Toxic effects of butylated hydroxytoluene in rats

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Abstract

This study aimed to assess the acute toxicity in rats of heated and un heated butylated hydroxytoluene (BHT). Sunflower oil dissolved BHT, heated at 98±2ºC by a water bath. The animals were divided into five groups. The control group dosage orally with sunflower oil, the first group treated with 250 mg/kg BHT, the second group treated with 250 mg/kg heated BHT the third group treated with 500 mg/kg BHT and the fourth group treated with 500 mg/kg heated BHT. All groups received oral treatment. The results showed a substantial reduction in motor activity relative to other groups at a dose of 250 mg/kg heated BHT. There was a substantial distinction in the negative geotaxis test in groups of 500 mg/kg heated and unheated BHT, while a cliff avoidance test in the heat treated dose of 250 and 500 mg/kg was observed in the cliff avoidance test compared to other groups. A significant reduction occurred in all groups in the pocketing and dorsal tonic immobility test. The pathological changes of heated BHT groups were more severe than those of un-heated BHT groups especially the dose of 500 mg/kg heated BHT. It represented by coagulative necrosis, muscle atrophy in heart, interstitial pneumonia, serofibrinous exudate, pulmonary emphysema in lung and neuronal degeneration, microgliosis, myelin vacuolation and satellitosis in the brain. The study concluded that heated BHT at a dose of 250 and 500 mg/kg had toxic effects to motor and neurobehavioral activity, and histopathological changes in the brain, heart, and lung.

Keywords: Butylated hydroxytoluene (BHT), Neurobehavioral test, Histopathological changes, Rats

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Introduction

Butylated hydroxytoluene (BHT) is the most commonly used antioxidant in consumer products worldwide as an antioxidant, including canned food, baby sweets, potato chips, silage, cosmetics and plastics. The baked or cooked product is now stored in a warehouse where the temperature can vary considerably before shipping, next the product is transported home where it can be reheated or stored for uncontrolled lengths of time before consumption. Home storage is largely uncontrolled so that when the packaging integrity is broken, the product will be exposed to a variety of temperatures and humidity along with increased oxygen exposure (1). The dose of 0.5 mg/kg is considered acceptable for human health conception (2). However, excessive intake of sweets and canned and fast food, especially re-heating, which already using sunflower oil for deep fried, leads to harmful health effects. The long-term treatment of BHT is capable of producing oxidative and metabolic changes similar to certain pathological disorders (3). Taking doses greater than 500 mg/kg/day produces some pathological, enzymatic, and fatty changes with some carcinogenic conditions in rodents (2).

Oxidation responses adversely influence food taste, color and texture and may decrease dietary value (4). Lipid oxidation is a complicated series of chemical, modifications resulting from lipid-oxygen interaction (5). Because of their elevated fat content 200-300 g kg⁻¹, pre-cooked meat products are prone to lipid oxidation, antioxidant compounds are often added to pre-cooked meat goods to
safeguard the taste and extend the quality of shelf life. BHA and BHT synthetic phenolic antioxidants are approved for use in meat products at a fat content of 0.2 g kg⁻¹; however, latest consumer concerns about fat content are efficient (6).

Studies conducted on laboratory animals indicate that BHT has ant oxidative in very low dose while the toxic effects are appeared according to the given dose. (3) Previous research has confirmed that the accumulation of BHT in the adipose tissue is more prevalent in humans than in rats and may be due to differences in the metabolism of BHT (7). The study of acute toxicity in different animals showed that the oral LD₃₀ value of BHT in rats was more than 2930 mg/kg (8). One of the causes of death in high doses was hemorrhagic death due to inhibition of hepatic prothrombin synthesis and metabolic interaction with vitamin K, also several study show BHT has been cause hepatocyte hypertrophy in mice, vacuolar, and necrosis of hepatic cells in mice (9).

There are several studies for bad BHT effects on organs of laboratory animals but we don’t find any study for the research in published literature show any effect of heat treatment (cooking) of BHT in animals organs and motor neurobehavioral activity. There for this study was done to evaluate the acute toxicity of BHT in doses 250 and 500 mg/kg dissolved in sunflower oil in the form of heated and unheated treated and study this effect on motor and neurobehavioral activity and its relationship with the histopathological effects on the brain, heart, and lung.

Materials and methods

Butylated hydroxytoluene is used as form of Sigma-B1378, E321. The dose was dissolved in sunflower oil from the local markets. The volume of dose administration was 2 ml/kg B.W, butylated hydroxytoluene dissolved in sunflower oil heated in a boiling water bath of 98 ± 2 °C for 15 minutes and then left to cool until used. This method is similar to deep-fried cooking food in the kitchen (10).

Animals

Twenty-five albino male Wistar rat aged 2 months, weighing 250-300 g, rats in cages under uniform conditions in room temperature, water, and food in the animal house of the College of Veterinary Medicine, Mosul University.

Experimental design

The rats were divided into five groups each group consists of 5 rats. All doses were given orally by gavage needle. The first group considered as control group treated with sunflower oil only. The second group treated with BHT 250 mg/kg BW. The third group treated with heated BHT 250 mg/kg BW. The fourth group treated with BHT 500 mg/kg BW. The fifth group treated with heated BHT 500 mg/kg BW. Treatment continued for four consecutive days on the fifth day, acute toxic effects were measured, the measurements included neurobehavioral and motor activity changes were performed within the open field for each animal and the changes were record.

Neurobehavioral measurements

Open field activity test (motor neurobehavioral activity)

This test was done by using a wooden box designed for this purpose. Its dimensions are 90 x 60 x 30 cm. Its floor is divided into 24 square. The square side is about 15 cm. In an isolated room, each animal was subjected to this test alone. The number of squares was calculated. Center the box in a dark room and the test took 3 minutes per animal and this measurement tests the general movement of the rat inside the box (11).

Negative geotaxis test

The test is based on placing the animal upside down and counting the time it takes for the animal to rotate 180 degrees in its entire body to the top of the slanted surface and the maximum duration of one minute (60 seconds) for each animal. This test measured vestibular function and neuromuscular activity of the animal (12).

Pocking head test

The test is carried out using a plastic surface of 30 cm diameter and 20 cm high. It contains 10 circular holes. The test was done by observing the animal and calculating the number of attempts to inserted into the holes. The test time is 3 minutes for animal. This test measured the animal curiosity and the degree of familiarity with its surroundings (13).

Cliff avoidance test

Each animal was placed on a high edge height 160 cm, and then the animal was monitored and the time taken by the animal was taken away from the edge and rotation (avoidance or non-avoidance fall). The maximum time given to each animal was two minutes for rotation. It records a failure to avoid the edge of the high place (14).

Dorsal tonic immobility test

This test measures the degree of fear and tension in the animal, which is conducted by holding the animal from the skin fold of the neck under the base of the skull and in a vertical position and then calculated the time period of the animal in silence until the start of resistance by the movement of the lower limbs or head and the maximum time is one minute. When the animal is failing to silent the attempt is tray again (15).

Fixation and tissue sections preparation

After the end of the of experiment, the rats were anesthetized by ether until died for the purpose of organs collection (brain, heart, lung) and then organ washed with tap water and placed in 10% neutral buffer formalin which
prepared according to Luna (16). Then specimens were dehydrated by series of ethanol alcohol, cleared by xylene, impregnated and embedded in paraffin wax (melting point 60°C in an oven for 4-6 hours. Then the specimens were blocked in paraffin wax, a serial transverse section of 4-micron thickness was cut by using rotary microtome. The section then floated in the water bath at 37-40°C and mounted on a slide, then stained with hematoxylin and eosin stain (17).

**Statistical analysis**
Results were analyzed by one-way analysis of variance then result was followed by the least significant difference test by SPSS program. The level of significant is P<0.05 (18).

**Results**
The results of the neurobehavioral and motor activity test showed a delay at the starting latency in animals treated with heated BHT at a dose of 500 mg/kg compared to other groups (Table 1), and a decrease in the number of crossed squares in heated 250,500 mg/kg and unheated 500mg/kg when compared with control group, there was a significant decrease in rearing in heated and unheated 250 mg/kg as compared to control group and group of unheated 500 mg/kg (Table 1).

There was a significant decrease in number of head poking and time of tonic immobility test in all groups as compared to control group (Table 2), while there was a significant increase in negative geotaxis time in heated and unheated 500 mg/kg BHT as compared to control group and 250 mg/kg (Table 2). The time of cliff avoidance was a significant increase in groups treated with a heated BHT in a dose of 250,500 mg/kg as compared to the control group (Table 2).

The gross pathological changes of rats organ treated with heated BHT at dose 250 and 500 mg/kg of body weight showed congestion of blood vessels and swelling of brain (Figure 1), hypertrophy and congestion of heart, congestion of lung, paleness of some lung lobes and necrotic foci on its surface (Figure 2), comparing with control groups. Gross lesions of un-heated BHT at dose 250 and 500 Mg/Kg of body weight also showed congestion of the heart, brain, and lungs of rats but in less degree compared with the control group.

Table 1: neurobehavioral and motor activity of rat after 4 days of BHT treatment

| Treatments          | Mean± standard error (n=5) | Starting latency | Crossed squares/ 3 minutes | Rearing/ 3 minutes |
|---------------------|---------------------------|------------------|----------------------------|--------------------|
| Control             | 2 ± 0.3                   | 50 ± 4           | 7 ± 1                      |
| Heating BHT 250 mg/kg | 3 ± 1                    | 29* ± 2          | 3 *± 0.9                   |
| Un heating BHT 250 mg/kg | 3 ± 0.5                  | 34 ± 7           | 5 ± 0.98                   |
| Heating BHT 500 mg/kg | 8* AB ± 1                | 13* AB ± 6       | 9*AB ± 1                   |
| Un heating BHT 500 mg/kg | 2C ± 0.4                 | 12* AB ± 5       | 11*AB ± 2                  |

*: Significant difference with control group (P<0.05), A: significant difference with heating BHT 250mg/kg, B: significant difference from un-heating BHT 250mg/kg, C: significant difference from heating BHT 500mg/kg.

Table 2: Neurobehavioral measurement of rat after 4 days of BHT treatment

| Treatments          | Mean± standard error (n=5) | Number of Head poking/ 3 minutes | Time of negative geotaxis/ second | Time of cliff avoidance/ second | Time of tonic immobility response/ second |
|---------------------|---------------------------|----------------------------------|----------------------------------|---------------------------------|------------------------------------------|
| Control             | 18 ± 3                    | 2± 0.5                           | 1.5± 0.3                         | 46± 4                           |
| Heating BHT 250 mg/kg | 6*± 0.5                   | 2 ± 1                            | 5* ± 1.5                         | 26*± 2                          |
| Unheating BHT 250mg/kg | 4* ± 1                    | 3 ± 1.1                          | 2.5*± 0.7                        | 33± 7                           |
| Heating BHT 500mg/kg | 5* ± 1                    | 5*± 1.1                          | 8* ± 1.1                         | 14*AB ± 1                       |
| Unheating BHT 500mg/kg | 5* ± 1                    | 5*± 1.3                          | 3*± 1.2                          | 20*AB ± 1                       |

*: Significant difference with control group (P<0.05), A: significant difference with heating BHT 250mg/kg, B: significant difference from un-heating BHT 250mg/kg, C: significant difference from heating BHT 500mg/kg.

Microscopically, lesions of heart, lung and brain tissue in rat treated with un-heated BHT at dose 250 and 500 mg/kg of body weight represented by vacuolar degeneration, coagulative necrosis and edema in heart (Figure 3 and 4), bronchopneumonia, emphysema and fibrinous exudate in lungs with peribronchial and alveolar wall infiltration of inflammatory cells (Figure 5 and 6) and vasogenic edema, congestion of blood vessels, focal microgliosis and neuronal degeneration in the brain (Figure 7 and 8) comparing with control group. Whereas the lesions of these organs of rats treated with heated BHT at dose 250 and 500 mg/kg of body weight were more severe than un-heated.
BHT at same doses especially dose 500 mg/kg comparing with the control group. These lesions include shows zener necrosis, edema and infiltration of inflammatory cells (neutrophils and macrophage) in the heart (Figure 9 and 10), interstitial pneumonia representing by infiltration of inflammatory cells, serofibrinous exudate filled alveoli, emphysema and present of hemosiderin pigment in the lungs (Figure 11 and 12) and neuronal degeneration, congested blood vessels, microgliosis, myelin vacuolation, satellitosis and atrophy in purkinje fibers in the brain (Figure 13-15).

Figure 1: Gross section of brain of rat treated with heated BHT at dose 250 mg/kg B.W shows congestion of blood vessels (A) with swelling of brain (B).

Figure 2: Gross section of heart and lung of rat treated with heated BHT at dose 500 mg/kg B.W shows hypertrophy and congestion of heart (A) congestion of lung (B), paleness of some lung lobes and necrotic foci on its surface (C).

Figure 3: Photomicrograph of rat heart treated with un-heated BHT at dose 250 mg/kg B.W shows coagulative necrosis (A), edema (B) and pyknosis of nucleus. H&E stain (180X)

Figure 4: Photomicrograph of rat heart treated with non-heated BHT at dose 500 mg/kg B.W shows vacuolar degeneration (A), edema (B) pyknosis of nuclei (C). H&E stain (165X).

Figure 5: Photomicrograph of rat lung treated with non-heated BHT at dose 250 mg/kg B.W shows emphysema (A), fibrinous exudate (B) and infiltrations of inflammatory cells (C). H&E stain (56X).
Figure 6: Photomicrograph of rat lung treated with non-heated BHT at dose 500 mg/kg B.W shows thickening of epithelium lining the bronchiole with desquamation (A), emphysema (B), and infiltration of inflammatory cells (C). H&E stain (100X).

Figure 7: Photomicrograph of rat brain treated with heated BHT at dose 250 mg/kg of body weight shows congested blood vessels (A) and vasogenic edema(B). H&E stain (165 X).

Figure 8: Photomicrograph of rat brain treated with non-heated BHT at dose 500 mg/kg B.W shows focal microgliosis (A) and neuronal degeneration (B). H&E stain (110X).

Figure 9: Photomicrograph of rat heart treated with heated BHT at dose 250 mg/kg B.W shows zenker necrosis (A), edema (B) and infiltration of inflammatory cells (neutrophils and macrophage) (C). H&E stain (180X).

Figure 10: Photomicrograph of rat heart treated with heated BHT at dose 500 mg/kg B.W. show zenker necrosis and pyknosis of nucleus (A), edema (B) and infiltration of inflammatory cells (C). H&E stain (180X).

Figure 11: Photomicrograph of rat lung treated with heated BHT at dose 250 mg/kg B.W shows interstitial pneumonia representing by infiltration of inflammatory cells (A), serofibrinous exudate (B) and emphysema (C). H&E stain (100X).
Discussion

The neurobehavioral tests were study depending on the lack of relevance to any previous experience and that the animals did not develop a strong reaction for themselves. At the same time, these tests do not require the study of emotional mechanisms directly to some extent (19). The results for treatment with BHT showed decreased in the open field activity as well as other neurobehavioral tests that were measured specially heated BHT 250, 500 mg/kg these showed a negative effect on the animals movement by decreased number of squares passed by and the rearing, this result has confirmed to histopathological changes of brain these effect may occur due to heated BHT led to formation of free radicals that have a bad effect on the brain and its function, that agreement with another study that show free radicals can create from sources like lipid peroxidation that can be initiated by heat and photochemical processes in foods. These free radicals may undergo a number of responses that affect the sensory, functional and toxicological properties (20).

The basis of walking in mammals is generated by a group of neurons in the spinal cord and this fiber is inhibited by another fiber from the brain stem, heated and non-heated BHT effect on these brain fibers, and may cause inhibiting for dopamine that has significant impact on the nervous system function (21), lead to decreased all of the motor activity and time of immobility in the dorsal tonic immobility test.

It seems that the high dose of BHT, cause injury of the nervous tissue that was appeared in the brain tissue examination in our study which is agreement with Karkhah and et al. (22) mention neuropathy led to the effects on the degree of animal interest in the environment has decreased and the length of time required for rotation in the avoidance of the high edge has been increased. Our explanation may
be due to the hypothalamic pathway being affected, here is that the neural information follows the second pathway from the cerebral cortex to the amygdala, speed of information transmission is slower and takes longer and the response is slower than before (23), represented by delayed in avoid the edge of the slope.

BHT is the most commonly used as synthetic antioxidant it has many effects on vital organs such as heart, liver, brain, lung, stomach even on reproduction specially its use in high dose or exposure to high degree of heat lead to change the function of parent material BHT to very toxic metabolite so the heating leads to produce primary and secondary oxidative components such as peroxy and hydroxide (24).

The result of the study showed histological changes of the myocardium represented by necrosis, edema. Zenker necrosis this is due to administration of BHT is capable to induce oxidative and metabolic alteration on heart (25) also when concentration of BHT was elevated it caused marked cell lysis, this indicates that BHT in large concentrations cause injury to myocardial cells this result match with Leslie and et al. (26) most toxic effect of BHT comes from its metabolism so the cyp450 metabolite BHT and by transformation alter BHT to the toxic metabolite and produce phenoxy radical, this metabolite reacts with other intrinsic components in the body by interacting with several metabolic pathways like oxidation and reduction reaction and interact with oxygen molecules so BHT-OOH is more toxic 10 times than BHT itself.

The histopathological changes of the lungs showed sloughing of epithelial lining the bronchi bronchopneumonia, edema, emphysema, and fibrinous edema that result agreement with another study which found that acute pulmonary toxicity and tumor promotion by the food additive 2,6-di-tert-butyl-4-methylphenol (BHT) are well documented to cause lung lesions (27,28). These suggest the possibility that BHT may exert their promoting effects by inducing oxidative stress, normal cells are destroyed more readily which allows neoplastic cells to expand their proliferation (29,30). Our interpretation for that is the high doses or reheated of BHT are produce lung damage due to some BHT metabolites that are conjunction with SH-groups for proteins like cysteine protein and also inhibit glutathione formation that causes lung toxicity.

Conclusion

We conclude from this study that the acute treatment with a dose of 250 mg and 500 mg/kg showed toxic effects on neurobehavioral and motor activity of rats, especially the heat-treated form of BHT, this effect coincided with the appearance of pathological effects on the brain, heart, and lung.

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Conflict of interest

There is no conflict of interest.

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The effects of butylated hydroxytoluene and butylated hydroxyanisole on the biochemical and histological parameters of the rats.

Aim: To investigate the effects of butylated hydroxytoluene and butylated hydroxyanisole on the biochemical and histological parameters of the rats.

Introduction: Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly used as antioxidants in food products. However, their effects on the biochemical and histological parameters of the rats are not well understood.

Methods: Forty rats were randomly divided into four groups (n=10 per group). The first group was the control group and the other three groups were treated with different concentrations of BHT and BHA. The biochemical parameters were determined using a biochemical analyzer, while the histological parameters were assessed using histological staining methods.

Results: The results showed that the treatment with BHT and BHA had a significant effect on the biochemical and histological parameters of the rats.

Conclusion: The results suggest that BHT and BHA should be used with caution in food products to avoid negative effects on the biochemical and histological parameters of the rats.