EVALUATION OF THE POSSIBLE ENDOCRINE DISRUPTIVE EFFECT OF BUTYLATED HYDROXYANISOLE, BUTYLATED HYDROXYTOLUENE AND PROPYL GALLATE IN IMMATURE FEMALE RATS

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Abstract

We evaluated the possible endocrine disruptive effect of butylated hydroxyanisole (BHA) (300mg/kg bw), butylated hydroxytoluene (BHT) (75mg/kg bw) and propyl gallate (PG) (405mg/kg bw), compounds extensively used as antioxidants in foods, food packaging, cosmetics and pharmaceuticals using the immature rat uterotrophic assay. To investigate their estrogenic and/or antiestrogenic effect we used 17-21 days old Wistar female rats that were given suspensions of the studied compounds for three consecutive days. Absolute and relative uterus weights were significantly decreased by all three compounds, while endometrial epithelium thickness was significantly affected only by propyl gallate when compared to the negative control. The data obtained from the present study suggests that BHA, BHT and PG indeed have endocrine disruptive effects on Wistar prepubescent female rats, the most aggressive compound being PG.

Rezumat

S-a evaluat posibilul efect perturbator endocrin al butilhidroxianisolului (300mg/kg corp), butilhidroxitoluenului (75mg/kg corp) şi propil galatului (405mg/kg corp), compuşi folosiţi ca antioxidanţi în industria alimentară, a ambalajelor, cosmeticelor, produselor farmaceutice, folosind testul uterotrofic la şobolani. Pentru a investiga efectele estrogenice/antiestrogenice s-au folosit femele de şobolan Wistar de 17-21 de zile cărora li s-au administrat suspensii ale compuşilor aflaţi în studiu timp de trei zile consecutive. Greutăţile absolute şi relative ale uterului au scăzut semnificativ la toate loturile aflate în studiu, iar grosimea epiteliului endometrului a scăzut semnificativ faţă de lotul control doar

la animalele care au primit propil galat. Datele obţinute în acest studiu indică faptul că BHA, BHT şi PG au efect perturbator endocrin la concentraţiile testate, efectul cel mai pronunţat fiind în cazul propil galatului.

Keywords: butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, immature rat uterotrophic assay

Introduction

An endocrine disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. The family of endocrine disrupting chemicals (EDCs) includes pharmaceutical estrogenic compounds, antioxidants, phytoestrogens, pesticides, plastic manufacturing chemicals, detergents, heavy metals, preservatives from cosmetics [2, 4-7, 14, 15, 25].

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are extensively used as antioxidants in foods, food packaging, cosmetics and pharmaceuticals. In the past years, all three compounds raised concerns regarding their possible endocrine disrupting effect. The existing *in vitro* studies indicate that BHA (E320) presents weak estrogenic effect and also anti-androgenic properties. BHT (E321) was proved to be less estrogenic than BHA, and based on the cell proliferation assays it was included in the nonestrogenic chemicals list. After being tested *in vitro* PG (E310) was found to be one of the strongest ligands among the xenochemicals that are known as ERα binders, but without inducing any transactivation activity at the concentrations tested. BHA was also found to have antiestrogenic properties in one *in vivo* study. [1, 10, 12, 13,17, 18, 20, 24, 27, 29].

Studies that estimate the daily intake of BHA and BHT showed that through an average diet the population can get close to the ADI (acceptable daily intake) and that a fraction of the population might be exposed to doses superior to ADI [11, 28].

The objective of this work was to assess the effects of BHA, BHT and PG at concentrations higher than the average diet exposure on genital female tract using the immature rat uterotrophic assay [8, 23].

Materials and Methods

Chemicals

BHA, BHT, PG, 17-β estradiol were purchased from Sigma-Aldrich (USA), buffered formalin from Chempur (Poland).

Animals and housing

17-21 days Wistar female rats were purchased from the Practical Skills and Experimental Medicine Center of the ‘‘Iuliu Hatieganu’’ University of Medicine and Pharmacy, Romania. The rats were kept in standard conditions of temperature, humidity, day/night cycle and they had access to food and water *ad libitum* throughout the experiment. Body weight and clinical signs were recorded daily.

Experimental protocol

The experimental protocol was in compliance with the institutional and European guidelines for laboratory animal experiments, being approved by the Ethics Committee of the University. The protocol included three experimental groups and two control groups, each consisting of 10 animals. Chemicals were administrated once per day for three consecutive days in the morning between 9-10a.m. The doses studied represented three times NOAEL (No Observable Adverse Effects Level) of each compound. The tested compounds and the negative control (vehicle) were given as an oral suspension at doses of 300 mg/kg bw for BHA, 75mg/kg bw for BHT and 405mg/kg bw for PG while 17-beta estradiol (positive control) was given by subcutaneous injection into dorsal surface at a dose of 20μg/kg in sun flower oil. The rats were weighed and sacrificed using diethyl ether at 24hrs after the last treatment. Besides the genital tract, the liver, spleen and also kidneys were removed and weighed.

Tissue collection

After removal, the genital tract (ovaries, oviduct, uterine horns, body, cervix and vagina) was weighed and immediately immersed for fixation in 10% buffered formalin for at least 24 hours. The sectioning and trimming were performed in accordance with the goRENI standards [26]. Longitudinal sections through the uterus, the utero-cervical junction, cervix and vagina (as a single unit) as well as transversal section through the midhorn were prepared.

Tissues were embedded in paraffin according to standard histological techniques [3].

Histopathological and morphometric assessment

Slides containing H&E (hematoxylin and eosin) stained tissue samples were blindly evaluated by two pathologists for pathologic changes using conventional light microscopy (Olympus BX 51 microscope equipped with Olympus SP 350 digital camera). Pathological assessments were

evaluated according to standardized National Toxicology Program (NTP) pathology codes.

Morphometric analysis was performed on midhorn cross sections of both uterine horns for all animals (n = 10 per treatment group) using Olympus Stream Basic image analysis software. As previously described, we quantified the length of basal lamina underlying the luminal epithelium (LE) and corresponding areas of LE, stroma, and myometrium for multiple representative sectors of each section [9, 16]. Total luminal and glandular circumferences were also quantified.

Morphometric analysis was limited to LE cell height, stromal thickness, myometrium thickness and full-thickness of uterine horns, as these parameters capture the most sensitive histological endpoints in the uterotrophic assay [9,16]. For each animal we averaged the cell height/thickness of at least three locations on the H&E slides.

Statistical analysis on all morphometry data were performed using Shapiro-Wilk normality test followed by one way ANOVA and by the two-sample t-test, using R' software. The results were expressed in mean±standard error (S.E.M.).

Results and Discussion

Body weight gain, organ weights

Except for the controls, both negative and positive, no significant increase or decrease in body weight gain was observed in the treated groups.

As seen in the Figure 1 a-c, the relative liver, kidneys and spleen weights were not affected by the treatment with BHA, BHT and PG. Relative uterine and ovaries weights (Figure 1d) were significantly decreased by all the tested compounds compared to the negative control, propyl gallate having the most visible effect.

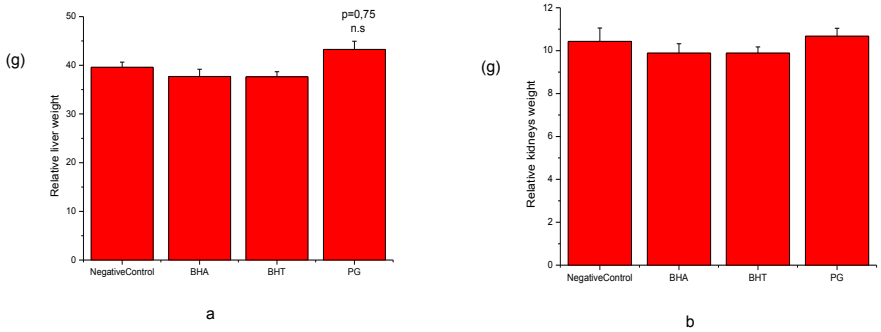

Figure 1 a-b

The relative weight of: a) liver, b) kidneys after the 3 days treatment

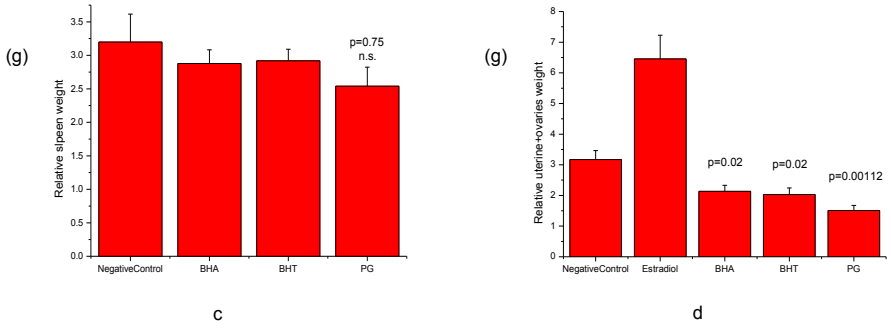

Figure 1 c-d

The relative weight of: c) spleen and d) uterus+ovaries after the 3 days treatment

Histopathology

The histological sections from the negative control animals, showed no estrogenic changes (Figure 2A and 2B). On the other hand, examination of the uterus, cervix and vagina of estradiol treated rats, revealed several characteristic, estrogenic changes (Figure 2C and 2D). In the uterus, there was mild edema of the endometrium characterized by separation of the endometrial stromal cells, accompanied by moderate thickening (hyperplasia) of the uterine mucosal and endometrial glandular epithelia. Apoptotic endometrial epithelial cells were also observed as well as mitoses and some lymphocytic infiltrate in the same layer. In all estradiol treated animals uterine luminal circumference was increased, with more pronounced invaginations of endometrial epithelium. Infiltrating neutrophils were present in the uterine and cervical stroma. The cervical and vaginal epithelium responded to estradiol stimulation by squamous hyperplasia and cornification of the epithelium.

Rats treated with BHA alone had minimal histopathologic changes in the uterus, with no changes in the cervix and vagina. Thus, in one animal we noticed moderate increase of luminal circumference, with more pronounced invaginations of endometrial epithelium into the stroma as well as a minimal endometrial stroma edema and minimal neutrophilic infiltrate (Figure 2E and 2F). From the BHT treated female rats, just in one case we observed a minimal endometrial stroma edema (Figure 2G and 2H), with no other visible morphological changes in the evaluated reproductive tract segments. There were no visible morphological changes in the uterine (Figure 2I and 2J), cervical and vaginal segments of the PG treated animals.

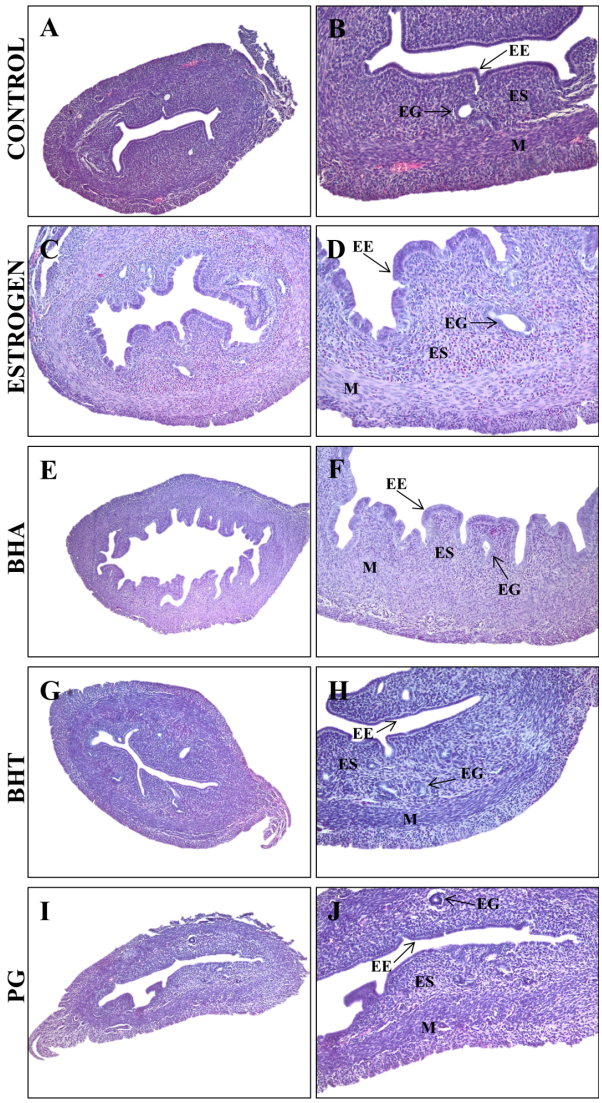

Figure 2

Histopathologic alterations in the rat uterus in control, estradiol, BHA, BHT or PG treated animals for 3 days from PND (post natal day) 17–21. Control Tissues (**A** and **B**): Note normal morphology of the uterus in a control rat; Estradiol treatment: (**C**), note moderate increase of luminal and glandular circumference, with increased invaginations of endometrial epithelium and increased number of uterine glands; (**D**), note moderate edema of the endometrial stroma (ES), moderate hyperplasia of endometrial epithelium (EE) and endometrial gland epithelium (EG), accompanied by myometrial (M) hypertrophy; a moderate transmural infiltrate with neutrophils (eosinophilic aspect) is also observed; BHA treatment: (**E**), note moderate increase of luminal circumference, with more pronounced invaginations of endometrial epithelium into the stroma; (**F**), note minimal edema of endometrial stroma (ES), no hyperplasia of endometrial epithelium (EE) and endometrial gland epithelium (EG), accompanied by minimal myometrial (M) hypertrophy; a minimal, transmural neutrophilic infiltrate is also present; BHT treatment: (**G**), note no changes in the uterine morphology; (**H**), note minimal edema of endometrial stroma (ES); PG treatment (**I** and **J**): absence of visible morphological changes; Left column, H&E 4×, original magnification; Right column, H&E 20x, original magnification.

Morphometric analysis

Endometrial epithelial hyperplasia of the uterine mucosa, stroma, myometrium and that of the uterine wall are considered histological markers of endocrine action [19, 21, 22]. As expected, endometrial epithelium cell height (EECH) was significantly increased in estradiol treated rat group, as compared to the other four groups. Interestingly, EECH of animals from BHT and PG treated groups was significantly decreased as compared to that of BHA treated group. Endometrial stroma (ES) of estradiol treated animals was significantly thicker than that of animals from BHA and PG treated group. Moreover, the PG treated rats had a significantly thinner ES as compared to that of BHT treated ones. Myometrial thickness was significantly increased in the estradiol treated group and BHT treated group as compared to the control group and to the PG group. The myometrium of animals from the PG treated group was significantly reduced as compared to that of the BHA treated group. A similar trend was observed regarding the uterine wall thickness, where the animals from the estradiol, BHA and BHT treated groups had significantly thicker walls as compared to the control group and to the PG group.

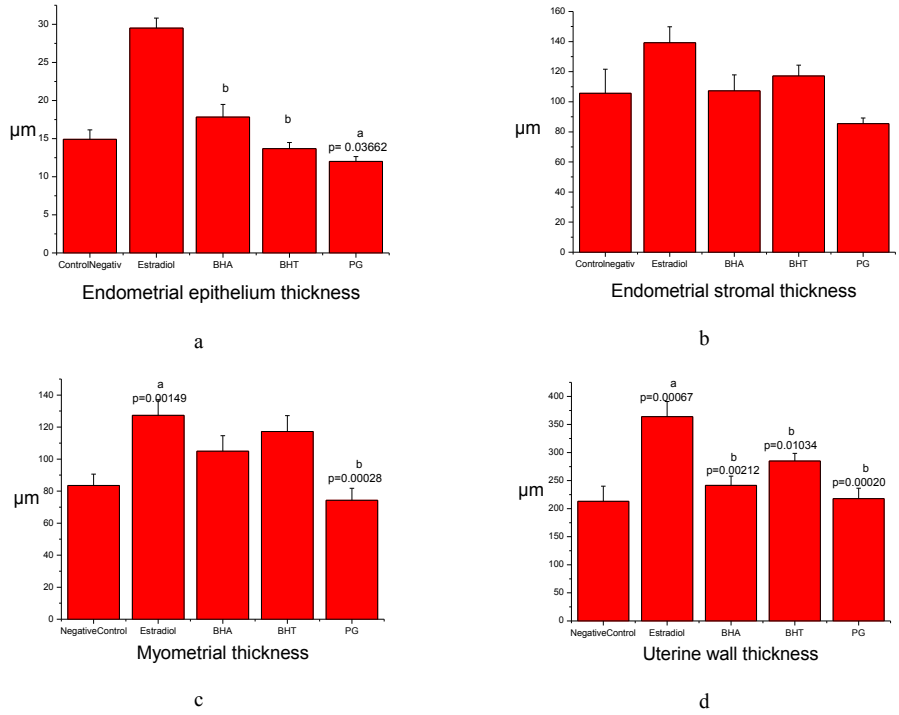

Figure 3

Morphometric analysis of changes in uterine cellular and compartmental structures.

Morphometric methods were used to quantify morphological indexes in histological cross sections from the midhorn uterine wall of Wistar female rats. Average endometrial epithelium cell height (EECH), stromal thickness (ES), myometrial thickness (M) and uterine wall thickness (U) were calculated for each animal. For EECH: (a) marked groups have significantly lower values as compared to the negative control group; (b) marked groups have significantly lower values as compared to the estradiol treated rats. For ES: no significantly differences between the animals from control groups, both positive and negative and the treated animals. For M: (a) marked groups have significantly higher values as compared to the untreated control group; (b) marked groups have significantly higher values as compared to estradiol treated group. For U: (a) marked groups have significantly higher values as compared to the untreated control group; (b) marked groups have significantly lower values as compared to estradiol treated control.

For all groups a confidence level of 95% ($p < 0.05$) was considered significant.

Information about the *in vivo* effects of BHA, BHT and PG on prepubescent female Wistar rats and uterine development is unknown or sparse. Consistent with previously published data about the presumed endocrine disrupting effects of the substances used in our study we conclude from the present *in vivo* study that BHT, BHA and PG have the ability to decrease the relative uterine weight of prepubescent female rats, PG having the most severe effect (Figure 1d). The mentioned data may be correlated with the morphometric analysis results. Thus, the endometrial epithelium cell height was decreased in the groups treated with BHT and PG, PG treated group being the one were the decrease was statistically significant ($p<0.05$) (Figure 3a).

According to previously published studies [13], BHA was not expected to have any influence on the endometrial epithelium cell height when compared to control, but in our study this parameter was increased, though not statistically significant, showing a possible estrogenic effect (Figure 3a)[13].

Other results yielded by the morphometric analysis can be associated with the decreased uterine weight such as the decreased endometrial stroma in the PG treated group and the significantly reduced myometral thickness, also in the PG treated group (Figure 3c and 3d).

Conclusions

The data obtained from the present study suggest possible endocrine disruptive effects at the dose of 3x NOAEL for BHA, BHT and PG in Wistar prepubescent female rats, with statistically significant changes showing the antiestrogenic properties of PG.

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