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PROTECTIVE EFFECT OF KOLAVIRON ON BROMATE-INDUCED TOXICITY ON RAW U937 CELLS AND MACROPHAGES

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Abstract

Bromate is used as an additive in bread making and also a disinfection by-product during water purification. However, its reported toxicity seems to limit its usage. The protective effect of kolaviron on bromate-induced toxicity on the cell line U937 and U937-derived macrophages was investigated. In the first instance, cell culture was supplemented with kolaviron before incubated with bromate. Later cell viability and the production of reactive oxygen species (ROS) were investigated using the MTT reduction and DCHF-DA assays, respectively. In the other experiment, the U937 cells were first differentiated to the macrophage form using phorbol-12-myristate-13-acetate. After that, culture having the U937-derived macrophages was supplemented with kolaviron before exposure to bromate. Later, the production of TNF-α and IL-6 was assessed via cytokine ELISA. The expression of the transcription factors iNOS and NF-κB was also determined via RT-PCR. It revealed that kolaviron protected the cells from bromate-induced cytotoxicity and ROS production significantly compared to controls ($p < 0.05$). Pre-incubated the U937-derived macrophages with kolaviron also reduced the bromate-mediated production of TNF-α and IL-6. Kolaviron also reduced bromate-induced expression of iNOS and NF-κB in the macrophages. The kolaviron-mediated response on cytotoxicity, production of ROS and TNF-α was concentration-dependent ($p < 0.05$). The findings show that the reduction by kolaviron on bromate-induced cytotoxicity and production of reactive oxygen species could be linked to the modulation of the inflammatory response.

INTRODUCTION

Bromate is an oxidizing agent used in bread making as a dough conditioner [1]. It is a by-product of hypochlorite treatment and ozonation during treatment of water for consumption [2, 3]. Bromate (as potassium bromate) is also used in hair-colouring, cosmetics and as a food additive in some countries [3-5]. However several studies have reported that bromate causes severe toxicity in experimental models. It has been revealed that bromate is carcinogenic, neurotoxic, and affects thyroid function upon continuous exposure [3, 6, 7]. Bromate also reduces the nutritional quality of bread as it degrades vitamin A2, B1, B2 and niacin thus, its use is banned in some countries [8].

According to the World Health Organization (WHO), almost 80% of the world's population still rely on plants, herbs and herbal formulations as first-line treatment for various ailments [9]. It has been argued that factors contributing to this trend could be poverty, illiteracy, et cetera. However, the consumption of botanical products has been encouraged because less side effects are produced related to their consumption as compared to modern chemotherapy. The medicinal potentials of a lot of plants have been investigated but almost negative correlation is

found between the reported bioactivities and their consumption.

Garcinia kola Heckel (Clusiacaea) is found mainly in Western and Central Africa and highly valued. Various parts of the plant are consumed to alleviate coughs, colic, diabetes, liver diseases, headache, bronchitis, malaria, gonorrhea and also consumed as an aphrodisiac [10, 11]. Kolaviron is a biflavonoid complex extracted from Garcinia kola seeds that exhibit various bioactivities in experimental models such as anti-hepatotoxic, anti-nephrotoxic and anti-carcinogenic effects thus could be beneficial to health [12, 13]. This study investigates the protective ability of kolaviron on bromate-induced cytotoxicity and production of reactive oxygen species (ROS) in the human monocyte cell line U937. The ability of kolaviron to alleviate some bromate-induced alterations on U937-derived macrophages was also investigated.

MATERIALS AND METHOD

Chemicals

Potassium bromate, dimethyl sulfoxide (DMSO), RPMI-1640 media (Roswell Park Memorial Institute), phorbol-12-myristate-13-acetate (PMA), *L*-glutamine, penicillin-streptomycin, fetal calf serum (heat inoculated), 2′,7′-dichlorohydrofluorescein diacetate (DCHF-DA), and 3-(4,5-dimetylthiazol-2-yl),-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemicals (USA). The human macrophage cell line U937 was obtained from the European Collection of Cell Cultures (Salisbury). All antibodies and biotinylated cytokines were products from Pharmingin. All other reagents and chemicals were of analytical grade. Solutions and buffers were prepared using Milli-Q (18 $m\Omega.cm^{-1}$) water and stored at room temperature unless otherwise stated.

Extraction of Kolaviron

The seeds of *Garcinia kola* were purchased from a local market and certified. The seeds were peeled, sliced and sundried for about a week. The dry seeds were pulverized using an electric blender and further dried at 40^0C in an oven. Extraction was done according to Iwu [14]. Briefly, pulverized samples were defatted using petroleum ether (low boiling point) in a soxlet overnight. The defatted dried marc was repacked and extracted with acetone. The acetone layer was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The ethylacetate layer was concentrated to give a golden yellow solid termed kolaviron, which is a biflavonoid complex.

Cytotoxicity Test

The cell line U937 was grown in complete RPMI medium as reported by Okoko and Oruambo [15]. Briefly, cells were grown in RPMI supplemented with fetal calf serum (heat inoculated), *L*-glutamine (0.02M), penicillin-streptomycin and grown at 37^0C under 5 % CO_2 atmosphere. Cell numbers were maintained at 5 x 10^4 cells.mL^{-1}. Cells were later incubated with or without kolaviron (10 µg/mL or 25 µg/mL) for 24 h before exposure to 4 mM bromate (as potassium bromate). Instead of 4 mM bromate, control cells were supplemented with equivalent volumes of RPMI-1640. After 1 h of incubation at 37^0C, cell viability was assessed via the MTT reduction assay as reported [16]. Briefly, MTT was added to each culture to a final concentration of 0.5 mg/mL and incubated for 1 h at 37°C. The MTT layer was aspirated and culture supplemented with DMSO. Absorbance was finally measured at 570 nm using a microplate reader.

Production of Reactive Oxygen Species (ROS)

The production of ROS was assessed according to the method of Koga and Meydani [17]. Briefly, cell culture (maintained at 5 x 10^4 cells/mL) was aspirated and incubated with or without kolaviron (10 µg/mL or 25 µg/mL) for 24 h. Media was removed and replaced with 50 µM DCHF-DA (in RPMI-1640) and incubated for 30 min at 37°C. Cells were later washed with phosphate-buffered saline (0.02 M, pH 7.4) and incubated with bromate (4 mM) for 1 h. Fluorescence of cells was measured at excitation and emission wavelength at 485 nm and 530 nm, respectively. The final value was expressed as % production of ROS.

Production of TNF-α and IL-6

Cell line U937 was first differentiated to the macrophage form using PMA as described [15]. Media were removed and replaced with or without kolaviron and incubated for 24 h at 37^0C. Cells were subsequently incubated with or without bromate (4 mM) and the production of TNF-α and IL-6 analysed via cytokine capture ELISA as described [15].

RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and quantified by measuring absorbance at 260 nm using NanoDrop 1000 Spectrophotometer. The cDNA was synthesized using a Revert Aid cDNA synthesis kit according to the manufacturer's proctocol. For RT-PCR, 1 µg of the resulting cDNA was used to amplify regions specific to iNOS and NF-κB in an ABI Prism 7500 system (Applied Biosciences) with primer pairs listed in Table 1. Real-Time PCR data were analyzed and presented as fold change in expression to the GAPDH housekeeping gene of same sample.

Table 1. Primers pairs for RT-PCR

mRNA	Primer sequence (5′-3′)
iNOS	FP: GTGCCACCTCCAGTCCAG
	RP:GCTGCCCCAGTTTTTGATCC
NF-κB	FP:GCCTTGCATCTAGCCACAGAG
	RP:GATGTCAGCACCAGCCTTCAG
GAPDH	FP:GTCGGAGTCAACGGATTTGGTC
	RP:CTTCCCGTTCTCAGCCTTGAC

Statistical Analysis

Values are expressed as mean ± SEM (n = 6). Data were analyzed using analysis of variance followed by Duncan's multiple range test. Significance was set at $p < 0.05$. All the statistics were carried out using Past3 data analysis package.

RESULTS AND DISCUSSION

The toxicity of bromate has been ascribed to DNA damage principally due to the formation of adducts [18]. It is believed that this oxidative damage is a result of bromate activation that involves reductants such as glutathione which generates active intermediates that form adducts with DNA. This results in single-strand breaks, double strand breaks, mutations, various sugar modifications, and free bases from nucleic acid [4, 19, 20]. It is believed the oxidative damage to DNA is accompanied by lipid peroxidation and oxidative stress and is thought to be the mode of bromate-induced carcinogenicity [21, 22]. As revealed in Figure 1, bromate caused significant cell death compared to controls ($p < 0.05$). Bromate causes serious damage to lipids and proteins via oxidative modifications and has been implicated in various organ disorders [23-26]. It has been reported that the DNA damage (due to the formation of adducts) is an initiation event in cell death which could be necrosis and apoptosis [27]. This supports the significant cytotoxicity caused by bromate in the current experiment. However, incubating the cells with kolaviron before exposure to bromate enhanced the number of viable cells (Figure 1). Incubating the cells with 25 µg/mL kolaviron significantly enhanced number of viable cells than 10 µg/mL ($p < 0.05$). Flavonoids (e.g. kolaviron) protect cells by inducing the expression of phase 2 detoxification proteins such as glutathione, γ-glutamylcystein ligase, glutathione s-transferase and NAD(P)H:quinine oxidoreductase in different cell systems [28, 29]. As revealed in Figure 2, incubating the cells with bromate caused significant ROS production compared to untreated controls ($p < 0.05$). Incubation of cells with kolaviron before exposure to bromate reduced ROS production, which was concentration-dependent ($p < 0.05$). Flavonoids also have the potential as cytoprotective agents by their direct antioxidant property, thereby suppressing oxidative stress [30-32]. This may involve neutralizing free radicals and other toxic reactive oxygen species, reducing and/or chelation of redox active metals, and neutralizing high reactive toxic electrophiles [33, 34].

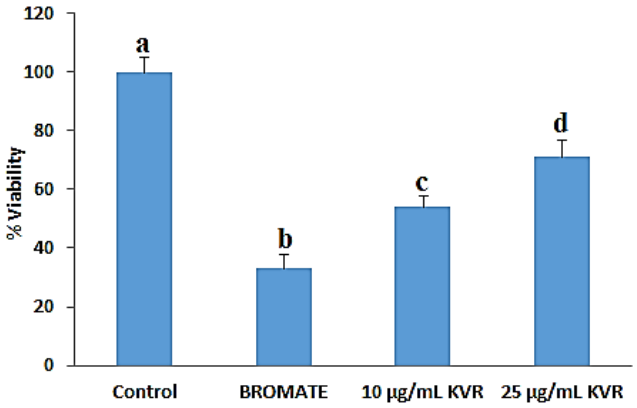

Figure 1. Effect of kolaviron on bromate-induced cell death in U937 cells. BROMATE, cells treated with bromate only. 10 µg/mL KVR, cells treated with 10 µg/mL kolaviron; 25 µg/mL KVR, cells treated with 25 µg/mL KVR before incubating with bromate. Each column represents mean ± SEM (n = 6). Means having different superscript letters differ significantly ($p < 0.05$).

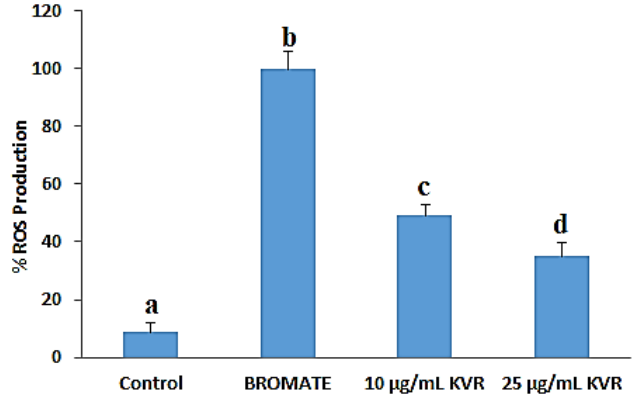

Figure 2. Effect of kolaviron on bromate-induced production of ROS in U937 cells. BROMATE, cells treated with bromate only. 10 µg/mL KVR, cells treated with 10 µg/mL kolaviron; 25 µg/mL KVR, cells treated with 25 µg/mL KVR before incubating with bromate. Each column represents mean ± SEM (n = 6). Means having different superscript letters differ significantly ($p < 0.05$).

The production of pro-inflammatory cytokines by macrophages is key as part of the inflammatory process in the fight against pathogens since cytokines play important roles in many physiological processes, including regulating the immune system [35]. But if the production of these cytokines is deregulated, they contribute significantly to the pathophysiology of a range of disorders which includes organ failure, septic shock, rheumatoid arthritis, asthma, cancer, chronic obstructive pulmonary diseases, viral

infections, autoimmune diseases, hypotension and other systemic responses which could partly be traceable to the production of ROS [35-38]. Activation of macrophages releases cytokines, nitric oxide and other factors which could be detrimental to health. As shown in Figure 3, bromate caused significant production of both TNF-α and IL-6. Pre-incubating the U937-derived macrophages with kolaviron before treatment with bromate reduced both cytokines' production compared to cells treated with bromate only ($p < 0.05$). While the kolaviron effect was concentration-dependent for the production of TNF-α ($p < 0.05$), it was not concentration-dependent for the production of IL-6 ($p > 0.05$).

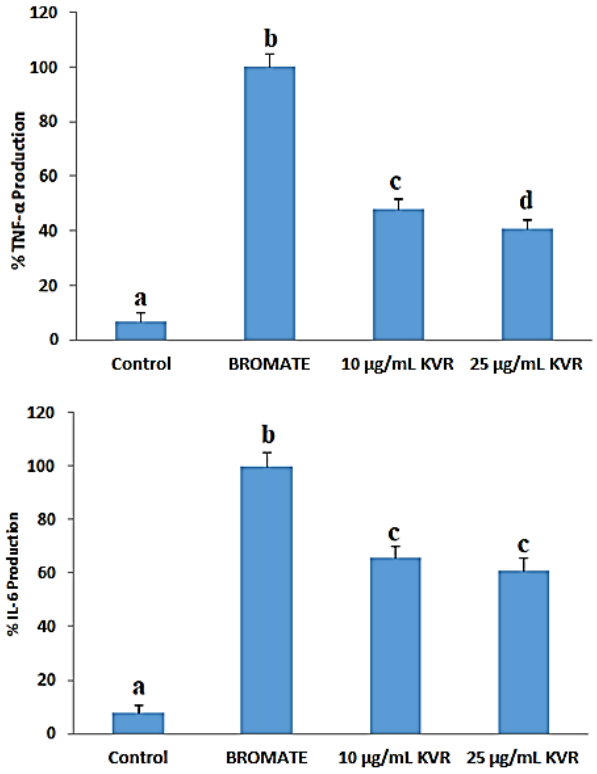

Figure 3. Effect of kolaviron on bromate-induced secretion of TNF-α and IL-6 in U937-derived macrophages. BROMATE, cells treated with bromate only. 10 µg/mL KVR, cells treated with 10 µg/mL kolaviron; 25 µg/mL KVR, cells treated with 25 µg/mL KVR before incubating with bromate. Each column represents mean ± SEM (n = 6). Means having different superscript letters differ significantly ($p < 0.05$).

Nuclear transcription factor kappa-B (NF-κB) is one of the most important transcription factors released by activated macrophages that induces the transcription of iNOS and other pro-inflammatory mediators [39]. This could be responsible for the bromate-mediated upregulation of the expression of both factors when compared to controls ($p < 0.05$) (Figure 4).

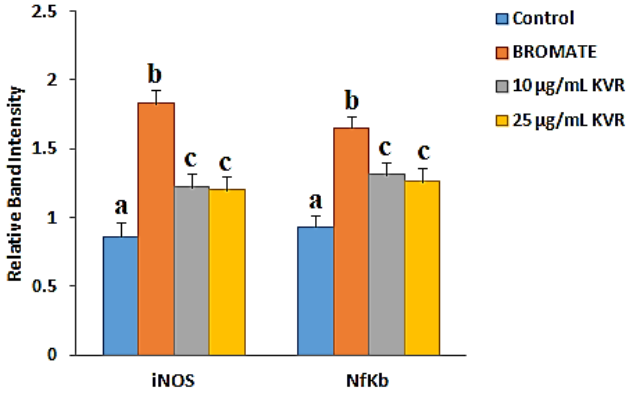

Figure 4. Expression of iNOS and NF-κB in U937-derived macrophages. Values are % fold expressions over housekeeping gene GAPDH for RT-PCR of mRNA isolated from U937-derived macrophages. BROMATE, cells treated with bromate only. 10 µg/mL KVR, cells treated with 10 µg/mL kolaviron; 25 µg/mL KVR, cells treated with 25 µg/mL KVR before incubating with bromate. Each column represents mean ± SEM (n = 6). Means having different superscript letters differ significantly ($p < 0.05$). (Comparisons not made between different markers)

The production of nitric oxide (NO) from arginine is catalyzed by iNOS thus the upregulation of NF-κB will increase levels of NO which can cause organ damage [37]. The RT-PCR data also revealed that pre-incubating the macrophages with kolaviron before exposure to bromate significantly reduced the expression of both factors closer to control levels. However, the effect of kolaviron was not concentration-dependent ($p > 0.05$). It has been reported that kolaviron downregulates the expression of iNOS and NF-κB which is associated with the inhibition of cytokine production [40, 41]. The induction of iNOS could lead to the production of NO which reacts with superoxide anion to produce a more potent oxidant peroxynitrite thus the downregulation of iNOS is an efficient mechanism to curb oxidative stress. Thus a major mechanism by which kolaviron reduces bromate-mediated oxidative damage could be by downregulating the expression of iNOS. The modulation of inducible transcription factor NF-κB triggers a cascade of signaling events involving an integrated sequence of protein-regulated steps, some of which are potential key targets for intervention in treating inflammatory conditions and one of those key targets is the production of pro-inflammatory cytokines [42, 43]. Flavonoids have been shown to directly inhibit the activation/expression of NF-κB by reducing the phosphorylation of the inducible factor [42] and this could be a mechanism of the kolaviron-mediated response. However, this is a subject for further investigation.

CONCLUSION

It has been demonstrated that kolaviron reduces bromate-induced cytotoxicity, production of ROS and activation of U937-derived macrophages. It could be inferred that the cytotoxicity caused by bromate could be a consequence of its effect on the inflammatory response. Hence we conclude that the inhibition of the bromate-induced inflammatory response protects against cell death and production of ROS.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript

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