Fish short-term reproductive assay for evaluating the estrogenic property of a commonly used antioxidant, butylated hydroxyanisole

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Fish short-term reproductive assay (FSTRA) is a tier one assay for screening the endocrine disrupting property of a compound. This study analysed the end points like variations in Gonado–Somatic index (GSI), sperm quality and count, serum hormone levels (FSH, LH, estrogen and testosterone) to study the endocrine disrupting properties of butylated hydroxyanisole (BHA). A fresh water teleost, *Anabas testudineus* was used as animal model in this study. LC50 value of BHA was 250 mg/kg body weight in fish. Effects of four different doses of BHA were studied – 2, 5, 50 and 75 mg/kg body weight. BHA is found toxic at high doses of 250 mg/kg body weight and above. All the four doses of BHA caused significant impact on GSI, sperm count and quality. Serum hormone assay by ELISA showed significant variations in treated groups compared to control group.

Keywords: *Anabas testudineus*, butylated hydroxyanisole, endocrine disruption.

ONE of the controversial issues today is the increasing evidence that many xenobiotic chemicals interfere with the endocrine system, which leads to reproductive and developmental abnormalities in humans and animals. Many xenobiotic contaminants mimic or modulate the action of endocrine hormones. Indeed many laboratory experiments show that exposure to these chemicals can impair permanently the reproductive functions in adults and make irreversible changes in the developing stages of an organism[1].

Endocrine disruptors are chemicals which alter or mimic the hormones and disrupt the body's normal functions. This disruption can be through alteration of the normal hormone levels, halting or stimulating the production of hormones or changing the way the hormone travels through the body. Exposure to these endocrine disruptors can occur through direct contact or through ingestion. Exposure to endocrine-disrupting chemicals in the environment has been associated with abnormal thyroid function in birds[2] and fish[3]; decreased fertility in birds[4], fish[5], shellfish[6] and mammals[7]; decreased hatching success in fish[8], birds[9] and turtles[10]; demasculinization and feminization of male fish[11], birds[12] and mammals[13]; defeminization and masculinization of female fish[14], gastropods[15] and birds[12]; and alterations of immune functions in birds[16] and mammals[17].

Butylated hydroxyanisole (BHA) is a mixture of two isomers (2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole) (Figure 1). BHA, an antioxidant has been extensively used as a food preservative with E number E320. BHA is added to the packaging material in order to provide protection to the food inside the package through volatilization of the antioxidant[18]. In Europe the use of BHA is permitted to a maximum limit of 200 mg/kg expressed on the fat content of the product[19].

The European Commission on Endocrine Disruption listed it as a category-1 priority substance that may cause endocrine disruption. The extent of endocrine disruption caused by BHA is not well studied in any animal model. Tier one assays using *in vitro* cell lines reported BHA as an estrogenic compound. The aim of the present study is to explain the estrogenic properties of BHA. The concerns over decrease in sperm count and viability of the sperm due to endocrine disruption have been mentioned in literature. Production of high quality gametes relies on the correct concentration of sex hormones and any alteration in the hormone level induces the changes in gamete quantity and quality. So we can use gamete quality test as an indicator of endocrine disruption. The sex hormone concentrations can also be tested for establishing the presence of endocrine disrupting property[20].

Common freshwater fish *A. testudineus* was used as experimental model. Fishes weighing 30 ± 5 g were acclamatized to laboratory conditions by maintaining them in 50 litre tanks for 15 days. The fishes were divided into 5 groups, consisting of 20 fishes per group. The first group was the control group which was kept in dechlorinated water. The LC50 value of BHA was found to be 250 mg/kg bodyweight for *A. testudineus*; sub-lethal concentrations of 2, 5, 50 and 75 mg/kg body weight were selected as experimental doses. The fishes were exposed to various doses of BHA by intra-peritoneal injection, twice a week for two months.

Sample fishes were anesthetized prior to handling in a bath of clove oil. Body weight and length were measured.

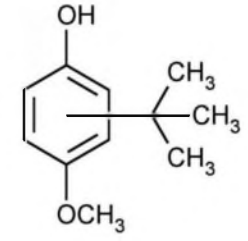

Figure 1. Structure of butylated hydroxyanisole.

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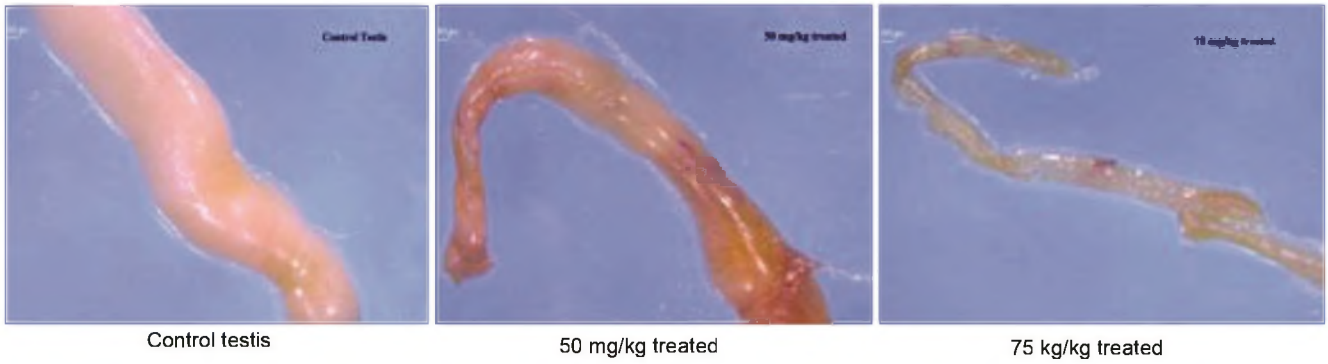

Figure 2. Effect of various doses of BHA on Gonadal morphology.

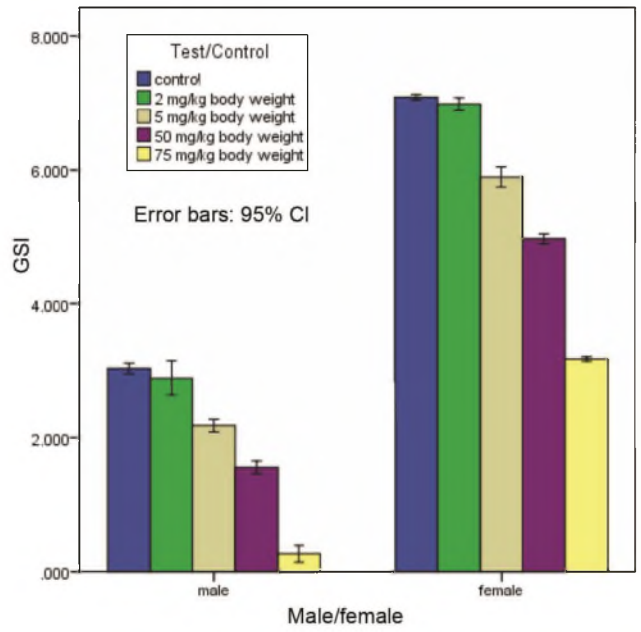

Figure 3. Effect of different doses of BHA on the Gonado–Somatic index.

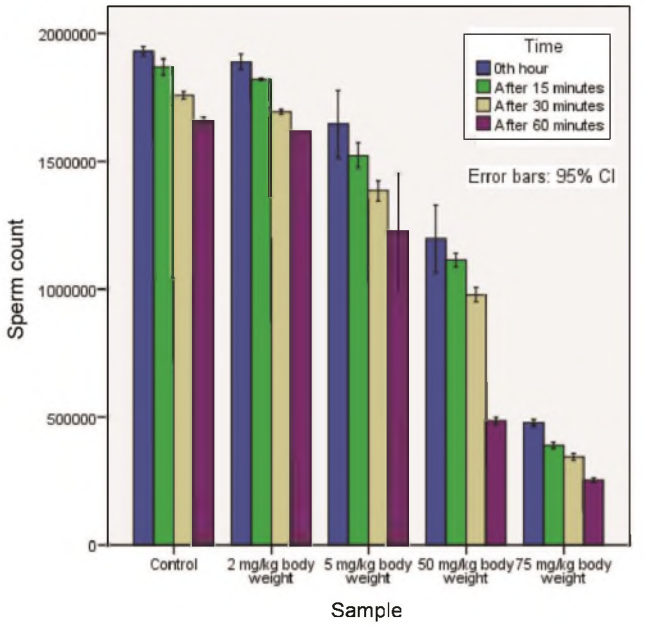

Figure 4. Effect of different doses of BHA on sperm count.

Blood was taken from the caudal vein and transferred to tubes for further studies. The gonads were taken and weighed to calculate the Gonado–Somatic index (GSI).

GSI is used as one of metrics for reproductive status. Body weight of the fish was taken and a ventro-lateral incision was done on anesthetized fish and the gonads were removed and weighed. GSI was calculated as gonadal weight (to the nearest 0.1 mg) divided by body weight (mg), multiplied by 100%.

For sperm quality analysis, milt was collected by gently rubbing the abdomen and diluted 100 times with water. The tests were performed by taking 10 µl sample on a counting chamber and observing it under phase contrast microscope.

Sandwich ELISA was performed for quantification of LH, FSH, testosterone and estradiol in serum using kits provided by Bioassay Technology Laboratory. The kit uses ELISA based on the Biotin double antibody sandwich technology to assay the fish hormones. The wells are pre-coated with monoclonal antibody. According to the number of samples, coated wells were taken and 40 µl of sample, 10 µl E2 antibodies and 50 µl streptavidin-HRP were added to them. They were then covered with seal plate membrane, shaken gently to mix the contents and incubated at 37°C for 60 min. After incubation the solution in the well was removed and filled with washing solution, liquid was drained after 30 s, repeated the washing procedure was repeated to five times and it blotted the plate. 50 µl of chromogen A, and 50 µl chromogen B were added, shaken well to mix and incubated for 10 min at 37°C away from light for colour development. 50 µl stop solution was added to stop the reaction. Absorbance (OD) of each well at 450 nm wavelength was measured within 10 min of adding the stop solution.

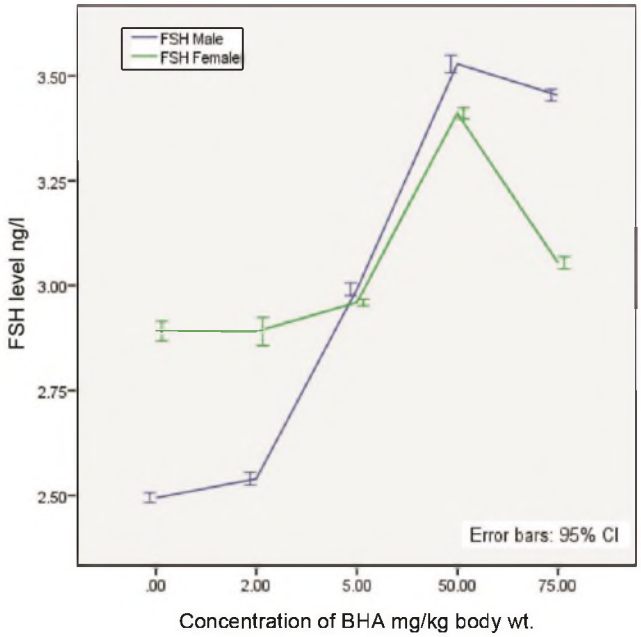

Figure 5. Effect of various doses of BHA on serum FSH level.

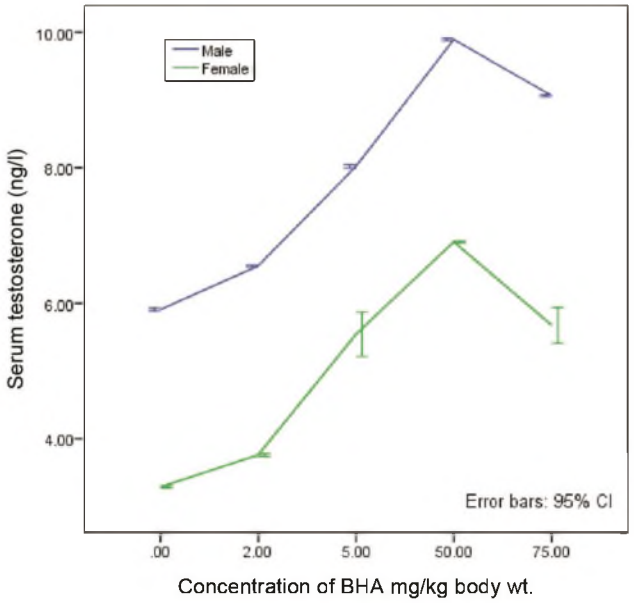

Figure 7. Effect of various doses of BHA on serum testosterone level.

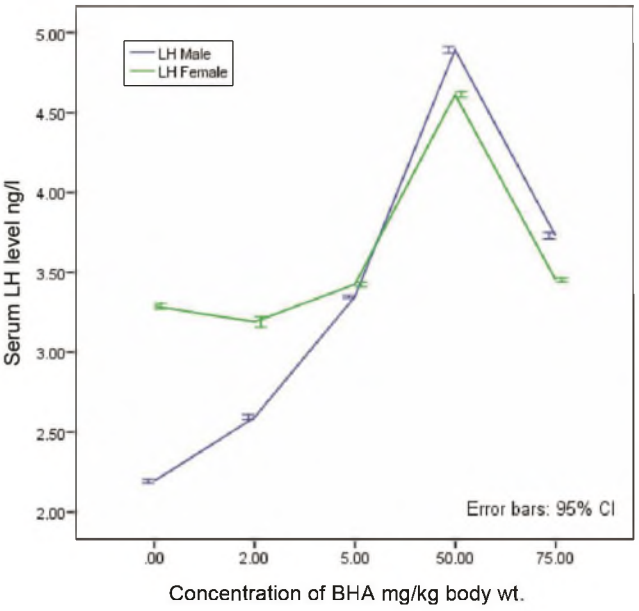

Figure 6. Effect of various doses of BHA on serum LH level.

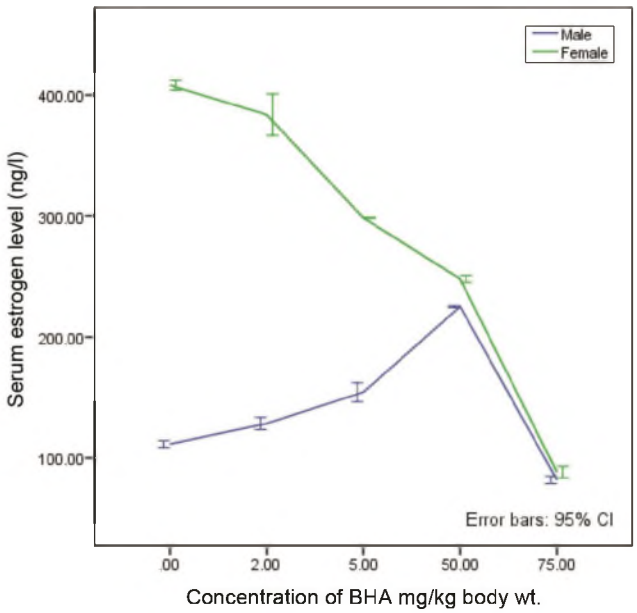

Figure 8. Effect of various doses of BHA on serum estrogen level.

Gonadal (testis) size and weight significantly decreased at 50 mg/kg body weight and 75 mg/kg body weight test organisms group within 30 and 60 days of administration of BHA (Figure 2). In the male fishes treated with 75 mg/kg body weight, after 60 days atrophied testis was found, its structure became thread like and atrophy continued even in the breeding season. By contrast, atrophied testis was not found in control male and vehicle male. GSI was calculated. As in Figure 3, GSI showed significant variation on administration of high doses of BHA.

Sperm quality analysis showed sperm count reduction, impairment in sperm motility in fish treated with 75 mg/kg body weight of BHA (Figure 4).

Serum hormone ELISA showed variations in the level of FSH, LH, estrogen and testosterone in male and female fishes treated with various doses of BHA compared to the control.

FSH and LH level in the serum of male fish was found to increase compared to the control fish on administration of BHA (Figures 5 and 6). At two low doses of BHA

(2 mg/kg body weight and 5 mg/kg body weight) there was a gradual and significant increase in FSH and LH level and at 50 mg/kg concentration, maximum level of serum FSH and LH was found. The FSH and LH level in male started decreasing on administration of 75 mg/kg body weight of BHA. In female fish also a similar pattern of increase on administration of two low doses of BHA and significantly high increase at a higher concentration 50 mg/kg body weight and a sudden decline in the hormone level at 75 mg/kg body weight was observed.

Serum testosterone level in control fish was found to be 5.9 ng/l. On administration of BHA dose of 2 mg/kg body weight, it was found to increase to 6.5 ng/l (Figure 7). Testosterone level increased significantly on administration of 5 mg/kg body weight and at high dose of BHA (50 mg/kg body weight) there was maximum increase of 9.8 ng/l. However at 75 mg/kg body weight dosage of BHA, testosterone level decreased to 9 ng/l. In female control fish, testosterone level was 3.2 ng/l. It increased to a maximum concentration of 6.9 ng/l, on administration of 50 mg/kg body weight of BHA. Testosterone level decreased to 5.7 ng/l at a BHA dose of 75 mg/kg body weight.

Estrogen (estradiol) level in control male was found to be 110 ng/l. On administration of BHA it increased from 130.5 ng/l at a dose of 2 mg/kg body weight to 225.2 ng/l at 50 mg/kg body weight (Figure 8). It dropped to 81.9 ng/l at 75 mg/kg body weight dosage. Estrogen level in control female was 408 ng/l and it decreased to 381 ng/l at 2 mg/kg body weight of BHA and dropped down to 298.5 ng/l at 5 mg/kg body weight of BHA. There was a further decrease at a concentration of 50 mg/kg body weight and a drastic decrease of estrogen level to 88.7 ng/l at BHA dose of 75 mg/kg body weight.

There are no sufficient data available regarding the endocrine disrupting properties of BHA. Existing reports based on *in vitro* studies suggested BHA as an estrogenic compound[20]. Findings of this study suggest that, BHA acts as a possible endocrine disruptor in animals including humans, impairs reproduction and sperm quality. This study will provide a baseline data on the estrogenic effect of BHA in an animal model. More studies are needed in mammalian models to conclude the exact effect of this compound as an endocrine disruptor.

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