Possible Disrupting Effect of Different Doses of Butylated hydroxyanisole (BHA) on Thyroid Follicular Cells of Adult Male Albino Rats: Anatomical, Histological and Biochemical Study

Original Article

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ABSTRACT

Background: Butylated hydroxyanisole (BHA) is a synthetic antioxidant which is absorbed mainly through gastrointestinal tract following oral administration. It was introduced to the food industry as a safe preservative and to the pharmaceutical industry in cosmetics manufacturing and as an additive to fat-soluble vitamins to prevent rancidity. The US Food and Drug Administration (FDA) approves BHA as a safe food additive in a maximum oral dose of 150 mg/ kg, meanwhile it is a potential allergen.

A new question has been raised if BHA is a potential human carcinogen? another potential hazard is also a recent concern as the compound has been linked with some endocrine disruption. The endocrine effect of BHA is associated with sex hormones, a little has been known about the exact effect neither on them nor on other hormones.

Aim of the Work: This work aimed to assess the possible disrupting effect of different doses of BHA on thyroid follicular cells.

Materials and Methods: This study was conducted on 30 adult male albino rats. 12 as control and 18 divided equally to 3 groups and received 10, 100 and 500 mg/kg body weight of BHA dissolved in corn oil orally for 42 days. All animals were examined for body and thyroid weight, thyroid hormones and oxidative markers levels. Semithin and ultrastructural examination of thyroid follicles were done for all. All data were statistically analyzed.

Results: All data revealed dose defendant disrupting effect of BHA on thyroid and follicular cells. Variable follicular shapes and multiple layers of vacuolated cells and dilated endoplasmic reticulum. Histological findings were confirmed by both anatomical and biochemical examinations.

Conclusion: BHA has a possible thyroid disrupting effect in long term intake. This effect is dose dependent and significantly recognized in moderate and high doses intake.

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Key Words: BHA, endocrine disrupting substance, follicular cells, morphometric.

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BACKGROUND

Butylated hydroxyanisole (BHA) is a synthetic antioxidant. This waxy compound is a synthetic mixture of the isomers 3-tert-butyl-4-hydroxyanisole (3-BHA) and 2-tert-butyl-4-hydroxyanisole (2-BHA) with the active substance constituting more than 98.5%. The compound is absorbed mainly through gastrointestinal tract following oral administration, rabidly metabolized and then excreted through both urine and stool[1]. It was introduced to the drug and food industry as a safe food additive and preservative. Through its antioxidant effect it has a superior value in keeping preserved and canned food fresh and tasty for a good amount of time through preventing oxidation and then rancidity of lipids. Additionally, it is used in food packing materials due to volatilization of the antioxidant[2,3]. Moreover, it is used in pharmaceutical industry in cosmetics manufacturing and as an additive to fat-soluble vitamins. It is specifically used in the market as an additive to animal feeding stuff. With this special use, work has been recently done to assess its safety on different animal species. It was reported as being safe in most of them but with the uncertain degradability and metabolism of BHA in cats, it couldn't be marked safe as an additive in their food supplies[4]. Apart from the previous uses, the BHA is used also in rubber and plastic manufacturing as proved to increase their longevity and working life[1,2].

The major route of BHA human intake is through food ingestion and the estimated daily dose is different with residence, age, dietary habits and others[5]. The US Food and Drug Administration (FDA) approved BHA as a safe food additive. Although BHA is considered safe for most of animal species in a maximum oral dose of 150 mg/ kg,

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it carries the risk of skin and eye irritation as potential allergen. Still the maximum dose usage might carry also an environmental risk[2].

A new question has been raised to the research world after an association between a chronic administration of a high oral BHA and a consequent rodent forestomach tumor. Is BHA a potential human carcinogen or just a harmless synthetic antioxidant[6]?. Another potential hazard of BHA is also a recent concern as the compound has been linked with some endocrine disruption. The endocrine effect of BHA is associated with sex hormones, however a little has been known about the exact effect neither on them nor on other hormones. Therefore more in *vivo* and in*vitro* studies are needed to answer the question and reconsidering the compound as a safe antioxidant with its wide range of uses and high risk of human and animal exposure[7]. Hence the aim of this work was to assess the possible endocrine disrupting effect of BHA on follicular cells of thyroid gland of adult male albino rats at different doses of oral intake.

MATERIAL AND METHODS

Chemicals

BHA (2,3)-tert-butyl-4-hydroxyanisole [25013-16-5], 99.9%, and corn oil were purchased from Sigma–Aldrich Chemical Company, St. Louis, MO, USA.

Animals and experimental design

Thirty healthy adult male albino rats (weighting 200–230 g) were used for the present work. All procedures followed the guidelines for the care and handling of animals after approval of the study protocol by the ethical committee of Alexandria Faculty of Medicine.

The animals were housed in standard polypropylene cages under the same laboratory conditions of light and temperature of 20–22°C with free access to standard laboratory food and water. They were acclimatized for one week prior to the onset of the experiment.

The rats were randomly assigned into two main experimental groups as follows:

Group I (the control group): 12 male albino rats which were further subdivided into 2 equal subgroups (aubgroup Ia and Ib); 6 rats each. Rats of subgroup Ia are negative control. Subgroup Ib animals received 1 ml of corn oil orally via orogastric-tube daily for 42 days.

Group II (BHA-treated group): 18 male albino rats were further subdivided into 3 equal subgroups (subgroup IIa, b and c); 6 rats each. They received the treatment of BHA dissolved in corn oil via orogastric tube in morning daily doses of 10, 100 and 500 mg/kg/ day for 42 days respectively[8].

Methods

Anatomical study

- Each rat was weighed twice; at day 0 of the experiment and just before euthanization.
- After euthanization, the weight of the thyroid gland of each animal was measured.
- All data were tabulated and statistically analyzed.

Biochemical study

Determination of serum T3, T4 and TSH: Blood samples were taken from rats' orbital sinus, at day 0 (baseline level) and at day 42 just before sacrification. Blood samples were collected in sterile tubes and immediately centrifugation was done at 400 x g (times gravity) for 5 min and serum was kept at −20 °C for further analysis of triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) levels by ELISA[9,10] in medical biochemistry department Alexandria faculty of medicine.

Markers of oxidative stress (OS): Parameters of OS profile were assayed by colorimetric technique using commercial kits (Biodiagnostic, Egypt) according to the manufacture instructions. The protein content of the supernatants was determined using Lowry's method[11]. oxidative damage parameter Superoxide dismutase (SOD) & Maliondialdehyde MDA (nmol/gm tissue) were estimated[12].

Histological study

All experimental rats' groups at the end of designed period were euthanized by decapitation under light ether anesthesia. The thyroid glands were immediately excised then the tissues were processed for electron microscopic examinations.

Electron microscopic study

Specimens were cut by sharp razor blade into thin slices (1mm3) and immediately immersed in fixative (4% glutaraldehyde solution for 20 h), then the specimens were washed with buffer solution and fixed in osmium tetroxide 1% concentration buffered with 0.1 M phosphate buffer at 7.4 pH for 1 hr. 1 µm thick sections were mounted on glass slides and stained with toluidine blue and photographed by light microscopy (Olympus using 200 & oil immersion lenses). By using an ultramicrotome (Leica, Glienicker, Berlin, Germany), ultrathin sections (80-90 nm) were obtained. Sections were stained with uranyl acetate and lead citrate[13]. Ultrathin sections examined and photographed with transmission electron microscope (Jeol- JEM- 100 CXII; Jeol, Tokyo, Japan) in the Electron Microscopic unit. Faculty of Science Alexandria University.

Histomorphometric study

The colloid area & the height of follicular cells from five sections from each experimental subgroups' rats were randomly selected & examined using light microscope. Thyroid follicles section was enclosed inside the standard measuring frame; then the area % of the colloid and follicular cell height were measured from the semithin-stained sections of the thyroid gland of each single animal per group at magnification of 100 using the Image

Analyzer (Olympus BX41TF, Tokyo, Japan) at the Center of Excellence for Research in Regenerative Medicine and Application "CERRMA", faculty of medicine Alexandria University. Such data were subjected to statistical analysis and represented as tables and histograms.

Statistical analysis

Analysis of data was done using IBM SPSS software package version 20 and the following were calculated: Range (minimum and maximum), mean and standard deviations. For normally distributed data, comparison between the three studied groups was analyzed using F-test (ANOVA) and confirmed using Post Hoc test (LSD). *p-value* less than 0.05 was considered statistically significant[14].

RESULTS

One animal of subgroup IIIc had died at the 4th week of the experiment with premortem manifest loss of appetite and lethargy. It was replaced with full course of the drug administration as designed to keep the number of the group standardized for statistics.

Anatomical results

Weighing the rats at the beginning and by the end of the study showed weight gain of both subgroups IIb and IIc. Significant body weight gain in subgroup IIc was less than those of subgroup IIb.

The thyroid gland weight as estimated just after sacrifice and before processing for histological examination showed the heaviest in subgroup IIb (statistically significant), insignificantly higher in subgroup IIa and significantly higher as compared with normal and still less than subgroup IIb in subgroup IIc (Table 1, Figure 1).

Biochemical results

T3& T4 levels declined in all treated subgroups (IIa, b, c). The decrease was dose dependent as more marked with the highest dose. Only subgroups IIb and IIc showed significant decline. TSH serum level was reciprocally elevated in subgroups IIa and IIb. The change was significant only in the last. Marked decline in TSH level was reported in subgroup IIc (Table 2, Figure 2).

Regarding the antioxidant enzyme and lipid peroxidation, there was a significant increase in MDA and decrease in SOD with moderate (subgroup II b) and high (subgroup IIc) doses of BHA. The changes were dose dependent and more significant as doses increased. The changes in subgroup Ia were insignificant (Table 3, Figure 3).

Histological results

Light microscopic results (toluidine blue stain)

Group I (control group): Semithin sections showed rounded thyroid follicles lined by a single layer of cuboidal follicular epithelium surrounding lumen filled with colloid. The lining follicular cells appeared with rounded to oval active nuclei. Also, large pale parafollicular cells were observed. Small blood capillary depicted in between the follicles (Figure 4 a-c).

Group II (BHA-groups): Toluidine blue-stained semithin sections showed rounded thyroid follicles in subgroup IIa. were large, rounded and lined by a single layer of follicular cells. Multiple layers of follicular cells were also noticed in some follicles (Figure 5 a,b). Nuclei were oval surrounded by minimal vacuolated cytoplasm in few follicles while others appeared normal. (Figure 5 b-c).

Subgroup II b, follicles showed apparent increase in the number & height of their lining follicular cells. These cells were high cuboidal with vacuolated cytoplasm and rounded vesicular and darkly stained nuclei can be noticed (Figure 6 a-b). Follicles had multiple layers of follicular cells on one side while other follicles were fused together (Figure 6 b-c). Mast cell with its typical basophilic cytoplasmic granules was depicted.

Semithin sections of subgroup IIc, showed irregular variable shapes of thyroid follicles (Figure 7 a). Follicular cells appeared in some follicles as flat to irregular cuboidal cells while other cells were detached in the lumen (Figure 7a-b). Frequently seen densely stained nuclei and extensive vacuolated cytoplasm in (Figure 7b-c). Also, mast cells were observed in (Figure 7 b inset).

Histomorphometric study

The mean colloid area% of the thyroid follicles in semithin sections were significantly lowered in experimental subgroup IIb and marked decreased were noticed in subgroup IIc relatively compared to the control group. On the other hand, the mean follicular cell height significantly increased in subgroup IIb more than subgroup II a & II c (Table 4, Figure 8).

Electron Microscopic results

Control group

Follicular cells appeared with central rounded nuclei, apical microvilli projecting into the lumen, cytoplasm depicted multiple lysosomes & rough endoplasmic reticulum. Tight junctions between follicular cells was clearly recognized (Figure 9 a-c).

BHA group

Electron photomicrographs of subgroup IIa (Figure 10 a &b) revealed double layers of follicular cells with rounded nuclei, while other cells appeared with irregular small nucleus as in (Figure 10- b). Follicular cells appeared with dilated endoplasmic reticulum (Figure 11a,b), lysosomes & mitochondria.

Subgroup IIb showed follicular cells with irregular nuclei, decreased amount of lysosomes as compared to the control group, and dilated endoplasmic reticulum with retained secretion (Figure 12 a-c). Other follicular cells revealed electron dense small nucleus and fragmented

chromatin, dilated endoplasmic reticulum with retained secretion in cytoplasm was noticed (Figure 13 a-c).

Examination of subgroup IIc rats thyroid follicles showed irregular bizzar shaped follicles (Figure 14a) lined by vacuolated swollen cells with empty cytoplasm and irregular elongated electron dense nucleus (Figure 14 b-c). Some follicles were completely destructed lined by flat follicular cell with flat dense nuclei, scattered detached small cells due to loss of cell junctions. Loss of colloid secretion and congested capillaries were frequently seen (Figure 15a-d).

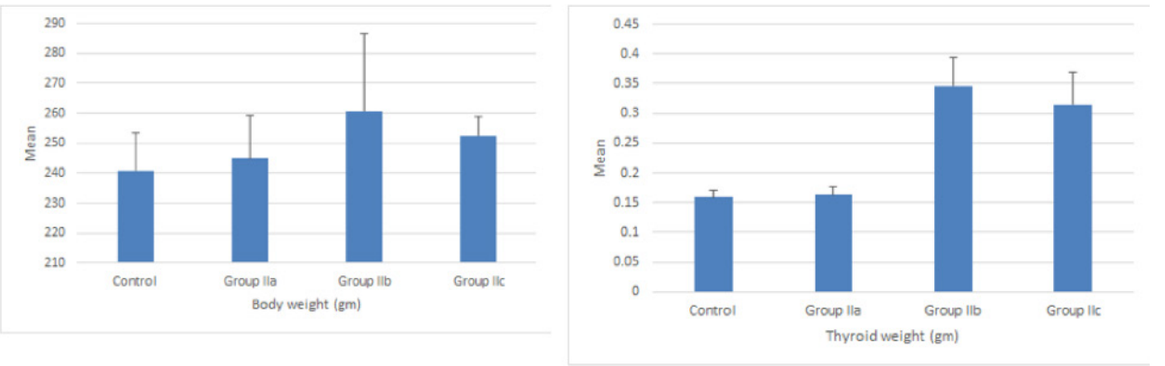

Fig. 1: Body weight (gm) and thyroid weight (gm) in the different studied groups.

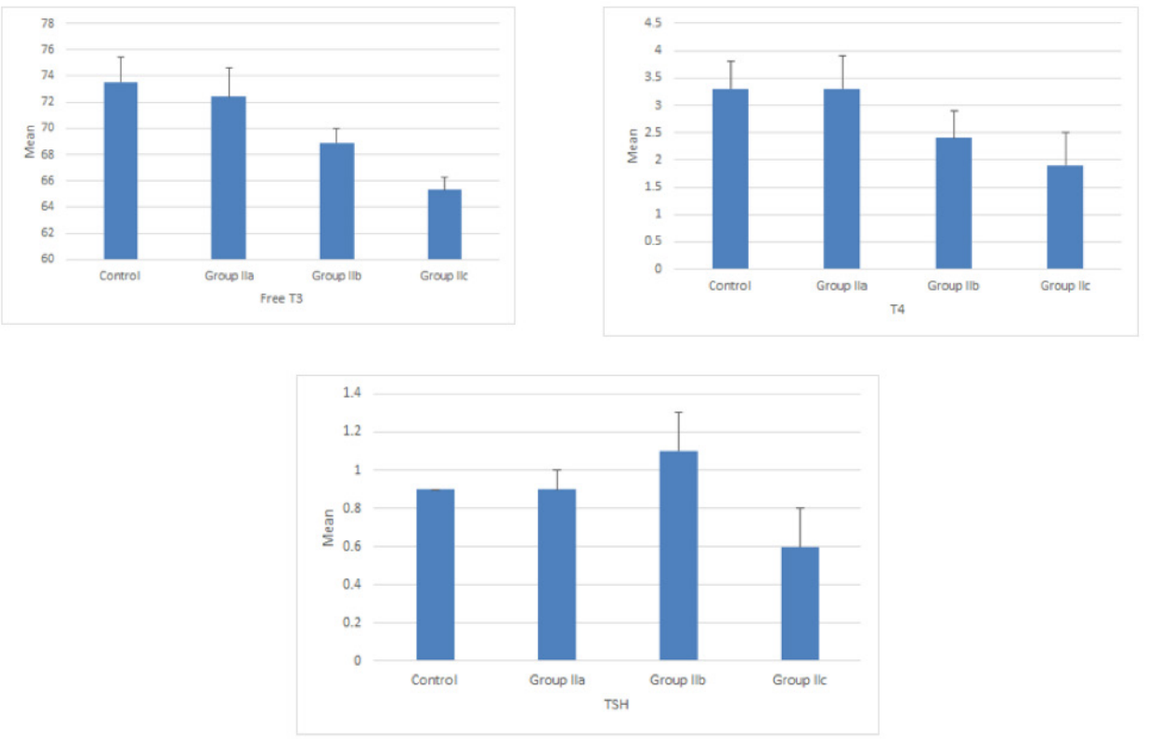

Fig. 2: FreeT3, T4 and TSH in the different studied groups.

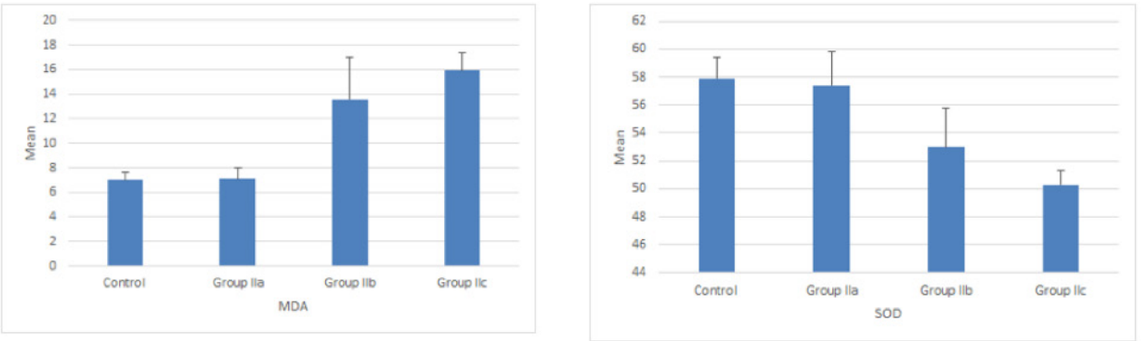

Fig. 3: MDA and SOD in the different studied groups.

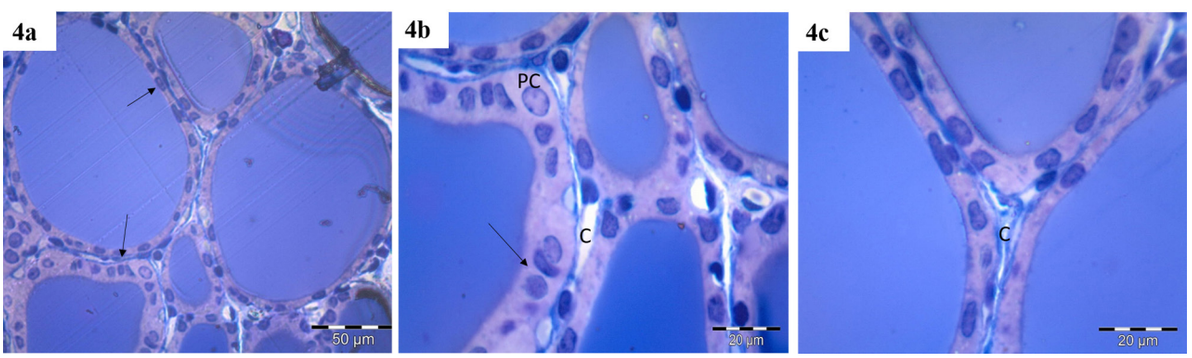

Fig. 4 a-c: Semithin sections of control groups thyroid follicles show: Fig. 4a: rounded follicles filled with colloid and lined by cuboidal follicular cells (↑) with oval nuclei. Figs. 4b-c: high magnification revealed single layer of follicular cells (↑). Parafollicular cells (PC) is seen insinuated between them & basement membrane. Notice blood capillary(c) in between adjacent follicles (Mic.Mag. a X200 b&c X1000)

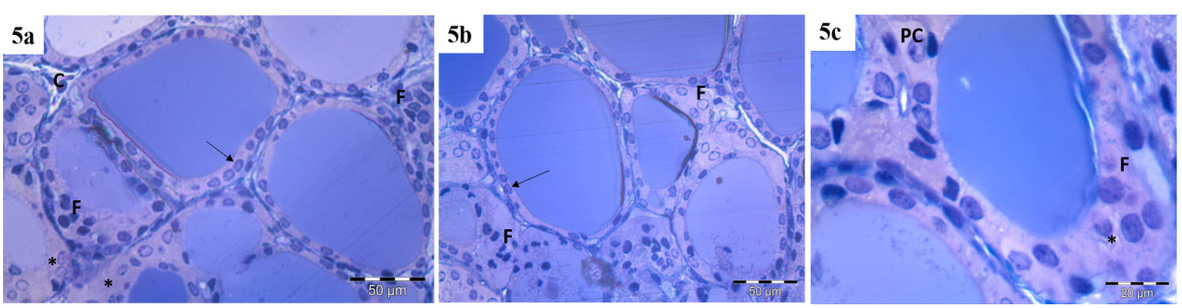

Fig. 5a-c: Photomicrographs of semithin sections of rats thyroid follicles subgroup IIa show some follicles are rounded lined by single layer of follicular cells (↑), while other follicles reveals irregular outline & lined by multiple layers of cells (F). Nearby follicles showing pale vacoulated cytoplasm.(*). A capillary (c) & parafolicular cells (PC)can be seen (Mic.Mag. a&b X200 c X1000)

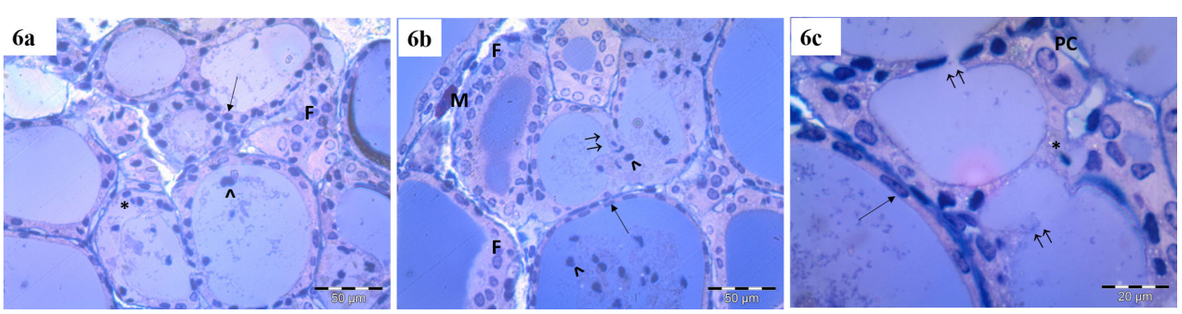

Fig. 6 a-c: Photomicrographs of subgroup IIb semithin section of rat's thyroid follicles show Fig. 6a: follicles of variable shapes & diameter lined by single layer of cells while others were lined by multiple vacuolated cells (F) Fig. 6b-c: fused follicles (↑↑) with detached follicular cells in the lumen (^), vacuolated swollen follicular cells (*) (PC) parafollicular cells (M) mast cells can be noticed (Mic Mag.6 a,b x200, cx1000)

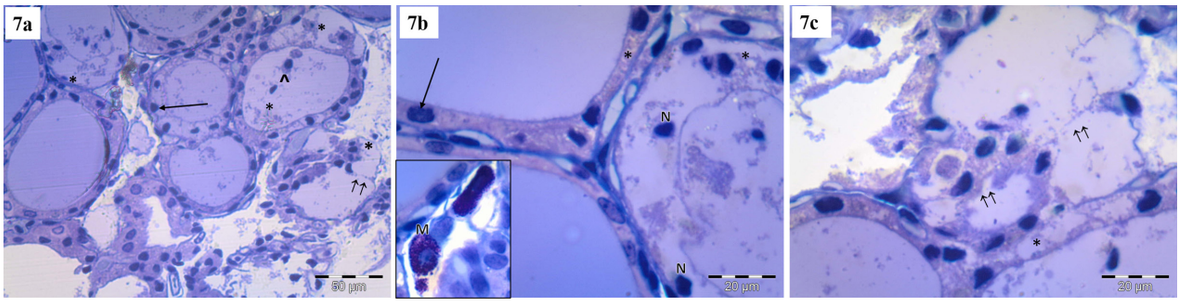

Fig. 7a-c: Photomicrographs of semi thin section of subgroup IIc rat's thyroid follicles show multiple irregular follicles while other were incomplete (↑↑) 7b: swollen follicles with rarified cytoplasm (*) and small darkly stained nuclei (N) 7c: incomplete irregular follicles and loss of its architecture (↑↑). (M) Mast cells can be observed between follicles in (fig 7b inset) (Mic Mag a x200 , b-c x1000)

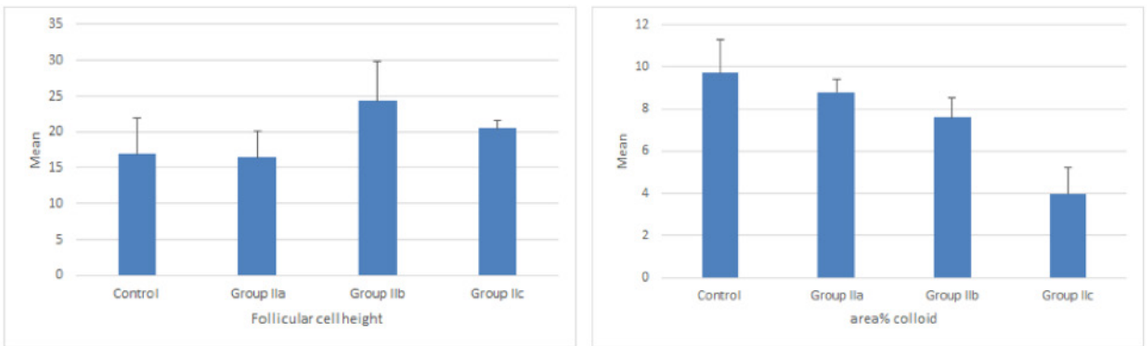

Fig. 8: Follicular cell height and area% colloid in the different studied groups.

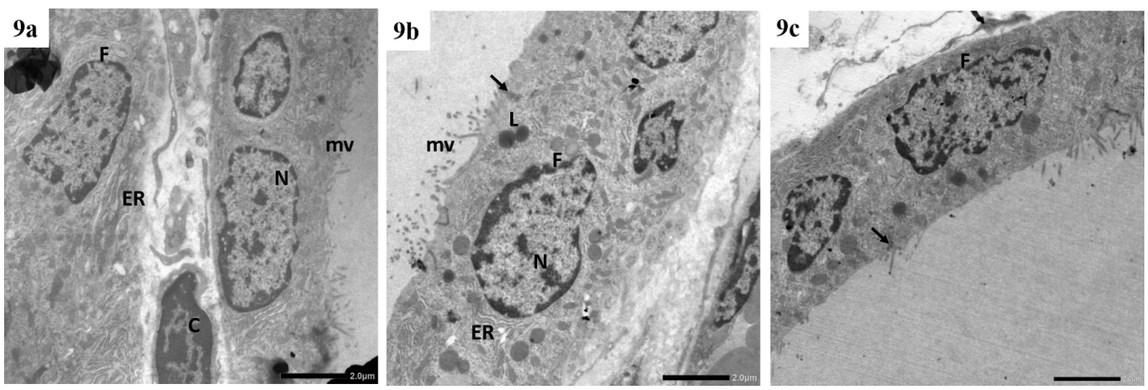

Fig. 9 a-c: Electron photomicrographs of control groups show follicular cells (F) with central rounded nuclei (n), apical microvilli (mv) projecting into the lumen, multiple lysosomes (L) & rough endoplasmic reticulum (ER). Notice tight junctions (↑) between follicular cells. Blood capillary (C) in between follicles (Mag X 3000).

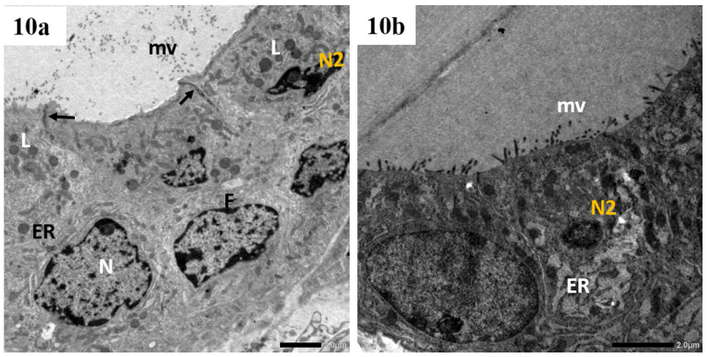

Fig. 10 a-b: Electron micrographs of subgroup IIa reveal (Fig.10 a) double layer of follicular cells (F) with rounded nuclei (N) lysosomes (L) microvilli (mv), tight junction (↑) and endoplasmic reticulum (ER) Electron dense small nucleus (N2) with dilated endoplasmic reticulum (ER) were seen in (Fig.10 b) (Mic Mag a-b x 3000)

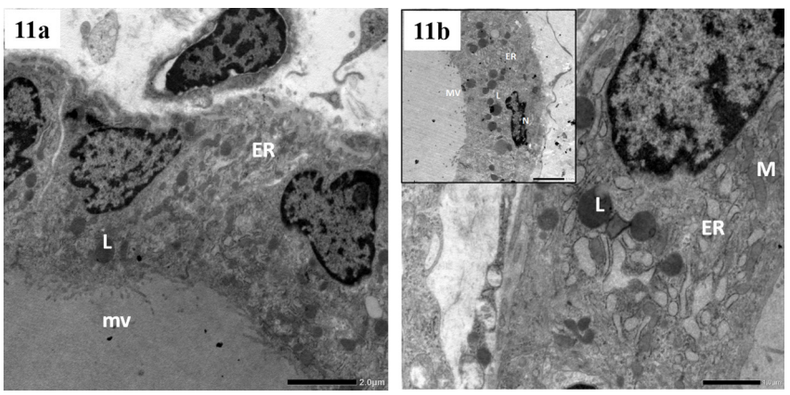

Fig. 11 a-b: Electron micrographs of subgroup IIa show single layer of follicular cells with irregular to flat nuclei (n) (inset b). Notice dilated endoplasmic reticulum (ER) emphasized in (Fig. 11b) (M) mitochondria ,(L) lysosomes, (mv) microvilli (Mic Mag ax2000-b x 5000)

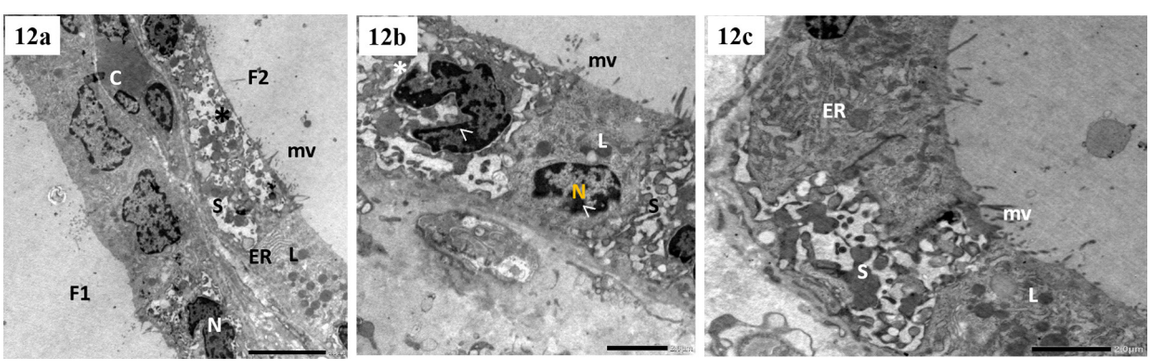

Fig 12 a-c: Electron micrographs of subgroup IIb showed two adjacent follicles lined by follicular cells with dilated endoplasmic reticulum (ER) while adjacent (F2) showing marked dilation with retained secretion (s). few lysosomes can be seen (L) (Fig.12 b,c,d) marked dilatation of (ER) irregular nuclei (N) with marginated chromatin (^)in (Fig. 12 c) (Mic Mag ax1500,b&c x3000)

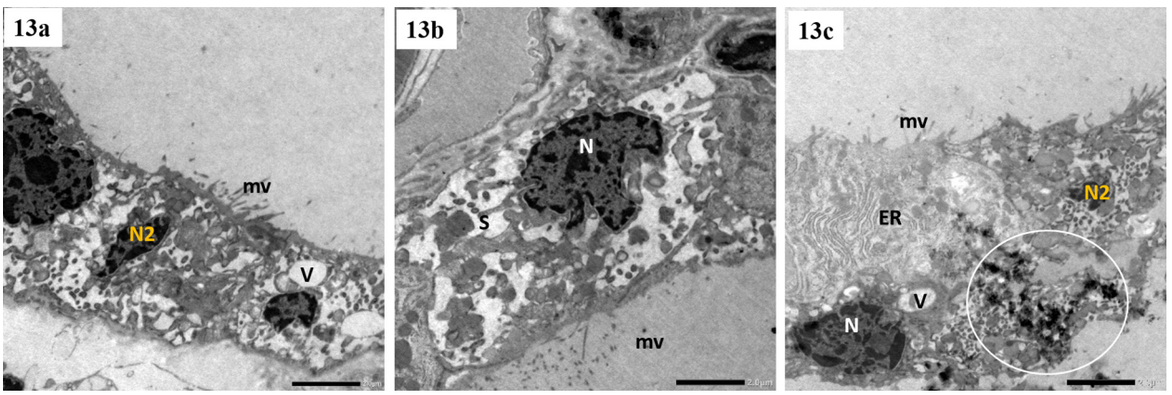

Fig.13 a-c: Electron micrographs of subgroup II b follicles show marked dilatation of endoplasmic reticulum(ER), rarified cytoplasm and vacuoles (v), electron dense nuclei(N) and tall microvilli (mv) projecting into the lumen were reported in (Fig.13 b). (Fig.13 c) reveal thick wall follicle with basal nucleus (N) abnormal distribution of chromatin. Other follicular cells reveal electron dense small nucleus (N2) ,fragmented chromatin & retained secretion among rarified cytoplasm were depicted (circle). Apical dilated endoplasmic reticulum (ER) can be seen. (Mic Mag x3000)

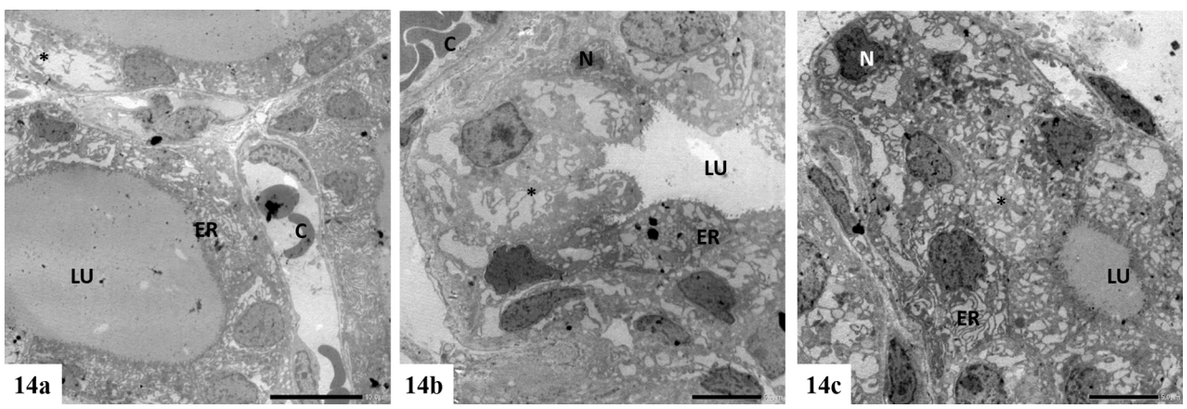

Fig. 14 a-c: Electron micrographs of subgroup IIc depict swollen vacuolated follicular cells bulging in apparently narrow lumen (Lu) Cells in (Fig 14C) showed irregular small dense nuclei (N) surrounded by dilated endoplasmic (ER). Congested capillary(c) can be noticed in (fig 14 a-b) (Mic Mag. a x800,b&c x1200)

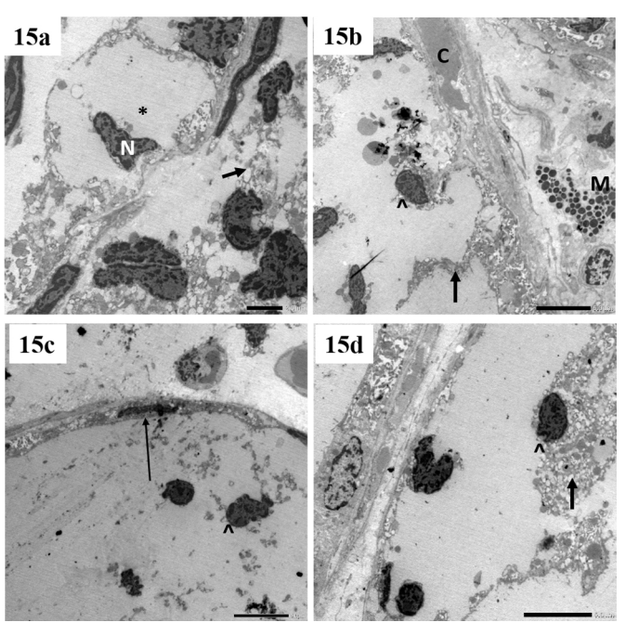

Fig. 15 a-d: Electron micrographs of subgroup IIc show (Fig.15a): Swollen follicular cell with empty cytoplasm (*) and irregular elongated nucleus (N) notice abnormal alignment of follicular cells and loss of cell junctions. (Fig. 15b): detached abnormal follicular cells in the lumen (^) Notice capillary congestion (c), & mast cell (M). (Fig. 15 c-d): thin wall follicular cells with flat dense nucleus (↑) & detached follicular cells (^). Abnormal secretion in the lumen (thick arrow) (Mic Mag a x2000, b -c x1200 &d x1500)

Table 1: Body weight (gm) and thyroid weight (gm) in the different studied groups

	Control group (Group I)	Subgroup IIa	Subgroup IIb	Subgroup IIc	ANOVA *P value*
Body weight (gm)	240.9±12.4	245.0±14.1	260.8±25.7	252.6±6.3	
P1		0.272 N.S.	0.034*	0.0159*	6.25
P2			0.075 N.S.	0.091 N.S.	0.023*
P3				0.200 N.S.	
Thyroid weight (gm)	0.159±0.013	0.163±0.014	0.346±0.049	0.315±0.054	
P1		0.28 N.S.	0.001*	0.001*	10.8
P2			0.001*	0.001*	0.002*
P3				0.13 N.S.	

Data was presented as mean±S.D.
P1 comparison between control group and other subgroups.
P3 comparsion between subgroup IIb and IIc.
P was significant if < 0.05
P2 comparison between subgroup IIa and both IIb, IIc.
* Signfiicant difference

Table 2: Free T3, T4 and TSH in the different studied groups

	Control group (Group I)	Subgroup IIa	Subgroup IIb	Subgroup IIc	ANOVA *P value*
Free T3	73.9±1.2	71.0±0.9	68.9±1.1	65.3±1.0	
P1		0.001*	0.001*	0.001*	88.9
P2			0.00052	0.00000	0.0001*
P3				0.00001	
T4	3.5±0.5	3.0±0.5	2.5±0.5	1.9±0.6	
P1		0.041*	0.0011*	0.001*	12.19
P2			0.04121	0.00095	0.001*
P3				0.02626	
TSH	0.9±0.0	0.8±0.1	1.1±0.2	0.6±0.2	
P1		0.001*	0.0734	0.001*	15.87
P2			0.00093	0.01972	0.001*
P3				0.00018	

Data was presented as mean±S.D.
P1 comparison between control group and other subgroups.
P3 comparsion between subgroup IIb and IIc.
P was significant if < 0.05
P2 comparison between subgroup IIa and both IIb, IIc.
* Signfiicant difference

Table 3: MDA and SOD in the different studied groups

	Control group (Group I)	Subgroup IIa	Subgroup IIb	Subgroup IIc	ANOVA *P value*
MDA	7.0±0.6	7.1±0.9	13.6±3.4	15.9±1.5	
P1		0.44 N.S.	0.001*	0.001*	28.5
P2			0.001*	0.001*	0.0003*
P3				0.001*	
SOD	57.9±1.5	57.4±2.4	53.0±2.8	50.3±1.0	
P1		0.31 N.S.	0.001*	0.001*	31.3
P2			0.001*	0.001*	0.0001*
P3				0.01*	

Data was presented as mean±S.D.
P1 comparison between control group and other subgroups.
P3 comparsion between subgroup IIb and IIc.
P was significant if < 0.05
P2 comparison between subgroup IIa and both IIb, IIc.
* Signfiicant difference

Table 4: Follicular cell height and area% colloid in the different studied groups

	Control group (Group I)	Subgroup IIa	Subgroup IIb	Subgroup IIc	ANOVA *P value*
Follicular cell height	17.0±5.0	16.5±3.7	24.4±5.4	20.5±1.1	
P1		0.41 N.S.	0.01*	0.04*	9.2
P2			0.001*	0.01*	0.001*
P3				0.03*	
area% colloid	9.7±1.6	8.8±0.6	7.6±0.9	4.0±1.2	
P1		0.09 N.S.	0.001*	0.001*	19.52
P2			0.001*	0.001*	0.001*
P3				0.001*	

Data was presented as mean±S.D.
P1 comparison between control group and other subgroups.
P3 comparsion between subgroup IIb and IIc.
P was significant if < 0.05
P2 comparison between subgroup IIa and both IIb, IIc.
* Signfiicant difference

DISCUSSION

WHO identified the endocrine disruptor compounds (EDCs) as exogenous substances or mixtures that would interact with the endocrine system and its hormonal mechanism of actions in a way that seriously affect health. The list of these compounds is increasing every year. Their effects are dose, duration and mean of exposure dependent. The adverse effects might be modulated, to some extent, by the natural body reaction that would limit their hazards[7,15]. Many hypotheses tried to explain EDCs mechanisms and subsequently their possible effects. It might be one or more mechanism that ends in their disrupting effect. Interaction with hormone receptors with subsequent cellular response is a possible explanation. Another theory is supposing them as being antagonists; besides this receptor-mediated response. EDCs might also interfere with the hormone transport or with the metabolic processes[16,17]. A concern has been raised about the possible endocrine disrupting effect of some synthetic antioxidants including BHA as being interacting with sex hormones[18]. Butylated hydroxyanisol (BHA) is a popular synthetic antioxidant with wide uses especially as food preservative for products that need long preservation as preventing rancidity of oil content. Additionally, it enters in cosmetics and pharmaceuticals industry. BHA is generally reported for decades as a safe compound, however it has recently started to be classified as a suspicious endocrine-disrupting compound[14].

The current in-*vivo* study included anatomical, biochemical and histological assessment methods to identify the possible effect of oral intake of different doses of BHA; 10, 100 and 500 mg/ kg body weight on the thyroid functions of the experimental animals.

Anatomical assessment showed that rats received the low dose of BHA (10 mg/ kg body weight), had no significant changes as compared to the control groups in their body weight. While those received the moderate dose (100 mg/ kg body weight) of BHA gained weight significantly. This weight gain concurred with hormonal level changes denoting hypothyroidism with subsequent myxedematous changes. Experimental animals received the high dose (500 mg/ kg body weight) of BHA showed significant increase in body weight but lower than those received moderate doses denoting severe concomitant general toxicity going in hand with the anorexia, diminished water intake and lethargy noted in animals of this group during the last 2 weeks of the study.

As for the estimate of thyroid gland weight by the end of the study and just after sacrifice of the animals, non-significant weight gain has been reported with the low dose intake. Moderate and high doses showed significant increase in the gland weight as compared to the normal. The increased weight of the gland is attributable to tissue changes reveled in microscopic examination; congestion and edema.

The T3 & T4 serum levels were declined significantly with positive feedback effect on TSH that markedly increased by the end of the experimental period with moderate dose intake (subgroup IIb). With high dose intake (subgroup IIc), there was marked decline on both T3 & T4 serum levels however TSH serum level was declined also in this group with no feedback elevation that might be attributable to concomitant pituitary effect. A further study is needed for detailed examination of this possible effect. The biochemical assay of the antioxidant enzymes and lipid peroxidation confirmed the thyroid disrupting effect of the drug as identified by significant increase in MDA and decrease in SOD with moderate (subgroup IIb) and high (subgroup IIc) doses of BHA. The changes were dose dependent and more significant as doses increased. The changes in subgroup Ia were insignificant.

Histological examination of subgroup IIa showed that the thyroid follicles were large, rounded and lined by single layer of vacuolated follicular cells and multiple layers of follicular cells in some follicles. Increase in the number of layers and height of the lining follicular cells with extensive vacuolated cytoplasm and apparent dilatation of endoplasmic reticulum in subgroup II b semithin sections were noted. Semithin sections of subgroup II c showed more disrupting effects as: variable thyroid follicles. Follicular cells appeared with various shapes while some

cells were detached in the lumen. Dense nuclei and extensive vacuolated and empty cytoplasm were observed. These results were also confirmed by morphometric study that revealed significant decrease in area colloid % in subgroup IIb &IIc compared to control group and subgroup IIa. On the other hand follicular height in subgroups IIb and IIc depicted significant increase due to vacuolated swollen cytoplasm despite the multiple detached cells.

Electron photomicrographs of experimental groups revealed double layers of follicular cells with rounded small nuclei and dilated endoplasmic reticulum as in subgroup IIa. These changes became more evident in other high doses groups as in the form of bizarre- shaped irregular thyroid follicles lined by vacuolated swollen cells with darkly stained nuclei, decreased amount of lysosomes and dilated endoplasmic reticulum with retained secretion.

Multiple follicles in subgroup IIc were completely destructed while others were lined by flat follicular cell with flat dense nuclei. Scattered detached small cells & loss of colloid secretion were also marked in subgroup IIc.

These results were confirmed by the decline biochemical levels of free T3 and T4 in experimental groups. However, TSH depicted no significant changes in subgroup IIa, significant increase in subgroup IIb and significant decrease in subgroup IIc compared to the control group that also explained the changes of oxidative stress markers used in this study.

All the 3 methods of assessment denoted that BHA had a thyroid disrupting effect that was determined through histological assessment and confirmed by both anatomical examination and biochemical assays. With BHA administration for 6 weeks, the effect was dose dependent. Low dose administration revealed mild histological changes with insignificant effect on body and thyroid weights and on hormonal and oxidative markers assay. Although changes were insignificant, a more profound effect with prolonged administration is expected in view of the present histological changes specially that both anatomical and biochemical markers were affected even if it was insignificant. Moderate (100 mg/ kg body weight) and high (500 mg/ kg body weight) doses administration for 6 weeks revealed histological with anatomical and biochemical significant effects. With the highest dose, general toxicity and expected multiple glandular disruption is expected especially with reported declined TSH serum level.

The current findings agreed with the hypothesis of Pop A *et al*.[7] who suggested a possible endocrine disrupting effect of BHA in humans but with lack of sufficient data at their hands, it wouldn't possible to draw it with certainty.

In accordance of the current data, Jeong SH *et al*.[8], reported a similar histological finding on the thyroid gland of female rats administrated BHA at 500 mg/ kg body weight for 13 weeks (a longer period than the current study design). The findings lack ultrastructural detailed examination of the gland and also toxicity is reported at shorter duration. Agreeing with the current study, Klopčič I& Dolenc MS[19], as studied the effect of BHA in combination with 2-methylresorcinol (2MR) and avobenzone (AVB) based on the fact that they usually used together in personal care products and cosmetics, reported that the tested compounds AVB, 2MR, and BHA showed anti-androgen-, (anti)-glucocorticoid-, and (anti)thyroid hormone-like effects. Moreover, mixtures of two or three of them had different potential endocrine consequences.

Mechanism of action

The possible toxicity of BHA might be explained by having a potent cytotoxic effect as explained by Park *et al*.[20] on their study. They suggested an antigrowth effect of the compound that would arrest the cell cycle and then negatively affect the regulatory protein expression. Both phosphoinositide 3-kinase/protein kinase B and extracellular signal-regulated kinase 1/2 were diminished in the astrocytes with increased the levels of pro-apoptotic proteins, such as BAX, cytochrome c, cleaved caspase 3, and cleaved caspase 9. The level of anti-apoptotic protein BCL-XL was diminished. BHA treatment caused increase in the cytosolic calcium level and then the expression of endoplasmic reticulum stress proteins. This explanation mimicked the current results' ultrastructural finding of dilated ER in group IIb and more marked dilatation with increased dose of BHA administration in subgroup IIc.

BHA has a similar biochemical structure of bisphenol A (BPA). Both are phenol substrates and ends up into phenoxyl radicals with subsequent comparable effects and mechanism of action[21]. Another hypothesis was carried out by the work of Sheng ZG *et al*. who reported a thyroid disrupting effect of BPA similar to and also explaining the biochemical data reported in the present work. They explained the thyroid disrupting effect of low concentration of BPA through suppression of thyroid receptor (TR) mediated transaction that executed the thyroid hormones (TH) mediated integrin through a non-genomic action[22].

CONCLUSION

BHA has a possible thyroid disrupting effect in long term intake. This effect is dose dependent. Low dose administration of BHA although insignificantly affects the thyroid gland, still has a potential disrupting effect with prolonged administration. Moderate and high doses administration of the compound for long period expected to have serious effects on the thyroid functions with resultant hypothyroidism and myxedema. High dose administration might have concomitant serious effects both generally and on other endocrine glands possibly the pituitary. More studies are recommended to evaluate it. The usage of BHA as a food preservative for dietary products with long shelf life as fried potatoes and snakes is crucial and should be reconsidered especially that the consumers of these products are usually children at growing age.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Kolar B, Kouba M, López-Alonso M, Puente SL, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Lundebye AK, Nebbia C, Renshaw D, Innocenti ML, Gropp J. Safety and efficacy of butylated hydroxyanisole (BHA) as a feed additive for all animal species. EFSA J. 2018; 16(3): e05215
2. G.M.Williams, M.J.Iatropoulos, J. Whysnera. Safety Assessment of Butylated Hydroxyanisole and Butylated Hydroxytoluene as Antioxidant Food Additives. Food and Chemical Toxicology 1999; 37 (9- 10): 1027- 38
3. F. Shahidi, Y. Zhong. Antioxidants: regulatory status. (sixth ed.)F. Shahidi (Ed.), Bailey's Industrial Oil and Fat Products, vol. 1, John Wiley & Sons (2005), pp. 491-512
4. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Maryline Kouba, Mojca Kos Durjava, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Gabriele Aquilina, Georges Bories, Jürgen Gropp, Carlo Nebbia, Matteo Lorenzo Innocenti. Safety of butylated hydroxy anisole (BHA) for all animal species. FSA J. 2019;17(12): e05913
5. EFSA, EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). Scientific Opinion on the re-evaluation of butylated hydroxyanisole – BHA (E 320) as a food additive EFSA J.2011; 9 (10): 2392
6. Felter SP, Zhang X, Thompson C. Butylated hydroxyanisole: Carcinogenic food additive to be avoided or harmless antioxidant important to protect food supply? Regul Toxicol Pharmacol. 2021; 121:104887
7. Pop A, Kiss B, Loghin F. Endocrine disrupting effects of butylated hydroxyanisole (BHA - E320). Clujul Med. 2013; 86(1):16-20
8. Jeong SH, Kim BY, Kang HG, Ku HO, Cho JH. Effects of butylated hydroxyanisole on the development and functions of reproductive system in rats. Toxicology. 2005; 208(1):49-62
9. Kuriyama SN, Wanner A, Fidalgo-Neto AA, Talsness CE, Koerner W, Chahoud I. Developmental exposure to low-dose PBDE-99: tissue distribution and thyroid hormone levels. Toxicology. 2007; 242(1-3):80-90.
10. Badr El Dine FMM, Nabil IM, Dwedar FI. The effect of Tributyltin on thyroid follicular cells of adult male albino rats and the possible protective role of green tea: a toxicological, histological and biochemical study. Egypt J Forensic Sci. 2017; 7(1): 7
11. Lowry OH, Roserrough NJ, Farr AL, Randall RJ; Protein measurement with folin phenol reagent. J Biol Chem., 1951; 193: 265-275
12. Ohkawa H, Ohishi N, Yagi Y. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95(2):351-8
13. Hayat MA (ed) (2000). Chemical fixation. In: Principles and techniques of electron microscopy: biological applications. 4th edn. Cambridge University Press, UK, pp. 4-85
14. Dawson-Saunders Band Trapp R. Basic and clinical biostatics. 3rd ed., Lang Medical Book, McGrow Hill Medical Publishing Division. 2001; 161-218
15. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-Disrupting Chemicals: an Endocrine Society scientific statement. Endocr Rev. 2009; 30(4):293-342
16. Wuttke W, Jarry H, Seidlova-Wuttke D. Definition, classification and mechanism of action of endocrine disrupting chemicals. Hormones (Athenes). 2010; 9(1):9-15
17. Waring RH, Ayers S, Gescher A J, Glatt H-R, Meinl W, Jarratt P, Kirk C J, Pettitt T, Rea D, Harris R M. Phytoestrogens and xenoestrogens: The contribution of diet and environment to endocrine disruption. J Steroid Biochem Mol Biol, 2008; 108(3- 5):213-20
18. Nilsson R. Endocrine modulators in the food chain and environment. Toxicol Pathol. 2000; 28(3):420-431
19. Klopčič I, Dolenc MS. Endocrine Activity of AVB, 2MR, BHA, and Their Mixtures. Toxicological Sciences. 2017; 156 (1): 240–251
20. Park S, Lee JY, Lim W, You S, Song G. Correction to Butylated Hydroxyanisole Exerts Neurotoxic Effects by Promoting Cytosolic Calcium Accumulation and Endoplasmic Reticulum Stress in Astrocytes. J Agric Food Chem. 2019; 67(40):11277
21. Babu S , Uppu S, Claville MO, Uppu RM. Prooxidant actions of bisphenol A (BPA) phenoxyl radicals: implications to BPA-related oxidative stress and toxicity. Toxicol Mech Methods. 2013;23(4):273-80
22. Sheng ZG ,Tang Y, Liu YX, Yuan Y, Zhao BQ, Chao XJ, Zhu BZ. Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. Toxicol Appl Pharmacol. 2012; 259(1):133-42

الملخص العربى

التأثير المعطل المحتمل لجرعات مختلفة من هيدروكسيانيزول بوتيل (BHA) على الخلايا الحويصلية للغدة الدرقية في ذكور الجرذان البيضاء البالغة: دراسة تشريحية ونسيجية وكيميائية

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الخلفية: هيدروكسيانيزول بوتيل (BHA) هو أحد مضادات الأكسدة الاصطناعية التي يتم امتصاصها بشكل رئيسي من خلال الجهاز الهضمي بعد تناوله عن طريق الفم. تم ادخاله إلى صناعة الأغذية كمادة حافظة آمنة وأيضا في الصناعات الدوائية في تصنيع مستحضرات التجميل وكمضاف لمنع التزنخ للفيتامينات التي تذوب في الدهون. وافقت إدارة الغذاء والدواء الأمريكية (FDA) على العقار كمضاف غذائي آمن بجرعة فموية قصوى تبلغ ١٥٠ مجم / كجم.
لقد تم طرح سؤال جديد عما إذا كان العقار مادة مسرطنة محتملة للإنسان؟ هناك خطر محتمل آخر هو أيضًا مصدر قلق حديث حيث تم ربط المركب ببعض اضطرابات الغدد الصماء. يرتبط تأثير الغدد الصماء للعقار بالهرمونات الجنسية، وقد عُرف القليل عن التأثير الدقيق عليها وعلى الهرمونات الأخرى أيضا.
الهدف من البحث: يهدف هذا البحث إلى تقييم التأثير المحتمل للجرعات المختلفة من هيدروكسيانيزول بوتيل (BHA) على الخلايا الحويصلية للغدة الدرقية.
المواد وطرق البحث: أجريت هذه الدراسة على ٣٠ من ذكور الجرذان البيضاء البالغة. ١٢ جرذ كمجموعة محكمة و١٨ جرذ مقسمة عشوائيا بالتساوي إلى ٣ مجموعات فرعية وتلقت كل مجموعة منهم ١٠ و١٠٠ و ٥٠٠ مجم / كجم من وزن الجسم من هيدروكسيانيزول بوتيل (BHA) المذاب في زيت الذرة عن طريق الفم لمدة ٤٢ يومًا. تم فحص جميع الحيوانات لمعرفة وزن الجسم والغدة الدرقية وهرمونات الغدة الدرقية ومستويات التأكسد. تم أيضا إجراء فحص ميكروسكوبي لأنسجة والخلايا الحويصلية للغدة الدرقية.
النتائج: كشفت جميع البيانات عن وجود تأثير للجرعات المختلفة من مركب هيدروكسيانيزول بوتيل (BHA) على والخلايا الحويصلية للغدة الدرقية بالفحص الميكروسكوبي. تم تأكيد النتائج النسيجية من خلال الفحوصات التشريحية والكيميائية الحيوية.
الاستنتاج: ان مركب BHA له تأثير محتمل في اضطراب الغدة الدرقية عند تناوله على المدى الطويل. هذا التأثير يعتمد على الجرعة و قد تم رصده بشكل فارق احصائيا في الجرعات المتوسطة والعالية.