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A general Mechanistic Model of Bromate Cytotoxicity Mediated by DNA Damage

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ABSTRACT

Potassium bromide ($KBrO_3$) is widely used in bread production, cosmetics and water disinfection. $KBrO_3$ is a DNA oxidizing agent that induces base modifications as 8-Oxo-deoxyguanosine (8-oxodG), also induces chromosomal aberrations, micronuclei and γH2AX foci which are the endpoints of DNA double-strand breaks (DSBs). Therefore, the cytotoxic and genotoxic effects of $KBrO_3$ are mainly related to DNA damage. Conversely, the role of DNA damage in the cytotoxic effects of $KBrO_3$ has been poorly understood in conjunction with that of protein damage. Herein, we illustrated the cytotoxic effects of $KBrO_3$ through DNA damage-dependent and DNA damage-independent mechanisms. For this proof of concept, we analyzed the sensitivity of DNA repair-proficient and –deficient cells and subsequently measure DNA and protein damage after treatment with $KBrO_3$. Our results show that the DNA repair genes *DNA-PKcs, XRCC3* and *RAD51D* are strongly required for cell survival and essential to alleviate the cytotoxic effect of $KBrO_3$. The quantitative analysis of DSBs upon treatment with $KBrO_3$ shows a significant increase in the yields of DSBs suggesting that DSBs are critical DNA damage induced by $KBrO_3$ and accounts for DNA damage-dependent cytotoxicity. Furthermore, thioredoxin 1 oxidation was not observed upon treatment with potassium bromide, strongly exclude the involvement of DNA damage-independent cytotoxicity based on protein damage. Based on the current findings we suggested a mechanistic model of bromate cytotoxicity that is mediated by DNA but not protein damage**.**

INTRODUCTION

Potassium bromide ($KBrO_3$) is a potent oxidizing agent that has been used as food additive and is widely used in bread production, cosmetics and a drinking water disinfection byproduct (Ekop *et al*., 2008; IARC, 1986 and Naglaa Bayomy *et al*., 2016). Additionally, $KBrO_3$ acts as a bleaching agent that lends bond strength by improving the elasticity that results in soft and white bread (Preethi Grace, 2016). In Japan, $KBrO_3$ is used in beer making and added to some Japanese pastes of fish and cheese (Ueno *et al*., 2000). Although the World Health Organization (WHO) banned the use of potassium bromate in 1993 and some countries have been removed potassium bromide from the list of permitted food additives as reported by

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the National Agency for Food and Drug Administration and Control (NAFDAC) in 2003, a lot of people still using potassium bromide in a wide range of food production and water disinfection. The international agency for research on cancer has also listed potassium bromate as a human carcinogen (IARC, 1999). Several studies were reported that potassium bromide is nephrotoxic, neurotoxic and carcinogenic as it causes lipid peroxidation (LPO) and oxidative DNA damage (Ahmad *et al.*, 2012b). A histopathological examination of rats treated with potassium bromate showed congestion in the liver and brain edema (Waheeba Ahmed *et al.*, 2016). Potassium bromide has been reported to affect gene expression in renal tissues and chronic exposure induces carcinomas in rats, hamsters and mice (EPA, 2001; Limonciel, 2012 and Kolisetty, 2013). The genotoxic effect of bromate is directly related to DNA damage. By contrast, the role of DNA damage in the cytotoxic effects of bromate has been poorly understood in conjunction with that of protein damage. To address this issue, we systematically assessed the sensitivity of cells deficient in various DNA repair genes to different doses of potassium bromide. The results of this study indicate that cell survival against potassium bromide is mainly depended on particular DNA repair genes. Treatment of cells with potassium bromide significantly increases the yields of DSBs. Moreover, we demonstrate that the oxidation of thioredoxin (Trx) 1 is not accounts for the DNA damage-independent cytotoxicity. A mechanistic model of bromate cytotoxicity mediated by DNA but not protein damage is proposed on the basis of the current results.

MATERIALS AND METHODS

Chemicals and Cells:

Potassium bromide, Iodoacetic acid (IAA) and auranofin were purchased from Sigma-Aldrich. Iodoacetamide (IAM) and dithiothreitol (DTT) were obtained from Tokyo Chemical Industry. CHO (Chinese hamster ovary) cells were derived from gene chemistry laboratory at a graduate school of science, Hiroshima University, Japan. The DNA repair-proficient and -deficient CHO cells are listed in Table 1.

Table 1: CHO cells and their mutations related to DNA repair.

Cell	Mutation	Repair defect
CHO cells		
AA8	wild type	none
EM9	*XRCC1*	BER
UV5	*ERCC2 (XPD)*	NER
UV41	*ERCC4 (XPF)*	NER
51D1	*RAD51D*	HR
irs1SF	*XRCC3*	HR
V3	*DNA-PKcs*	NHEJ

BER: Base excision repair; NER: Nucleotide excision repair; HR: Homologous recombination; NHEJ: Non-homologous end-joining

Cell Culture and Cytotoxicity Assay:

CHO Cells were cultured in Eagle's minimum essential media (Wako Pure Chemical Industry), supplemented with 10% inactivated fetal bovine serum (Corning) and L-glutamine. Cells were maintained in a humidified incubator at 37°C with a 5% CO2 atmosphere. Colony formation assay was used as a measure for bromate cytotoxicity. Cells were plated in 10 cm culture dishes (Corning) and incubated for 12 hr. Then, cells were incubated for 1 hr in a fresh medium containing 2.5, 5 and 10 mM of potassium bromide. After treatment, cells were washed twice with a fresh medium and allowed to form colonies for 5–6 days. Finally, colonies with more than 50 cells were scored and the survival curves were plotted.

Analysis of Apoptosis:

Apoptosis of potassium bromide-treated cells was measured by using the MEBCYTO apoptosis kit (Medical & Biological Laboratories) in accordance with the manufacturer's commandment. The groups of annexin V-FITC-positive cells and propidium iodide (PI)-positive cells were counted on a Fluoview FV10i fluorescence microscope (Olympus). Approximately 600 cells were counted for each sample. Dead or dying cells were classified into three sets: early apoptotic cells (annexin V-FITC-positive), late apoptotic cells (PI- and annexin V-FITC-positive) and necrotic cells (PI-positive).

Analysis of DNA Damage :

The induction of DSBs was used as a measure of DNA damage induced by potassium bromide. DSBs were analysed by static-field gel electrophoresis (SFGE) as previously explained in (Schneider *et al*., 1994; Mahmoud Shoulkamy *et al*., 2012 and Ming Zhang Xie *et al*., 2016). Briefly, CHO cells in the mid-logarithmic phase were treated with 10 mM potassium bromide and incubated for 1 hr or irradiated with X-rays (10 Gy) as a positive control. After the incubation period, cells were collected by using a scraper in cold phosphate-buffered saline (PBS) and embedded in 1% Insert agarose plugs (Lonza) at a density of 10^4 cells/plug. For cell lysis, the agarose plug containing cells were incubated in a lysis buffer containing 1% sarkosyl, 0.5 M EDTA and 0.5 mg/ml proteinase K for 24 hr at 50°C and then equilibrated in 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA at room temperature. The plugs were inserted into the wells of 0.6% Seakem Gold agarose gel (Lonza) and subjected to electrophoresis at a field strength of 0.6 V/cm in 0.5 × TBE buffer [45 mM Tris, 45 mM boric acid and 1 mM EDTA] for 36 hr at room temperature. After electrophoresis, the gel was stained with ethidium bromide, and the fluorescence image of the gel was recorded on FAS-III (Nippon Genetics). The image was analyzed by ImageJ software version 1.47. To measure the yields of DSBs, the quantity of DNA eluted from the plug relative to total DNA (i.e., eluted and retained DNA) was calculated.

Analysis of Protein Damage:
Protein Extraction and Gel Mobility Standards Preparation:

Analysis of the redox state of cytosolic thioredoxin (Trx) 1 was used as the measure of protein damage. The redox state of Trx 1 was detected using a modified urea-polyacrylamide gel electrophoresis (urea-PAGE) and western blot analyses (Bersani *et al*., 2002; Zhang *et al*., 2014 and Ming Zhang Xie *et al*., 2016). Cells were treated with 10 mM potassium bromide and incubated for 1 hr. Meanwhile, cells were incubated 24 hr with 5 μM auranofin for positive oxidation of Trx 1. Treated cells were subjected to post-incubation time in a fresh medium for 24 hr. Cells were washed twice by phosphate-buffered saline (PBS) immediately or after 24 hr post-incubation time. To lyse cells, 300 μl of urea buffer [8 M urea, 100 mM Tris-

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HCl (pH 8.2) and 1 mM EDTA] containing 30 mM IAA was dropped to the cell monolayers. The cell lysate was collected and incubated for 15 min at 37°C. The cell debris was removed by centrifugation and proteins were precipitated by adding 10 volumes of cold acetone-1 M HCl (98:2, v/v). The pellet was washed three times with cold acetone-1 M HCl-H$_2$O (98:2:10, v/v/v). Proteins were dissolved in 200 µl of urea buffer containing 3.5 mM DTT and incubated for 30 min at 37°C. Protein extracts were mixed with 10 mM of IAM and incubated for 15 min at 37°C and finally, protein concentration was measured by using BCA kit (Thermo Scientific). For the preparation of gel mobility standards, cells were harvested in 300 µl of urea buffer containing 3.5 mM DTT and incubated for 30 min at 37°C to reduce all the cysteine residues to the sulfhydryl form. After precipitation, proteins were treated with either 30 mM IAA, 10 mM IAM or 15 mM IAA+15 mM IAM at 37°C for 15 min.

Separation and Detection of Protein:

Sixty µg of proteins were separated using 8 M Urea-PAGE. Two acrylamide concentrations (12, 2.5%) have been used for separating and stacking gels respectively. A constant current of 5 mA for 4 hr was applied to separate protein samples during electrophoresis. Upon completion of the electrophoretic separation, the gel was washed three times in 50 mM Tris-HCl (pH 8.3) and rinsed for 10 min in Towbin buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.1% SDS]. Protein samples were transferred to a PDVF membrane and incubated in TBST [20 mM Tris-HCl (pH 7.6), 140 mM NaCl and 0.1% Tween] at 4°C for 30 hr. For western blot analysis, the membrane was incubated with rabbit polyclonal anti-Trx1 (1:1000, Cell Signaling C63C6) at 4°C overnight. The excess of primary antibody was removed by washing the membrane twice with TBST for 10 min. Anti-Trx 1 antibody was detected by incubation with HRP-conjugated goat anti-rabbit IgG (1:9000, Abcam ab6721) at room temperature for 1 hr. The membrane was washed with TBST and incubated with the ECL western blotting substrate (Promega). Chemiluminescence was detected on a ChemiDoc XRS+ system (BIO-RAD). The intensity of protein bands was measured by ImageJ software version 1.47.

RESULTS

The Sensitivity of CHO Cells to Potassium Bromide:

In order to get a first global hint about the cytotoxicity of KBrO$_3$, The repair-proficient and -deficient CHO cells were treated with various doses of KBrO$_3$, and their sensitivities were analyzed by colony formation assay (Fig. 1). The mutants tested here included those deficient in base excision repair (BER) EM9 (*XRCC1*), nucleotide excision repair (NER) UV5 (*ERCC2/XPD*) and UV41 (*ERCC4/XPF*), homologous recombination (HR) 51D1 (*RAD51D*) and irs1SF (*XRCC3*) and non-homologous end joining (NHEJ) V3 (*DNA-PKcs*) (Table 1). The sensitivity results show that both HR (irs1SF) and NHEJ (V3) mutants were highly sensitive to potassium bromide and share similar sensitivity (Fig. 1). In contrast, NER mutants (UV41) and (UV5) were not sensitive to potassium bromide (Fig. 1). Nevertheless, BER (EM9) and HR (51D1) exhibit moderate sensitivity. The shared sensitivity of NHEJ (V3) and HR (irs1SF) mutants signifying that both HR and NHEJ repair genes are essential for cell survival upon treatment with potassium bromide and strongly recommended DSBs formation. Furthermore, the sensitivity observed with BER (EM9) mutant to potassium bromide suggesting the formation of base damage or DNA single-strand breaks (SSBs). These data illustrate that HR and NHEJ repair

genes are essential in alleviating the cytotoxic lesions induced by potassium bromide (Fig. 5).

Investigation of DSBs Induced by Potassium Bromide Using SFGE:

To investigate whether potassium bromide induces DSBs, DNA repair-proficient (AA8) and -deficient (V3 and irSF1) cells at mid-logarithmic phase were treated with 10 mM potassium bromide for 1 hr. Cells were collected immediately after treatment, and the induction of DSBs was analyzed by SFGE (Fig. 2). The formation of DSBs was clearly seen within all the treated cells compared to the untreated control (Fig. 2, upper panel). The yields of DSBs were quantified (Fig. 2, lower panel), DSBs were significantly increased with potassium bromide treatment and the yields were comparable to that formed with 10 Gy of X-ray irradiation. These results indicating that exposure of cells to potassium bromide significantly induce DSBs.

Cells Undergo Apoptosis Upon Treatment with Potassium Bromide:

It is well understood that cells undergo apoptosis when they are exposed to intensive DNA damage (Susan, 2007). To get insights on the cell's apoptosis, a cytological analysis of potassium bromide treated cells indicated that apoptosis was initiated at 24 hr after treatment and enhanced further at 48 hr (Fig. 3). The fraction of apoptotic cells induced at 24 and 48 hr after treatment increases in V3 (*DNA-PKcs*) and irSF1 (*XRCC3*) mutants relative to wild-type (AA8). The correlation between apoptosis data (Fig. 3) and the sensitivity data (Fig. 1) confirming the important role of DNA repair genes *DNA-PKcs and XRCC3* in cell survival.

Protein Damage Is Not Accounted for Potassium Bromide Cytotoxicity:

Protein damage was assessed by analyzing cytosolic Trx1 oxidation using a modified urea-PAGE and western blot analyses as described in materials and methods. To assess the effect of potassium bromide on Trx1, AA8 cells were treated with 10 mM potassium bromide for 1 hr. Proteins were extracted, separated and blotted to a PVDF membrane for western blot analysis. For positive oxidation of Trx1, AA8 cells were treated with auranofin (5 µM for 24 hr). Auranofin is known as an inhibitor of Trx reductase (TrxR) and hence impairs the reduction of oxidized Trx1 that increases the fraction of oxidized Trx1 (Becker *et al.*, 2000). Trx1 was separated into several distinct bands according to its different redox states (Fig. 4, upper panel). As shown in Fig. 4 (upper panel) with the untreated cells, Trx1 was detected either in a fully reduced form (band 1, Cont lanes) or in slightly oxidized forms (bands 2 and 3, Cont lanes). Moreover, no shift of the bands was seen immediately or 24 hr after treatment with 10 mM potassium bromide (bands 1, 2 and 3, 0 and 24 hr under $KBrO_3$ lanes). Fig. 4 (lower panel) shows the fraction of oxidized Trx1 (= total Trx1 − fully reduced Trx1), the percentages of oxidized Trx1 for potassium bromide treated cells were similar to that of untreated control (around 40%) and did not change with 0 and 24 hr after treatment. In the other side, treatment of AA8 cells with auranofin, an inhibitor of TrxR (5 µM for 24 hr) causes a clear shift of bands and therefore increases the percentage of oxidized Trx1 (70%) (Fig. 4). These results show that no accumulation of oxidized Trx1 was observed at 0 and 24 hr after potassium bromide treatment, indicating that Trx1 was not a target of DNA damage-independent cytotoxicity (Fig. 5).
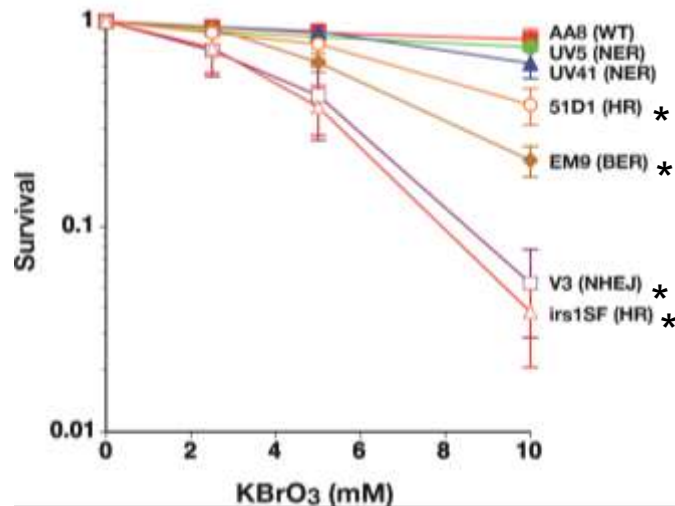
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Fig. (1): **Analysis of cytotoxicity of KBrO$_3$.** CHO cells deficient in various DNA repair genes and AA8 (wild-type) were treated with the indicated potassium bromide concentrations for 1 hr, and their survival was measured using colony formation assay. Data are the means of 3 independent experiments with standard deviations. Statistically significant differences of cell sensitivity at 10 mM concentration (t-test, $p < 0.05$) between the wild type and the mutants are indicated by an asterisk.
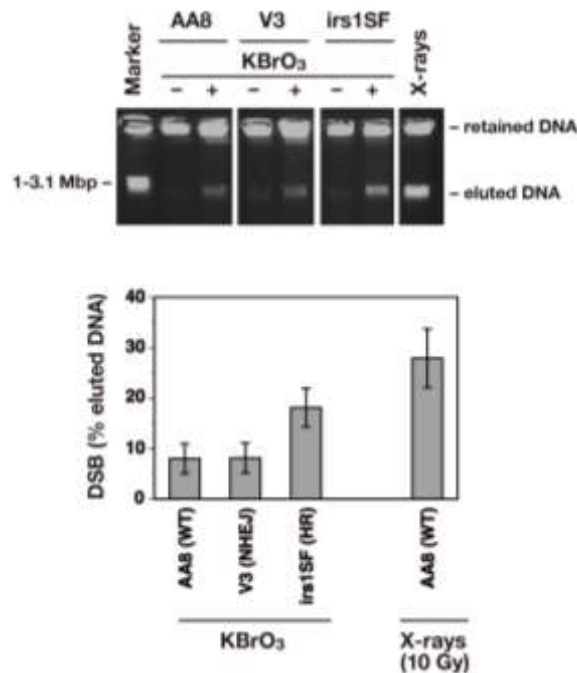

Fig. (2): Analysis of DSBs induction in CHO cells upon treatment with KBrO$_3$. CHO cells (AA8, V3 and irs1SF) were treated for 1 hr with 10 mM potassium bromide as determined from Fig. 1. Cells were collected immediately after treatment and DSBs were analyzed by SFGE (upper panel). The quantity of DNA eluted from the plug relative to total DNA (*i.e.*, eluted and retained DNA) was calculated and the percentage of DSBs was plotted (lower panel). Data points are means of three experiments with standard deviation. X-rays irradiated cells (10 Gy) were used as a positive control and analyzed in parallel with potassium bromide treated cells.
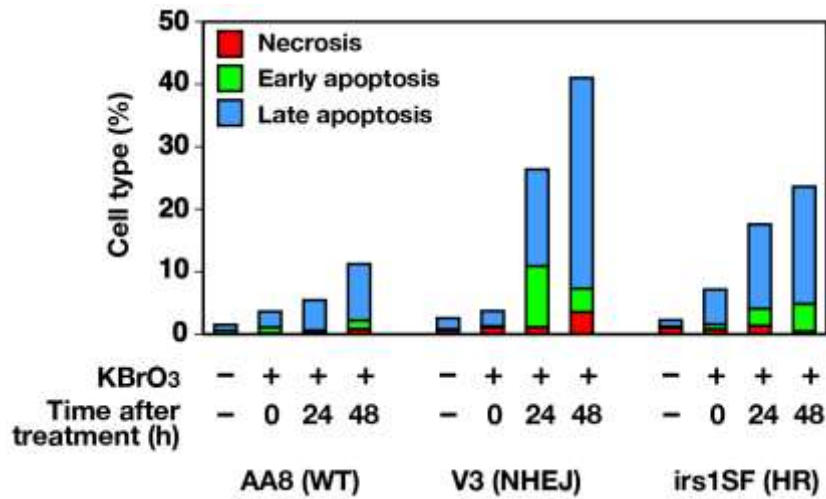
Fig. (3): Analysis of apoptosis in potassium bromide treated cells. CHO cells (AA8, V3 and irs1SF) were treated without (-) or with (+) 10 mM potassium bromide for 1 hr and subjected to post-incubation in a fresh medium for the indicated time (0, 24 and 48 hr). Dead or dying cells were divided into three fractions: necrotic (PI-positive, red), early apoptotic (annexin V-FITC-positive, green), and late apoptotic (PI- and annexin V-FITC-positive, blue). Data are the means of two independent experiments.
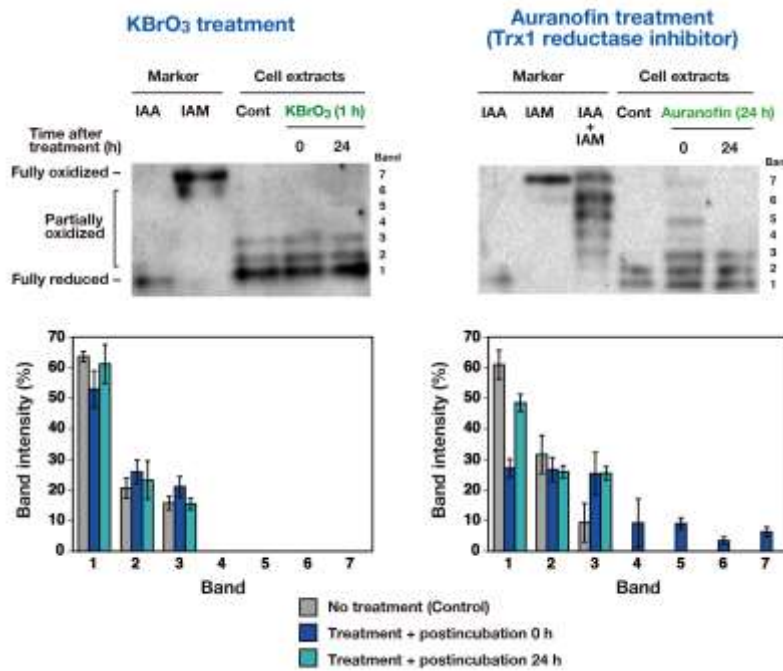

Fig. (4): **Analysis of protein damage by measure the effects of potassium bromide on the redox state of Trx1.** AA8 cells were treated without (Cont) or with 10 mM potassium bromide for 1 hr and the redox states of Trx1 with 0 and 24 hr after treatment were analyzed using modified western blot (upper panel). In the upper panel, Lanes with IAA, IAM, and IAA+IAM were mobility standards prepared as explained in materials and methods. The bands were numbered from the bottom (band 1, fully reduced Trx1) to top (band 7, fully oxidized Trx1). For positive oxidation of Trx1, AA8 cells were also treated with 5 μM auranofin for 24 hr and the redox states of Trx1 with 0 and 24 hr after treatment were analyzed. In the lower panel, the percentages of oxidized Trx1 with 0 hr and 24 hr after treatment were plotted. Data were derived from densitometric analysis of the western blots (upper panel) and are the means of 3 independent experiments with standard deviations.
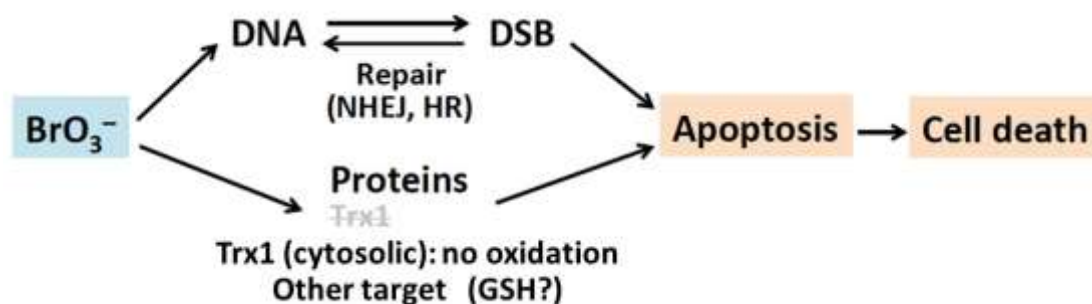
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Fig. (5): A general mechanistic model of bromate cytotoxicity mediated by DNA but not protein damage. Bromates diffuse into cells but don't react with cytosolic (Trx1) proteins. A fraction of bromates that escaped from cytosolic proteins diffuse further and reaches into a nucleus to react with DNA inducing DSBs which is the critical DNA damage associated with bromate cytotoxicity.

DISCUSSION

. Potassium bromide is a food additive product that is widely used around the world. It is well known that bromates cause congestion in the liver and brain edema in rats (Waheeba *et al*., 2016) and affect gene expression in renal tissues and chronic exposure induces carcinomas in rats, hamsters and mice (EPA, 2001; Limonciel, 2012 and Kolisetty, 2013).The cytotoxic effects of bromate are directly related to lipid peroxidation (LPO) and oxidative DNA damage (Ahmad *et al*., 2012b). Potassium bromide induces 8-Oxo-deoxyguanosine (8-oxodG) which is an oxidized derivative of deoxyguanosine (Ballmaier *et al*., 2006). In addition, bromates induce chromosomal abnormalities, micronuclei and γH2AX foci (Priestley *et al*., 2009). Consequently, the cytotoxic and genotoxic effects of bromate are mainly related to DNA damage. However, the role of DNA damage in the cytotoxic effects of bromate has been poorly understood in comparison with that of protein damage. In the current study, we aimed to give insights about the DNA damage-dependent and DNA damage-independent mechanisms of bromate cytotoxicity. We found that, NHEJ (*DNA-PKcs*) and HR (*XRCC3*) DNA repair genes are strongly required for cell survival upon potassium bromide treatment. Thus, DNA repair genes are essential for alleviating the cytotoxic effect of bromate. It is well established that potassium bromide is a DNA- oxidizing agent that induces particular DNA base damage 8-oxodG which is one of the important product of DNA oxidation (Ballmaier *et al*., 2006) this data is consistent with our sensitivity data, in which the BER repair deficient cells (EM9, *XRCC1*) show a moderate sensitivity to potassium bromide indicates the formation of base damage. Previous studies mentioned that bromates may induce DSBs as a result of the processing of 8-oxodG lesions (Sage *et al*., 2011 and Sedelnikova *et al*., 2010). Moreover, several researchers have also mentioned that DSB-specific endpoints as chromosomal aberrations, micronuclei and γH2AX foci have been observed upon exposure to potassium bromate (Kaya *et al*., 2007; Luan *et al*., 2007; Platel *et al*., 2010 and Priestley *et al*., 2009). Nevertheless, in these studies, there is no quantitative data for DSBs formation. Therefore, a quantitative analysis of DSBs after potassium bromide treatment (10 mM for 1 hr) was done. Our results show a significant increase in the yields of DSBs compared with untreated cells, indicating that DSBs are critical DNA damage induced by bromate and the repair of such damage is essential for alleviate the cytotoxic effect of bromate. Both cell sensitivity and DSBs data strongly suggest the DNA damage-dependent

cytotoxicity. Regarding the DNA damage-independent cytotoxicity, we found that no accumulation of oxidized Trx1 immediately and 24 hr after potassium bromide treatment, indicating that Trx1 was not a target of DNA damage-independent cytotoxicity. To my knowledge, this is the first study presents a quantitative data for DNA (DSBs) and protein (Trx 1 oxidation) damages induced by bromate. Based on the current findings, we suggested a mechanistic model of bromate cytotoxicity that is mediated by DNA but not protein damage (Fig. 5). In this model, bromate diffuses in cells and doesn't react with cytosolic proteins (Trx 1), thus a large fraction of bromate diffuses further into the nucleus and reacts with DNA inducing DSBs which is a critical DNA damage associated with bromate cytotoxicity. The current findings provide a good insight into bromate toxicity that associated with DNA damage. However, further studies need to check weather bromate can react with other protein targets that can cause severe damage and impairs protein function.

ACKNOWLEDGMENTS

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ARABIC SUMMARY

نموذج عام لميكانيكية التسمم الخلوي عند التعرض للبرومات عن طريق حدوث تلف فى الحمض النووي

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يستخدم بروميد البوتاسيوم ($KBrO_3$) على نطاق واسع في إنتاج الخبز ومستحضرات التجميل وتطهير المياه. وبروميد البوتاسيوم هو عامل مؤكسد للحمض النووي (DNA) حيث يقوم بعمل تحورات فى القواعد النيتروجينية مثل (8-oxodG) ، كما يسبب عيوب للكروموسومات وتكون النوية الصغيرة وكذلك بؤر γH2AX التي هي من أهم النقاط النهائية لحدوث كسور مزدوجة للحمض النووي(DSBs) ولذلك فإن التسمم الخلوي والجينومي الناتج عن تعرض الخلايا لبروميد البوتاسيوم يرتبط بشكل أساسي بحدوث تلف فى الحمض النووي(DNA). وعلى النقيض من ذلك ، فإن دور حدوث تلف فى الحمض النووي في التأثيرات السامة للخلايا لم يكن مفهومًا بشكل جيد بالإقتران بتأثير التلف الذى يحدث فى البروتين . لقد قمنا فى هذه الدراسة بتوضيح التأثير السمي لبروميد البوتاسيوم من خلال آلية تعتمد بشكل أساسي أو لا تعتمد على التلف الذى يحدث فى الحمض النووي. من أجل إثبات هذا المفهوم ، قمنابعمل تحليل لحساسية الخلاياالتى تحتوى على عيوب فى بعض الجينات المسؤلة عن إصلاح التلف الذى يحدث فى الحمض النووي ومقارنتها بخلايا تعمل بكفاءة لإصلاح هذا التلف ثم قمنا بقياس التلف الناتج فى الحمض النووي وكذلك الذى حدث فى البروتين بعد تعرض الخلايا لجرعات من بروميد البوتاسيوم .وقد أظهرت النتائج أن جينات إصلاح الحمض النووي DNA-PKcs و XRCC3 و RAD51D مهمة وبشدة لبقاء الخلية حية وضرورية للتخفيف من التأثير السام لبروميد البوتاسيوم داخل الخلية كما يُظهر التحليل الكمي للكسور التى تحدث فى الحمض النووى (DSBs)عند تعرض الخلية لبروميد البوتاسيوم زيادة معنوية في كمية الكسور مقارنة بالخلايا التى لم تتعرض لبروميد البوتاسيوم مما يوحي بأن هذه الكسور هي التلف الحرج الذى يحدث فى الحمض النووي (DNA) و يؤكد أن هذا التلف من أهم الأسباب لسمية بروميد البوتاسيوم وعلاوة على ذلك ، لم يلاحظ أكسدة لبروتين السيانيد ١ (Trx)عند معالجة الخلايا ببروميد البوتاسيوم ، مما يستبعد بقوة التلف الذى يحدث للبروتين. إستناداً إلى النتائج الحالية ، إقترحنا نموذجاً ميكانيكياً للسمية الخلوية للبرومات التي تتم بواسطة التلف الذى يحدث فى الحمض النووي (DNA) دون البروتين.