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EFFECT OF THE ANTIOXIDANT BUTYLATEDHYDROXYTOLUENE ON THE GENOTOXICITY AND CYTOTOXICITY INDUCED BY ARSENIC TRIOXIDE IN MALE MICE

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Key Word: Genotoxicity, Cytotoxicity, Arsenic trioxide, Apoptosis, BHT.

ABSTRACT

The present study aims to evaluate the effect of antioxidant on the genotoxicity and apoptosis induced in mice by arsenic trioxide (As_2O_3) in normal adult male SWR/J mouse. Animals were treated intraperitoneally (ip) with single doses (2.65, 5.35 or 10.70 mg kg^{-1} b.wt. of As_2O_3 and decapitated 24 h later. Another group of animals were treated firstly with 60 mg kg^{-1} b.wt. of the antioxidant agent butylated hydroxytoluene (BHT) 1 h prior to As_2O_3 doses administration. The three treated single doses of As_2O_3 significantly ($P<0.05$) increased the rate of sister chromatid exchanges (SCEs), micronucleus (MNs) formation, DNA ladder and apoptosis as compared with the negative control. The three treated doses with BHT were significantly ($P<0.05$) increased the rate of MNs as compared with the effect of the individual single doses. With agarose gel electrophoresis, DNA ladder were clearly detected in all the treated mice. The data recorded revealed that, administration of As_2O_3 had a negative effects as represented in SCEs, MNs and DNA ladder test. Thus, we have demonstrated that arsenic trioxide play direct or indirect role in the induction of genotoxicity and apoptosis in mice cells.

INTRODUCTION

With respect to human health hazard, arsenic consider one of the most dangerous environmental toxins (Toribio and Romanya, 2005). And it has become evident that increasing human activities have modified the natural cycle of metals and metalloids (Alarifi et al., 2009). Arsenic is released into the atmosphere from both natural and anthropogenic sources (Reichard *et al.*, 2007) to contaminate air, water, food, and soil (Chowdhury *et al.*, 2008, Lee et al 2008) and has become a threat to the all livening organism including the human race (Manna *et al.*, 2007). In spite of various risk arsenic trioxide is still use against some cancer diseases (Florea and Büsselberg, 2008).

Apoptosis is an active form of cell death which is plays a crucial role in the development and maintenance of cell homeostasis and may in fact be a key mechanism in the development of toxicity (Lee et al., 2008). Apoptosis is known to be activated by a cascade of factors and to be placed under tight genetic regulation. It has now been recognized as an important determinant of cell degeneration in many toxic events (Yasin et al., 2003). Apoptosis may occur via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. Over the past few years, there has been

increasing recognition of (1) the important role of cell death in determining appropriate cell number and (2) how a lack of cell death under physiologic conditions can contribute to cellular transformation and malignant cell growth. Cell death permits the selective elimination of excess cells and permits the maintenance of tissue homeostasis in morphogenesis and in the immune system. The majority of such cell deaths share common characteristics, such as fragmentation of DNA as a biochemical hallmarks of apoptosis (Ho et al., 1999; Qurtam et al., 2009). Despite the large number of studies conducted concerning arsenic toxicity, the effects remain poorly understood (Doop *et al.*, 2004; Florea and Büsselberg, 2008). But several assays performed *in vivo* and *in vitro* on mammalian cells have shown that exposure to arsenic induces chromosomal aberrations and formation of micronuclei (Bhattacharya *et al.*, 2005, Klein *et al.*, 2007).

The present investigation was undertaken in an effort to determine the effect of butylated hydroxytoluene as an antioxidant on the genotoxicity and cytotoxicity induced by arsenic trioxide in male mice.

MATERIALS AND METHODS

All of the experimental procedures were conducted in the central lab of teachers college, king Saud university, Saudi Arabia in 2009.

1-1 Experimental animals :

Normal SWR/J male mice, 8-10 weeks old and weighing 25-30 g were used throughout the study. Animals were maintained and bred under standard laboratory at a temperature of 22±1°C, a relative humidity of 45±5% and photoperiod cycle of 10/14 hours. Mouse food (commercially available in Saudi Arabia) and water were offered *ad libitum*.

1-2 Treatments :

A total of 45 males were used and divided into 9 groups, each containing 5 males. Group-1 was subjected to (i. p.) injection (0.2 ml/10g b.wt.) of sterile normal saline as a negative control. Groups2, 3 and 4 were subjected to i.p. injection of As_2O_3 in single various dose levels (2.65, 5.35 or 10.70 mg kg^{-1} b.wt. which corresponds to 0.25, 0.50 or 1 LD_5, respectively). Groups5, 6 and 7 were treated with the same doses as in Groups2, 3 and 4 plus 60 mg kg^{-1} b.wt of the synthetic antioxidant agent butylated hydroxytoluene (BHT) one hour prior to As_2O_3 treatment. Group-8 was treated with only 60 mg kg^{-1} b.wt of BHT (Plumb et al., 2000). Group-9 was treated with the organic solvent Tween-80 (0.2 ml/10g b.wt.), which was used to dissolve the BHT.

1-3 Chemicals Used :

Arsenic trioxide, Butylated hydroxytoluene (BHT), Tween-80 were obtained from (Sigma, UAS). The methods of (Preston et al. (1987) were used for the chromosomal preparations. And the method of (Latt et al. (1981) was used for the staining.

1-4 Scoring :

I- Sister chromatid exchanges (SCEs) :

Fifty well-spread and clear metaphase from each slide (giving 50×5=250/group) were examined to detect (SCEs) (Allen et al., 1978).

II- Micronucleus test :

Slide preparation :

Femoral bone marrow cells flushed out from femur by syringe with foetal Calf Serum (FCS) and smeared on clean glass slides. Thereafter these cells are fixed with absolute methyl alcohol for 15min.

Staining :

Slides were stained by immersion in phosphate buffer solution and followed by treatment with acridine orange (AO) for 1 min. Slides were then treated with phosphate buffer solution for 10 min followed by an additional treatment with fresh phosphate buffer solution for 15 min. Slides were embedded with DPX, covered and then immediately examined using an FL EPI-Fluorescence microscope (Nikon, Eclipse E600W, Japan) at 530 wavelength.

Scoring :

One thousand polychromatic erythrocytes (PCEs) - oil reddish - from each slide, (giving 1000×5=5000/group) were examined in this study to evaluate the number of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs) in normochromatic erythrocytes (NCEs) – bright reddish. The ratio of MNPCEs to MNNCEs was used as an indicator of chromosomal changes, while %PCEs was used as an indicator of apoptogenicity (Garcia et al., 2001).

III- DNA extraction and electrophoresis analysis:

DNA were isolated from ten gram of mice livers by using Genomic Prep Cells and tissue DNA isolation Kit, (Amersham, Biosciences, USA). Concentration and purity of DNA measured by GeneQuant pro, Amersham, USA). Ten µg of each DNA sample were mixed with 2µl of 5x loading dye (5x Tris-acetate-EDTA (TAE), 50% glycerol, 0.2% bromophenol blue), and the fragments were resolved by elrctrophoresis through a 1% agarose minigel (Hoeffer HE 33 Mini Submarine Electrophoresis Unit) (Sambrook et al., 1989) in 1x TAE buffer. Approximately 70 min at 75v (Electrophoresis power supply-Eps 301(Amersham,USA). DNA staining in ethidium bromide (1µg/ml), followed by scanning (UV-transilluminator Hoefer,USA) and photography by Polaroid Gel Camera,UK.

Statistical analysis :

The data obtained in this study from SCEs and MNs tests were statistically analyzed with SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) using the Mann-Whitney U-test.

RESULTS

1- Sister chromatid exchanges (SCEs) :

The data in Table 1 show the SCEs following single treatment with three different doses of arsenic trioxide alone or in combination with BHT.

Single treatment with each of the three doses of arsenic trioxide induced a significant ($P<0.05$) increase in the rate of SCEs compared with the control (Table 1). On the other hand and as compared with the arsenic treated group no significant effect was observed on the rate of SCEs when arsenic trioxide was administrated in the combination with arsenic trioxide alone. The treatment with BHT alone significantly ($P<0.05$) increase the rate of SCEs compared with control.

11- Micronucleus (MNs):

The data in Table 2 show that the single and combined treatment at medium and high doses of arsenic trioxide induced a significant ($P<0.05$) decrease in the rate of %PCEs and the three single doses of arsenic trioxide induced a significant ($P<0.05$) increase in the number of MNPCEs compare with control. The three combined treatment with BHT induced a significant ($P<0.05$) decrease in the number of MNPCEs compared with single doses.

111- DNA Ladder

The results of agarose gel electrophoresis of DNA extracted from treated mice are shown in Fig 1. DNA

ladder formation was clearly detected after 24 hours of cell death process at all single and combined doses including treated with BHT alone compared with the control . On the other hand, DNA ladder formation was never detected in the control samples.

DISCUSSION

The genotoxic effect of arsenic compounds has been reported *in vitro* and *in vivo* in several publications (Chou et al., 2008; Florea and Büsselberg, 2008; Alarifi et al., 2009). Many studies have been pointed to genotoxic effects of investigated arsenic trioxide (As_2O_3) (Alarifi et al., 2009; Qurtam et al., 2009). The results of SCEs test showed significant increase in the rate of SCEs after treatments with the three treated doses of arsenic trioxide as compared with the control group. This results confirm the few earlier studies which used SCEs assay to evaluate the genotoxicity effects of arsenic in human and animals tissue culture (Lee et al., 1985; Bernstam and Nriagu, 2000; Chou et al., 2008; Han et al.; 2008).The present study showed that the single treatment with the medium and high treated doses of arsenic trioxide induced a significant increase in the rate of PCEs% and all the treated doses of arsenic trioxide induced a significantly increase in the number of MNPCEs which means induction of genotoxic effects in mice bone marrow cells. These finding, however are in accordance with (Adler, 1984; Hayashi et al., 1994; Jagetia and Reddy, 2002). Also, are in agreement with the finding of previous studies which has showed increased micronuclei in bladder epithelial cells for people exposed to arsenic in drinking water, and cells cultured of chaises ovary hamster (Rahman et al., 2001; Martínez, 2005). Both arsenic and its metabolites have a variety of genotoxic effects, which may be mediated by oxidants or free radical species (Jhala et al., 2008). Arsenic is a prooxidant and thus may cause lipid peroxidation, protein and enzyme oxidation, GSH depletion and DNA adherence. Furthermore, arsenic generates reactive oxygen species (ROS) which are known to induce poly ADP-ribosylation, which is implicated in DNA repair, signal transduction and apoptosis (Bhattacharya and Bhattacharya, 2007).The DNA damage caused by arsenic trioxide can be accounted for by the experimental evidence of its genotoxic effect. Its mode of action may include: (i) inhibition of various enzymes involved in DNA repair and expression; (ii) induction of ROS capable of inducing DNA damage and considerable accumulation of ROS in a variety of animal cells (Wang et al., 2004; Patlolla and Tchounwou,2005; Bishayi and Sengupta, 2006). Furthermore, several intranucleolus changes produced from activation of caspases enzymes such as active Dnase and Lamina-A degradation as apoptosis markers (Kang et al., 2006; Mclaren et al., 2006, Yu et al., 2008). Studies showed As_2O_3 induced apoptosis signals from the cell surface to the nucleus of lymphocytes through fragmentation of DNA, activation of caspase. Arsenic play a dual roles as anti-cancer and inducing of gentotoxicity and cytotoxicity, its these two apparently opposite effects on human life may share a common molecular mechanism. Extensive DNA damage lead to cell dysfunction and apoptosis, this kind of cell death takes in a tissue or organ with distinct biochemical and morphological markers of apoptosis (Nguewa et al., 2003). Previous studies demonstrated that the activity of caspase that cleavage Lamina is required for the disintegration of nuclei in the late stages of apoptosis. The Lamina-cleavage caspase-6 is sufficient to drive nuclear events to shutting down nuclear processes followed by apoptotic execution because of lamina proteins bind specifically to most nuclear

envelope proteins, histones, transcriptional regulators, gene expression regulators. Furthermore, lamina filaments interfere with chromosome segregation during mitosis. Mostly the lamina cleavage links in the apoptotic pathway and precedes DNA fragmentation (Takahashi et al., 1997; Chen et al., 2000; Cohen et al., 2001; Bjerke and Roller, 2006). Anyway, the relationship between arsenic dose-response and its toxic effects still unclear. Most studies showed that the dose-response depend on exposure protocol, exposure period and the treated dose (Yih and Lee, 1999).

In the present investigation exposure to arsenic trioxide in combination with BHT did not induce any significant changes in the rate of SCEs as compared with exposure to arsenic trioxide alone. As shown in our result BHT alone significantly increase the rate of SCEs and this finding agreed with some studies which are revealed its potential to induce genotoxicity (Grillo and Dulout, 1995) in contrast some studies considered genotoxic as shown in our MNs test. The three combined treatment with BHT induced a significant ($P<0.05$) decrease in the number of MNPCEs compared with single doses. The protective action of antioxidants was quite clear in this study particularly in MNs test perhaps due to the low BHT concentration employed, in contrast previous study which has been used 30 mg kg^{-1} b.wt which was unclear (Qurtam et al., 2009).

Conclusion:

Arsenic trioxide induce genotocxicity and cytotoxicity according to direct or indirect mechanism and had different potential cellular targets. In terms of DNA ladder for apoptosis detection the point need more extensive morphological studies via electronmicroscope. Also, the present data investigate the effect of the antioxidant BHT on the Genotoxicity and Cytotoxicity Induced by arsenic trioxide in male mice.

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Table 1 : Sister chromatid exchange frequency in bone marrow cells of mice treated with arsenic trioxide (As_2O_3) alone and in combination with butylated hydroxytoluene (BHT).

Group	Treatment (Dose mg/kg)	Sister chromatid exchanges (SCE_S)					
		mean / animal ± S.D.					mean / group ± S.D.
		i	ii	iii	iv	v	
1	-ve control	5.57±2.10	6.50±2.20	5.15±2.12	4.90±2.20	4.30±1.40	5.54±0.54
2	As_2O_3 (2.65)	9.14±3.42	9.40±2.92	8.60±4.22	8.20±4.34	9.36±3.00	8.83±0.46 [a]
3	As_2O_3 (5.35)	7.16±1.80	8.12±2.22	10.86±2.60	9.50±3.50	10.60±3.16	9.30±2.90 [a]
4	As_2O_3 (10.70)	13.15±3.88	11.00±4.07	8.40±3.25	10.84±3.5	10.30±3.42	10.74±1.50 [a]
5	As_2O_3 + BHT (2.65+60)	7.54±2.90	6.92±2.99	7.70±3.22	9.60±3.71	8.08±2.30	7.70±1.23 [a]
6	As_2O_3 + BHT (5.35+60)	10.82 ±3.20	10.54±3.28	9.44±3.10	10.70±3.5	10.11±2.90	10.32±1.23 [a,e]
7	As_2O_3 + BHT (10.70+60)	9.30 ±3.20	13.16±5.87	14.81±6.23	9.94±3.35	12.34±3.80	11.90±2.70 [a,e]
8	BHT (60)	8.98±3.20	8.20±3.44	6.51±2.22	5.20±2.11	8.10±3.23	7.77±0.58 [a]
9	Tween-80	5.84 ±3.16	6.24±2.88	5.90±2.13	6.15±2.10	6.00±3.11	6.40±0.30 [a]

a: Significant difference from group 1 at $p < 0.05$ e: Significant difference from group 8 at $p < 0.05$
The number of experimental animals of each group: 5 and the number of examined cells of each animal: 50

Table 2: Effect of sodium arsenite ($NaAsO_2$) alone and in combination with butylatedhydroxytoluene (BHT) on micronucleus induction in bone marrow cells of SWR/J mice.

Groups	Test substance	Number of examined PCE_s	% PCE_s	Micronucleated cells per 1000	
				PCE_s	NCE_s
1	**-ve control**	5000	60.88	0.50	0.22
2	**As_2O_3 (2.65)**	5000	51.10	1.59 [a]	0.50
3	**As_2O_3 (5.35)**	5000	48.20 [a]	4.40 [a]	1.50
4	**As_2O_3 (10.70)**	5000	42.22 [a]	8.50 [a]	2.60 [a]
5	**As_2O_3 + BHT (2.65+60)**	5000	59.97	0.40 [b]	00.00
6	**As_2O_3 + BHT (5.35+60)**	5000	45.33 [a]	0.75 [c]	0.75
7	**As_2O_3 + BHT (10.70+60)**	5000	38.10 [a,e]	3.10 [d]	1.12
8	**BHT (60)**	5000	60.00	1.30	1.38
9	**Tween-80**	5000	50.20	-	-

PCE_s: Polychromatic erythrocytes NCE_s: Normochromatic erythrocytes
BHT: Butylated hydroxytoluene
a: Significant difference from group 1 at $p < 0.05$ c: Significant difference from group 3 at $p < 0.05$
d: Significant difference from group 4 at $p < 0.05$ e: Significant difference from group 8 at $p < 0.05$

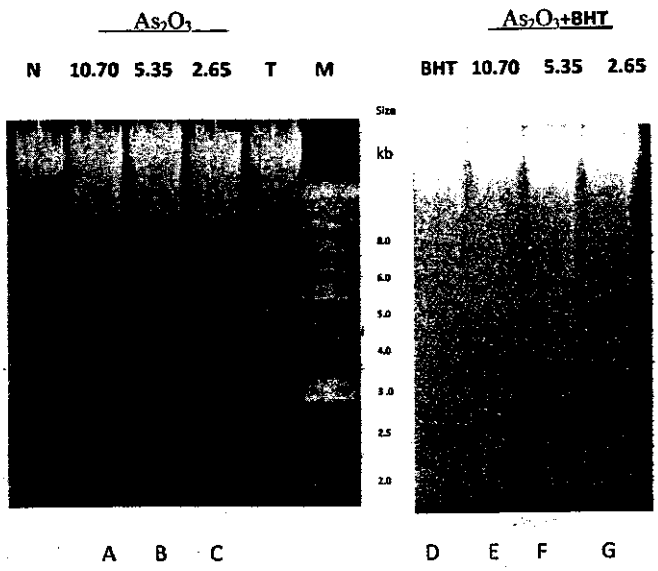

Fig. 1 Agarose gel electrophoresis of DNA isolation from livers of mice treated with arsenic trioxide alone (A,B,C) or in combination with BHT (E, F,G). N:untreated; T:tween-80; M:marker; BHT:butylatedhydroxytoluene(I). The numbers shown above the gel represent the used doses(mg/kg).

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المجلة المصرية الألمانية لعلم الحيوان
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تأثير مضاد الأكسدة بيوتيلاتيد هيدروكسي تولوين (BHT) على السمية الوراثية والخلوية المحفزة بواسطة ثالث أكسيد الزرنيخ في ذكور الفئران

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تهدف هذه الدراسة إلى تقييم تأثير مضاد الأكسدة على السمية الوراثية والخلوية المحفزة بواسطة ثالث أكسيد الزرنيخ (As_2O_3) في ذكور الفئران البالغة من السلالة SWR/J. حقنت الحيوانات في التجويف البطني بالجرعات (٢,٦٥ ؛ ٥,٣٥ أو ١٠,٧٠ ملجم/كجم من ثالث أكسيد الزرنيخ ثم قتلت الحيوانات بعد ٢٤ ساعة. مجموعة أخرى عوملت أولاً بـــ ٦٠ ملجم/كجم من مضاد الأكسدة BHT قبل ساعة من حقنها بثالث أكسيد الزرنيخ. الجرعات الثلاث المنفردة زادت معنوياً ($P<0.05$) من معدل التبادلات الكروماتيدية الشقيقة (SCEs)، تكون النويات الدقيقة (MNs)، سلم الدنا والموت الخلوي المبرمج مقارنة بالمجموعة الضابطة. جرعات الزرنيخ المشتركة مع BHT زادت معنوياً من معدل النويات الدقيقة مقارنة بالمجموعة المنفردة. تم تحديد سلم الدنا بوضوح من خلال الترحيل الكهربائي للدنا عل جل الأجاروز في كل الفئران المعاملة مقارنة بالمجموعة الضابطة. كشفت هذه الدراسة أن المعاملة بثالث أكسيد الزرنيخ قد أدت إلى تأثيرات سلبية كما تشير نتائج اختبارات الـــتبادلات الكروماتيدية الشقيقة، تكون النويات الدقيقة و سلم الدنا. وبهذا فقد بينت الدراسة أن ثالث أكسيد الزرنيخ يلعب دوراً مباشر أو غير مباشر في تحفيز السمية الوراثية والموت الخلوي المبرمج في خلايا الفئران.