

ENDOCRINE DISRUPTING EFFECTS OF BUTYLATED HYDROXYANISOLE (BHA - E320)

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Abstract

Butylated hydroxyanisole (BHA) is extensively used as antioxidant in foods, food packaging, cosmetics and pharmaceuticals. In the past years, it raised concerns regarding its possible endocrine disrupting effect. The existing in vitro studies indicate that BHA presents a weak estrogenic effect and also anti-androgenic properties while an in vivo study found it to have antiestrogenic properties.

There is no sufficient data available at the moment to draw a conclusion regarding the safety of BHA when referring to its endocrine disrupting effect.

Since a fraction of the population might be exposed to doses superior to the acceptable daily intake (ADI), it is important to gather more in vitro and in vivo data concerning the potential effects that BHA might have alone, but also in mixtures with natural hormones or other endocrine disrupting compounds.

Keywords: butylated hydroxyanisole, endocrine disrupting effect, safety concern.

Introduction

According to the World Health Organization an endocrine disrupter 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” [1].

The number of the substances discovered to act as endocrine disruptors is increasing every year. Endocrine disrupting compounds (EDCs) have different origins and structures; they can be synthetic pharmaceutical estrogenic compounds such as diethylstilbestrol, pesticides of different classes, plastic manufacturing chemicals, detergents, heavy metals, cosmetics preservatives, antioxidants, natural occurring compounds (phytoestrogens) [2,3,4].

Numerous types of in vitro and in vivo studies tried to explain EDCs mechanisms, to monitor their presence and to study their effects. EDCs might act via more than just one mechanism. They might interact with a hormone receptor and activate a cellular response or act as antagonists; besides this receptor-mediated response, EDCs might also interfere with the hormone transport or with the metabolic processes [1-5].

Everyday exposure to this class of compounds, through food, air, water, cosmetic products can lead to

different kind of effects, a special attention being given to the ones that can accumulate in the environment and also in the fat tissue.

The consequences of EDCs exposure depend on the moment of the exposure, the period of exposure, the possibility for the normal homeostatic mechanisms to compensate the effects. The endocrine disrupting compounds are being incriminated of diminishing the quality and quantity of sperm, increasing the incidence of testicular, prostate and breast cancer, but also of inducing malformations of the male reproductive system, endometriosis, alterations of the thyroid and central nervous system functions, implication in the etiology of type 2 diabetes [1,2,6].

Most of the time, there is a significant difference between the high concentrations of the endocrine disruptors needed to produce effects in laboratory and the real concentrations found in environment or human blood or tissue.

Rajapakse et al. studied the effect of a mixture of xenoestrogens-estradiol, where all the xenoestrogens were at a level well below their no-observed-effect concentration (NOEC). The study has shown that EDCs at concentrations that alone would not produce any measurable effect were able to modulate the effects of 17 β -estradiol. The obtained result would encourage the idea that the biological activity of xenoestrogens should not be seen as insignificant just because of their low potency compared to estrogens. The

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number of the xenoestrogens in the environment and humans is likely to be large and by ignoring the combination effects it is possible to significantly underestimate the effects associated with the exposure to xenoestrogens [7].

Kang et al, went further more and besides analyzing the effect of estradiol-xenoestrogen mixture, they investigated the estrogenicity of a two weak estrogens mixture and found additive results, but the combination of the compounds at high concentrations gave a lower estrogenic response than the one expected by summing the individual activities [8].

Estimation of BHA exposure

Butylated hydroxyanisole (BHA) is a mixture of two isomers (2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole). The 3-isomer is considered to be a better antioxidant and represents 90% of the commercial BHA.

BHA is extensively used in bulk oils and oil-in-water emulsions. It proved to be a very effective protector in animal fats, but relatively ineffective in vegetable oils. BHA can be added to packaging materials in order to provide protection to foods inside the package through the volatilization of the antioxidant [9].

In Europe, the use of BHA is permitted in several foods like bouillons, gravies, dehydrated soups and dehydrated meat, individually or in combination with other antioxidants. The maximum limit is set to 200 mg/kg expressed on the fat content of the product [10].

According to the Directive 2006/52/EC of the European Parliament and of the European Council, when combination of gallates, BHA and BHT are used, the individual levels must be reduced proportionally [11].

The FDA limitations for BHA when used alone or in combination with other antioxidants varies as follows: between 2 ppm (in beverages and desserts prepared from dry mixtures) and 1000 ppm (in active dry yeast) for BHA only; between 10 ppm (in potato granules) and 200 ppm (in emulsion stabilizers for shortenings) for BHA and BHT in combination [12].

Different antioxidants (e.g. BHA and BHT) are present in cosmetic products also [13]. According to the Household Products Database, BHA is contained in 42 personal care products [14].

Recent estimates of BHA and BHT daily intakes showed that ingestion of these compounds through an average diet can get close to their acceptable daily intake (ADI). An important aspect is that the additional intake of BHA or BHT through pharmaceuticals could result in exceeding the ADI [15,16].

The exposure levels to additives by food intake were estimated in several countries, the results indicating that a fraction of the population might be exposed to doses superior to ADI. Given that there are significant differences regarding the eating behavior and manufacturing practices

between different countries, the results of the evaluation could not be extrapolated from one region to another [17].

There are several methods that were used to estimate the intake of BHA. The estimated intake expressed as % of the ADI was between: 1-100 of % ADI in case of estimation based on poundage data (disappearance) (the highest value was obtained in Spain probably due to the fact that in this country BHA addition was allowed for both solid foods and beverages); 3-50 of %ADI in case of assessments based on household economic surveys and sales data; 1-95 of %ADI in case of assessments based on model diets and 6-500 of %ADI based on individual dietary records. The estimates obtained based on the GSFA (General Standard for Food Additives) significantly overestimate the actual intakes in any country because it is presumed that the highest levels of additive are used in all foods [18].

In vitro evaluation of BHA endocrine disrupting effects

In recent years, concern has been expressed regarding the possible endocrine disrupting effect of BHA [19]. Several *in vitro* assays were performed to evaluate the potential of this antioxidant to mimic or interfere with the effects of sex hormones.

Soto et al. developed the E-SCREEN assay as a tool to identify potential estrogenic compounds. This assay is based on the proliferative effect of estrogens on target cells (MCF-7 breast cancer cells) and measures as an end point the cell number achieved in the presence of the test substance. The test is performed using a positive (in the presence of 17 β -estradiol) and negative (in the absence of estrogen) control.

Soto et al. evaluated the estrogenic potential of several antioxidants and plasticizers using the E-SCREEN *in vitro* test. BHA proved to be a weak estrogen at the tested concentration (50 μ M). In order to characterize the intensity of the estrogenic (proliferative) effect compared to that of 17 β -estradiol (E_2), the relative proliferative effect (RPE %) and relative proliferative potency (RPP %) were calculated. RPE was calculated as 100 x (PE-1) of the test compound/(PE-1) of E_2 , while RPP % was defined as the ratio between E_2 and test compound doses needed to produce maximal cell yields x 100. The calculated values for BHA for these two parameters were as follows: RPE = 30%, RPP = 0.00006 [20].

Jobling et al. also investigated the proliferative effect of BHA on MCF-7 human breast cancer cell line. Based on the observed cell proliferation, the tested compound proved to be a weak estrogenic [21].

The same cell line was used by Okubo and Kano to study the estrogenic activity of 66 food additives, including BHA. They also evaluated the capacity of these compounds to compete with E_2 for binding to the human ER α and ER β estrogen receptors. BHA induced cell

proliferation and had the capacity to compete with E_2 for binding to the estrogen receptors. The C_{max} (the concentration of test compound giving maximal cell proliferation) for BHA was 5×10^{-5} M, while in case of E_2 the maximum cell yield was achieved at 3×10^{-11} M. The RPE of BHA, defined by Okubo et al as the ratio between the maximal cell yield for the test compound and $E_2 \times 100$, was 66.8%. By using RT-PCR, the authors evaluated the gene expression of ER α and PR (progesterone receptor), following the treatment of MCF-7 cells with BHA. The selected antioxidant induced a decrease in gene expression of ER α and an increase in that of PR in a time-dependent manner (sampling at 0, 24 and 48 h) [22].

In a study using two transfected U2-OS (human osteoblasts devoid of endogenous estrogen receptors) ER α and β reporter gene cell lines, ter Veld et al. evaluated the estrogenic potency of several food-packaging-associated plasticizers and antioxidants. This model line allowed distinguishing the ER α and ER β agonist/antagonist properties of the tested xenobiotics, since these cells do not express simultaneously both type of ER. They stably express either ER α or ER β in addition to 3xERE-tata-Luc as a reporter gene. The luciferase assay showed that BHA was estrogenic in both the ER α and ER β cells. It is noteworthy that when compared to E_2 , the potency of BHA in the ER α was weaker compared to the ER β -mediated effect. The E_2 equivalency factors ($EEF_{10} = EC_{10 \text{ estradiol}}/EC_{10 \text{ test compound}}$) of BHA for the responses in ER α and ER β cell lines were of 5.2×10^{-8} and 7.7×10^{-7} , respectively. This difference regarding the estrogenic activity mediated by the two type of ER can be very important since it was suggested that binding of xenobiotics to ER β might modulate and reduce the ER α -mediated response. That could mean that activation of ER β could diminish or counteract the negative consequences of ER α mediated effects. Besides evaluating BHA alone, he was also tested in the presence of E_2 (used in concentrations which are relevant for real human exposure: 5 and 100 pM for the ER α and ER β cell lines). The exposure to the $E_2 + BHA$ combination revealed that the effects of the two compounds on the ERs are additive. This interaction is very important since it means that even if man is exposed to very small concentrations of xenobiotics (concentrations which are not associated with endocrine disrupting effects if exposure involves only one xenobiotic) given the additivity of the effects on ERs, each endocrine disruptor will contribute to the whole estrogen body burden. Given that real life human exposure to xenoestrogens involves simultaneous exposure to a great number of potential endocrine disruptors from the environment, foods, cosmetics and pharmaceuticals, it is very important to evaluate the possible interactions (additive, synergism, antagonism) between the different chemicals, or between these chemicals and endogenous hormones [23].

Amadasi et al. tried to identify food additives

with xenoestrogenic potential based on structure-activity relationship using an integrated in silico method, followed by an *in vitro* approach. BHA was identified as a potential ER α ligand. It presents the structural elements essential for the interaction with the binding domain of the ER α . These elements consist on the phenolic group in para position (which mimics that of E_2) and the aromatic ring acting as a hydrogen bond donor to the carboxyl group of Glu353 and as an acceptor from the guanidinium group of Arg394 [24].

Schrader et al. developed an androgen-responsive reporter gene assay by co-transfecting human PC-3 androgen-insensitive prostatic carcinoma cells with a human wild-type androgen receptor cDNA expression vector and a MMTV-firefly luciferase reporter gene construct. They used this cell line to evaluate the (anti)androgenic potential of BHA. The compound showed androgenic properties when tested alone, but it had the capacity to antagonize the activation of AR by DHT (dihydrotestosterone), without affecting cell viability. This study indicated clearly that BHA could act as androgenic antagonist. In case of BHA, the existing studies indicate that it presents dual properties: anti-androgenic and estrogenic, two effects which could account for some undesired effects on human subjects and wildlife [25].

***In vivo* evaluation of BHA endocrine disrupting effects**

Kang et al. evaluated the (anti) estrogenic and (anti) androgenic properties of BHA by *in vivo* tests. The (anti) estrogenicity was evaluated in immature female rats exposed to BHA alone or in combination with E_2 for three consecutive days. BHA at all doses (50, 100, 250 and 500 mg/kg) significantly reduced the absolute and relative uterine weights when administered alone. When administered in a dose of 500 mg/kg, BHA also significantly decreased E_2 -stimulated increase of uterine and vaginal weight. The body weight gain was significantly decreased at 250 and 500 mg/kg BHA and 500 mg/kg BHA + 2 μ g/kg E_2 , while the liver weight was increased in both BHA and BHA+ E_2 treatment groups. The anti (androgenic) effect was evaluated through the Hershberger assay in castrated male rats orally exposed to BHA alone or in combination with testosterone propionate (TP) for 10 days. BHA alone and in combination with TP did not induce significant effects on androgen-dependent accessory sex organs (seminal vesicle/coagulative glands, glans penis, Cowper's gland, ventral prostate gland and levator ani plus bulbocavernosus muscle) weights. A significant increase was seen in the relative TP-stimulated ventral prostate weight at 250 mg/kg BHA, but the absolute and formalin-fixed weight was not significantly modified. No significant changes were observed in the testosterone and thyroxine levels in serum or in body weight gain for BHA alone, but simultaneous exposure to BHA (250 mg/kg)

and TP has led to increased body weight gain. BHA was able to increase relative liver and adrenal gland weight, but did not influence relative kidney and thyroid weights. TP alone and in combination with 250 mg/kg BHA decreased relative adrenal gland weight. All these changes induced by BHA alone or in combination with E₂ or TP suggest that the tested compound has anti-estrogenic activity in immature female rats and negligible effect on the androgenic activity in castrated male rats. The increased metabolism of estradiol by BHA was suggested as a possible explanation of the suppressive effect of BHA on uterine weight and E₂-stimulated weights of the uterus and vagina [26].

According to a previous study of Zhu et al., BHA was able to enhance estrogen metabolism (by the increase of liver microsomal glucuronidation and NADPH-dependent oxidation of E₂ and estrone) and to inhibit uterotrophic action in CD-1 mice [27].

Jeong et al studied the reproductive and developmental toxicity of BHA by exposure of male and female Sprague-Dawley rats during the pre-gestation, gestation and lactation periods and of their offspring until 13 weeks old via gavage with BHA (doses: 0, 10, 100 and 500 mg/kg/day). They reported some changes in hormonal levels, as follows: reduced serum testosterone (in mature male and male offspring) and thyroxine levels (in mature male rats) at 100 and 500 mg/kg/day BHA; increased serum cholesterol (the precursor of steroid hormones) with decreased serum thyroxine (female offspring), at 500 mg/kg/day. At 100 and 500 mg/kg, BHA was capable of decreasing the weights of vagina, testes and ventral prostate. In the meantime, the weights of liver, adrenal and thyroid gland increased. A negative impact of BHA was observed on the reproductive function, such as delayed sexual maturation (indicated as vaginal opening and preputial separation) at 500 mg/kg BHA, shortened estrous cycle, lower mating rate, slower sperm motility, smaller sperm size. The effects on vaginal opening and vagina weight suggested an anti-estrogenic activity of BHA [28].

In a previous study performed by the same research group, BHA showed weak binding affinity to the human androgen receptor in transformed yeast expressing human androgen receptor and responsive elements [29].

Limitations of present evaluation status

The problem we are facing is the everyday exposure to this compound from different types of products (food, cosmetics, pharmaceuticals), and more than that, its possibility to accumulate in the fat tissue [30].

A variable in the analysis and comparisons performed so far is the method used for the quantification of the estimated intake of the compound. All the models used have some weak points (for example, just because the products are “disappearing” from the shelf, it does not necessarily mean that they are consumed entirely); therefore some errors might appear and interfere with the

conclusions of the research.

Also, there are aspects that should not be ignored when considering the routes of exposure. For example, since EDC mixtures could be used in cosmetic products, for the evaluation of the risk of dermal absorption, the penetration enhancers and retardants should also be taken into consideration [31].

The *in vitro* tests represent a very powerful tool in identifying potential endocrine disruptors. They permit the identification of endocrine disruptors which exert their effects through specific receptor mediated mechanisms, but do not allow the identification of disruptors that act by modifying the metabolism of endogenous hormones. Also, they are not useful if it is not the parent compound that is responsible for the endocrine disruption, but its metabolites.

Besides that active parent compound problem, each *in vitro* study appears to have weaknesses. The yeast based assays are simple to perform, but there is a difference in permeability between the yeast cell wall and the mammalian cell membrane and also a lack of response to some estrogens and antiestrogens can be observed. The competitive ligand binding assay shows the compound's ability to compete with the endogenous ligand, but does not present any information regarding the initiation of inhibition of gene transcription. Cell proliferation assays have as interferences other mitogens besides estrogens that can influence the proliferation. A more specific and quicker way to investigate the estrogenic/antiestrogenic response is by using the reporter assay based on stably transfected cell line. The only inconvenient here would be using cells expressing both alpha and beta receptors, instead of cell expressing only one receptor [32].

Conclusion

Based on the existing data, it is difficult to draw a conclusion regarding the safety of BHA when referring to its endocrine disrupting effect.

BHA might be responsible for different endocrine disrupting effects in humans, but the lack of sufficient evidence does not allow any direct link towards this antioxidant. It might act alone, or together with physiological hormones or any other EDCs to which the population is exposed on a daily base.

A correlation between the epidemiological data (plasma levels in humans), the *in vitro* and *in vivo* tested concentration and also the *in vitro* and *in vivo* observed effects would be useful in predicting the impact of BHA on the population.

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