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Original Research Article

Interaction of Butylparaben with Thyroid Hormone Receptors (TRα1 and TRβ1) and Level of its mRNA Transcript in Rat liver

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Abstract: Nuclear receptors are susceptible to endocrine disrupting chemicals (EDCs) and there is growing concern that environmental EDCs can alter the thyroid hormone receptors (TRs) activities and thereby affecting the downstream signaling processes. Butylparaben (BP), a widely used synthetic preservative with estrogenic potency has been reported to alter the normal thyroid homeostasis. However the effect of BP on TRs is not clearly understood yet. This study examined the effect of BP in three different doses (1mg/kg BW/d, 5 mg/kg BW/d and 10mg/kg BW/d) on the hepatic mRNA transcript of TRα1 and TRβ1 in rats after 7 days and 21 days of exposure. Molecular docking of BP with TRs was performed by using AutoDock 4.2.6 program. The qPCR findings showed down regulation of TRα1 in all BP treated rats regardless of the duration of exposure. It was observed that BP at all the concentrations cause several folds increase in TRβ1 expression. Significant upregulation was caused at 1mg/kg BW/d (98.96 ± 5.77fold increase) and at 5mg/kg BW/d (63.54 ± 4.48 fold increase) in 7 days and 21 days of exposure respectively ($p<0.05$). 17β-estradiol (0.01mg/kg BW/d) used as positive control also showed similar results. BP interacted with the amino acids of ligand binding domain (LBD) of both TRα1 and TRβ1 by hydrogen bonds and hydrophobic interactions with favourable low binding energies (ΔG). Differential effects of BP on TRα1 and TRβ1 expression may be due to specific binding interactions of BP with TRs. Present study has established new insight into the molecular mechanism of BP related thyrotoxicity in rats.

Key words: Butylparaben, EDCs, LBD, nuclear receptors, thyrotoxicity, TRα1, TRβ1.

Introduction

Recently there has been increase of concern regarding thyroid disorders amongst various population across the globe. Population based studies reported that around 42 million people of India suffer from thyroid problems, predominantly hypothyroidism and in North Indian population occurrence of hypothyroidism is more (Unnikrishnan and Menon, 2011). Interestingly, it has been reported that in young women of southern part of India, one in eight has thyroid gland problem (Velayutham *et al.,* 2015). Further, congenital hypothyroidism has been reported to be occurring in one out of 2,640 neonates in India (Unnikrishnan and Menon, 2011). The National Cancer Registry Program (NCRP) which is conducted by the Indian Council of Medical Research (ICMR) has documented more thyroid cancer cases of women (Unnikrishnan and Menon, 2011). It is reported that about 0.3 billion people all over the world suffer from thyroid gland malfunctions (Bajaj *et al.*, 2016).

Rapid globalization and growing population increase the basic needs of livelihood of people. To fulfil the demands, longer sustainability of the consumer products is becoming necessary. As a result there have been innumerable chemicals in use as preservatives. There are reports that many of these

chemicals are established endocrine disruptors like butylparaben (Pop *et al.,* 2018). Over 90 years, parabens have been used as antimicrobial ingredient to enhance the self-life of personal care stuffs, medicines and edibles (Kizhedath *et al*., 2019). Scientific investigations have raised concern about the safety regarding use of parabens because of the harmful consequences in biological processes, especially on the endocrine system (Garcia *et al*., 2017; Schreiber *et al*., 2019). The endocrine disrupting properties of parabens are reported to be mainly due to their estrogenic nature. Butylparaben has been found to be one of the most effective estrogenic parabens and established as 10,000 times less potent than estradiol (Engeli *et al*., 2017).

As Thyroid gland depends on external sources for synthesis of its hormones, the gland is vulnerable to the environmental toxicants (American Academy of Pediatrics, 2014). Extensive exposure to xenobiotics, which have been loaded to our environment through different means, is a major cause of increasing thyroid deformities (Lee *et al.,* 2010; Ghassabian and Trasande, 2018). Experimental evidences confirmed xenobiotics as leading cause of thyroid carcinoma (Nettore *et al*., 2018). Thyroid hormones (THs) are crucial for normal growth and maturation, metabolic activities, reproduction, developmental processes and many more (Yamada-Okabe *et al*., 2004). These diverse biological functions are mediated by binding of THs to their nuclear TRs (Power *et al.,* 2001). A broad range of thyroid disrupting chemicals (TDCs) like polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), bisphenol A (BPA), polybrominated biphenyls (PBBs) and phthalates have been reported to change thyroid hormone signalling pathways by modulating TRs (Zoeller, 2005). TRs are encoded by TRα and TRβ genes and different isoforms of TRs are generated through alternative splicing of the primary transcripts (Lazcano *et al.,* 2019). TRs are nuclear receptors specific for their endogenous ligand T3, biologically active form of THs and the relative expressions of isoforms of TRs (TRα1, TRα2, TRβ1 and TRβ2) are found to be tissue specific (Zoeller, 2010). Several TDCs elicited their toxicological effects by mimicking THs, thereby altering signalling cascades (Moriyama *et al.,* 2002). Agonistic and antagonistic binding with the ligand-binding domain (LBD) of TRs affected THs as well as their transcriptional activity (Jugan *et al*., 2010). Based on some cross-sectional studies and experimental data it has been suspected that parabens may have deleterious effects on thyroid physiology (Decker, 2015; Aker *et al.,* 2018), in addition to the other reported endocrine disrupting effects (Vo *et al.,* 2010). It is observed that there has been very little information available regarding the effect of BP on TRs expression. In this study we looked into the expression of TRα1 and TRβ1 in rat liver (Wistar strain) exposed to short-term (7days) as well as long-term (21 days) treatment of BP using qPCR. To explore the binding affinity of BP towards the TRα1 and TRβ1, molecular docking was performed.

Materials and methods

Animals

Female wistar rats (9 weeks old) weighing 120-130g were kept in the animal house facility of this University maintaining humidity (45-55%), temperature (20-22°C) and photoperiod (12:12-h). They were provided pellet diet (soya free) and drinking water *ad libitum*. In view of the fact that women have been reported to be more susceptible to thyroid disorders than men, we considered female wistar rats to evaluate the effect of BP (Allahabadia *et al.,* 2000; Rahbari *et al.,* 2010).

Test chemical and administration

A stock solution of butylparaben (>99% purity, Sigma Aldrich) was prepared by dissolving it in absolute ethanol. The required doses were then prepared from the stock by diluting with olive oil (HiMedia). Rats were assorted to each group (n=6) randomly. Animals were administered with three doses of BP i.e., 1mg/kg BW/d, 5mg/kg BW/d and 10 mg/kg BW/d (represented as BP1, BP5 and BP10 respectively). Control group (C) received olive oil with 0.5% alcohol. A positive control group (E2) was considered which received 0.01mg of 17β-estradiol (E2)/kg BW/d. 100 µl aliquot of each dose was administered subcutaneously (s.c) under the scruff region of

the rats in respective groups for 7 and 21 days. The overnight fasted rats were given injections daily in the morning hrs (8AM - 9AM). On the day of sacrifice animals were euthanized by cervical dislocation under mild ether anaesthesia. Liver was dissected out and immediately freeze in liquid nitrogen (LN_2) and stored at - 80°C until further processing.

RNA isolation and Real time PCR

RNA was isolated from liver tissue using Trizol-C reagent (Sisco Research Laboratories Pvt. Ltd., India). The total RNA content was extracted from the cell homogenate via phase separation by chloroform, RNA precipitation and washing by isopropanol and ethanol (75%) respectively (Rio *et al.,* 2010). Quantity and integrity of RNA were evaluated subsequently (Qubit 3 Fluorometer, invirogen by Thermo Fisher Scientific and Agarose gel Electrophoresis). Following instructions of RT-PCR kit (SRL, India), RNA was reverse-transcribed to its complementary DNA strand. All the primer sequences were self-designed with Primer Blast NCBI, which were then obtained from IDT MolBiogen and subsequently standardised for their optimal efficiency. The primer sequences for TRα1 are 5′- CTAGCTCTCCCACCTGCCA- 3′ (F), 5′- AGGGGACAGCACCCATAGTA- 3′ (R); for TRβ1 5′- CAGGATCCGTGGTTTCCCTC -3′ (F), 5′ - AGCTCTGGCATTCCCTTATTCA- 3′ (R); for housekeeping gene GAPDH 5′- AGTGCCAGCCTCGTCTCATA- 3′ (F), 5′- GGTAACCAGGCGTCCGATAC- 3′ (R). For quantification of gene expression by Real-time PCR (QIAGEN, Rotor-Gene Q), a total reaction volume of 12.5 µl was prepared with SYBR Green master mix (Bio-Rad). The changes in relative fold with respect to the housekeeping gene were measured by a method of relative quantification ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Molecular docking of BP and TH receptors

Molecular docking of butylparaben with TRα1 and TRβ1 was performed to check any possible binding affinities. The SDF file of the 3D conformer of BP (PubChem CID- 7184) was got from PubChem database (https://pubchem.ncbi.nlm.nih.gov). File conversion to PDB format was by means of Open Babel software (http://openbabel.org). Crystal structures of rat TRs were not available in RCSB Protein Data Bank (http://www.rcsb.org/pdb/). Hence rat TRα1 protein was modelled from human TRα1 protein (PDB ID: 1 NAV A, Query cover: 63%, Percent Identity: 99.62%) and rat TRβ1 protein was prepared from chain A of human thyroid hormone receptor beta (PDB ID: 1BSX A), which showed percent identity 99.62% and query cover 52% in blast results. Swiss model (https://swissmodel.expasy.org), a protein structure homology-modelling server was used to model rat TRs. The new models were selected only after checked in PROCHECK and Ramachandran plot (SAVES server, https://servicesn.mbi.ucla.edu/SAVES/). The template protein 1NAV A was co-crystallized with IH5 in ligand binding domain (LBD) and the amino acid residues were Phe218A, Ile 222A, Met259A, Ser 277A, Leu292A and His 381A. In the template protein (1BSX A), the LBD was co- crystallized with 3,5,3′ Triiodothyronine (T3), which interacted with three amino acid residues His 435A, Ile276A and Leu346A in the LBD. Structure comparison and pair wise alignment were done to find out the amino acid residues (His471A, Ile 312A and Leu382A) of LBD in the new receptor protein rat TRβ1 by using UCSF Chimera program (https://www.cgl.ucsf.edu/chimera/). The already attached ligands IH5 and T3 were removed and subsequently using Auto Dock Tools (ADT), the protein and the ligand were modified for docking. Molecular docking of BP into rat TRα1 and TRβ1 were carried out individually using AutoDock 4.2.6 program. A grid box (Dimension 64×42×68, Spacing: 0.375Å, Coordinates: x, y, z are 48.108, 18.812, 19.772 respectively for docking of BP and TRα1; Dimension: 50×52×40 Å, Spacing: 0.375Å, Coordinates: x, y, z are 18.926, 43.038 and 32.094 respectively for docking of BP and TRβ1) was constructed to cover the amino acid residues and to obtain the finest conformational state. Lamarckian Genetic Algorithm (LGA) was applied for performing docking. Finally, LigPlot$^+$ program (version v.1.4.5) was used to analyse and find out the interactions of BP with the amino acid residues of LBD of TRα1 and TRβ1. Docking was also performed

for the reference compounds. Already attached ligands IH5 (PubChem CID- 9863447) and T3 (PubChem CID- 5920) with the reference compounds human TRα1 (PDB ID-1NAV A) and human TRβ (PDB ID-1BSX A) respectively were removed and again docked back and the binding interactions were accessed by LigPolt tools.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). For all the statistical analysis SPSS software (Version 16) was used. Comparisons of treatment groups with control (C) group were made using the one-way analysis of variance (ANOVA) by way of post hoc comparisons followed by Tukey's multiple comparison tests. Statistical significance level was measured at P< 0.05 for all the results.

Results

Effect of BP on TRα1 and TRβ1 gene expression

The qPCR analysis showed down regulation of TRα1 genes when rats were exposed to BP for 7 and 21 days. All animals showed several fold decrease of TRα1 expression [0.016 ±0.001 (BP1), 0.01±0.00063 (BP5), 0.058±0.009 (BP10), 0.013±0.0033 fold decrease (E2) ($p<0.05$)] compared to control (1±0) after 7 days of dosing (Fig 1A). 21 days treatment with BP showed down regulation of TRα1 like E2 treated rats (Fig 1B). Though hepatic TRβ1 gene was up regulated in all animals of 7 days of dosing, a significant up regulation pattern ($p<0.05$) was observed in BP1 treated group (98.96±5.77 fold increase) as in E2 treated animals it was 76.31±2 fold increase (Fig 1C). Animals exposed for 21 days with BP5 (5mg/kg BW/d) showed significant up regulation of TRβ1 (63.54±4.48 fold

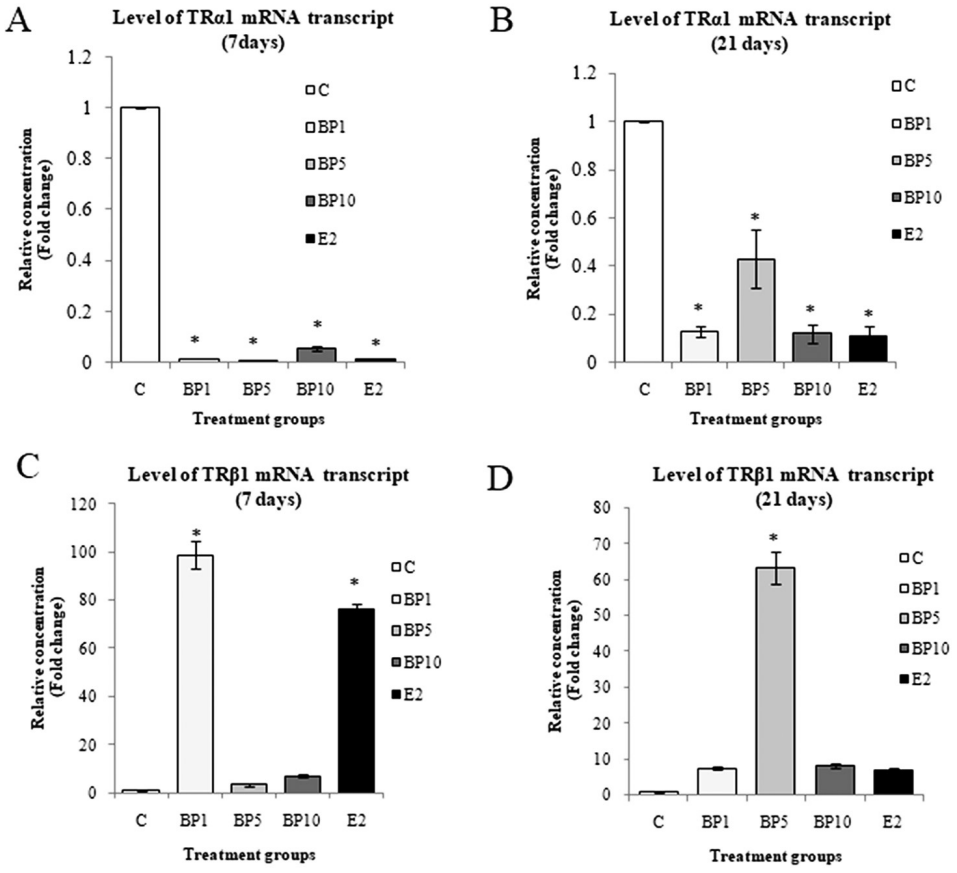

Fig. 1. Changes in the level of TRα1 (A & B) and TRβ1 (C & D) mRNA transcripts in liver of rats exposed to 1mg/kg BW/d (BP1), 5mg/kg BW/d (BP5) and 10mg/kg BW/d (BP10) of BP and 0.01mg/kg BW/d of 17β-estradiol (E2) for 7 and 21 days through subcutaneous route. Control animals were administered with the vehicle (olive oil) only (C). The qPCR results are expressed as relative concentration (fold changes). All the data are expressed as mean ± SEM. One-way ANOVA was performed followed by Tukey's test and statistical significance was measured against control (*$p<0.05$).

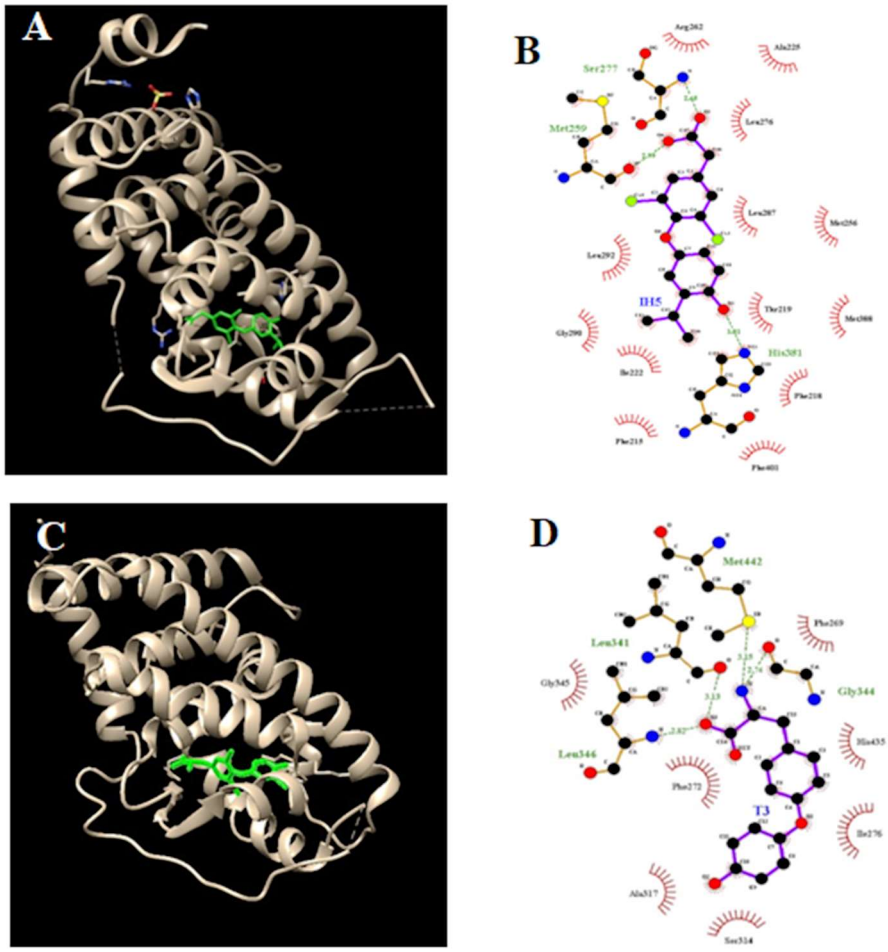

Fig. 2. Docking results of co-crystal reference compounds shown in ligplot images. (A) Structure of humanTRα1 (PDB ID-1NAV A) co-crytallized with IH5 molecule (green). (B) ligplot image of co-crystal IH5 with hTRα1 (reference compound of ratTRα1) showing formation of hydrogen bonds (green dash line) with Ser277(2.66Å), Met259 (2.99Å) and His381 (3.01Å) and hydrophobic interactions (red color arc with spokes) with Phe218, Ile222 and Leu292 between IH5 and ligand binding domain (LBD) of hTRα1. (C) Structure of humanTRβ (PDB ID-1BSX A) co-crystalized with T3 (green). (D) Ligplot images of co-crystal T3 with hTRβ (reference compound of ratTRβ1) showing hydrogen bonding (green dash line) with Leu346 (2.82Å) and hydrophobic interaction (red arc with spokes) with His435 and Ile 276 between T3 and LBD of hTRβ.

increase, $p<0.05$). The effect of BP1 and BP10 was not significant (Fig 1D).

Molecular docking of BP with TRα1 and TRβ1

Docking of IH5 with hTRα1 (reference compound of ratTRα1) showed formation of hydrogen bonds with Ser277A (2.66Å), Met259A (2.99Å) and His381A (3.01Å) and hydrophobic contacts with Phe218A, Ile222A and Leu292A of LBD with a binding free energy (ΔG) -10.52 kcal/mol (Fig 2A & 2B). Ligplot analysis of co-crystal T3 with hTRβ (reference compound of ratTRβ1) showed hydrogen bonding with Leu346A (2.82Å) and hydrophobic interaction with His435A and Ile276A with a binding free energy (ΔG) – 6.81 kcal/mol (Fig 2C & 2D). BP showed a strong binding affinity towards the amino acid residues in the LBD. Depending on the lowest binding free energy (ΔG) and highest number of interactions, protein-ligand complexes were considered. The most favourable TRα1-BP protein-ligand complex which showed interactions with four out of the six amino acids in LBD had the lowest binding free energy (ΔG-6.39kcal/mol). BP formed hydrogen bonds with Phe218A (distance 2.77Å) and Ser277A (distance 2.87Å) and

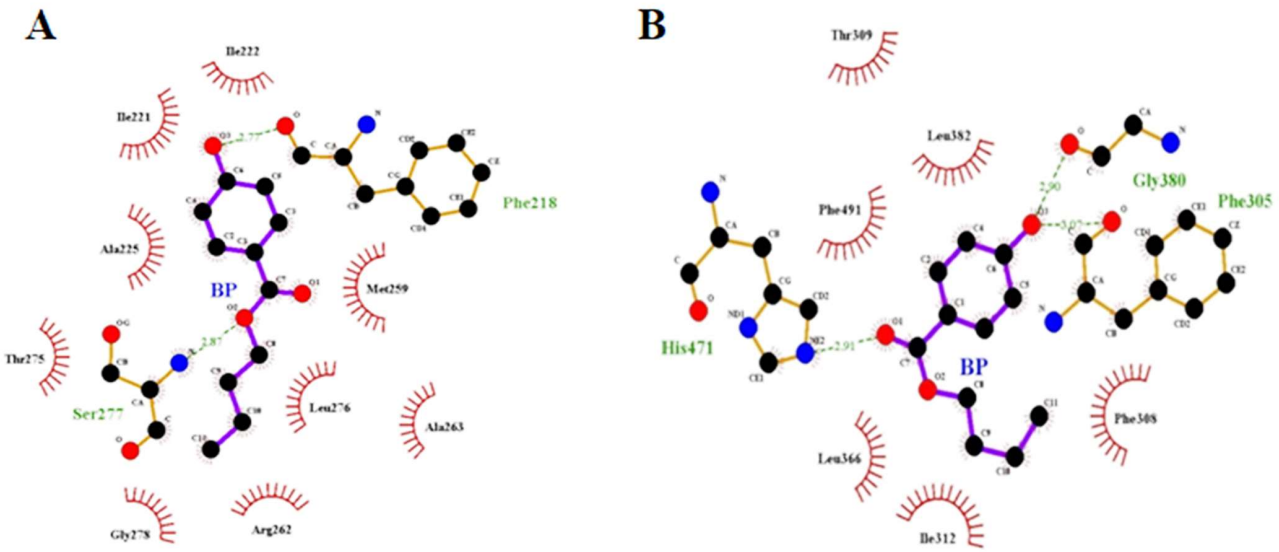

Fig. 3. Ligplot images showing interation of butylparaben (BP) with the amino acid residues of LBD of rat TRα1 (A) and TRβ1 (B).The green dash line indicates hydrogen bonds and the hydrophobic interactions are indicated by red color arc with spokes. (A) BP forms hydrogen bonds with Phe218 (2.77Å) and Ser277 (2.87Å) and hydrophobic interactions with Ile222 and Met259 in the LBD of TRα1. (B) Hydrogen bond with His471 (2.91Å) and hydrophobic interactions with Leu382 and Ile312 are esttablished between BP and LBD of TRβ1.

hydrophobic interactions were made with Ile222A and Met259A (Fig 3A). BP interacted with all the three amino acid residues in the LBD of TRβ1 which were found to interact with T3 in the human TRβ previously. In the best complex with lowest binding energy (Δ G -5.66 kcal/mol), BP interacted with His471A through hydrogen bond having a distance of 2.91Å and hydrophobic interactions with Ileu382A and Leu312A in the LBD of TRβ1 (Fig 3B). The LigPlot analysis confirmed that butylparaben (BP) fits favourably into the LBD of both the TRs.

Discussion

Parabens have been used since mid 1920s as preservatives in many products (Decker, 2015). Butylparaben (BP) is widely used preservative in the present time (Kizhedath *et al*., 2019). Although human exposure to BP is ubiquitous, many toxicity assessments reported very low systemic exposure and toxicity in human (Aubert *et al*., 2012). However enormous numbers of scientific studies on the properties of BP showed that endocrine system including the thyroid is susceptible to BP (Vo *et al.,* 2010). The THs regulate physiological processes via binding to the TH receptors (TRα and TRβ), which are ligand-inducible transcription factors belonging to the nuclear receptor (NR) superfamily (Arukwe and Jenssen, 2005). Earlier evidences regarding thyroid disrupting effects of BP were restricted to a few cross-sectional investigations and some with experimental supports. Urinary level of BP was associated with the altered level of serum THs during pregnancy (Aker *et al.,* 2016; Aker *et al.,* 2018). Data obtained from National Health and Nutrition Examination Survey (NHANES, 2007-2008) showed that reduce levels of THs was linked with increase levels of BP in adult women. Increase in butylparaben (each ng/ml) causes a -1.07 reduction in ng/dL T3 and -0.12 decreases in µg/dL T4 levels in adult women (Decker, 2015). A cross-sectional study also reported association of urinary BP level with THs changes in the US population (Koeppe *et al.,* 2013). Kitagawa *et al.* (2003) performed a yeast two-hybrid assay to determine the TRs-binding activity and reported no interaction of butylparaben with TRs. Later in an *in vitro* study of Taxvig *et al.* (2008) BP showed increase proliferation of GH3 cells in T-screen assay indicating BP as a week TR agonist.

The results of the present study about interaction of BP with TRα1 and TRβ1 establish an unusual pattern of gene expression. While BP at all three concentrations (1mg/

kg BW/d, 5mg/kg BW/d and 10mg/kg BW/d) down regulated the TRα1 expression in both 7 days and 21 days treated rats, but interestingly the expression of TRβ1 gene was found to be significantly up regulated in both the duration of treatment. The results demonstrated strong binding affinity of BP with both the TRs. This differential expression pattern may be due to different level of attraction towards the receptors of THs. Level of TRβ1 up regulation was varied depending on the days and doses of BP exposure. Therefore, perhaps a non-monotonic dose response curve of TRβ1 expression challenged the traditional concept of "the dose makes the poison" like many other TDCs did as reported by Vandenberg *et al.* 2012. At present there are many evidences that BP could interact with the nuclear receptor superfamily such as ER-α, ER-β and progesterone receptors to exert its effects (Wróbel and Gregoraszczuk; 2014; Zhang *et al.,* 2016a). A comparative study on 17 parabens showed that they are selective agonists for ERβ over ERα and the size and bulkiness of the alkyl chain of the parabens determine their interactions with the two ERs (Watanabe *et al.,* 2013). ERα and ERβ showed marked variations in binding affinity and activation towards ER ligands (Kuiper *et al.,* 1997, 1998). Coumestrol, genistein, apigenin, naringenin, and kaempferol were found to bind with ERβ by comparatively stronger competition with endogenous estradiol compare to ERα, but a dual role of zearalenone on ERs was found where it act as agonist for ERα and for ERβ showed mixed agonist-antagonistic properties (Kuiper *et al.,* 1998). Dose dependent agonistic/antagonistic activities of certain phytoestrogens on ERs were well documented. Zearalenone and resveratrol were antagonistic on both ERα and ERβ at high doses (Mueller *et al.,* 2004).

Many exogenous substances have receptor binding properties and can alter the normal endocrine homeostasis by binding with LBD of receptors. Unlike the DNA binding domain, the LBD is variable among the TRs isoforms, indicating affinities towards diverse groups of xenoestrogens (Lazcano *et al.,* 2019). In our study we found that BP can directly fit into the LBD of TRs. The low binding energies (ΔG) with strong interactions with the amino acid residues in LBD were in accordance with the previous reports on other TDCs (Zhang *et al.,* 2016b). In a recent study four compounds i.e., 2,4,5-trichlorophenoxyacetic acid, bisphenol A, 2,22 ,4,42 -tetrahydroxybenzophenone and 2,4- dichlorophenoxyacetic acid were identified as TRβ1 binders (Zhang *et al.,* 2016b). BPA inhibits the downstream transcriptional activity of TRs by replacing T3 with a transcriptional repressor in the TR, resulting in suppression of the target genes (Moriyama *et al.,* 2002). However experimental findings also explained involvement of non-genomic mechanisms in the alteration of TRs transcription (Sheng *et al.,* 2012).

The results of the present study on effects of butylparaben on thyroid hormone receptors (TRα1 and TRβ1) firmly establish an unusual pattern of receptor interaction and gene expression. Butylparaben regulated the expression of both the receptor genes in a differential manner which was found to be TRs specific. BP interacts with the ligand binding domain (LBD) of the TRα1 and TRβ1 by forming hydrogen and hydrophobic interactions with the amino acid residues constituting the LBD. Butylparaben may be viewed as a potential contributor to the development of thyroid problems and one of the possible routes of thyrotoxicity found to be through interacting with thyroid hormone receptors.

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