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Tributyltin chloride leads to adiposity and impairs metabolic functions in the rat liver and pancreas

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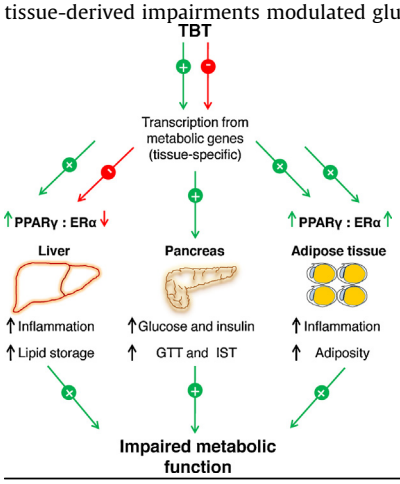
HIGHLIGHTS

- Tributyltin chloride modulates adipose tissue-specific in female rats.
- Tributyltin chloride up-regulated ER-alpha expression *in vivo*.
- Tributyltin chloride down-regulated ER-alpha expression in 3T3-L1 cells.
- Tributyltin chloride impairs liver and pancreas morphophysiology.

GRAPHICAL ABSTRACT

Diagram of the tissue–tissue cross-talk in tributyltin chloride (TBT) and metabolic homeostasis. TBT derived from exogenous sources stimulates (green line) or inhibits (red line) transcription from metabolic genes (tissue-specific). TBT stimulates PPARγ and inhibits ERα protein expression followed with hepatic inflammation and lipid storage. Interestingly, TBT stimulates both PPARγ and ERα protein expression in adipose tissue associated with inflammation and adiposity. Additionally, liver and adipose tissue-derived impairments modulated glucose tolerance (GTT) and insulin sensivity (IST) tests.



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ABSTRACT

Tributyltin chloride (TBT) is an environmental contaminant used in antifouling paints of boats. Endocrine disruptor effects of TBT are well established in animal models. However, the adverse effects on metabolism are less well understood. The toxicity of TBT in the white adipose tissue (WAT), liver and

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pancreas of female rats were assessed. Animals were divided into control and TBT (0.1 μg/kg/day) groups. TBT induced an increase in the body weight of the rats by the 15th day of oral exposure. The weight gain was associated with high parametrial (PR) and retroperitoneal (RP) WAT weights. TBT-treatment increased the adiposity, inflammation and expression of ERα and PPARγ proteins in both RP and PR WAT. In 3T3-L1 cells, estrogen treatment reduced lipid droplets accumulation, however increased the ERα protein expression. In contrast, TBT-treatment increased the lipid accumulation and reduced the ERα expression. WAT metabolic changes led to hepatic inflammation, lipid accumulation, increase of PPARγ and reduction of ERα protein expression. Accordingly, there were increases in the glucose tolerance and insulin sensitivity tests with increases in the number of pancreatic islets and insulin levels. These findings suggest that TBT leads to adiposity in WAT specifically, impairing the metabolic functions of the liver and pancreas.

1. Introduction

Organotin chemicals (OTs) are a diverse class of widely distributed xenobiotics (Fent, 1996; Graceli et al., 2013). These organometallic pollutants are used as biocides in antifouling paints (Barnes and Stoner, 1959; Grün and Blumberg, 2006), although use for this purpose has been restricted in recent years, on the basis of their various toxic effects (IMO, 2001; Oberdõrster and McClellan-Green, 2002; Graceli et al., 2013). OTs are markedly toxic to oysters and other non-target molluscs and are considered to be endocrine-active environmental chemicals. For instance, the tributyltin chloride (TBT) is inducers of imposex, the imposition of male sex characteristics on female snails (Fent, 1996; Oberdõrster and McClellan-Green, 2002). The mechanism by which TBT cause imposex is unclear, but TBT-induced inhibition of an aromatase, a cytochrome-P450 that converts testosterone into estrogen, seems to be involved (Oberdõrster and McClellan-Green, 2002).

Several investigations have shown that exposure to OTs cause hepatic, neural, immune and reproductive toxicity (Wiebkin et al., 1982; Kletzien et al., 1992; Tafuri, 1996; Grote et al., 2006; Grondin et al., 2007) in various mammalian experimental models by accumulation of TBT and their metabolites, as dibutyltin (DBT) and inorganic tin (iSn) (Krajnc et al., 1984; Dorneles et al., 2008). The iSn is poorly absorbed by the gastro-intestinal tract (GIT) and is associated with OTs metabolization into iSn by mammals (Appel, 2004). It has been suggested that an important fraction of iSn may be present in the bodies of mammals, as a result of OT contamination, which strengthens the importance of the total tin determination for evaluating the exposure of mammalian to OTs (Appel, 2004; Dorneles et al., 2008).

Among other effects, reports on their toxicity indicate that TBT promote adipogenesis *in vivo*,*in vitro* and *in utero* (Grün et al., 2006; Kirchner et al., 2010; Penza et al., 2011). Furthermore, TBT alters the stem cell compartment by sensitising multipotent stromal stem cells to differentiate into adipocytes, similar to the actions of the obesogen class of environmental chemicals (Grün and Blumberg, 2006; Kirchner et al., 2010).

Obesogens can be functionally defined as chemicals that inappropriately alter lipid homeostasis and fat storage, metabolic set points, energy balance, or the regulation of appetite and satiety to promote fat accumulation and obesity (Grün et al., 2006; Grün and Blumberg, 2007). The obesity and metabolic disorders related in the developed world are not associated only to overeating or inactivity, although these are clear factors (Newbold et al., 2009). Previous studies supported that a role of environmental factors in the development of obesity, such as environmental obesogens (Grün et al., 2006; Newbold et al., 2009; Zhuo et al., 2011; Chamorrro-Garcia et al., 2013). The rise in obesity coincides with an exponential increase in the use of industrial chemicals over the last 40 years. Numerous xenobiotics have attracted attention for their potential contribution to the increased obesity rate (Heindel and vom Saal, 2009; de Cock and van de Bor, 2014).

The white adipose tissue (WAT) is the principal modulator of metabolic function in mammals. WAT plays a pivotal role in regulating the cascade of paracrine events necessary for energetic metabolism, immune process and reproductive function (Guerre-Millo, 2002; Kershaw and Flier, 2004; Badman and Flier, 2005; MacLaren et al., 2008; Monget et al., 2008). In females, the granulosa cells in ovary secrete estrogen (E_2), which acts a important modulation in the typical distribution of body fat and WAT metabolism, mediated by two nuclear estrogen receptors (ERs), ER alpha (ERα) and beta (ERβ) (Danilovich et al., 2000). The enlarged fat mass deposition that occurs in women as they enter menopause and the growth of fat mass reported in various rodent models of E_2 deficiency represent the clearest physiological examples of the anti-adipogenic action of E_2 (Danilovich et al., 2000; Heine et al., 2000). E_2 modulates WAT increasing lipolysis through control of the expression of genes that regulate lipogenesis, adipocyte differentiation and metabolism (Cooke and Naaz, 2004; Pallottini et al., 2008).

Despite these discoveries of TBT and E_2 actions in WAT, few studies have explored the effect of TBT signaling directly in ERs on mammalian metabolic function (Grün and Blumberg, 2006; Penza et al., 2011). Consequently, herein, the aim of this study was to determine the association of TBT-induced adverse effects on the parametrial and retroperitoneal WAT (*in vivo*), 3T3-L1 cells (*in vitro*) and the metabolic functions of the liver and pancreas associated with the impairment of the E_2 levels in female rats.

2. Material and methods

2.1. Experimental animals and treatments

Adult female Wistar rats weighing approximately 230 g (12 week old) were housed in polypropylene cages under controlled temperature and humidity conditions with a 12-h light/dark cycle and free access to water and food. The rats were divided into two groups: (1) The tributyltin chloride group (TBT, $n = 10$), treated daily with tributyltin chloride (0.1 μg/kg/day of TBT diluted in vehicle consisting of 0.4% ethanol; Sigma, St. Louis, MO) for 15 day by oral administration (Lang Podratz et al., 2012); and (2) The control group (CON, $n = 10$) received the vehicle following the same protocol used for the TBT-group. Dose and route of TBT exposure were chosen based on previous work in our laboratory (dos Santos et al., 2012; Lang Podratz et al., 2012) and others (Rodrigues et al., 2014). Whole body composition of rodents could be changed with TBT exposure (Grün et al., 2006; Penza et al., 2011); hence, the weights of control and TBT rats were assessed twice a week for the full period of the study. All experiments were performed in accordance with the Biomedical Research Guidelines for the Care and Use of Laboratory Animals available on line at (http://www.cfmv.org.br/portal/legislacao/resolucoes/resolucao 879) and followed the recommendations of the American Veterinary Medical Association Guidelines, 2007 (available online

at http://www.nih.gov). All procedures were approved by the Committee for Animal Experiments of the University of Espirito Santo (CEUA number 047/10).

2.2. Measurements of hormones and tin

During the morning of the proestrus phase, female rats were anesthetized with ketamine (30 mg/kg, im) and xylazine (3 mg/kg, im) and the blood samples were obtained from the decapitation. Blood samples were collected and immediately centrifuged to obtain serum, which was kept at −20°C for subsequent measurements of progesterone (P_4), estrogen (E_2), testosterone (test) and insulin by radioimmunoassay according to the manufacturer's directions (Diagnostic Prod. Corporation, LA, CA) (Lang Podratz et al., 2012; Rodrigues et al., 2014).

In addition, the measurements of the blood tin levels were performed according to the protocol developed by Dorneles et al. (2008). Briefly, the tin concentrations in the serum samples of whole blood were measured in duplicate using a Model ZEEnit 700 atomic absorption spectrometer equipped with a transversely heated graphite tube atomiser and a Zeeman effect background correction system (Analytik Jena, Jena, Germany). The samples were weighed directly on the graphite platforms using an internal analytical micro-balance. The sample introduction into the graphite tube was performed using a Model SSA 600 automatic solid direct sampling system (Analytik Jena, Jena, Germany). A Sn hollow cathode lamp was used as the line source (Analytik Jena, Jena, Germany). The measurements were performed using the integrated absorbance at 224.6 nm. We used Pd (stock solution 10000 $\mu g\,ml^{-1}$ – Merck) and $MgNO_3$ (stock solution 1000 $\mu g\,ml^{-1}$ – SCP Science®) as modifiers. In each measurement of the samples or standards, the modifier was added (10 μg Pd + 6 μg $MgNO_3$). All samples were analysed directly, without dilution. For dilution of the modifiers, we used a solution of 0.2% (v/v) ultra-pure HNO_3 and ultrapure water obtained using an Elga – Purelab Ultra system (Marlow, UK).

2.3. Hepatic enzymes and lipid profile

The serum glutamic pyruvic transaminase (GPT) and glutamic-oxaloacetic transaminase (GOT) activities, total cholesterol (CT), low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL) were measured using colorimetric kits according to the manufacturer's directions (Bioclin®, Belo Horizonte, MG, Brazil).

2.4. Collection and weighing of organs

To obtain the target organs at the end of 15th day of TBT-treatment, during the proestrus phase, the animals were lightly anesthetised with ketamine and xylazine. The liver, pancreas, parametrial and retroperitoneal WAT (PR and RP WAT, respectively) were removed and weighed. The extent of the hypertrophy for each organ was estimated for each animal by calculating the ratio of the organ weight to the animal's body weight (dos Santos et al., 2012).

2.5. Tissue preparation

The animals were perfused with saline containing heparin (10 U/ml) via the left cardiac ventricle followed by infusion with 4% formaldehyde in phosphate-buffered saline (PF4%-PBS). The liver, pancreas, PR and RP WAT were removed and fixed in PF4%-PBS pH 7.4 for 24–48 h at room temperature. After fixation, the tissues were dehydrated in a graded ethanol series, cleared in xylol, embedded in paraffin at 60°C and subsequently sectioned into 5 μm slices. The sections were stained with haematoxylin and eosin (H&E) (Penza et al., 2011).

2.6. Histomorphometry

For histomorphometry, an image analysis system composed of a digital camera (Evolution, Media Cybernetics Inc. Bethesda, MD) coupled to a light microscope (Eclipse 400, Nikon) was used. High quality images (2048 × 1536 pixels) were captured with Pro Plus 4.5.1 software (Media Cybernetics). All the quantifications were performed by a two independent observers.

2.7. Adipocyte morphometry

After the WAT sections were H&E stained, the randomly acquired digital images (50 adipocytes/animal in 40X objective) were analysed using the Image-Pro Plus version 7.0 software (Media Cybernetics, Silver Spring, MD, USA). The major and minor adipocyte diameters were measured to determine the mean diameter of the adipocytes (Ludgero-Correia et al., 2012). In addition, the adipocyte quantification was performed on 20 high-powered images of randomly selected areas of each H&E-stained (20X objective) and expressed as the number per unit area (mm^2), as described by Yu et al. (2013).

2.8. Mast cells in WAT

PR and RP WAT sections were stained with Toluidine Blue according to standard protocol (Sigma–Aldrich Co., LLC). Each of these sections was used to obtain 20 photomicrographs (40X objective). The number of positively stained cells (*i.e.*, cells containing purple cytoplasmic granules) within the WAT were evaluated. The areas of WAT to be analysed were randomly selected with the exception that fields containing medium-sized blood vessels were carefully avoided. The number of positively stained cells was then expressed per unit area (mm^2), as described by Arzi et al. (2010) and dos Santos et al. (2012).

2.9. Immunohistochemistry in WAT

The 3 μm tissue sections from PR and RP WAT were deparaffinised as described previously and then subjected to antigen retrieval for 40 min using 10 mM citrate buffer, pH 6.0 at 95°C. Thereafter, the slides were rinsed in PBS pH 7.4 and the endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 20 min (Anwar et al., 2001). The sections were incubated with a blocking solution containing 1% BSA for 1 h at room temperature. The sections were incubated with the ERα primary antibody (1:200, sc-542, SCBT, CA) overnight at 4°C. After washing with PBS, the sections were incubated for 30 min in MACH 4HRP-Polymer (MRH534, Biocare Medical, LLC, Concord, CA). The slides were developed with the 3, 3′-diaminobenzidine tetrahydrochloride substrate (Dako, SP, Brazil) and counterstained with Mayer's haematoxylin. To provide a negative control for the ERα immunostaining, the primary antibody was omitted, and the positive control was provided by the use of uterine tissue. During the microscopic analysis, an overview was performed to qualify the slides, and then photomicrographs of the stained cells were captured at 1000X magnification using a system that included a light microscope and a digital camera, as described before.

2.10. Cell culture in 3T3-L1 cells

For the *in vitro* study, the 3T3-L1 cell line was used as described previously by de Oliveira et al. (2013). After cell confluence, the differentiation process was initiated by culture for 3 days in DMEM

containing 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) (Sigma–Aldrich Co., LLC), 1 μM dexamethasone (Sigma–Aldrich Co., LLC), and 1 μg/ml insulin (Sigma–Aldrich Co., LLC). After this period, the cells were maintained for 7 days in DMEM containing 10% FBS and 1 μg/ml insulin. Following the period of cell differentiation, the adipocytes were subjected to hormone depletion for 24 h in DMEM supplemented with charcoal-stripped fetal serum (Sigma–Aldrich Co., LLC). Subsequently, the cells were treated with TBT (10 nM or 100 nM), E_2 (10^{-7}M, Sigma), TBT (10 or 100 nM) + E_2 (10^{-7}M), ICI 182780 (1 μM, Fulvestrant, Sigma–Aldrich Co., LLC, antagonist of estrogen receptor), ICI 182780 (1 μM) + E_2 (10^{-7}M), ICI 182780 (1 μM) +TBT (10 nM) for 24 h. The untreated group was considered the control group (CON).

2.11. Oil red O staining of the 3T3-L1 cells

After 10 days of differentiation, the cells were washed twice with PBS, fixed with 37% formaldehyde for 30 min at room temperature, and then washed twice more with PBS. After fixation, the cells were stained for 2 h at room temperature with Oil red O solution (5%, Sigma–Aldrich, St. Louis USA) and then washed twice with distilled water (Sigma–Aldrich Co., LLC). The cell differentiation was evaluated by the presence of lipid droplets stained with Oil Red O positive as described previously by de Oliveira et al. (2013). Fifteen random fields from each well were photographed under phase contrast microscope and analysed using Image J. The images were converted into high-contrast back and white images to visualise the lipid droplets and scored as the percentage area per field (Baptista et al., 2009).

2.12. Western blotting of 3T3-L1 cells

After the treatment, the 3T3-L1 cells were harvested into lysis buffer (500 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5% deoxycholate of sodium), the lysate was centrifuged, the supernatant was collected and total protein content was determined by the Bradford's assay (Bradford, 1976), as described previously by de Oliveira et al. (2013). All the extracts were solubilised and SDS-PAGE and immunoblotting were performed as described below for the ERα protein.

2.13. Liver morphology

The liver morphometric analysis was performed using an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines of known length coupled to a conventional light microscope (Olympus BX51; Olympus Latin America-Inc. SP, Brazil). The area fraction of the granulomas was determined by the point-counting technique across 20 random non-coincident microscopic fields at a magnification of X200 (Maron-Gutierrez et al., 2011).

2.14. Tissue preparation and Liver Oil Red O staining

The livers were fixed in PF4%-PBS, then cryopreserved with a sucrose solution and frozen. Sections of 6 μm were obtained and fixed in cold acetone for 5 min (Gracelli et al., 2012). The sections were briefly rinsed in PBS pH 7.4 and subsequently incubated with Oil Red O stain (4 g/L, 60% isopropanol, Sigma–Aldrich) for 15 min, washed and counterstained for 2 min in haematoxylin stain (Grün et al., 2006; Zhuo et al., 2011). Representative photomicrographs were captured at 200X magnification using a system of a light microscope and a digital camera, as described above.

2.15. Protein extraction and western blotting

The proteins were obtained from the liver, PR and RP WAT samples of the control and TBT rats, and 100 μg of protein was resolved on SDS-PAGE gels (10%) and then transferred onto nitrocellulose membranes (Bio-Rad Hercules, CA) as described previously Gracelli et al. (2012). The membranes with the liver, PR and RP WAT proteins were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20 solution (TBST) for 1 h then washed once for 10 min in TBST. After that, the samples were incubated with antibodies to the ERα (1:500, sc-542, SCBT, CA), peroxisome proliferator-activated receptor gamma (PPARγ, 1:500, sc-7273, SCBT, CA) or βactin (1:1000, sc-130656, SCBT, CA) in the blocking solution overnight at 4 °C. The liver membranes were also incubated with the antibody to the rat ED1 protein of macrophages (ED1, 1:500, AbD Serotec, Raleigh, NC), using the same protocol as described above. After incubation with the primary antibody, the membranes were washed three times for 10 min each with TBST. The ERα and βactin proteins were detected using a secondary anti-rabbit IgG alkaline phosphatase conjugate (ERα-1:1000 and βactin-1:5000, respectively, Sigma–Aldrich Co., LLC). The ED1 and PPARγ proteins were detected using a secondary anti-mouse IgG alkaline phosphatase conjugate (1:1000, Sigma–Aldrich Co., LLC). The bands for the ERα, PPARγ and ED1 proteins and the βactin for each sample were visualised by a colour development reaction using nitroblue tetrazolium chloride (NBT) and 50 mg/mL of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (All from Life Technologies, Rockville, MD). The ERα, PPARγ, ED1 and βactin bands were analysed by densitometry using Image J software. The relative expression was normalised by dividing the ERα, PPARγ and ED1 values by the corresponding internal control values (βactin).

2.16. Glucose tolerance and insulin sensitivity tests

For the glucose tolerance test (GTT), D-glucose (2 mg/g of body weight) was intraperitoneally injected into the rats after an overnight fast (Santos et al., 2010). The glucose levels in the tail vein blood samples were monitored at 0, 15, 30, 60, and 120 minutes after injection using an Accu-Chek glucometer (Roche Diagnostics Corp, Ind). The insulin sensitivity test (IST) was performed in rats without overnight fasting by intraperitoneal injection of insulin (0.75 U/kg body weight; Sigma, St. Louis, Mo). The tail vein blood samples were taken at the same time points, 0, 15, 30, and 60 min after injection of insulin or, until at the time point that the glucose levels were similar between groups analyzed, as for measurement of the blood glucose levels.

2.17. Statistical Analysis

The normality of the data (Kolmogorov–Smirnov test with Lilliefors' correction) was tested. The comparison between the control and TBT-treated groups was performed using the unpaired *t*-test or Mann–Whitney *U*-test for parametric and nonparametric data, respectively. A two-way ANOVA test was used to evaluate the interaction between treatment and time of exposure for the body weights. A one-way ANOVA and post-hoc Tukey's test was used to evaluate the interaction between estrogen and TBT treatments on the accumulation of lipid droplets in the 3T3-L1 cells (GraphPad Prism 5.03; GraphPad Software, La Jolla, CA, USA). All results are shown as the mean ± SEM. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Effect of TBT on body weight and total body fat

To determine the importance of TBT exposure in the regulation of WAT homeostasis, we monitored the body weights of the TBT-treated rats for 15 days and found that there was a significant increase by the 15th day of treatment ($n = 10$, $p \leq 0.05$, Fig. 1A). Consistent with the growth curves, the TBT group had increased visceral adiposity as reflected by increases in the weights (fat mass/body weight) of both the parametrial and retroperitoneal fat pads ($n = 10$, $p \leq 0.05$, Fig. 1B). The food intake and the weights of the perirenal and mesenteric fat pads of the TBT-treated rats were not different from those of the control rats (data not shown).

3.2. TBT rats have low serum estrogen levels

Morning serum samples were obtained from control and TBT rats, and serum estrogen, progesterone and testosterone hormone levels were assessed (Table 1). Serum estrogen and progesterone levels in TBT rats were reduced and increased, respectively, when compared to control rats ($n = 6$–8, $p \leq 0.05$), however the testosterone levels did not change.

3.3. TBT rats have high serum tin levels

Serum samples were obtained from control and TBT rats and serum tin levels were determined using the atomic absorption spectrometer. TBT rats presented higher serum tin levels when compared to control rats (CON: 4.0 ± 1.0; TBT: 41.0 ± 2.0 ng/g, $n = 6$–8, $p \leq 0.05$).

3.4. Hepatic enzymes and Lipid profile

Serum biochemistry parameters levels were evaluated in control and TBT animals (Table 1). Serum GPT and GOT levels were higher in TBT when compared to control rats ($n = 6$–8, $p \leq 0.05$), however there were no significant changes in the liver or pancreas weights (data not shown), or in the CT, TG, LDL or HDL values ($n = 6$–8, $p \geq 0.05$).

3.5. Histomorphology of WAT

The histomorphology of PR and RP WATs are shown in Fig. 2. The adiposity induced by TBT was demonstrated by an increase in the diameter (CON-PR: 0.85 ± 0.16, TBT-PR: 1.30 ± 0.19; CON-RP: 0.49 ± 0.15, TBT-RP: 0.81 ± 0.12 μm, $n = 5$, $p \leq 0.05$, Fig. 2G) and a reduction in the number (CON-PR: 1.74 ± 0.07, TBT-PR: 1.16 ± 0.07; CON-RP: 1.09 ± 0.01, TBT-RP: 0.77 ± 0.05 adipocytes/mm^2, $n = 5$, $p \leq 0.05$, Fig. 2H) of adipocytes, which were surrounded by inflammatory cells, when compared to respective control fat tissue (Fig. 2B and E). In addition, there was a significant increase in the number of mast cells within both the TBT-PR (1.32 ± 0.11 mast cells/mm^2, $n = 5$, $p \leq 0.05$, Fig. 2C) and RP (0.81 ± 0.09 mast cells/mm^2, $n = 5$, $p \leq 0.05$, Fig. 2F) WATs, when compared to respective control fat tissue (RP: 0.85 ± 0.09; RP: 0.51 ± 0.07 mast cells/mm^2, $n = 5$, Fig. 2I).

3.6. Modulation of ERα and PPARγ protein expression in WAT

The ERα protein expression analysis was performed in both PR and RP WAT, in which they were assayed using the immunochemical and immunoblotting procedures. In the immunochemical assay, both CON and TBT-PR ($n = 3$, Fig. 3A and B) and RP ($n = 3$, Fig. 3C and D) WATs were shown to express ERα with predominant brown positive staining observed in the nuclei of the adipocytes. Moreover, we observed positive staining for ERα in only the cellular membrane of the adipocytes from both the TBT-PR and RP tissues (Fig. 3B and D). The negative controls demonstrated no brown staining and only negative (blue) staining for PR WAT and uterus sites was observed (Fig. 3E and F). The positive control for this assay (uterine tissue) showed the expected brown nuclei staining (Fig. 3G). In addition, the immunoblotting assay showed that the values for the expression of the ERα protein in the TBT-PR and RP WATs were 1.32 ± 0.06 and 1.47 ± 0.18 ($n = 5$, $p \leq 0.05$, Fig. 3H), times greater than those of the control from the same tissue. The values for the expression of the PPARγ protein in the TBT-PR and RP WATs were 1.20 ± 0.07 and 1.30 ± 0.08 ($n = 5$, $p \leq 0.05$, Fig. 3I), compared to the control for the same tissue.

3.7. Modulation of Adipogenesis and ERα protein expression in the 3T3-L1 cells

To assess the direct action of TBT on adipose cells, the 3T3-L1 preadipocytes were exposed to TBT and E_2 under basal and differentiated conditions. After 24 h, the lipid accumulation was visualised using Oil Red O staining. In the control cultures, normal lipid vacuoles were evident ($n = 3$, Fig. 4A). However, the E_2 and TBT10nM treatments resulted in decreases and increases in the formation of lipid vacuoles, respectively ($n = 3$, $p \leq 0.05$, Fig. 4B, C and G, respectively). Interestingly, the lipid accumulation was reduced after the combined E_2+TBT10nM treatment ($n = 3$, $p \leq 0.05$, Fig. 4D and G), when compared to control. The ICI treatment did not change the lipid accumulation (image not shown). In contrast,

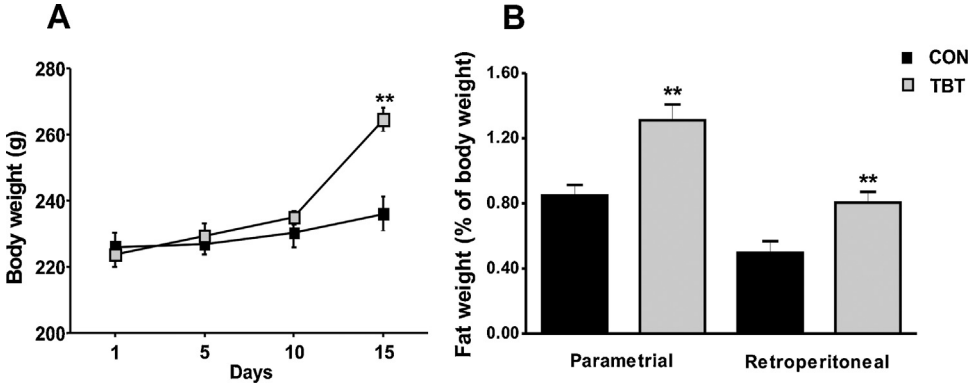

Fig. 1. The effects of TBT on body weight and fat development in female rats. (A) Body weights after 15 days of control or TBT-treatment (0.1 μg/Kg/day). (B) Parametrial and retroperitoneal fat pad weights are expressed as [fat weight (mg)/ body weight (g)] × 100. The values are expressed as the mean ± SEM. ($n = 10$). *$p \leq 0.05$ *vs* Control (two-way ANOVA and Tukey's test). Tributyltin chloride: TBT.

Table 1
Summary of Biochemical Parameters.

	Sexual hormones			Hepatic enzymes		Lipid profile		
	E_2 (pg/ml)	P_4 (ng/ml)	Test (ng/ml)	GPT (U/l)	GOT (U/l)	CT (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
CON	48.1 ± 6.3	4.3 ± 0.5	4.8 ± 0.8	41.0 ± 1.1	203.5 ± 3.6	48.0 ± 1.3	22.7 ± 2.0	13.7 ± 0.2
TBT	31.3 ± 3.8*	7.2 ± 1.3*	4.5 ± 0.3	46.7 ± 1.8*	217.9 ± 3.5*	46.4 ± 2.9	24.8 ± 3.8	12.1 ± 0.7

The values are expressed as the mean ± SEM (n = 6–8). E_2: estrogen. P_4: progesterone. Test: Testosterone. GPT: glutamic pyruvic transaminase. GOT: glutamic-oxaloacetic transaminase. CT: total cholesterol; LDL: low density lipoprotein cholesterol; HDL: high density lipoprotein cholesterol.
* $p \leq 0.05$ *vs.* CON (t-test).

for the cells treated with either ICI+E_2 or ICI+TBT10nM, the lipid accumulation was reduced (n = 3, $p \leq 0.05$, Fig. 4E, F and G, respectively) when compared to ICI treatment. In order to confirm that E_2 and TBT modulated the adipocyte differentiation, the expression of the adipocyte-specific protein ERα in the 3T3-L1 cells was assessed. The presence of the ERα protein was observed in the 3T3-L1 cells as measured by the immunoblotting assay (Fig. 4H). Additionally, the cells treated with same concentrations of E_2 and TBT presented an increase and decrease in the levels of the ERα protein expression (E_2: 1.48 ± 0.20; TBT: 0.48 ± 0.20, n = 3, $p \leq 0.05$, Fig. 4H). None of the other treatments conditions changed the levels of ERα protein expression in 3T3-L1 cells (E_2+TBT: 1.10 ± 0.15, ICI: 1.18 ± 0.3, ICI + E_2: 1.10 ± 0.4 and ICI + TBT: 1.08 ± 0.3, respectively, n = 3, $p \geq 0.05$). The last lane shows the positive control, a sample of normal female rat uterine tissue. In addition, the results for the treatment with TBT at the 100 nM concentration were similar to the control, however this concentration was very toxic to the cells (cells were in suspension and not viable, data not shown).

3.8. Liver inflammation and lipid accumulation

The hepatic tissues of the CON and TBT-treated groups exhibited apparent morphological differences. The CON livers

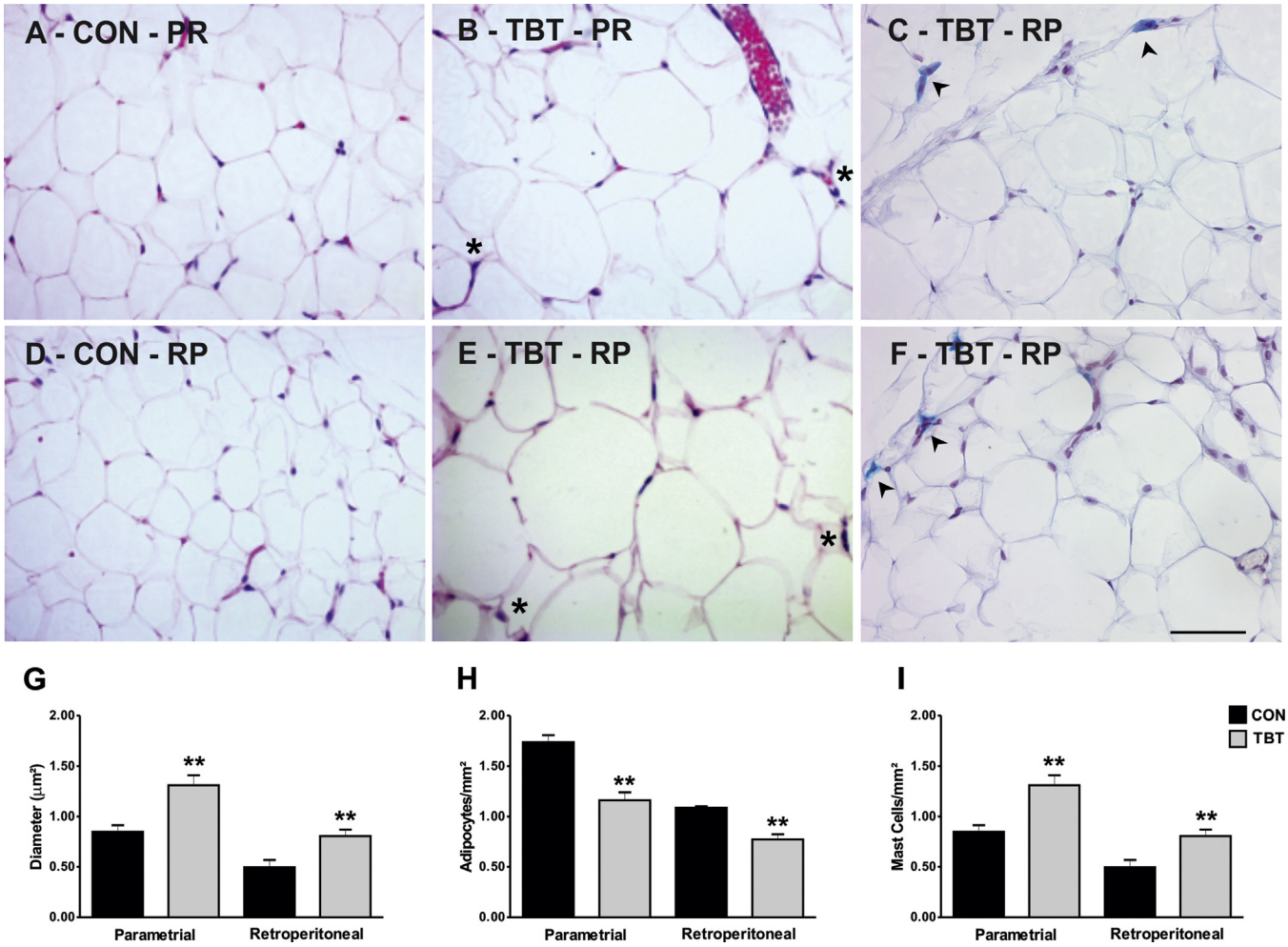

Fig. 2. Microphotographs of white adipose tissue (WAT) showing the adipocyte mean diameter, number and the number of mast cells in female rats. (A) PR and (D) RP WAT from control rats showing the normal aspect. (B) PR and (E) RP WAT sections from TBT-treated rats showing increases in the adipocyte diameter and exhibiting some surrounding inflammatory cells (asterisks). A, B, D and E were H&E stained (bar = 50 µm). (C) and (F) Mast cells are present in the WAT of TBT-treated rats (arrowhead, Toluidine Blue staining, C and F bars = 50 µm). (G) Graphical representation of the adipocyte mean diameter in the CON and TBT-treated rats. (H) Graphical representation of the number of adipocytes/ mm² in the CON and TBT-treated rats. (I) Graphical representation of the number of mast cells/mm² within the WAT of the CON and TBT-treated rats. The values are expressed as the mean ± SEM (n = 5). *$p \leq 0.05$ *vs* Control (one-way ANOVA and Tukey's test). PR WAT: parametrial white adipose tissue; RP WAT: Retroperitoneal white adipose tissue.

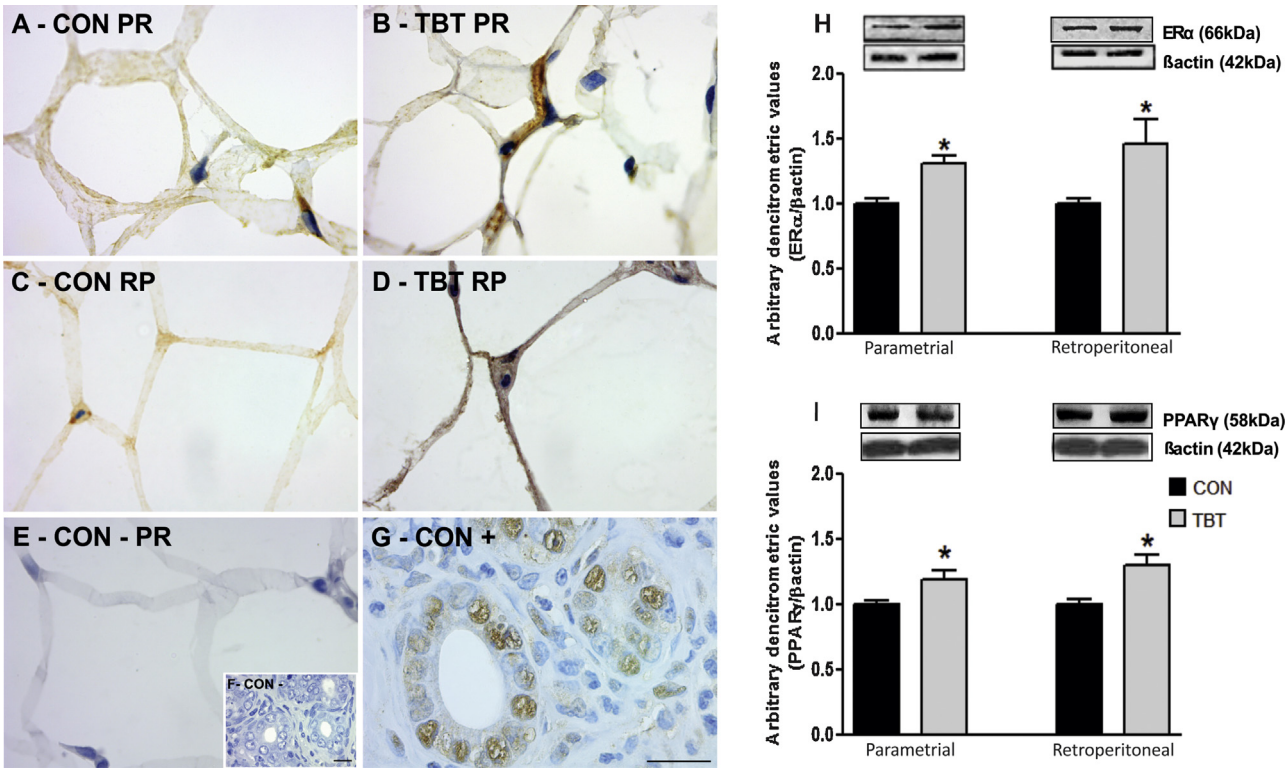

Fig. 3. Analysis of ERα protein expression in the WAT of control and TBT-treated female rats after 15 days (0.1 μg/Kg/day). TBT exposure increased the positive staining for ERα in WAT by the immunochemical assay. (A) and (C) Reported the representative immunostaining of PR and RP WAT from control animals showing positive staining in nucleus of adipocytes ($n = 3$). (B) and (D) Reported the representative immunostaining of PR and RP WAT from TBT-treated animals showing positive staining in nucleus and membrane of adipocytes ($n = 3$). (E) and (F) Showed the negative staining controls for detection of ERα using PR WAT and uterine tissue. (G) Reported the positive staining controls for detection of ERα using uterine tissue. Bars = 10 μm. (H) and (I) Reported the immunoblots for ERα and PPARγ in PR and RP WAT. The values are expressed as the mean ± SEM ($n = 5$). $^*p \leq 0.05$ *vs* Control (*t*-test).

exhibited a normal hepatic architecture with parenchyma constituted by polygonal cells joined to one another in anastomosing plates, with borders that face either the sinusoids or adjacent hepatocytes and portal space preserved ($n = 5$, Fig. 5A). However, hepatic tissue from TBT group, the hepatocytes in degeneration process, cells with morphological features of apoptosis, lipid droplets accumulation in hepatocytes cytoplasm ($n = 5$, Fig. 5B) and focus of inflammatory cells were observed ($n = 5$, Fig. 5C).

Consistent with the hepatic changes, TBT caused an increase in the area fraction of granulomas (280%, $n = 5$, Fig. 5D) and, the expression of the macrophage ED1 protein was 1.69 ± 0.18 ($n = 5$, $p \leq 0.05$, Fig. 5E) times that of the respective control.

In addition, the hepatic lipid accumulation was observed to be increased in the TBT group (217%, $n = 5$, $p \leq 0.05$, Fig. 6C, D and E). Furthermore, we detected an increase in the levels of the PPARγ protein (1.36 ± 0.15, $n = 5$, $p \leq 0.05$, Fig. 6F) and a reduction in the levels of the ERα protein (0.73 ± 0.7, $n = 5$, $p \leq 0.05$, Fig. 6G) in the livers of TBT-rats, relative to the values for the respective control animals.

3.9. Impaired glucose and insulin homeostasis

After an overnight (12-hour) fast, the TBT-treated rats exhibited elevations in blood glucose relative to the control rats (CON: 76.6 ± 12.0, TBT: 107.3 ± 7.5 mg/dl, $n = 4$–6, $p \leq 0.05$, Fig. 7A), and increased basal insulin levels (0.75 ± 0.07, $n = 4$–6, $p \leq 0.05$, Fig. 7B), compared to the control rats (0.50 ± 0.04 mcU/ml, $n = 4$–6, Fig. 7B). A higher value for the GTT at 15 min ($n = 4$–6, $p \leq 0.05$, Fig. 7C) and enhanced in IST at 30 min ($n = 4$–6, $p \leq 0.05$, Fig. 7D) were also observed in the TBT-treated group compared with the CON rats. Consistent with these observations, the TBT-treated group exhibited an increase in the number of pancreatic islets when compared to control pancreas (CON: 29.5 ± 4.5; TBT: 52.5 ± 1.5; $n = 4$-6, $p \leq 0.05$, Fig. 7G).

4. Discussion

In the present study, we showed that TBT was able to induce changes in the morphophysiology of female rat WAT, as well as pancreas and liver tissues. These effects were related to changes in adiposity and estrogen levels. The WAT remodelling included increases in the adipocyte size, decreases in the adipocyte number and changes in the numbers of mast cells, along with modulation of the protein expression of ERα and PPARγ in the WAT. Additionally, the exposure to TBT increased the quantity of lipid droplets as well as the levels of inflammation within the liver. The TBT exposure also increased the serum levels of glucose and insulin. These effects were correlated with increases in the numbers of pancreatic islets and the GTT and IST tests.

There is growing evidence supporting the concept that TBT exposure changes metabolic pathways and induces adipogenesis *in vivo* and *in vitro* (Table 2) (Grün et al., 2006; Kirchner et al., 2010; Penza et al., 2011; Zhuo et al., 2011; Graceli et al., 2013; Chamorro-García et al., 2013). When exposure occurs *in utero*, TBT affects the body fat deposition in mice, due to augmentation of adipogenesis via the PPARγ/RXRα interaction (Grün et al., 2006; Grün and Blumberg, 2006, 2007). In addition, TBT may affect WAT deposition in a gender- and time-dependent manner in peripubertal and sexually mature mice (Penza et al., 2011). Our current study, we observed that TBT increases the body weight and the PR and RP WAT accumulation after 15 days of treatment (Fig. 1A and B). In

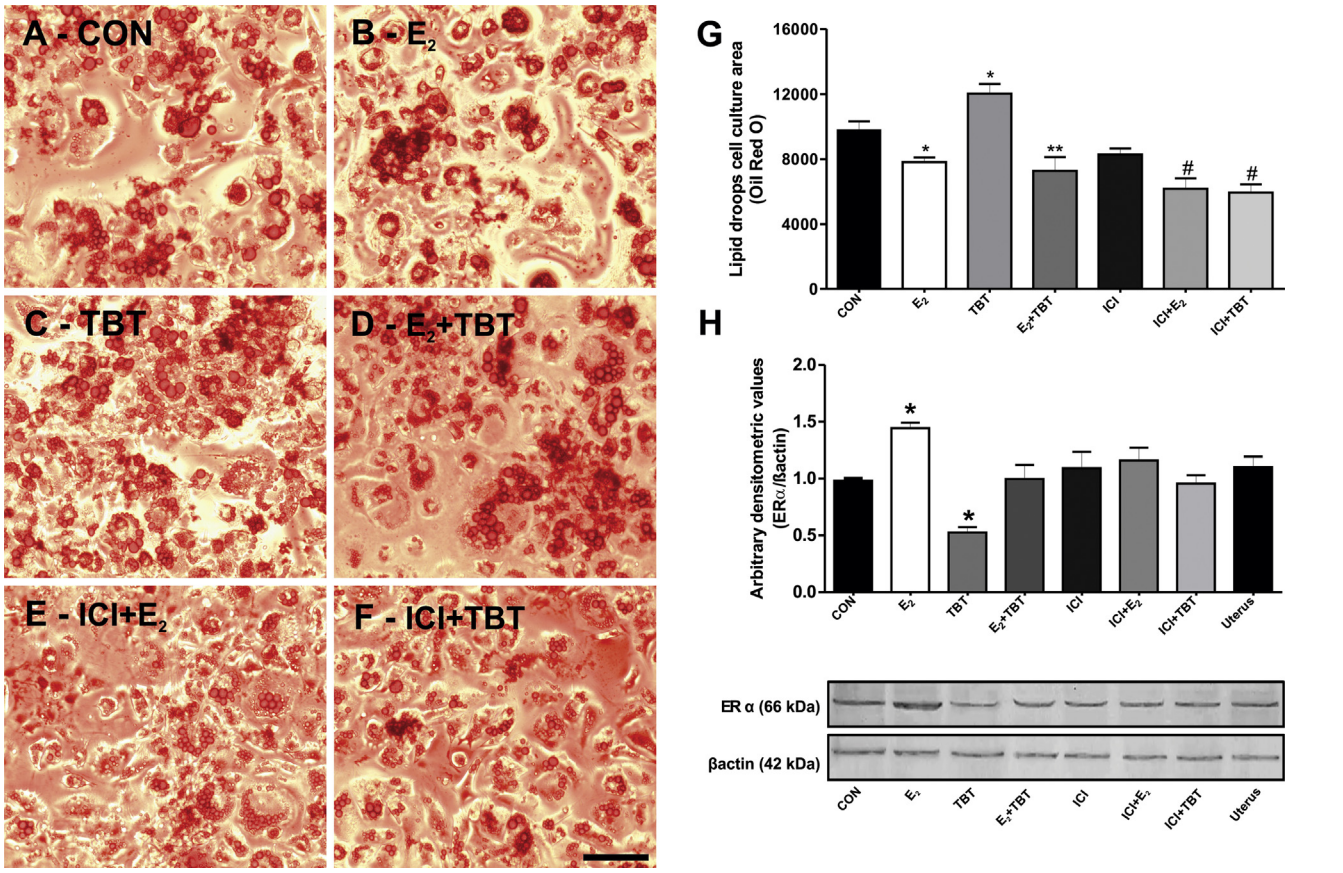

Fig. 4. Effect of E_2 and TBT exposure in adipocyte differentiation in 3T3-L1 cells. (A) 3T3-L1 cells were treated with vehicle (CON, DMSO, 0.1%), (B) E_2 (10^{-7} M), (C) TBT (10 nM), (D) TBT+E2, ICI 1827820 (1 μM, not shown), (E) ICI 182780+E_2 and (F) ICI 182780+TBT for 24 h. To assess the potential for adipocyte differentiation, the cultures were stained with Oil Red O to visualise the lipid accumulation in the respective treatment (A–F, bars = 100 μm) and quantified (G). (H) Western blot analysis for ERα protein expression in 3T3-L1 cells treated with the same conditions above was performed. The positive control for ERα protein expression by immunoblotting using uterine tissue is shown in the last column. The values are expressed as the mean ± SEM ($n = 3$). $^*p \leq 0.05$ *vs* Control. $\#p \leq 0.05$ *vs* ICI (one-way ANOVA and Tukey's test).

addition, Zhuo et al. (2011) reported that 5 μg/kg TBT for 45 days caused a body weight gain and an increase of the renal plus testicular peripheral adipose mass/body weight in mice. In another study, a 60-day exposure to TBT (0.5 μg/kg) caused an increase in fat mass in both genders (Penza et al., 2011). *In utero* exposure to TBT increased the mammary and inguinal WAT of the mouse pups, reflecting either an increase in lipid accumulation or an increase in the mature adipocytes (Grün et al., 2006).

Obesity is associated with an excessive increase of visceral WAT, specifically in adipocyte size and/or reduction in adipocyte number (Gimeno and Klaman, 2005; Heber, 2010; Yu et al. 2013). It is likely that the increase in WAT mass in obesity is associated with histological and biochemical changes, characteristic of inflammation (Ahima, 2006). Our results agree with previous results, we observed increases the size (15.7 and 14.5%) and decreases in the number (33 and 29%) of the adipocytes in the PR and RP WAT of the TBT-exposed rats (Fig. 2G and H). Similarly, Kirchner et al. (2010) reported that TBT treatment during the prenatal period increases the lipid accumulation and the number of cells containing lipid in mouse adipose-derived stem cells. This effect involves sensitisation of the multipotent stromal stem cells to differentiate into adipocytes, an effect that could likely increase adipose mass over time (Kirchner et al. 2010). In addition, Yu et al. (2013) reported a significant increase in visceral adiposity, reflected by enlargement of adipocyte size and reduction of adipocyte number per area in retroperitoneal fat pads of neuronal-specific androgen receptor knockout mice.

From previous studies, we learned that inflammation plays an important role in the development of obesity in different experimental models (Ahima, 2006; Liu et al., 2009 Altintas et al. (2011)). In obese mice (Altintas et al. (2011)) and humans (Liu et al., 2009) shown an increase in the mast cell numbers in WAT. Similar to the findings of Altintas et al. (2011) and Liu et al. (2009), we observed in TBT animals of this study, an approximate increase in PR and RP WAT mast cells number by 63% and 50%, respectively (Fig. 2I). Other study using the mast cell-deficient mice that were fed a western diet for 12 weeks gained less body weight and had reduced WAT inflammation with reduced insulin levels, compared with the wild-type controls (WT). Consistent with this result, WT mice that received the mast cell stabiliser also had an attenuated body weight gain (Liu et al., 2009). As obesity develops, the enlarging adipocytes secrete chemokines that attract immune cells. Macrophages are amongst the earliest immune cells to infiltrate metabolically active tissues (Solinas and Karin, 2010). Although the immune response is a result of interactions between multiple cell types, mast cells within WAT have also been implicated in the pathogenesis of obesity-related hyperinsulinaemia (Liu et al. 2009), consistent with our results reported in TBT-PR and RP WAT.

E_2, acting on both ERα and β, is recognised as an important regulator of metabolic homeostasis and lipid metabolism. To provide a few examples, E_2 was shown to regulate lipogenesis, lipolysis and adipogenesis in fat tissue (Murata et al., 2002; Cooke and Naaz, 2004; Bryzgalova et al., 2008). ERα is the predominant form found in the liver and WAT, whereas ERβ is the predominant

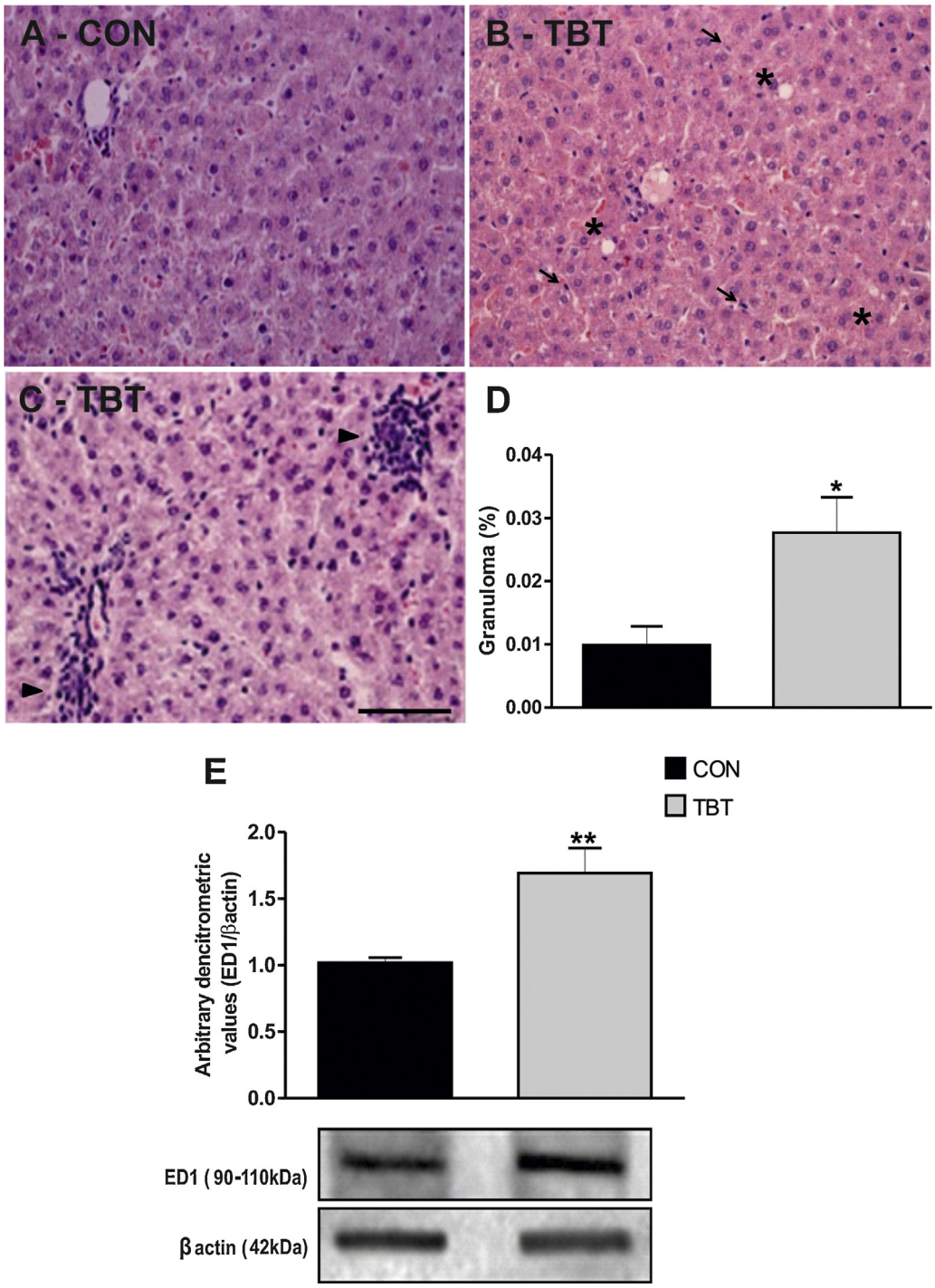

Fig. 5. Photomicrographs of H&E-stained liver parenchyma from control animals and treated with TBT for 15 days (0.1 μg/Kg/day). (A) Regular hepatocytes with a normal aspect from control animals. (B) Note the presence of lipid droplets (asterisks), apoptotic (arrow) and (C) inflammatory cells, as granulomas (arrowhead) in the TBT-treated animals. (D) Quantification of the fractional area of granulomas. ($n = 5$, bar = 100 μm). (E) Analysis of the levels of macrophages ED1 protein in the liver tissues of control female rats and treated with TBT. The values are expressed as the mean ± SEM ($n = 5$). *$p \leq 0.05$ *vs* Control (*t*-test).

form found in the ovaries and hypothalamus in rodents (Couse et al., 1997; Korach, 2000; Leitman et al., 2012). From previous studies, we learned that ERα plays a critical inhibitory role in the development of the adipose tissue (Cooke and Naaz, 2004; Bryzgaloya et al., 2008; Heine et al., 2000). Several studies have shown that increases in WAT after E_2 deficiency associated with changes in lipid metabolism signaling through ERα pathways (Heine et al., 2000). ERα was found in the nuclei and membranes of female human adipocytes, and the expression of the ERα increased in same WAT after E_2-treatment (Anwar et al., 2001). The translocation of ERα to the cell membrane may be explained by the pathways involved in the movements of the ERα/Shc/IGF-1R complex between cell cytoplasm and the plasma membrane. These interactions may be involved in the E_2-induced membrane association of ERα and the effect of this process on MAPK activation in MCF-7 cells (human breast adenocarcinoma cell) (Song et al. 2004). In our results, we observed a positive staining in the membranes of the adipose tissue in both types of WAT from the

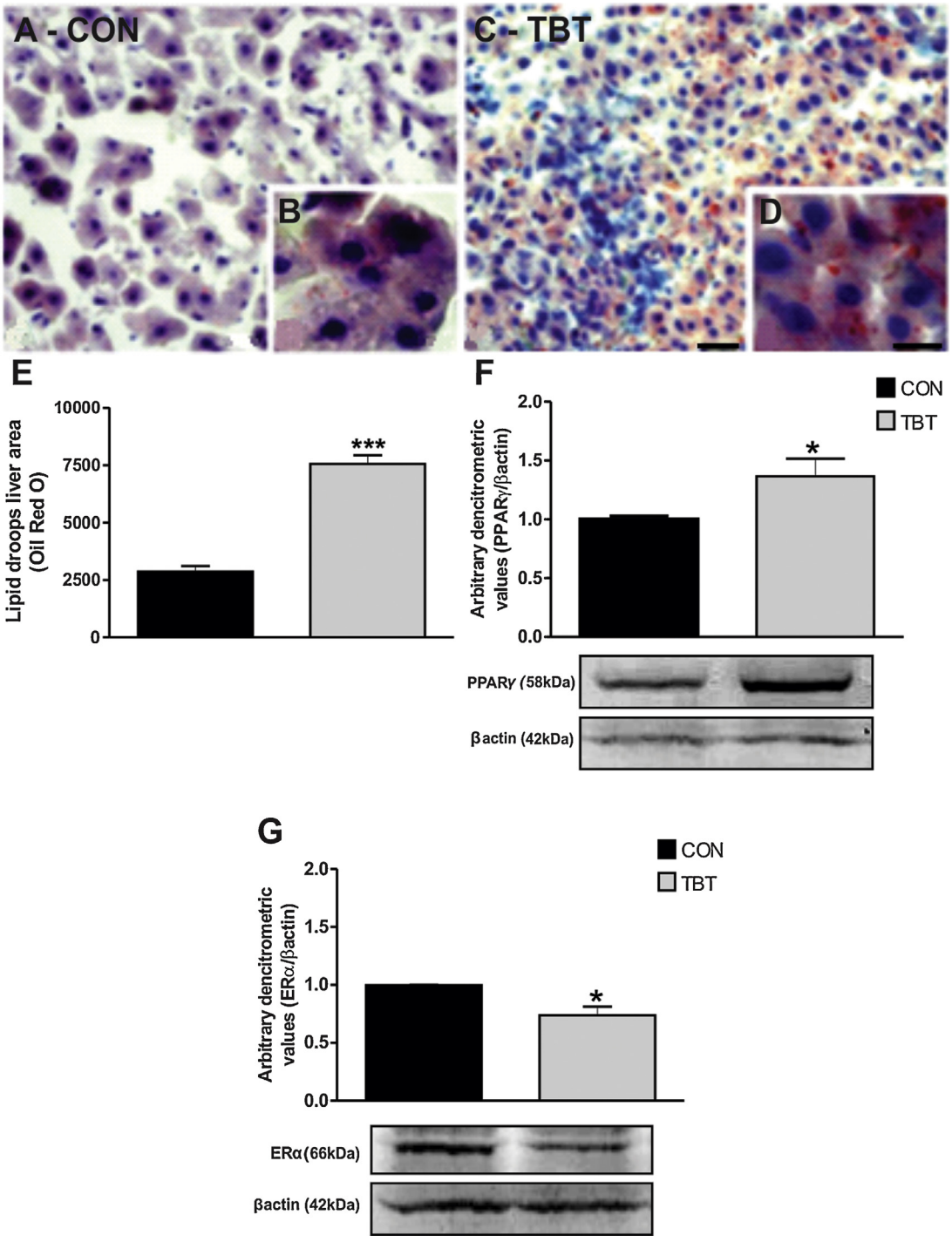

Fig. 6. Histological changes in the droplet lipids in the livers of the female rats treated with TBT for 15 days (0.1 μg/Kg/day). The sections of liver were stained with Oil Red O and counterstained with haematoxylin in control (A and B) and TBT (C and D). A and C bar = 20 μm; B and D bar = 10 μm. (E) Quantification of fractional area of the lipid droplet accumulation in the control and TBT groups ($n = 5$). (F) and (G) Analysis of PPARγ and ERα protein expression in the liver tissues from control female rats and treated with TBT. The values are expressed as the mean ± SEM ($n = 5$). $^{*}p \leq 0.05$ *vs* Control (*t*-test).

TBT-exposed animals (Fig. 3B and D). Alternatively, the localisation of ERα in adipocyte membrane may be due to the presence of another agonist, as observed for TBT in fat tissue (Penza et al. 2011). Consistent with these observations, in our study, the expression of the ERα and PPARγ proteins increased in the fat tissues of TBT-treated rats (Fig. 3H and I), even though the E_2 levels were reduced (Table 1). A reduction in estrogen levels was reported for us (Podratz et al., 2012; Santos et al., 2012) and our group (Rodrigues et al., 2014) in other studies, using the same dose and time of treatment with TBT. In addition, studies have shown that a reduction of the E_2 levels in ovariectomized rats (OVX) up-regulated the ERα expression in uterine (Medlock et al. 1991, 1994) and renal tissues (Mohamed and Abdel-Rahman, 2000), although the no significant changes in liver, cerebellum, brainstem, heart and aorta were observed in OVX or E_2 replacement rats (Mohamed and Abdel-Rahman, 2000). Therefore, regulation of ERα gene expression is tissue-specific and its expression in different tissues may be not only attributable a relationship between E_2 and ERα

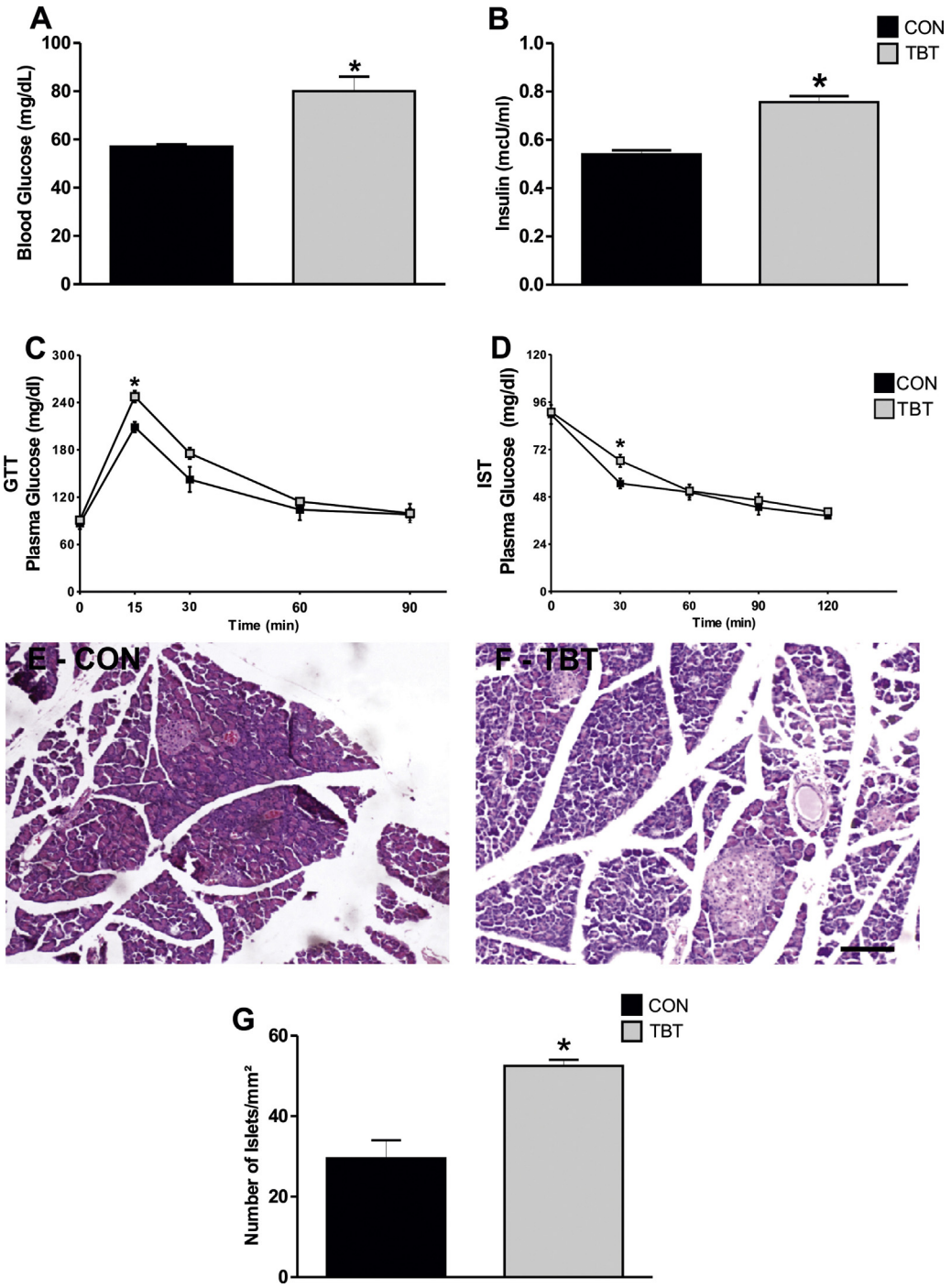

Fig. 7. Effect of TBT on blood glucose and morphophysiology of the pancreas. (A) Twelve-hour fasting blood glucose. (B) Serum insulin after a 12-hour fast. (C) Glucose tolerance test. (D) Insulin sensitivity. (E) and (F) H&E-stained pancreata were blindly scored for the numbers of pancreatic islets (bar = 10 μm). (G) Graphical representation of the number of pancreatic islets /mm^2 within the pancreas of the CON and TBT-treated rats. The values are expressed as the mean ± SEM (n = 4–6). *$p \leq 0.05$ *vs* Control (t-test or two-way ANOVA test).

pathways, but maybe it is influenced by other nuclear receptor pathways (Grun et al. 2006; Grun and Blumberg, 2007). Furthermore, other possibility that explain the obesogens effect of TBT in vertebrates, it was reported by inhibition of aromatase expression in a human granulose cell line and amphibian gonadal aromatase expression by TBT-PPARγ/RXR complex (Mu et al., 2000, 2001; Saitoh et al., 2001; Grun et al., 2006).

Previous studies reported that TBT up-regulated the ERα mRNA and protein expression in a dose-dependent manner (25, 50 and 100 nM) in MCF-7 cells and induced the migration of the expressed ERα from the cytoplasm to the nucleus (Sharan et al., 2013). TBT may be as a direct activator of ERs in mammalian adipose cells *in vitro* and *in vivo* in acute and long-term longitudinal treatments (Lee et al. 2005). TBT (0, 0.5, 5, 50, 500, 5000 μg/kg TBT for 16 h) modulated the activity of the ERs in a dose-dependent and tissue-preferential manner starting at 50 μg/kg in mouse pancreas, epididymal, renal, and brown fat (Penza et al. 2011). In a study of long-term TBT treatment (500 μg/kg for 21 days), the authors found evidence for tissue-specific kinetics of ERs regulation in mouse abdomen and the genital tract (Penza et al. 2011). TBT had

Table 2
Summary of Metabolic Changes induced by TBT.

	Animal/Dose				
Metabolic parameter	Mice (300 μg /kg)	Mice (0.5, 5, 50 μg/kg)	Mice (0.5, 5, 50, 500 μg/kg)	Mice (0.5, 5, 50 μg/kg)	Rat (0.1 μg/kg)
Body weight	↔	↑	↑	↔/↑	↑
Fat development	↑	↑	↑	↑	↑
WAT morphology	Impaired	↑	Impaired	Impaired	Impaired
WAT inflammation	NR	NR	NR	NR	↑
Liver morphology	Impaired	Impaired	NR	Impaired	Impaired
Liver inflammation	NR	NR	NR	NR	↑
Pancreas morphology	NR	NR	NR	NR	Impaired
Glucose *vs* control	NR	NR	NR	NR	↑
Insulin *vs* control	NR	↑	NR	NR	↑
GTT	NR	NR	NR	NR	Impaired
IST	NR	NR	NR	NR	Impaired
PPARγ/ ERs	+/NR	NR	+/+	+/NR	+/−
References	Grun et al. (2006)	Zhuo et al. (2011)	Penza et al. (2011)	Chamorro-Garcia et al. (2013)	This study

TBT: Tributyltin chloride. ↑: increased; ↓: decreased; ↔: unchanged or similar to control; NR: not reported; +: positive regulation; −: negative regulation; WAT: white adipose tissue. GTT: Glucose Tolerance Test; IST: Insulin Sensitivity Test.

agonistic activities for ERα in MCF-7 cells (Sharan et al., 2013) and for PPARγ *in vivo* and *in vitro* (Grün et al. 2006). These TBT actions in the adipose tissue are able to impair the role of E_2 in the fat metabolism (Korach, 2000; Tchernof et al., 2000; Shi et al., 2009). Moreover, TBT may be disrupted the signal pathways responsible for normal anti-adipogenic E_2 effects mediated by ERα. In agreement with our results, Penza et al. (2011) reported the adipogenic action of E_2 mimicked for xenobiotics, as TBT.

E_2 is known to have anti-adipogenic effects (Murata et al. 2002; Cooke and Naaz, 2004), and *in vitro* and *in vivo* studies have shown that TBT is an endocrine disruptor with respect to lipid metabolism (Grun et al., 2006; Grun and Blumberg, 2006; Kirchner et al., 2010; Penza et al., 2011; Graceli et al., 2013). In agreement with these previous findings, we observe in our results that E_2 reduces the lipid accumulation and increases an ERα expression in the 3T3-L1 cells (Fig. 4B, G and H). Nagira et al. (2006) demonstrated an increase in ERα expression in 3T3-L1 cells after E_2 treatment and inhibition of the lipoprotein lipase (LPL) expression and the fat accumulation in adipocytes that express ERα (Homma et al. 2000). Furthermore, we observed that TBT increases the lipid accumulation and reduces the ERα expression in 3T3-L1 cells were observed (Fig. 4C, G and H), demonstrating a direct effect of TBT in adipose cells. Penza et al. (2011) suggested that ERα could be modulated in 3T3-L1 cells by both E_2 and TBT action. TBT promoted adipogenesis in 3T3-L1 cells by direct transcriptional effects on RXR:PPARγ targets such as adipocyte-specific fatty acid-binding protein (aP2) mRNA. TBT also perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*, including effects on fatty acid transport protein (Fatp) and LPL gene expression in mouse WAT (Grün et al., 2006; Penza et al., 2011). The reduction of the E_2 pathways and ERα expression was associated with increased fat accumulation, and E_2 treatment was shown to inhibit fat accumulation in adipocytes (3T3-L1 cells) expressing ERα (Homma et al., 2000). Moreover, there was a reduction in the fat formation associated with the elevated ERα expression in 3T3-L1 cells after steroid saponin treatment (Xiao et al., 2010). In E_2+TBT-treatment a reduction in the lipid accumulation and normalization of the ERα expression were observed for us (Fig. 4D, G and H). In addition, Penza et al. (2011) reported that TBT might interfere in E_2 pathways, producing effects competitive with the action of E_2 on the adipogenic genes, possibly with a lower affinity of TBT for the ERs compared to E_2, as shown for ERα activation in 3T3-L1 cells. The adipogenic action of an exogenous ERs agonist/antagonist occurs through complex interaction with other nuclear receptors that regulate the adipogenic pathways, as TBT and RXR/PPARγ receptors (Grün and Blumberg, 2006, 2007). PPARγ is a key factor in adipogenesis and is highly expressed in adipose tissue (Rosen et al., 2000). The effect of the relationship between TBT with both the ERs and RXR/PPARγ receptor systems is not fully understood (Grün and Blumberg, 2006; Penza et al., 2011). Numerous studies reported that may depend on several factors, including the specific affinity, doses of TBT and E_2 for one of these receptors, time of exposure, experimental models, other nuclear receptors pathways, etc. (Grün and Blumberg, 2006; 2007; Penza et al., 2011; Graceli et al., 2013). Moreover, the negative modulation between PPARγ and ERα occurs at various levels and has been reported in several cell line models (Keller et al., 1995; Wang and Kilgore, 2002; Okubo et al., 2003; Suzuki et al., 2006; Yepuru et al., 2010). Furthermore, the PPARγ activation is also accompanied by a decrease in ER action (Dang and Löwik, 2004; Penza et al., 2006), may leading to adipogenic effects and/or impair in normal estrogenic effects after TBT exposure.

The liver serves a complex and integrative metabolic function in the mammalian body. Also, the liver is the main organ to metabolise and accumulate xenobiotics, as TBT (Wiebkin et al. 1982; Grondin, et al. 2007). TBT appears to be metabolized by hepatic cytochrome P450 into inorganic tin and high serum tin levels suggest TBT contamination in mammalian body (Dorneles et al., 2008). Our results agree with the previous findings, we observed higher serum tin levels in female rats treated with TBT. Furthermore, an increase in the production of reactive oxygen species and oxidative damage were detected in rat liver tissues treated with TBT (Ueno et al., 1994; Liu et al., 2006). In addition, Krajnc et al. (1984) demonstrated that female rats were fed with TBT at 0, 5, 20, 80 and 320 mg/Kg in diet for 4 weeks presented an incidence of area of liver necrosis with inflammatory reaction. Histological changes in the livers of the TBT-treated animals were observed, which may be involved in the development of the metabolic risks and inflammation (Fig. 5). Zuo et al. (2011) showed that treatment of mice with low doses of TBT for 45 days (0.5, 5, and 50 μg/kg) increased the lipid droplets and hepatocyte degeneration. TBT (0.3 mg/kg for 24 h) also induced expression of adipogenic modulators including C/EBPb, Fatp and Acac (acetyl-coenzyme A carboxylase) in mouse livers. *In utero* exposure to TBT increases the adiposity in mouse livers with a disorganisation of hepatic structures and increased accumulation of lipids droplets (Grün et al., 2006). Grondin et al. (2007) verified that TBT can activate the endoplasmic reticulum pathway of apoptosis in hepatocytes, through activation of calpain and caspase-12. Our current study, we also observed an increase in the lipid accumulation in the hepatocytes of TBT-treated rat followed by an increase in PPARγ protein expression (Fig. 6). From

previous studies, we learned that PPARγ is a master regulator of the formation of fat cells and their ability to function normally in the adult (Rosen et al., 2000). Furthermore, PPARγ is induced during adipocyte differentiation, and forced expression of PPARγ in nonadipogenic cells effectively converts them into mature adipocytes (Tontonoz and Spiegelman, 1994). Exposure to toxic xenobiotics, as TBT or heavy metals may lead to hepatic inflammation and dysfunction (Navab et al., 2008; Brenner et al., 2013). Our results showed that TBT was able to increase the hepatic granulomatous nodules and ED1 protein expression in the TBT-treated rats (Fig. 5). In addition, a non-alcoholic fatty liver disease, increases in the number of macrophages infiltrating the liver and elevated cytokine production lead to the initiation of hepatic inflammation and progression of lesion culminating in late phase in hepatic fibrosis, consequently in loose of parenchyma functionality (Bieghs et al., 2012; Bieghs and Trautwein, 2013). Macrophages are key players in metabolic homeostasis. They respond to metabolic cues and produce pro- and/or anti-inflammatory mediators to modulate metabolite programs. At the onset of weight gain, macrophages start to infiltrate the metabolic tissue and contribute to and perpetuate the inflammatory response, eventually leading to systemic insulin resistance/hyperinsulinaemia and the development of obesity in different models (Johnson et al., 2012). In this study, a reduction in the expression of the ERα protein in livers of the TBT-treated animals was observed for us (Fig. 6). Interestingly, ERα is the predominant ER isoform in hepatocytes and is the receptor that controls inflammation, lipid, glucose, protein, and cholesterol homeostasis in the liver (Gao et al., 2008). Furthermore, the ERα mediates the E_2-induced protection against liver inflammation (Evans et al., 2002). In the livers of ERα$^{-/-}$ mice, there is increased glucose production (Bryzgalova et al., 2006). Also, the treatment of mice with an ER agonist decreased the weight, fat mass, dyslipidaemia and cholesterol liver content in WT mice, but not in ERα$^{-/-}$ mice (Lemieux et al., 2005). Therefore, ERα role is essential for normal response in GTT and IST tests (Lundholm et al., 2008).

E_2 increases the insulin content of the rat pancreas and not only influences islet size but is also important in determining insulin release from the β cells (Godsland, 2005). E_2 has rapid effects on β cells where it regulates membrane depolarisation, Ca^{2+} influx, insulin secretion, and overall glycemia (Alonso-Magdalena et al., 2006). However, in women, insulin secretion after menopause does not appear to be different from that of premenopausal women. The overall effect is the maintenance of insulin levels, similar to that in premenopausal women (Godsland, 2005). In addition to this, E_2 is an important regulator of β cell inflammation and apoptosis, improving glucose homeostasis in diabetic rats (Yamabe et al., 2010). Nevertheless, an experimental model for pancreatitis has been developed in which an OTs, specifically dibutyltin dichloride, was used (Zhou et al., 2013). Zuo et al. (2011) showed that treatment of mice with low doses of TBT for 45 days (0.5, 5, and 50 μg/kg) induced hyperinsulinemia. Low doses of bisphenol A, an endocrine disruptor with estrogenic activity, and E_2 caused increased insulin secretion from mice β cells (Alonso-Magdalena et al., 2006). ERα$^{-/-}$ mice have increased fasting insulin and glucose levels. Thus, the absence or impairment of ERα actions results in islet dysfunction and hyperinsulinemia (Bryzgalova et al., 2006). Our current results reported that an increase in the serum insulin levels of TBT-treated rat followed by an increase in GTT and IST tests (Fig. 7). However, PPT (an ERα agonist) and E_2 had no effect on insulin secretion from the isolated islets of obese mice (Lundholm et al., 2008). Therefore, TBT could impair the E_2-regulated pathways that integrate the metabolic interactions among the WAT, liver and pancreas.

In conclusion, our findings provide evidence that the toxic potential of TBT leads adiposity in WAT associated with inflammation and ERα pathways. In addition, TBT alters the metabolic relationships among the liver, pancreas and WAT associated with a reduction in the E_2 levels, inducing the development of metabolic risks. This work provides increased clarity to our understanding of the mechanisms of actions of xenobiotic, as TBT and its role in metabolic disorders development. Furthermore, the outcomes of which might help in establishing new environmental protection policies.

Conflict of interest

The authors declare that there are no conflicts of interest related to this work.

Transparency document

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

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