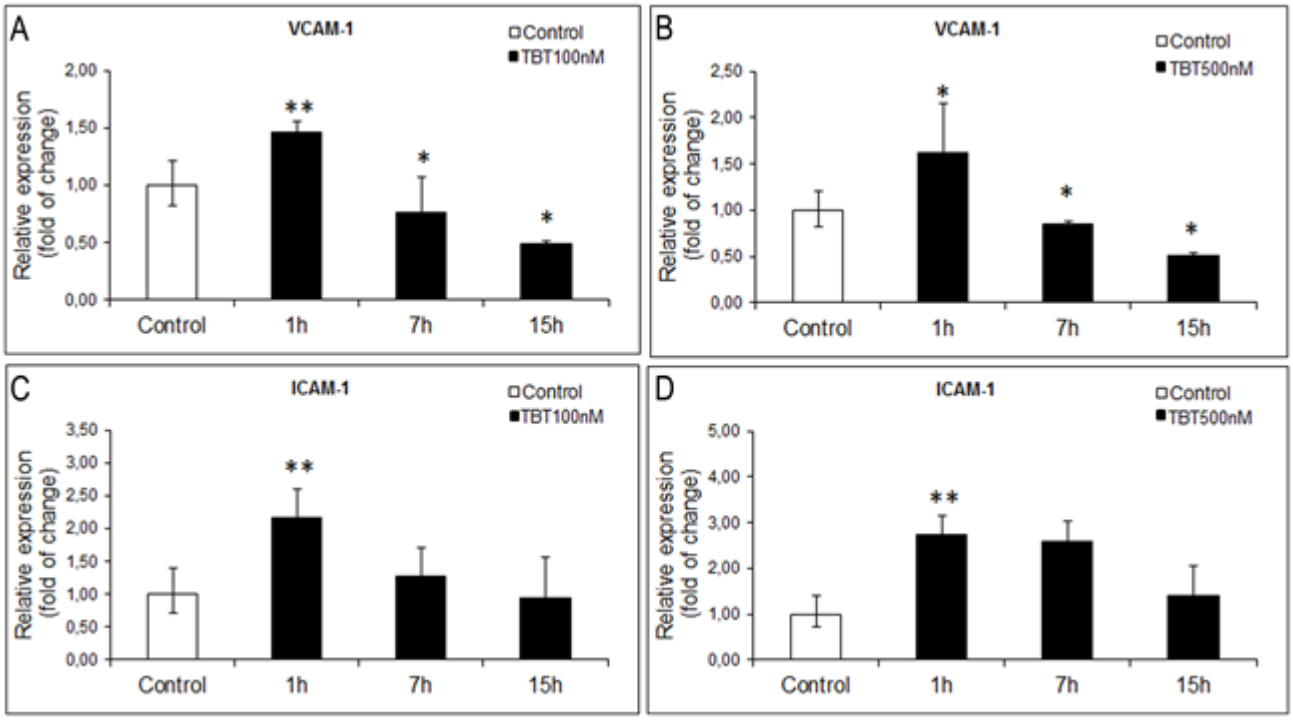


Figure 4. Effect of TBT exposure on expression levels of IL-6 gene in pAECs. treated with TBT 100 nM (A) and 500 nM (B) at different period (1, 7 and 15 hours). Relative expression was calculated as fold of change in respect to the control cells ($2^{-\Delta\Delta Ct}$ method). Error bar represents the range of relative expression.

**P< 0.01 and *P< 0.05

Interleukin 6 (IL-6)

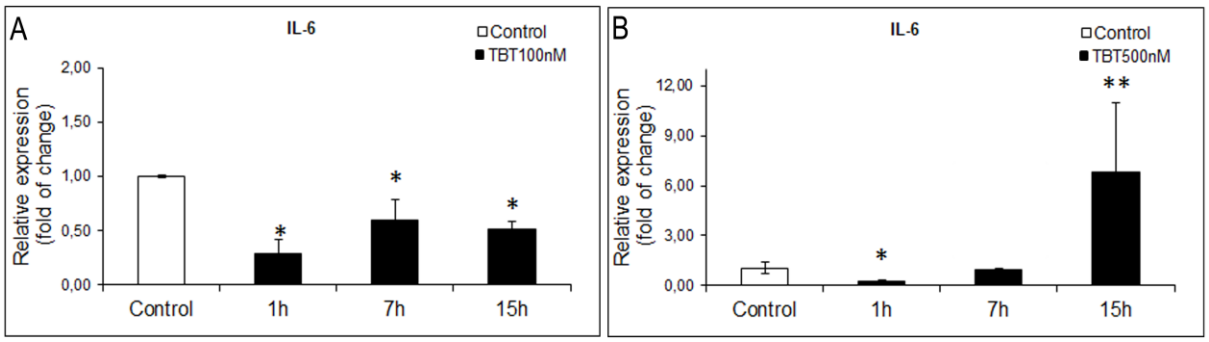


Figure 5 Effect of TBT exposure on expression levels of ESR-1 and ESR-2 genes in in pAECs treated with TBT (100 and 500 nM) at different period (1, 7 and 15 hours). Relative expression was calculated as fold of change in respect to the control cells ($2^{-\Delta\Delta Ct}$ method). Error bar represents the range of relative expression.

*P< 0.05

estrogen receptor 1 (ESR-1); estrogen receptor (ESR-2)

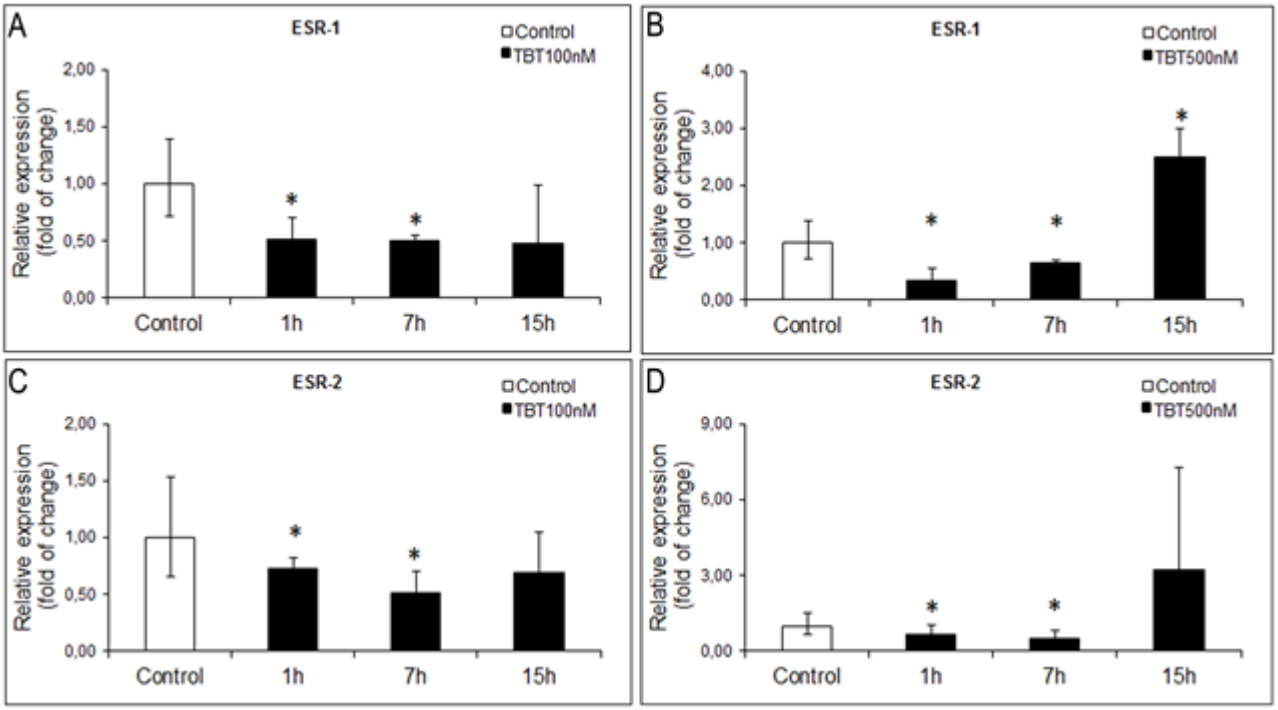
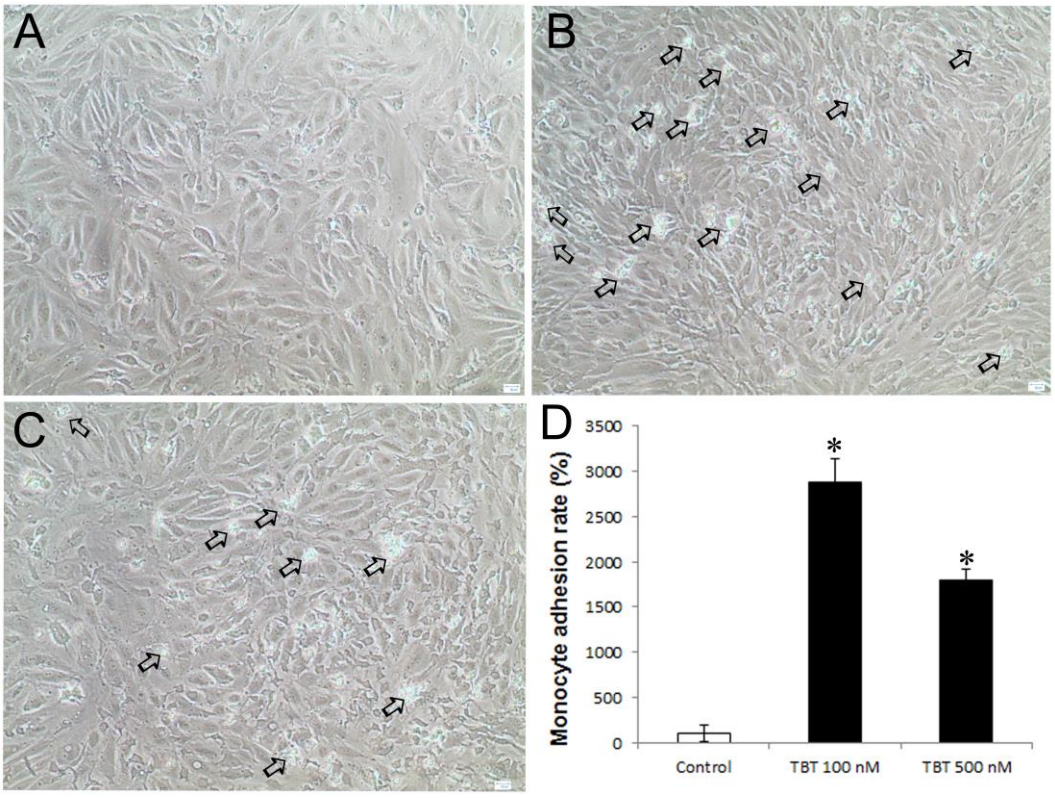


Figure 6 Effect of TBT on monocyte adhesion. A) pAECs in standard culture condition (control); B, C) pAECs treated with TBT 100nM or 500 respectively (10X). The arrows indicated adhered monocytes. D) Bars represent the mean Monocyte adhesion rate represented as the percentage compared with control group. *P< 0,01



579 Table 1: Sequences, expected PCR product lengths, and accession numbers in the NCBI
580 database of the swine primers for indicated genes.

Gene	Sequence (5'-3')	Length (bp)	Acc. No.
Estrogen receptor 1 (ESR-1)	For: CGGAGAGGAGGGAGAATGTTG	142 bp	NM214220
	Rev: GGCTGTTCTTCTTAGTGTGTTTAAT		
Estrogen receptor 2 (ESR-2)	For: AACCTTAACTCTCCTGTCTCCTAC	250 bp	NM001001533
	Rev: GCTGGCAATGGATGGCTAAAG		
Occludin (OCLN)	For: ATCAACAAAGGCAACTCT	157 bp	NM001163647.2
	Rev: GCAGCAGCCATGTACTCT		
Zonula Occludens-1 (ZO-1)	For: AGTGCCGCCTCCTGAGTTTG	147 bp	AJ318101
	Rev: CCATCCTCATCTTCATCATCTTCTACAG		
Vascular cell adhesion molecule (VCAM-1)	For: GAGGATGGAAGATTCTGGAATTTACG	172 bp	NM213891
	Rev: ATCACTAGAGCAGGTCATGTTCAC		
Intercellular adhesion molecule (ICAM-1)	For: GCCACTAACAATCACGCATAATG	212 bp	NM213816
	Rev: TGCTCACTGTAGTCCCTTCTG		
Interleukin-6 (IL-6)	For: CTGGCAGAAAACAACCTGAACC	94 bp	NM214399.1
	Rev: TGATTCTCATCAAGCAGGTCTCC		
β-Actina (Act)	For: ATCGTGCGGACATCAAGGA	169 bp	AJ312193
	Rev: AGGAAGGAGGGCTGGAAGAG		
GAPDH	For: TGGTGAAGGTCGGAGTGAAC	120 bp	AF017079
	Rev: TGTAGTGGAGGTCAATGAAGGG		
HPRT	For: GGACAGGACTGAACGGCTTG	115 bp	AF143818
	Rev: GTAATCCAGCAGGTCAGCAAAG		

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