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Commonly Used Food Preservative and Antioxidant BHT Alter the Expression of Gene in Steroidogenic Pathway and Affect Gonadal Architecture

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ABSTRACT: Terrestrial and aquatic life is being influenced by the exposure with various manmade chemicals. BHT is a common antioxidant used in food preservation, cosmetics, and industry. European federation of Endocrine disruption categories BHT as Number 1 priority substance causing endocrine disruption. Studies till date mainly focuses on the allergic, antioxidant, and carcinogenic properties of BHT. It has been recommended by the EFSA (European Food Safety Authority) to study the reproductive properties of the compound to ensure its safety as a food additive. In present study we have analysed the estrogenic/anti-estrogenic or androgenic effect of BHT using FSTRA assay (tier 1 screening assay used for identifying Endocrine Disruptions). Two test doses (T_1- 15 mg/Kg.bwt and T_2- 30 mg/Kg.bwt) of BHT were administered in fish twice a week. Aromatase gene expression was studied by RT PCR and Quantified the expression of gene in the gonad. We found an up regulation of aromatase gene. Aromatase gene is involved in the synthesis of aromatase enzyme which convert androgen in to estrogen. Histopathological observation of the testis and ovary of the treated fish compare to the control fish, the following pathological feature decreased number of mature sperms (MS), primordial germ cells (PGC), and increased number of impaired germ cells (IGC). General atrophy (GA) or testicular atrophy (TA), interstitial fibrosis (IFB), and Leydig cell hyperplasia (LCH) with vacant spaces in the seminiferous tubules (ST). Histopathology of ovary degeneration of vitellogenic oocyte (DVO), primary oocyte (PO), secondary oocyte (SO) and mature oocytes (MO) (A). Degeneration of vitellogenic oocyte or degeneration of oocyte (DVO or DO).

Keywords: BHT, Aromatase gene, FSTRA assay, RTPCR, Histopathological study.

INTRODUCTION

Antioxidants are natural or synthetic in origin and are extensively used in food, cosmetics, petroleum industries to scavenge the free radicals and their action. One such antioxidant is butylated hydroxytoluene (BHT), IUPAC name as 2,6-Di-tert-butyl-4-methylphenol, and the molecular formula, $C_{15}H_{24}O$. BHT is also synthesized as a natural product by cyanobacteria, phytoplankton, and lychee fruit's pericarp. A combination of BHA (butylated hydroxyanisole) and BHT are widely recommended as preservatives in food and cosmetics (U.S.FDA). The Phenolic ring in BHA and BHT contains di-tert-butyl groups and significantly acts as primary antioxidants. Inhibition of autoxidation of unsaturated organic compounds, prevention of free radical generation, and the oxidation process are the significant functions of these synthetic antioxidants (Lanigan and Yamarik, 2002; Yehye *et al.*, 2015).

The histopathological studies identified alternations in the architecture of internal organs, including hepatocytomegaly, non-neoplastic lesions, cytoplasmic vacuolation, hepatocellular degeneration, and necrosis in liver and pulmonary fibrosis and hyperplasia in lungs (Adamson *et al.*, 1977; Haschek *et al.*, 1979; Peraino *et al.*, 1977). BHT was also found to induce reticulum cell sarcomas (Clapp *et al.*, 1974), lung and ovarian tumor, social isolation, decreased sleeping, and aggressive behavior in animals (Stokes *et al.*, 1972). Exposure to BHT was found to cause hypersensitivity, allergy, skin irritation, depigmentation, and sensitization in some studies (Flyvholm and Menne, 1990; Goodman *et al.*, 1990). Anophthalmia condition in litters (Brown *et al.*, 1959) and significant weight loss in pregnant rats with fetal death (Ames *et al.*, 1956) were also reported.

BHT and BHA administration increased the uterine wall thickness, decreased the endometrial epithelium cell height, induced minimal endometrial edema in mice, and reduced the uterine weight in prepubescent female rats (Pop *et al.*, 2013). BHT was also found to alter the endogenous estrogen-responsive genes in the human uterine Ishikawa cell line. In cell proliferation assays, BHT was less estrogenic than BHA and is considered as anti-androgenic (Cooke *et al.*, 2013). It was reported that the addition of BHT in the cryopreservation technique increases the quality of artificially inseminated sperms (Patel *et al.*, 2015) and

the fluidity of the spermatozoa membrane (Memon *et al.,* 2011).None of these studies has described in detail the actual effect of BHT in gonads of males and females.

The European Commission on Endocrine disruptors categorized the compound as a potent endocrine disruptor with the E number 321. EFSA (European Food Safety Authority) Panel on Food Additives and Nutrient Sources added to Food (ANS) derived an ADI of 0.25 mg/kg/bw/day for BHT based on NOAEL (No Observed Adverse Effect Level). In the light of the existing reports, the compound can cause alternations in the levels of circulating endogenous hormones (Estrogen, Progesterone, and Testosterone) in the studied animal models (Cooke *et al.,* 2013; Paul *et al.,* 2018). All these studies recommend further validation of the actual reproductive effect of this compound in various animal models. The present study is relevant because the potential gonadal impacts of BHT are elucidated using histopathology as an investigatory tool.

Aromatase gene are also known as cytochrome p450 (cyp 19) and they produce aromatase enzyme. Cyp 19 gene plays a crucial role in the aromatization of androgen in to estrogen. In teleost fish, cyp19 gene has two isoforms cyp 19 Aromatase A and Cyp 19 Aromatase B. These gene products have been identified in several species of teleost including goldfish and catfish (Kazeto *et al.*, 2004). Cyp 19A aromatase enzyme is expressed in gonad and Cyp 19B aromatase enzyme is expressed in brine. In the present study we have analysed whether the estrogenic compound BHT has any effect in the normal steroidogenic pathway in the male and female fish, way baromatase gene expression with the help of using By RT PCR the expression of aromatase gene was analysed.

MATERIALS AND METHODS

In vivo experiments

Selection of experimental model and experimental design. The freshwater teleost, *Anabas testudineus*, Bloch (1792), usually known as climbing perch, were selected for the study. The mature fish (with 10-12 months of growth) were purchased from local breeders with dimensions of 30 ±5 g weight (W) and 10 ±5 cm length (L). The acclimatization was done for 15 days with 27 ±3 ºC temperature under the natural photoperiod condition and was fed ad libitum (Paul *et al.,* 2018). A total of 60 fishes were selected which were then divided into three groups of 20 fishes.

Preparation of test chemical and administration of Intraperitoneal injection (IP). Butylated hydroxytoluene (BHT) is a fat-soluble compound. Corn oil was used as a suitable vehicle for dissolving the BHT. Dose standardization was done in the laboratory. Two doses (T_1- 15 mg/kg/b.wt and T_2- 30 mg/kg/b.wt) were selected for the experiment. Fish short term assay was used for the study experiment were done on the basis of OECD guidelines (229) of fish short term reproduction assay is an in vivo screening assay for fish reproduction where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life cycle (21 days). The drug is delivered into the peritoneal cavity of the fish. Fishes were starved for 24 hours before the injection procedure to empty their digestive system. Coldwater anesthesia was used instead of chemical anesthesia. In a separate outer container, crushed ice cubes were added to bring down the temperature to 17 °C. Fishes were transferred into the container using a net. At 17 °C, fishes slowly stopped their movement and spread their pectoral fin horizontally. The test doses were calculated as per the bodyweight of the fish and were injected intraperitoneally (IP) in between the central portion of the pelvic fin. After IP injection, fishes were immediately transferred into warm water (~28.5 °C) tank for recovery (Kinkel *et al.,* 2010).

Group I, kept in dechlorinated water, served as the control. Group II was treated with 15 mg/kg/b.wt of BHT (T_1 dose), and group III was treated with 30 mg/kg/b.wt of BHT (T_2 dose) for a period of 21 days, respectively. The IP injection was done twice a week. Continuous evaluation of the behavioral and physiological status of the organism was noted during the study. After the stipulated period fishes were sacrificed, and the samples were collected for analysis. The Institutional Animal Ethical Committee [Mar Athanasius College (Autonomous), Kerala, India] approved the experiments in this animal model.

Sampling and histopathology analysis

Fishes were anesthetized using clove oil and were sacrificed by caudal concussion. The gonads of both sexes were collected and were transferred into 10% neutral buffered formalin for fixation and preservation (Copper *et al.,* 2018). Tissues were passed by increasing the gradient of alcohol series (from 30% to 100% each for 30 minutes) for dehydration after fixation and washing and were eventually cleared in benzene for 30 minutes.

Overnight cold infiltration and 30 minutes hot infiltration were done. Tissues were impregnated with paraffin wax at 58 ºC followed by embedding. After embedding, block sectioning was carried out using 4µ ultra-microtome (Leica, Germany). Spreading of the ribbon or sections was done on a clean glass slide coated with Mayer's albumin. Sections were dewaxed (in two series of xylene for 3 minutes each) and transferred into decreasing gradient of alcohol series (from 100 to 70% for two minutes each). Sections were stained with hematoxylin (3 minutes), differentiated with weak acid alcohol (1 minute) followed by blueing in diluted alkaline solution (1 minute), and then counterstained with eosin (<1 minute). Stained slides were dehydrated using an increasing alcohol gradient (from 50 to 100% for two minutes each). Rendering and clearing were done with xylene I and II (for 3 minutes each). Finally, sections were mounted on DPX with a coverslip (Van der Ven *et al.,* 2003). The histopathological examinations were done using a binocular research microscope attached with a CCD camera (Leica, Germany). Significant portions of the sections were photographed in 5X, 10X and 40X for description and comparison. The representative six areas of the tissues from each category were analyzed.

RT PCR Analysis of Aromatase gene expression

Isolation of mRNA. Total RNA Extraction Kit is designed for rapid purification of RNA from different samples using RNAiso Plus (TaKaRa Bio, Shiga, Japan). This product is a mixture of guanidine thiocyanate and phenol in a mono-phase solution which effectively dissolves RNA. After adding chloroform and centrifuging, the mixture separates into three phases: an aqueous phase containing RNA, the interphase containing DNA and an organic phase containing proteins. RNA is precipitated using isopropyl alcohol. Impurities are removed using ethanol and pure RNA is resuspended in RNAase-free water. 1 ml RNAiso Plus (TaKaRa Bio, Shiga, Japan) reagent is sufficient for isolating RNA from 50-100 mg of tissue. This advanced RNA isolation procedure is an improvement to the single-step RNA isolation using phenol and guanidine isothiocyanate developed by Chomczynski and Sacchi.

Sample preparation: Homogenized cells in 1 ml of RNAiso Plus (TaKaRa Bio, Shiga, Japan). Transfer the mixture to a 2.0 ml collection tube. Phase separation: Incubate the homogenized sample for 5 minutes at room temperature (15-25 ˚C) to permit the complete dissociation of nucleoprotein complexes. Add 200 µl of chloroform (not provided) per ml RNAiso Plus reagent used. Cover the sample tightly, shake vigorously for 15 seconds and allow the tube to stand for 10 minutes at room temperature (15-25 ˚C). Centrifuge the resulting mixture at 13,000 rpm for 15 minutes at 4 ˚C. Following centrifugation, the mixture separates into lower organic phase (containing protein), an interphase (containing cell debris and DNA) and upper aqueous phase containing RNA. RNA precipitation: Transfer the aqueous phase containing RNA to fresh tube and add 500 µl of isopropyl alcohol. Allow the sample to stand for 10minutes at room temperature (15-25 ˚C). Centrifuge at 13,000 rpm for 10 minutes at 4 ˚C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and the bottom of the tube. RNA wash: Remove the supernatant and wash the RNA pellet by adding 1ml of 75% ethanol. Pipette gently to resuspend the pellet and the centrifuge at 10,500 rpm for 5 minutes at 4 ˚C. RNA elution: Briefly dry the RNA for 10 minutes by air-drying. Resuspend the pellet with 50 µl of RNase-free water.

cDNA Synthesis. The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. The kit uses RevertAid Reverse Transcriptase (RT), which has lower RNase H activity compared to AMV reverse transcriptase. The enzyme maintains activity at 42-50 °C and is suitable for synthesis of cDNA up to 13 kb. The recombinant Thermo Scientific™ RiboLock™ RNase Inhibitor, supplied with the kit, effectively protects RNA from degradation at temperatures up to 55 °C. First strand cDNA synthesized with this system can be directly used as a template in PCR or real-time PCR. It is also ideal for second strand cDNA synthesis or linear RNA amplification. All components of the kit should be stored at -20 °C. Keep control RNA at -70 °C for longer storage.

Strand cDNA Synthesis After thawing, mix and briefly centrifuge the components of the kit. Store on ice. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order: Template RNA total RNA or poly(A) mRNA, or specific RNA 2 µg, Primer Oligo (dT) 18 primer or 1 µL, Water, nuclease-free to 12 µL total volume 12 L.

Add the following components in the indicated order: 5X Reaction Buffer 4 µL, RiboLockRNase Inhibitor (20 U/µL) 1 µL, 10 mMdNTP Mix 2 µL, RevertAid M-MuLV RT (200 U/µL) 1 µL and mix gently and centrifuge briefly. Foroligo (dT) primed cDNA synthesis, incubate for 60 min. at 42 °C. Terminate the reaction by heating at 70 °C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20 °C for less than one week. For longer storage, -70 °C is recommended.

Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses.

Real Time PCR Procedure. In a real time PCR protocol, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed. SYBR® Green is the most widely used double-strand DNA-specific dye reported for real time PCR. SYBR® Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double stranded DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback to intercalation-based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

Real Time PCR – Qiagen, ROTOR GENE Q Real-Time PCR Detection System, Reaction Volume – 10 ul, 2X Real Time PCR smart mix- 5 ul, Forward primer+Reverse primer- 2 ul Template cDNA – 1 ul, ddNFW – 2 ul Program Polymerase activation 95 °C 10 minutes Denaturation 95 °C 15 sec, Annealing/extension 55 °C 1 minute.

Total change can be identified by, ΔΔCT = ΔCT (a target sample) - ΔCT (a reference sample) = (CTD - CT B) - (CTC - CTA). The final result of this method is presented as fold change of target gene expression in a target sample relative to a reference gene.

The fold change is the expression ratio: If the fold change is positive, it means that the gene is upregulated. If the fold change is negative, it means it is down regulated.

RESULTS AND DISCUSSION

Investigation of changes in the tissue via histopathology due to BHT exposure

Histopathological examination of tissues was done by using a binocular research microscope attached with a CCD camera (Leica, Germany). The sections were analyzed, and images were captured in 5X, 10X and 40X magnification. Significant features were noted and described from 6 representative samples for each category. The categories for examination include control and two treated groups.

Histopathology of the testis

The architecture of the control testis (COT) was intact and normal. Well-arranged primordial germ cells (PGC) with a spherical nucleus, spermatogonia, primary spermatocytes (PST), and secondary spermatocytes (SST) were identified. Spermatids (SPD) with mature (MS) and immature sperms (IMS) in seminiferous tubules (ST) were seen prominently. STs were connected by connective tissues. Leydig cells (LC), were found adjacent to the STs. The nurse cells known as Sertoli cells (presence of dark nucleolus) were located in the ST. The typical structure of the testis was well packed in the control tissue (Fig. 1A). T1 testis that received 3 mg/Kg.b.wt of BHT for 21 days revealed the following pathological changes; prominently decreased number of MS,PGC and increased number of IGC (impaired germ cell) in STs. General atrophy (GA), interstitial fibrosis (IFB) and Leydig cell hyperplasia (LCH) were significant. LCH is a form of benign Leydig cell tumor (LCT) usually infiltrating between STs, while benign LCTs form a nodule that lacks tubules. LCH is usually characterized by an increased number of LCs, spermatogenic failure and atypia of the nucleus. Testicular atrophy (TA) or GA indicates the inhibition of spermatogenesis. Loss of testicular organization with vacant spaces was significantly identified in the STs (Fig. 1B).

Short-term T_2 testis received 30 mg/Kg.b.wt of BHT for 21 days showed the following changes; increased GA (TA), LCH and IFB than T_1 dose exposure. Testicular architecture was highly disrupted, and the number of IMS were increased. The standard architecture of STs and sperms were distorted. Infiltration of Leydig cell (LC) was identified12 mg/Kg.b.wt of BHT which significantly increased the features of GA, IFB and LCH compared to other treated groups. Decreased numbers of PGC, PST, SST, SPD and mature sperms were observed. The typical architecture of testis was found to have degenerated to its maximum. It can be an indication of damage in STs and may be due to the estrogenic activity of the compound (Fig. 1:C).

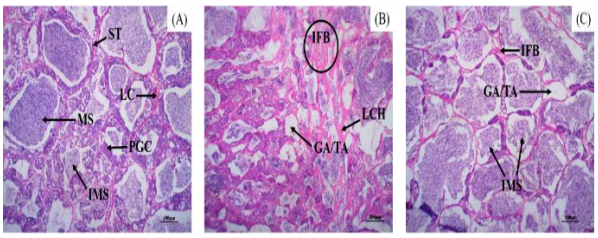

Fig. 1. Histopathology of *control testis (COT)* of *Anabas testudineus* shows seminiferous tubules (ST), Leydig cells (LC), primordial germ cells (PGC), mature (MS) and immature sperms (IMS) (A). Interstitial fibrosis (IFB), Leydig cell hyperplasia (LCH) and General atrophy (GA) or Testicular atrophy (TA) were identified in T1 testis(B), T2 testis (T2) (C).

Histopathology of the ovary

The structure of the control ovary (CO-OV) was normal. The primary growth stage or pre-vitellogenic stage and primary oocyte (PO) were identified by the presence of multiple nucleoli in the nucleus of the (germinal vesicle) oocytes. Increased size of the nucleus was another feature of PO. In H&E, stained cells were blue colored with a pink colored nucleus. The cortical alveoli stage and the secondary oocyte (SO) were represented by the ooplasm's increased granular structures. The nucleus was enlarged and the opaque appearance of oocyte was noted in the area that surrounded the nucleus. The vitellogenic stage and the vitellogenic oocyte (VO) were identified by the oocyte's increased size. Cortical vesicles appeared spherically on the periphery of the cytoplasm. The number and size of the yolk vesicle increased. The nucleus was irregular in shape. The presence of a vitelline membrane was observed. The maturation stage & mature oocytes (MO) were identified by the non-visibility of the nucleus. The entire cytoplasm was filled with granular structures. The membrane of the nucleus dissolved. The yolk vesicle gradually became more dominant. Degeneration of vitellogenic oocyte (DVO) was observed with vacant spaces in the ooplasm. The nucleus was pinkish, and later development progress disappeared. The aggregated structure represents the yolk granules. The large spherical empty structures surrounding the entire ooplasm represent the yolk vesicle. Oocytes were connected by connective tissues (Fig. 2A).

The T_1 (15 mg/Kg.b.wt) and T_2 (30 mg/Kg.b.wt) doses of BHT exposure in fishes were indicated as follows: Fig. 2:B, Fig. 2:D, T_1 ovary (T_1-OV) for 21 days and Fig. 2:E T_2 ovary (T_2-OV) for 21 days of study period. DO (degeneration of oocytes) or DVO was the major feature noted in all four treated categories. DO or DVO may not be an indication of a significant pathological condition because they were also observed in the control ovary. Some architectural differences in the

ovarian structure were observed in all treated categories. No significant evidence was observed in the treated ovaries. The overall architecture of the ovary was well arranged and the same was observed in all five categories.

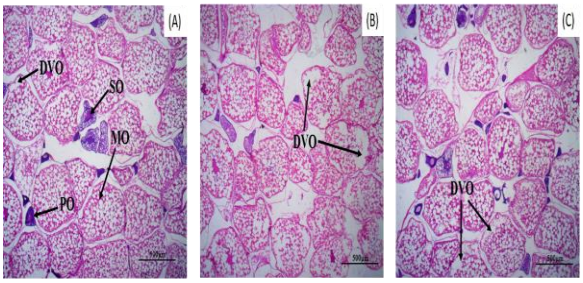

Fig. 2. Histopathology of *control ovary (CO-OV)* of *Anabas testudineus* shows degeneration of vitellogenic oocyte (DVO), primary oocyte (PO), secondary oocyte (SO) and mature oocytes (MO) (A). Degeneration of vitellogenic oocyte or degeneration of oocyte (DVO or DO) were frequently identified in in short-term T1 ovary (T1-OV) (B), T2 ovary (T2-OV) (C).

Effect of BHT on the expression of Aromatase gene in female and male

To determine the expression of Cyp 19 a gene, we performed RT PCR in gonadal tissue from *Anabus testudineus*. Using the RT PCR result, quantify the aromatase gene expression. Gonadal aromatase Cyp19a was exclusively expressed in the gonad. We examined both male and female fishes using gonadal tissue the fold change of treated group is higher than control group, As shown in the Fig. 3A Cyp19a mRNA was expressed up regulated in treated female (15 mg/kg/btw) compare with control fish. Fig. 3B. Aromatase gene expression in male also shows up regulation in treated male (15 mg/kg/btw) compare with control.

The endocrine disrupting (ED) properties of BHT in the animal model still needs validation. There are gaps in the research demonstrating the ED activities of BHT. In the present study, it is evident from the histological examinations that the given BHT doses disrupted the typical architecture of the testis.

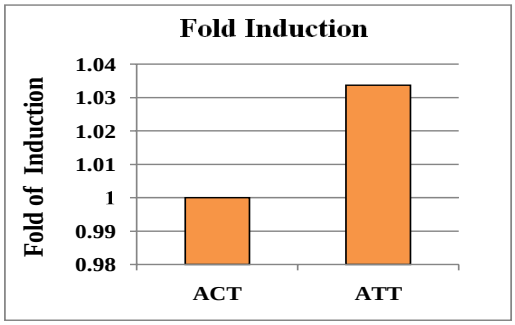

Fig. 3A. Expression of Cyp19amRNA in gonads in control and treated male fishes by real time RT PCR after exposure to 15 mg/kg/btw of BHT for 21 days. The result represent means ± SEM of triplicate test $p<0.01$. ACT: Aromatase gene in Control Testis, ATT: Aromatase Gene in Treated testis.

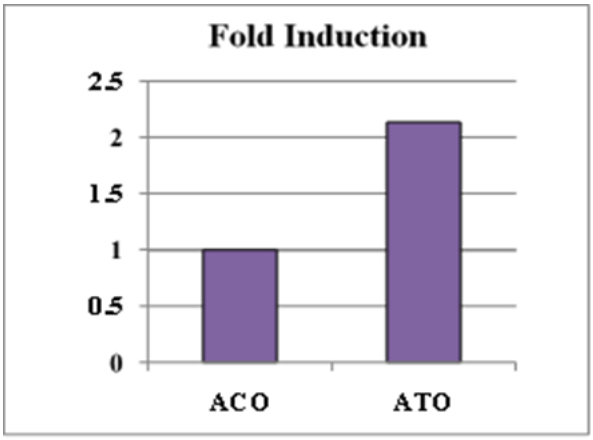

Fig. 3B. Expression of Cyp19a mRNA in gonads in control and treated female fishes by real time RT PCR after exposure to 15 mg/kg/btw of BHT for 21 days. The result represent means ± SEM of triplicate test $p<0.01$.

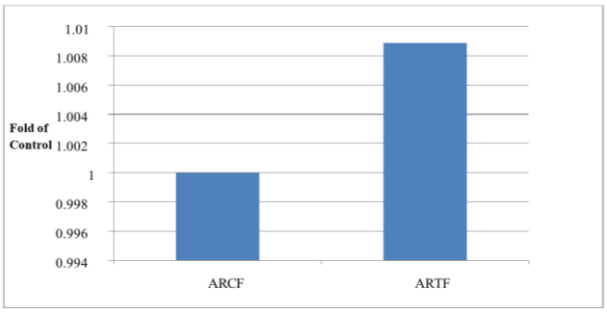

Fig. 4A. Expression of Cyp19a mRNA in gonads of control and treated female fishes by real time RT PCR after exposure to 30 mg/kg/btw of BHT for 21 days .The result represent means ± SEM of triplicate test $p<0.01$. ACT: Aromatase gene in Control Testis, ATT: Aromatase Gene in Treated testis.

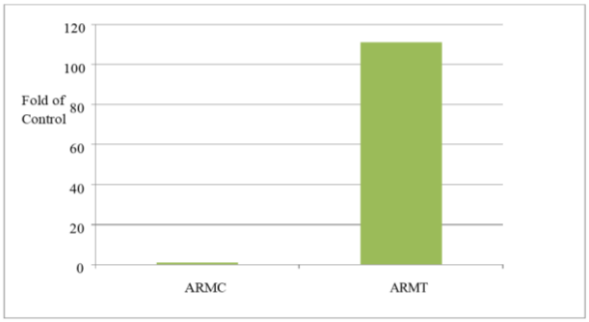

Fig. 4B. Expression of Cyp19a mRNA in gonads in control and treated male fishes by real time RT PCR after exposure to 15 mg/kg/btw of BHT for 21 days. The result represent means ± SEM of triplicate test $p<0.01$.

Significant pathological observations were not seen in the ovarian architecture through histopathological analysis. The lethal level of BHT is not yet calculated through I.P mode in fishes. T_1 and T_2 doses of BHT used in the study were derived from the existing literature. The combination of BHT+BHA is used as a synthetic antioxidant mainly because of their structural similarities. Some studies directly involved the ED properties of BHA rather than BHT.

In this study, no significant changes were observed in the ovarian architecture of fishes, but whereas there were significant pathological changes in the testis. The ovary was well arranged with typical structures in T_1 and T_2 doses of study. DVO or DO was observed with no other significant changes. Pop *et al.* (2013) identified the case of minimal endometrial stroma edema, a significant reduction in the endometrial epithelium cell height and increased myometrial thickness in BHT treated immature female rats. No other visible morphological changes in reproductive tract segments were observed in the previous study. The significant changes noted in testis were the following in all treated categories; LCH, IFB and the disruption of the tissue's normal architecture. BHT and BHA were proved to have weak anti-androgenic properties. The mixture of BuPB + BHA, BuPB + BHT and BHA + BHT showed significant anti-androgenic activity and none of these compounds were identified with androgenic activity in the MDAkb2 cell line study (Pop *et al.*, 2013). The first study of BHT in the human uterine cell line demonstrated that the BHT inhibits estrogen-responsive gene expression even at lower concentrations. In a study (Wada *et al.*, 2004), BHT was proved to promote estrogen activity in 293 T cells. The (anti) estrogenic and (anti) androgenic activity of BHA was evaluated (Kang *et al.*, 2005). BHA was identified as a weak estrogen (Jobling *et al.*, 1995; Soto *et al.*, 1995) and it was found out that BHT increases the gene expression of ERα and PR (progesterone receptor), respectively onMCF-7 human breast cancer cell line (Okubo and Kano, 2003). The existing works of literature on BHA indicate the dual properties of the same. In studies, both estrogenic and anti-androgenic action of BHA were identified. BHA might act as an androgenic antagonist (Pop *et al*., 2013).

A study conducted in Common carp and Japanese Eel (Hwang *et al.*, 1992) demonstrated that the uptake of BHT was similar to that of nonylphenoletoxylated, sodium linear alkylbenzene and sodium alkylsulfate in fish. The deposition of metabolites and the hepatomegaly effect of BHT was studied in Rainbow Trout and Common carp, resulting in the radioactivity in the liver and other organs. BHA and BHT also act like anti-androgens and BHA may alter the estrogen signalling (Cooke *et al.*, 2013). The weak estrogenic effects and the anti-androgenic properties of BHA were extracted from the existing in vitro studies. Based on the cell proliferation assays, BHT shows less estrogenic effects than BHA (Amadasi *et al.*, 2009; Freitas and Fatibello-Filho, 2010; Kang *et al.*, 2005; Lanigan and Yamarik, 2002; Okubo and Kano, 2003).

Endocrine disrupting properties of compound was identified by RT PCR aromatase gene expression testing which is widely accepted one. Aromatase gene complex otherwise called ‘estrogen synthase’. Cyt 19a is a key steroidogenic enzymes, it is a terminal enzyme, which catalysis the irreversible conversion of androgen in to estrogen (Tang, 2017). Cyt 19a gene is also known as gonadal aromatase which are need for embryo development, onset of puberty, brain sex differentiation, gametogenesis, development of secondary sex characters, immune response, differentiating gonad, sex differentiation and reproduction (Trant, 2001). Androgen and estrogen hormonal balance to be crucial role in the sexual differentiation in developing teleost fish. In the developmental period of female fish if aromatase enzyme inhibition take place it may lead to the development of phenotypic male fish. Steroid hormones biosynthesis is a main target by EDCs particularly Cytochrome P450 dependent enzymes. Many aromatic Inhibitors like ketoconazole, propiconazole, prochloraz cause down regulation of aromatase enzyme that means reduction of estrogen, estrogen reduction cause the lowering the amount of vitellogenin (Oliveira *et al.*, 2020). In zebra fish strong up regulation showed by Cyp 19 b gene by estrogenic compound (Kazeto, 2004). Aromatase mRNA level have been studied as an indicator of sexual differenation in fish. In this study we examined the expression of Cyp 19 a gene using highly sensitive method of quantify the expression of gene in gonad of both control and test group quantifying the gene expression by RT PCR. RT PCR is an innovative technique for measuring gene expression (Rees, 2003). It is most useful and effective method. In this study we can see the upregulation of both aromatase and vitellogenin gene expression that means the compound has estrogenic effect that is why in male fish of treated show high level of both gene are very low. Xeno-estrogen compounds can able to interfere the estrogenic pathway.

CONCLUSIONS

The gonadal effect of antioxidant butylated hydroxytoluene (BHT) in *Anabas testudineus* was investigated for short-term (14 days) and long-term periods (28 days). The examination was based on the histopathological observations of the tissues. Pathological significance was noted in the testis and was devoid in the ovary. The results obtained from this study provide a preliminary data for further evaluation of the effects of BHT on gonads. Long-term monitoring and continuous assessment in different animal models are imperative to obtain further insights into the properties of BHT and its effect on gonads. Aromatase gene expression study by RT PCR gives the information that the compound BHT effect the steroidogenic pathway.

Declaration of Competing Interest. There is no conflict of interest between the authors of this paper.

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