RESEARCH ARTICLE

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APOPTOSIS AND OXIDATIVE STRESS INDUCE BY FOOD PRESERVATIVE BUTYLATED HYDROXYTOLUENE IN THE LIVER OF ALBINO RATS: THE PROTECTIVE EFFECT OF VITAMIN E ACETATE

ABSTRACT:
The present study examined the apoptosis and the oxidative stress induced by the food preservative, butylated hydroxytoluene (BHT) in liver of albino rats and the protective role might be played by vitamin E was assessed. Apoptosis was evaluated via determination of cytochrome C release and the oxidative stress was assessed by determination of lipid peroxidation and the total antioxidant status in liver homogenate. It was found that feeding the animals 1% BHT supplemented food for 4 weeks resulted in a significant increase of cytochrome C release in medium containing liver mitochondria. This may provide evidence that apoptosis is a possible mechanism of cell death in liver tissues. Meanwhile, lipid peroxides levels measured as malondialdehyde (MDA) in liver tissue homogenates and antioxidant power significantly increased and decreased, respectively in the 1% BHT fed animal group. Feeding the animals 0.4% vitamin E acetate added to the 1% BHT supplemented food for 4 weeks resulted in a significant reduction in the level of cytochrome C release concomitant with an improvement of the antioxidant power and a reduction in the MDA induced by BHT alone. These findings may further suggest that BHT-induced apoptosis in liver is mediated, at least in part, by oxidative stress. Therefore, the protective effect of vitamin E acetate against BHT-induced hepatocyte apoptosis raised a great concern of adding vitamin E acetate to food and their preparations containing BHT.

KEY WORDS: Butylated Hydroxytoluene (BHT), apoptosis, antioxidant power.

INTRODUCTION:
Antioxidants may be present in foods as one of its component or may be added to preserve their lipid components from quality deterioration. Butylated hydroxytoluene (BHT), a free radical scavenger, may be present in foodstuffs as a result of migration from plastic packaging (WHO, 2000). It is the most commonly used food additive in preserved foods. Foods are stored according to usual household habits, freeze- dried, homogenized, and extracted three times with hexane containing BHT as a preservative (Botterweck et al., 2000). The use of BHT confers substantial benefits to man by improving the palatability of food and preventing oxidative spoilage of unsaturated fats and oils. It also provides protection against the pathological effects of reactive oxygen species associated with development of cancer, cardiovascular diseases and aging (Grillo and Dulout, 2006). BHT is also used in cosmetics and has several therapeutic effects (Simán and Eriksson, 1996). BHT was found to prevent hepatic damage induced by food oil (Terrazos-Luch et al., 1997) and to inhibit chemically induced lung tumor formation in rodents when applied prior to the carcinogen presumably by interfering with the carcinogen metabolism (Bomhard, et al., 1992). On the other hand, BHT was found to elicit toxic effects in experimental animals and humans (Guyton et al., 1993). It has the ability to enhance the growth of many types of neoplasm by acting as a promoting agent when applied after the carcinogen most probably by exerting selective cytotoxicity and killing normal cells which allow uninhibited clone expansion of neighboring initiated cells. It, also, causes stomach cancer in human (Baserga, 1997; Williams et al., 1999). Although apoptosis is a crucial process during development, maintenance of cell homeostasis and regulation of immune system, it is clear that derangement of apoptotic cascade and its regulation can

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occur in a variety of disease states. Apoptosis occurs in response to various apoptotic stimuli such as cytotoxic chemicals and ionizing radiation in a wide variety of cell types (Rudel, 1999).

Oxidative stress has been found to be a feature of hepatic toxicity associated with significant reduction in hepatic levels of vitamin E (Factor et al., 2000).

New evidence indicates that vitamin E in addition to its well characterized antioxidant function, can protect against cancer formation by enhancing immunological cell surveillance, as well as by affecting signal transduction pathways involved in regulation of cell proliferation and apoptosis (Brigelius-Flohé et al., 2002). The widespread use of BHT and the possibility to use BHT in the treatment of diseases, however, raises a demand on more data regarding the safety of the compound. The ability of the phenolic lipid soluble BHT compound to induce oxidative stress-mediated apoptosis in hepatic cells of albino rats and whether the lipid soluble antioxidant vitamin E acetate can interfere with the response of hepatocyte damage provide the aim of this study.

MATERIAL AND METHODS:

This study was carried out at the Zoology Department, Faculty of Science, Tanta University (2006-2007) on 80 Albino rats of both sexes weighing 180-200 g. Animals were kept under optimum conditions of humidity and temperature and fed the commercial pellet diet and water ad libitum.

Experimental design:

Butylated hydroxytoluene and vitamin E acetate were obtained from Sigma. Animals were fed diets containing 1% BHT for 4 weeks with and without vitamin E acetate which was added at a dose of 0.4% (Simán and Eriksson, 1996). The diets were prepared by dissolving each of the substance added and soaking pellet animal diet with an appropriate amount of the solution. The ether was then allowed to evaporate in a fuming house and the dry pellets were presented to the rats. The animals had free access to food and tap water throughout the experiment. Animals were divided into 4 equal groups of 20 animals each:

**Group 1:** received no supplemented food and served as control.

**Group 2:** animals were fed 1% BHT supplemented diet.

**Group 3:** animals were fed the 1% BHT supplemented diet with 0.4% vitamin E acetate.

**Group 4:** animals were fed 0.4% vitamin E acetate supplemented diet.

After 4 weeks, the animals were sacrificed by decapitation and livers were excised from the animals of each group.

I- Detection of apoptosis by determination of cytochrome C release:

Isolation of liver mitochondrial fraction: liver tissue was homogenized with a buffer containing 250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES-NaOH, pH 7.2, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,000 X g for 10 min to remove intact cells and nuclei and the supernatant was further centrifuged at 10000 X g at 4 °C for 10 min to precipitate the heavy membrane fraction of mitochondria. The mitochondrial pellet was suspended in a buffer containing 250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH2PO4, b5 mM sodium succinate, 25 uM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. Mitochondria were kept in ice and used within 2 h of preparation.

Cytochrome C release was studied in a medium containing, in mM: CaKαEGTA 1.9, K2EGTA 8.1, MgCl2 1.4, KH2PO4 3.0, dithiothreitol (DTT) 0.5, MES 100.0 (pH 7.1), imidazole 20.0, taurine 20.0, pyruvate 5.0, bovine serum albumin 2 mg/mL. Mitochondria at a final concentration of 4.2 mg/ml, were incubated in 1 ml of this medium for 30 min at 25°C and samples were taken for cytochrome C determination. The cytochrome C spectrum was recorded by a spectrophotometer. Optical density of clear supernatants was recorded against the medium as a reference at 414 nm (Appaix et al., 2000). Mitochondrial protein concentration was measured by the Biuret method using serum albumin as a standard.

II- Measurement of oxidative stress induced by BHT in liver:

1- Measurement of lipid peroxides:

One gram of each frozen liver sample was homogenized in 11 ml of 0.05 M phosphate buffer (pH 7.4) for 2 minutes. A half ml of liver homogenate was used to determine the level of the thiobarbituric acid reactive substances (TBARS) that were measured as malondialdehyde (MDA) according to the method of Mihara et al. (1980). The concentration of TBARS in the samples was expressed as MDA nmol /g wet liver.
2-Measurement of antioxidant power of liver:

The antioxidant power was measured by the ferric reducing antioxidant power (FRAP) according to Benzie and Strain (1999).

Reagent preparation:

Reagents included 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l 2, 4, 6-tripyridyl-s-triazine (TP) in 40 mmol/l HCl and 20 mmol/l FeCl₃·6H₂O solution. The working FRAP reagent was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml FeCl₃·6H₂O solution. 10 μl of sample was then added, along with 30 μl H₂O; final dilution of sample in reaction mixture was, therefore, 1/34. After mixing 3 ml of the working FRAP reagent with 400 μl tissue homogenate or standard solution in a test tube, warmed to 37°C and a reagent blank reading was taken (M1) at 593 nm; (A) reading were taken after 0.5 S and every 15 S thereafter during the monitoring period. The change in absorbance (Δ A₅₉₃nm) between the M1 readings was calculated for each sample and related to Δ A₅₉₃nm of a Fe standard solution tested in parallel. A blank reading with only the FRAP reagent was subtracted from the absorbance of the FRAP reagent with a sample to measure the actual FRAP value of each tube. The change in absorbance (ΔA) is proportional to the combined (total) ferric reducing / antioxidant power (FRAP value) of the antioxidants in the sample. The final results were expressed as micromole Trolox equivalents (TE) per gram wet liver (TE μmol/g, wet liver).

Statistical Analysis:

For statistical comparison the Student’s t-test was used and data are presented as means ± SEM. The data were analyzed for paired two-tailed distribution and the differences were considered significant at P < 0.05.

RESULTS:

I- Cytochrome C release induced by BHT in liver tissues:

Table 1 shows significant increase in the cytochrome C release representing possible apoptotic changes in the animals fed 1% BHT supplemented diet for 4 weeks compared to the normal control group. On the other hand, giving the animals 0.4% vitamin E acetate with diet along with 1% BHT supplemented food for 4 weeks effectively suppressed this apoptotic response. Meanwhile, samples obtained from vitamin E acetate (0.4%) fed group did not show any apoptotic effect by the vitamin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Cytochrome C release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 20)</td>
<td>(nmol/mg mitochondrial protein)</td>
</tr>
<tr>
<td>1</td>
<td>None (control)</td>
<td>0.040 ±0.003</td>
</tr>
<tr>
<td>2</td>
<td>1% BHT supplemented food</td>
<td>0.095 ± 0.007*</td>
</tr>
<tr>
<td>3</td>
<td>1% BHT + 0.4% Vit. E acetate</td>
<td>0.051 ±0.010**</td>
</tr>
<tr>
<td>4</td>
<td>0.4% Vit E acetate supplemented food</td>
<td>0.042 ±0.002</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM of 20 rats in each group.
*Significant difference from control group (p < 0.01).
** Significant difference from group 2 (p < 0.01).

II- Effect of BHT on the lipid peroxides and antioxidant power of liver:

Administration of 1% BHT supplemented diet for 4 weeks resulted in significant increase of total lipid peroxide levels measured as MDA in liver tissue homogenates (P < 0.05) and in significant decrease of antioxidant power of liver (P < 0.01) when compared with their corresponding control values. Meanwhile, giving the animals 0.4% vitamin E acetate with BHT supplemented diet daily for 4 weeks caused significant decrease in the peroxides (P < 0.05), as well as significant increase in TAS (P < 0.01) as compared with their corresponding values in the 1% BHT fed group (Tables 2&3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>MDA (nmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 20)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>None (control)</td>
<td>46.75 ± 2.79</td>
</tr>
<tr>
<td>2</td>
<td>1% BHT supplemented food</td>
<td>69.95 ± 6.67*</td>
</tr>
<tr>
<td>3</td>
<td>1% BHT + 0.4% Vit. E acetate supplemented food</td>
<td>47.65 ± 3.34**</td>
</tr>
<tr>
<td>4</td>
<td>0.4% Vit E acetate supplemented food</td>
<td>45.77 ±0.74</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM of 20 rats of each group.
* Significant difference from control group (p < 0.05).
** Significant difference from group 2 (p < 0.05).

Table 3. Effect of 1% BHT and vitamin E acetate on antioxidant power of rat liver

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>TAS (µmol/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 20)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>None (control)</td>
<td>223.7 ±10.7</td>
</tr>
<tr>
<td>2</td>
<td>1% BHT supplemented food</td>
<td>180 ± 6.6*</td>
</tr>
<tr>
<td>3</td>
<td>1% BHT + 0.4% Vit. E acetate supplemented food</td>
<td>233.2 ± 17.7**</td>
</tr>
<tr>
<td>4</td>
<td>0.4% Vit E acetate supplemented food</td>
<td>269.7 ±11.5*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM of 20 animals of each group.
*Significant difference from control group (p < 0.01).
** Significant difference from group 2 (p < 0.01).
DISCUSSION:

In contrast to David and Peter (1988) and Safer and Al-Nughamish (1999) who showed that BHT induced hepatic cell necrosis, the present data identified apoptosis as the possible mechanism of cell death in liver tissues of animals fed on 1% BHT supplemented diet. Although the mechanism through which BHT induces apoptosis in liver tissue is still unclear, several suggested mechanisms were reported. BHT induces apoptosis in tumorigenic and non-tumorigenic mouse and human lung cell lines through activation and down-regulation of protein kinase C and calpain (Miller et al., 1994; Lori et al., 1998) and that calpain mediates the induction of apoptosis in other cell types (Squier and Cohen, 1997). BHT may also activate calcium dependent endonuclease, resulting in DNA fragmentation which is a necessary step in the sequence of events in the process of apoptosis (Oikawa et al., 1998).

This study presents evidence that BHT induces apoptosis through induction of oxidative stress since feeding the animals on 1% BHT supplemented diet significantly increased lipid peroxides levels in liver tissue homogenates as compared to the control group. This is in accordance with the finding of Oikawa et al. (1998) who showed an increase of peroxides and H$_2$O$_2$ in cultured hepatocytes treated with BHT metabolites. Also, the oxidative metabolism of BHT generates several reactive species that mediate its adverse effects. Reactive oxygen species and other free radicals together with lipid peroxides have independently shown to exert toxic reactions, induce membrane lipid peroxidation and cellular damage (Rubbo et al., 1994; Szabó, 1996). So, ROS have been implicated as mediators in the process of apoptosis in response to many toxicants (Hsieh et al., 2001).

It has also been demonstrated that an increase of lipid peroxides level induces apoptosis by exerting early loss of cellular redox balance. This is based on the fact that among the multiple factors required for cell survival are molecular oxygen and antioxidants in the right proportion to maintain a delicate intracellular redox balance. A sustained perturbation of the latter may result in lethal oxidative cell injury and death that may cause apoptotic cell death (Wang et al., 2000).

On the other hand, Juan et al. (2000) demonstrated that mitochondrial oxidative stress mediated by lipid peroxides leads to decrease in the mitochondrial membrane potential and permeability transition which is an early event in the process of apoptosis. It involves the opening of the so called permeability transition pores and release of apoptogenic factors such as cytochrome C and procaspases 2, 3, 9 (Sultan and Sokolove, 2001). Moreover, it was suggested that, intracellular reactive oxygen species including H$_2$O$_2$, may act as second messengers in cell survival that may play a role in the DNA fragmentation and apoptosis (Kowaltowski et al., 2001). In the present study, feeding the animals on 1%BHT supplemented diet with 0.4% vitamin E acetate was effective in reducing the magnitude of oxidative stress induced by BHT as indicated by the decrease in the MDA level and the increase in the antioxidant power of liver homogenate. This effect of vitamin E may be due mainly to minimizing the role of ROS and lipid peroxides in reducing apoptosis. These findings might further support the assumption that BHT-induced hepatocyte apoptosis is mediated, at least in part, by oxidative stress.

In addition, the present data support the previous findings that vitamin E acetate may maintain the normal cellular redox balance and counteracts the causative events leading to apoptosis elicited by ROS. The decrease in cytochrome C release with the antioxidant vitamin E acetate, suggests that this antioxidant inhibits apoptosis by counteracting upstream events in the apoptosis cascade at the mitochondrial level (Cheng et al., 1998). Some synergistic in vivo interactions between BHT and vitamin E acetate that have been suggested by Kamal El-Din et al. (2000) may intensify the antioxidant properties of vitamin E acetate. So, the significant reduction in lipid peroxides level and the significant increase of TAS after adding vitamin E acetate may suggest a potential role in counteracting the deleterious effect of BHT. The present study suggests that vitamin E protects against apoptosis not only by scavenging reactive oxygen species, but also by inhibiting cytochrome C release, which means that its activity may exceed that of a mere antioxidant.

In conclusion, this study has shown that the commonly used food preservative BHT is hepatotoxic and may induce apoptosis in normal hepatocytes through oxidative sue mechanism and appreciated the protective effect of vitamin E acetate against BHT induced apoptosis in liver cells. Therefore, due to safety concerns, the present study recommends that vitamin E acetate is added to the food and other preparations containing BHT as a preservative.
REFERENCES:


الموت المبرمج لخلايا كبد الحيزان البيضاء والإجهاد النكاسدي الناتج عن تأثير إضافة المادة الحافظة بوتيل هيدروكسيلي تولوين للطعام: تأثير إضافة فيتامين E.

عماق مختار الأطرش
قسم علم الحيوان، كلية العلوم، جامعة طنطا

المضاف إلى هذه المادة الحافظة قد أحدثت تحسناً واضحاً في أداء هذا النغير لخلايا الكبد وكذلك انخفاضاً ذو دلالة إحصائية في الإجهاد النكاسدي لخلايا الكبد. وعلى هذا فإن إضافة فيتامين E إلى الطعام أو المستحضرات التي يستخدم في حفظها مادة بوتيل هيدروكسيلي تولوين يعتبر ذو فائدة كبيرة.

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