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Article

The effects of potassium bromate on the apoptosis and survivability of human cell lines

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SUMMARY

Potassium bromate ($KBrO_3$) is a food additive used by companies to increase oxidation in bread and fermented drinks, thereby improving texture or increasing carbonation. We hypothesized that $KBrO_3$ would significantly increase apoptosis and other forms of cell death in human cell lines since it has been shown to induce renal dysfunction and ulceration in mice. We used two major human cell lines, CCD-18 and U937 cells, to model chronic gut diseases. We used an MTT cell viability assay to assess damage after 24 hours and 7 days of $KBrO_3$ exposure. Within CCD-18 cells, we observed significant effects in the 24-hour incubation, where $KBrO_3$ reduced cell viability to ~82% at all concentrations. In U937 cells, we recorded a similar reduction in viability. CCD-18 cells after a 7-day treatment displayed similar results to the 24-hour trials, where we recorded reductions in cell viability to 77%–86%. In contrast, 7-day treatments on U937 cells reduced viability to ~50%, which suggested more severe damage. We performed a caspase-3 assay to assess the effects of $KBrO_3$ on apoptosis. In CCD-18 cells, we observed significant increases in caspase activity of 105% at 0.03 µM, and of 74% at 30 µM. In U937 cells, results fluctuated drastically and there was no clear conclusion. This may be attributed to necrosis taking over as the primary path for cell death. These results suggest that $KBrO_3$ may cause a range of detrimental effects on immune and epithelial cells and may contribute to chronic gut conditions like IBD.

INTRODUCTION

Potassium bromate is an inorganic chemical and a salt of bromate with the potential to be a powerful oxidizer (1). It acts on starches and proteins, removing sulfhydryl groups from gluten proteins and promoting the formation of disulfide linkages, thereby improving the properties of bread dough (2). These properties include the gelatinization, viscosity, and swelling characteristics (2). The chemical may also be used as an oxidizer for malt and barley drinks. The Food and Drug Administration (FDA) permits the use of 75 ppm (parts per million) (449.1 µM) of bromate salts including potassium bromate in malt and barley drinks, 25 ppm (149.7 µM) of which can remain as bromide residue (3). 75 ppm (449.1 µM) of potassium bromate is also the concentration limit placed by the FDA in bread (4). There are three main variables that control the final concentration of potassium bromate after baking: the amount added, the baking temperature, and the baking time. The FDA regulates bromated flour based on these three variables (5). In bread, potassium bromate degrades at 150°C–250°C (6). This degradation process decomposes the potassium bromate in baked goods into O_2 and potassium bromide, a far less harmful chemical (7). Potassium bromide is favorable for two reasons; it lacks the oxygen atoms required to produce free oxygen radicals that cause oxidative stress and is more stable, reducing the risk of decomposition into bromine. In fact, potassium bromide is used in some parts of the world as a sedative and antiepileptic agent (8). However, it cannot be assumed that all the bromate in bread is reduced to bromide, and there is a possibility that some residual bromate may be left in the finished baked product (9). This residual potassium bromate can cause various problems (10). Potassium bromate is rapidly absorbed into the human body, where it is decomposed by the kidney, liver, and digestive tract into bromite, hypobromite ions, and free oxygen radicals (11). This causes problems in three ways: the free oxygen radicals induce oxidative stress, the physical absorption and processing cause damage, and the bromine-containing ions can form new compounds like hydrobromic acid or free bromine, which are also toxins (11).

Research has found potassium bromate to be genotoxic, inflammatory, and generally cytotoxic, while inducing abnormal cell growth such as that of cardiac hypertrophy (12). In human blood lymphocytes, potassium bromate exposure for 48 hours at concentrations of 400 µM–550 µM induced sister chromatid exchange, micronuclei, and chromatid aberration (13). All these effects indicate significant genotoxicity in human cell lines. Potassium bromate also reduces the cell mitotic index, which implies the ability to reduce cell populations directly (13). This mitotic index is calculated by counting the number of mitotic cells per sample using contrast microscopy, then dividing by the total cells (14). If cells cannot reproduce by mitosis, the total cell count will slowly decrease, and the population will eventually disappear. It has also been found that potassium bromate acts as an irritant to the epithelial lining of Wister rats (15). Rats underwent angiogenesis and ulcer proliferation when exposed to potassium bromate (15). However, this research was done on a mammalian, non-human model organism, so potassium bromate may have different effects on human cells. In cell lines derived from mammals, potassium bromate induced the formation of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, via the generation of bromide radicals and oxides (16).

In this research, we attempted to show the effects of potassium bromate on human cells' apoptotic pathways and cell survivability. Apoptosis is one of the major intrinsic pathways of cell death, often referred to as programmed cell death, and plays an opposite role to mitosis in controlling

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cell populations (17). Importantly, it represents changes in an organism›s internal programming due to stimulus, which is important for understanding toxicity and disease (17). We assessed apoptotic activity by measuring the activity of the enzyme caspase (cysteine-aspartic proteases), specifically, caspase-3, since it mediates the final step in mitochondrial apoptosis in cells (18). We assessed survivability by measuring the reduction of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by certain cell nicotinamide adenine dinucleotide phosphate (NAD(P)H) dependent cellular oxidoreductase enzymes (19).

The lowest concentration administered simulated the minute amounts of bromate not fully degraded by the cooking process. The upper concentration limit of this experiment was based on the concentrations published in various *in vivo* studies, as well as the FDA permissible amount of potassium bromate in bread and malted drinks, which is 75 ppm or ~449.1 µM. We hypothesized that potassium bromate would significantly increase apoptosis, necrosis, and other forms of irritation or damage in human cells. Our results supported this hypothesis, where apoptotic markers in caspase-3 increased at nearly all concentrations, and general cell viability decreased as potassium bromate was added. Our study elucidated some negative effects of potassium bromate on human cell lines at minute concentrations, which suggests potential hazards to food safety and public health in countries that still permit its use. In this study, we elucidated the effects of potassium bromate on human cell lines, adding another link between *in vivo* rat studies, *in vitro* studies on primary animal cell cultures, and the effects of potassium bromate on humans.

RESULTS

Two different human cell lines were used to form a more comprehensive picture of potassium bromate's impact on the cells of the gut, especially in the context of the immune system. The CCD-18 cell line was derived from healthy human colon tissue and thus modeled direct damage to the epithelial cell lining in the human gut when exposed to potassium bromate (20). This cell line has empirically been used to model proliferation and differentiation of human colon cells (19). Since many chronic bowel conditions also affect the immune system in the gut, U937 human histiocytic lymphoma cells were used to model human immune cells (20, 21). This cell line was derived from a patient with histiocytic lymphoma, and can differentiate into various immune cells, making it an effective model of neutrophil, eosinophil, and macrophage cells (22). Similarly to CCD-18, U937 has historically been used in similar cell viability and proliferation studies (23). Much of the inflammation associated with chronic bowel conditions like IBS are caused by immune cells, especially macrophages, which often become polarized when exposed to external stimulants (24).

We assessed the effects of potassium bromate on human cell lines by measuring enzyme activity in two cell processes: the metabolization of MTT, and caspase activity as a measure of apoptosis. We assessed cell viability using the MTT assay. Since healthy mitochondria metabolize MTT into purple formazan crystals, quantifying the amount purple formazan produced indicates the productivity of cells' mitochondria (25). This is correlated with cell viability and proliferation. We measured the MTT metabolized by cells colorimetrically to yield an approximate viability value for all testing groups.

Short term cell viability trials

We first performed short-term (24 hour) treatments to examine the acute effects of potassium bromate on cell viability. We exposed human cell lines to 0.03 µM–300 µM potassium bromate on a 10x concentration gradient for 24 hours. In each trial, the ability of cells to metabolize MTT was measured to approximate viability. In CCD-18 cells, we noted a significant reduction in cell viability to around 82% for most concentrations (paired *t*-test, $p = 0.031 < 0.05$). At 0.3 µM and 300 µM, we observed no significant changes in cell viability (paired *t*-test, $p = 0.291$ & $p = 0.083$ respectively, statistical significance at $p < 0.05$). We recorded around 86% cell viability at the 0.03 µM, 3 µM, and 30 µM concentrations in U937 cells. We observed slightly reduced survival at the other concentrations, with 76% cell survival at 0.3 µM, and 77% cell survival at 300 µM (paired *t*-test, $p = 0.0002$ & p

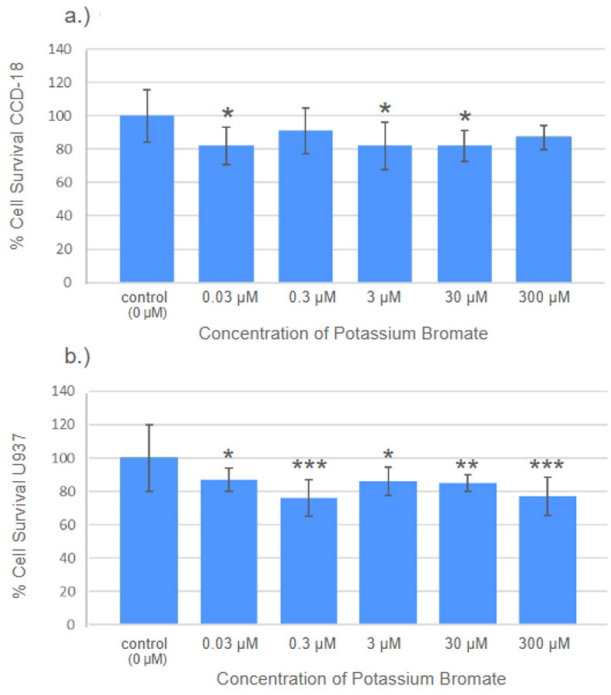

Figure 1: Viability after 24-hour exposure to potassium bromate in CCD-18 colon cells and U937 histiocytic lymphoma cells. We measured cell viability over 24 hours of treatment using a colorimetric MTT assay, where cell survival directly correlates to the % light absorption. The control received no bromate treatment, and experimental groups received bromate on a 10x concentration gradient from 0.03 µM to 300 µM. (A) In CCD-18, we observed significant effects in the 24hr incubation at the 0.03 µM, 3 µM, and 30 µM concentrations, with insignificant changes at all other concentrations (n=16 for all groups). (B) In U937 cells, we recorded a statistically significant decrease in cell viability for all concentrations of potassium bromate tested (n=16 for all groups). Both cell types showed no dose dependency. Error bars show ±1 standard deviation (SD). *P*-values were calculated using one-sample, two-tailed *t*-tests that compared each testing group to the control. Significance is indicated by the asterisks: a p-value <0.05 is notated with (*), a p-value <0.01 with (**), and a p-value <0.001 with (***).

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= 0.0005 < 0.05) (**Figure 1**). Although most concentrations of potassium bromate reduced the viability of both CCD-18 and U937 cells, cell viability never dropped below 75%, suggesting a limit for how quickly cells can take up oxygen and bromide/hypobromite radicals.

Long term cell viability trials

We conducted seven-day long-term trials to assess the effects of longer-term absorption of potassium bromate contaminants and to ensure there was ample time for the chemical to diffuse past the cell membrane. Similar to the acute trials, we exposed human cell lines to 0.03 µM–300 µM potassium bromate on a 10x concentration gradient, this time for seven days. We used each trial's mitochondrial activity to approximate viability. In CCD-18, we observed a significant reduction in mitochondrial activity at nearly all concentrations after the seven-day incubation, reducing cell viability to 77–86%. The lowest viability values we recorded were at 0.03 µM and 300 µM, with 78% (paired *t*-test, $p = 0.007 < 0.05$) and 77% viability, respectively (paired *t*-test, $p = 0.006 < 0.05$). From 0.3 µM–30 µM treatments, we observed cell viability remaining between 80%-86% (paired t-test, $p < 0.05$). These values are similar to the cell viability results observed in the acute trial. However, chronic exposure to potassium bromate resulted in a larger difference in viability in U937 cells. We recorded a roughly linear pattern from 0.03 µM–300 µM, where cell viability was reduced to 69%, then to 55%, then 51%, and finally to 46% viability (paired *t*-test, $p < 0.001$ for all values, statistical significance at $p < 0.05$) (**Figure 2**). While the chronic exposure results for CCD-18 yielded inconclusive evidence for a relationship between exposure time and damage, U937 cells seemed to readily take up potassium bromate as the exposure time increased, and this intake caused significant cell death leading to possible immune damage. The data for U937 cells supported a possible relationship between oxygen, bromide, and hypobromite radicals absorption and exposure time.

Cell apoptotic activity trials

One pathway of cell death that is related to autoimmune conditions or intracellular damage is apoptosis, and all human cells use caspase proteins to induce apoptosis (17). A keystone caspase variant responsible for regulating apoptosis in human cells is caspase-3, so quantifying the percent increase of caspase-3 activity in cells allows us to assess the impact of potassium bromate on apoptosis. Similarly to MTT, we colorimetrically measured caspase activity in all groups to yield approximate values for caspase activity changes. We exposed CCD-18 and U937 samples to 0.03 µM–300 µM potassium bromate on a 10x concentration gradient for 24 hours, then analyzed the treated samples for their apoptotic activity. We recorded somewhat similar results for both cell lines. We observed the highest increase in caspase activity for 0.03 µM potassium bromate, where CCD-18 exhibited a 69% increase (paired *t*-test, $p = 0.016 < 0.05$) and U937 exhibited a 105% increase (paired *t*-test, $p = 0.00002 < 0.05$). The difference in significance values can be attributed to the higher standard deviation in this U937 trial. We also observed a secondary, smaller increase at 30 µM, where CCD-18 exhibited a 49% increase (paired *t*-test, $p = 0.014 < 0.05$) and U937 exhibited a 74% increase (paired *t*-test, $p = 0.011 < 0.05$). All other caspase activity values across the concentration gradient were comparatively lower than observed at these two concentrations. We also observed significant caspase activity changes in CCD-18 cells treated with 3 µM potassium bromate (paired *t*-test, $p = 0.015 < 0.05$) (**Figure 3**).

DISCUSSION

This study aimed to investigate the effects of potassium bromate on human cell apoptosis and viability. We hypothesized that potassium bromate would significantly increase apoptosis, necrosis, and other cellular damage in human cells. We measured both apoptosis, by measuring caspase-3 activity, and cell survival, using a colorimetric MTT assay. We tested concentrations simulating both trace exposure and the legal limit using a 10x concentration gradient from 0.03 µM–300 µM. Our results partially supported our hypothesis. Although there were significant decreases in cell viability across all long-term trials and most short-term trials, there was no concrete evidence of an increase in apoptosis

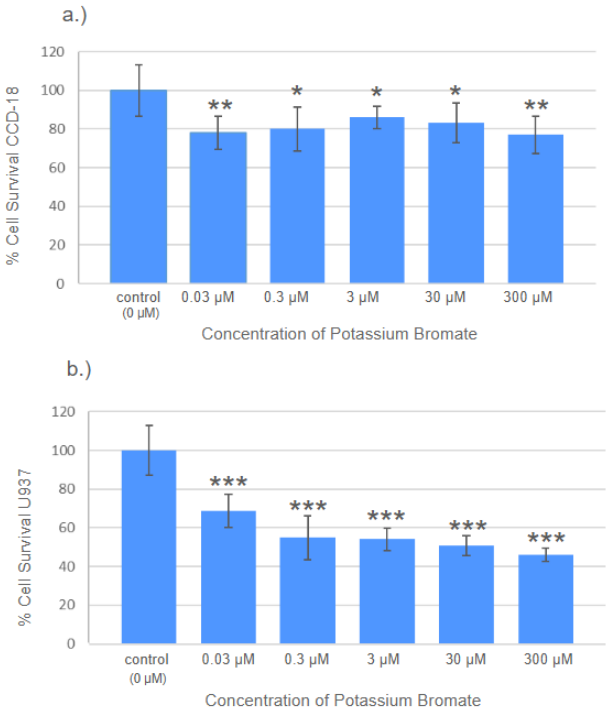

Figure 2: Viability after 7-day exposure to potassium bromate in CCD-18 colon cells and U937 histiocytic lymphoma cells. We measured cell viability over 7 days of treatment using a colorimetric MTT assay, where cell survival directly correlates to the % light absorption. The control received no bromate treatment, and experimental groups received bromate on a 10x concentration gradient from 0.03 µM to 300 µM. (A) In CCD-18, significant changes in cell viability were observed at all concentrations of bromate exposure, but there was no apparent dose dependency ($n=6$ for all groups). (B) In U937 cells, cell survivability decreased dose-dependently as concentration increased ($n=16$ for all groups except 0.03 µM, where $n=8$). Error bars show ±1 standard deviation (SD). *P*-values were calculated using one-sample, two-tailed *T*-tests that compared each testing group to the control. Significance is indicated by the asterisks: a *p*-value <0.05 is notated with (*), a *p*-value <0.01 with (**), and a *p*-value <0.001 with (***).

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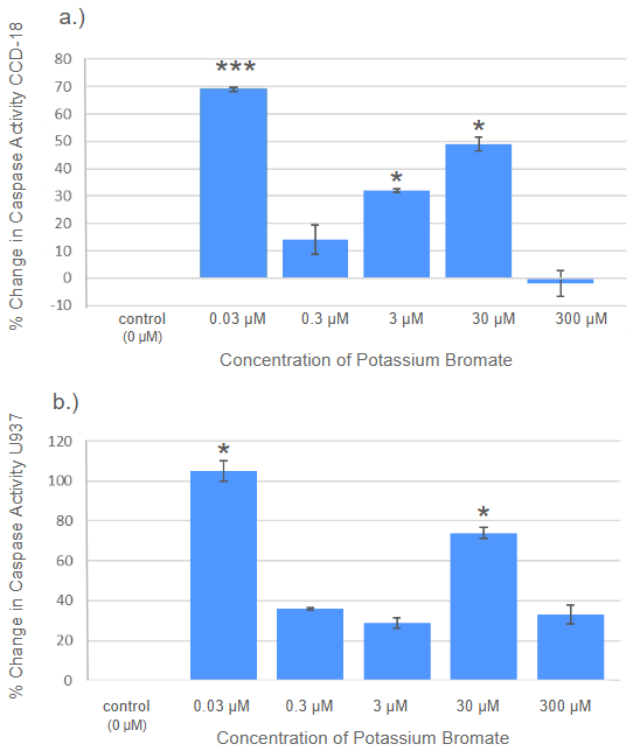

Figure 3: Apoptotic effects of 24-hour exposure to potassium bromate in CCD-18 colon cells and U937 histiocytic lymphoma cells. We measured apoptotic activity over a 24-hour treatment using a colorimetric caspase assay, where caspase activity is directly correlated with % light absorption. We did not treat the control with potassium bromate, and we treated experimental groups with potassium bromate on a 10x concentration gradient from 0.03 µM to 300 µM. (A) We observed significant changes in caspase apoptosis in CCD-18 at the 0.03 µM, 3 µM and 30 µM concentrations, with the greatest changes at the 0.03 µM and 30 µM concentrations (n=3). (B) We recorded inconsistent changes in caspase activity in the presence of potassium bromate in U937 cells, but similar to CCD-18 trials, the largest change recorded was at 0.03 µM and the second largest was at 30 µM (n=3 for all groups). Error bars show ±1 standard deviation (SD). P-values were calculated using one-sample, two-tailed T-tests that compared each testing group to the control. Significance is indicated by the asterisks: a p-value <0.05 is notated with (*), a p-value <0.01 with (**), and a p-value <0.001 with (***).

across all trials.

Our analysis using CCD-18 cell models has implications for the impact of potassium bromate exposure on a full human GI tract. In CCD-18 cells, potassium bromate appeared to significantly impact overall cell viability across all tested concentrations. The model exhibited evidence of cytotoxicity at low to moderate levels. Notably, the colon is capable of processing substantial amounts of potassium without excretion and likely handles dissociated potassium ions from potassium bromate in the same manner as other potassium ion containing species (26). This means potassium uptake by colonic cells is increased with an increase in the quantity of potassium consumed. Within the body, this process could lead to a localized concentration of bromate ions dissociated from potassium bromate in the colon. Considering the cytotoxic effects observed in this study, highly concentrated bromate ions could pose severe risks to colon health. This theory can be explored in the future using an *in silico* cell membrane model, radioactive ion uptake assays made to assess potassium channels, like the K+ ^{86}Rb flux assay, or future *in vivo* research, where it can be applied and tested on a full GI tract. Previous GI tract analysis in mice has shown potassium bromate to be a potent irritant, causing ulcerating and other tissue damage, so analysis of K+ ion uptake may be of interest (15). This rapid absorption may also contribute to the lack of significant differences in long-term and short-term data for CCD-18. In terms of the activity of apoptotic enzymes, potassium bromate induces a range of different effects, from protective effects to significant spikes in activity.

In U937 cells, we showed that caspase activity was highest at the 0.03 µM concentration of potassium bromate and second highest at the 30 µM concentration. This may indicate that potassium bromate has activated other pathways for cell death in U937 cells at concentrations other than 0.03 µM and 30 µM, disrupting the cells enough to divert pathways of cell death away from caspase 3-mediated apoptosis (27). Since we recorded less variability in cell viability than in apoptotic markers, there may be an alternate pathway of cell death acting in conjunction with caspase apoptosis. This can either be caspase-independent death pathways like necroptosis, or cell death induced by external factors not involving internal programming (28). Key signals of necrosis include the flooding of stress proteins, uric acid, ATP, and DNA into the extracellular space, all substances that would have been cleared out by organelles or not produced at all prior to lysis in controlled cell death (29). These substances should be quantified in future research to determine if necrosis is the alternate pathway of cell death. In U937 cells, potassium bromate caused nearly the same amount of cell death at all tested concentrations, as quantified by the MTT proliferation assay. This data suggests that the concentration of potassium bromate may not have much of an effect on the breakdown and absorption of MTT or the greater metabolic properties of cells but has a much larger effect on caspase activity. Regardless, our findings show that potassium bromate affects cell viability negatively through some combination of pathways at all tested concentrations. Additionally, the importance of long-term damage is supported by the 24-hour MTT assay, which showed that after longer incubation times, cell viability was significantly lower under the same potassium bromate treatments. An *in vivo* study on Nubian goats showed immune damage upon exposure to higher concentrations of potassium bromate (30). The study examined the percentage of lymphocytes, monocytes, and eosinophils through blood labs after a 6-week exposure to potassium bromate (30). The percentages of these immune cells were reduced significantly in goats exposed to potassium bromate as compared to control goats (30). However, these goats were exposed to 0.35 M–0.53 M (60–90 mg/kg body weight) potassium bromate, which are significantly higher concentrations than the concentrations and time scales used in this *in vitro* study. Even with these high concentrations and longer exposure times, the results of the study provide evidence for immune damage across systems, and similar effects were observed in our long term MTT trials on U937 cells. However, U937 is an isolated cell line, so analysis of

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human blood labs after extended exposure to potassium bromate may be necessary. This could be done on sample populations exposed to potassium bromate in daily life, such as the population detailed in an analysis of potassium bromate distribution in bread products in India (10). This would rule out variables like incomplete absorption of potassium bromate and its constituent ions in solution. There may also be an underlying mechanism of action where the body uses the immune system to process toxins like bromate. For example, T-cells have developed a complex method to mark microbes, toxins, and allergens, differentiating them from body cells and preparing them for removal by the liver, kidneys, lymph nodes, etc. (31). A system like this would not be modeled in a cell line study but would be better modeled by *in vivo* experimentation. Another factor could simply be that goats are not humans and thus have very different digestive toxin tolerances.

U937 cells, however, are a model often used for their similarities to lone healthy human immune cells, especially monocytes (20). When monocytes are disturbed by external factors like potassium bromate, their gene expression can be altered, and the total monocyte count can also be skewed (32). The fact that potassium bromate significantly impacted the survivability of U937 in long-term trials suggests that monocyte count may be decreased by exposure to the chemical, since U937 cells partially model human monocytes. Reactive oxygen species (ROS) caused by the dissociation of potassium bromate could be a potential cause of this decreased monocyte count (27, 33). Potassium bromate may also cause endothelial cell activation and monocyte-endothelial adhesion (34). This can be tested in future studies through assays such as the cell adhesion assay to confirm the involvement of this ROS-led pathway.

These potential links between potassium bromate, immune cell damage, and gut disease, combined with the direct damage to gut cells caused by potassium bromate exposure, raise reasonable concern that potassium bromate may contribute to increased incidence of gut-related diseases such as irritable bowel disease, Crohn's disease, celiac disease, and ulcerative colitis. Notably, there is a high prevalence of inflammatory bowel disease in the developed world, especially in the US and Great Britain, which have the loosest regulations on food additives (35). The European Union has banned all potassium bromate use in foodstuffs, but there is no study directly examining the effects of this 1990 ban on the overall health of the entire population of the European Union (36). While the developed world shows an increase in cases of irritable bowel diseases like Crohn›s disease, African, Asian, and Eastern European countries report fewer cases of such diseases (37). Although the data of this study did suggest a weak correlation between food additives like potassium bromate and the distribution of chronic autoimmune bowel diseases across populations, there is not enough analysis of global medical records and long-term clinical trials to confirm any causality. Furthermore, many factors may impact the rate of such diseases including lack of reporting/diagnosis, lifestyle, and care infrastructure, among other possible variables.

Potassium bromate has been tested through various *in vivo* studies, most of which yield results reflecting toxicity. One such study administered potassium bromate to mice and rats orally by dissolving it in drinking water for a period of 100 weeks (38). The dosage for the mice was between 0.479 mM and 4.79 mM (0.08 g/l and 0.8 g/l) and the rats between 0.120 mM and 2.39 mM (0.02 g/l and 0.4g/l) (38). It should be noted that these concentrations are around ten times higher than the highest concentration used in our study. All of the animals in the treated group produced tumors, although the number of tumors varied among the treated animals (38). The tumors were found in the kidneys, thyroid, and mesothelium (38). This data suggests two independent effects of potassium bromate. Firstly, a tumorigenic property that causes uncontrolled cell growth, possibly through cancerous mutations, and secondly, a property that causes either programmed or unprogrammed cell death. These mechanisms should be explored in future research.

Our research revealed some of the possible mechanisms of action behind this decrease in cell proliferation and mitosis, expanding on the research done on primary blood lymphocytes and vertebrate trials. Based on our findings, even the concentrations of potassium bromate allowed by the FDA could have significant effects on cell viability despite incomplete absorption by the human body. In the US, nearly 130 companies use potassium bromate in their products (39). Potassium bromate is also a problem outside the US; a study in India in 2020 showed that fully cooked bread on the streets of India still contained at most ~45 ppm of potassium bromate (269.46 μM), an amount that is higher than the FDA-permitted amount for malt and barley drinks, but lower than bread (10). However, we have shown that the FDA-permitted amount is lethal on a cellular level, suggesting that these quantities could still have dangerous effects on humans. Our research introduced the idea that potassium bromate may be a cause of irritable bowel disease and other chronic conditions especially prevalent in the developed world today.

Further experimentation can be done to elucidate the exact pathways by which potassium bromate affects cells. A recent report associated potassium bromate with certain genetic changes such as mutations and deletions when human cells were subjected to treatment times as low as four hours (40). *In vitro* investigations found that many external stimuli, such as chemical exposure, can modify organism epigenetics, including upregulations in gene expression, so future studies should investigate the effects of potassium bromate effects on similar epigenetic changes (41). These changes could also be a possible mechanism of action behind chronic irritable bowel diseases. Techniques like the enzyme-linked immunosorbent assay (ELISA) can be used to confirm cytokine expression changes in cells exposed to potassium bromate, which may explain the pathways behind cell death. Assessing these genetic pathways may also help to explain the two jumps in caspase activity in the 0.03 μM and 30 μM treatment groups for both CCD-18 and U937 cells. Replicates should also be carried out to confirm the consistency of the increases in apoptotic markers at the two concentrations.

Long-term trials modeling chronic health conditions, like IBD, can also be done. Clinical analysis of patients with chronic health conditions related to the digestive tract should also be performed to confirm a correlation between bromate consumption and autoimmune diseases of the gastrointestinal tract. Experimentation and analysis should

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be done to address contradictions in survivability and carcinogenicity data as well. Finally, future research should investigate possible agents that could reverse the effects of potassium bromate.

From this research, we conclude two major ideas. Firstly, through both immune irritation and cell damage, potassium bromate may induce changes in the tissue environment of the human gastric system, suggesting weak causality to various chronic gut conditions. Secondly, there are still many mechanisms of action behind potassium bromate's interactions with the body that need to be explored. This includes the exact ways by which potassium bromate induces tumorigenesis, cell death, and genotoxicity. Overall, this paper serves as another addition to the toxicological profile of potassium bromate, leaving more room for future research.

MATERIALS AND METHODS

Potassium bromate solution preparation

To prepare the potassium bromate stock solution, 10 mg of potassium bromate powder (Sigma-Aldrich #309087) was dissolved in 1000 µL of water, resulting in a 59.880 mM solution. This stock solution was further diluted to 5.988 mM, 598.8 µM, 59.88 µM, 5.988 µM, and 0.5988 µM concentrations by sequentially pipetting 100 µL of the preceding solution concentration into 900 µL of water. The final effective concentration ranges from 0.03 µM-300 µM because each dilution is further diluted by the 20 µL of media in each well.

Cell culture

U937 (histiocytic lymphoma), and CCD-18 (healthy colon) cells were obtained from ATCC (American Type Culture Collection), and potassium bromate was obtained from Sigma Aldrich. Cells were grown in cell culture flasks with EMEM (Eagle's Minimum Essential Media) supplemented by 10% fetal bovine serum (FBS) and 10 mL L-glutamine-penicillin-streptomycin solution per 500 mL of EMEM. All of the cell culture reagents were obtained from ATCC. To replace media, cells were transferred into centrifuge tubes and centrifuged for five minutes at 175 x RCF (relative centrifugal force) to isolate a pellet. The old media was then removed without disturbing the pellet, and the pellet was resuspended in new media. During this media replacement, cells were split into 6 and 96-well plates, containing approximately 20,000 cells/well and 5,000 cells/well respectively. The cells were incubated for 7 days at 37°C, 5% CO_2.

Potassium bromate treatment

CCD-18 cells and U937 cells were treated with potassium bromate at each dilution for either 24 hours for acute trials or 7 days for chronic trials. The media was replaced every 3-4 days and supplemented with the respective potassium bromate concentrations of each treatment. After the incubation period, the media was removed from the 6-well plates, and the cells were then removed from the six-well plates using trypsin (0.5 ml or 1 ml depending on the quantity of cells and media) to dislodge the cells from the bases of the plates. After soaking for five minutes, the wells were rinsed five times using 1 ml of media, and the cells were collected and centrifuged at 131 x RCF. U937 cells were rinsed immediately after incubation without the addition of trypsin and centrifuged at 131 x RCF. After a pellet was isolated, the media was removed and replaced, the cells were resuspended, and 50 µL of caspase or MTT assay lysis buffer (1000 µL of assay buffer and 10 µL of triton X lysis agent) was added. The cells were then stored at -20°C, if it was not possible to complete the assay on the same day. Cells were then processed as per the MTT (Sigma-Aldrich #11465007001) or caspase assay procedures (G-Biosciences #BAQ007).

Assay procedures

The MTT assay, procedure, and materials were from Sigma-Aldrich's Cell Proliferation Kit I (MTT) (Sigma-Aldrich #11465007001). The chemical MTT is processed by mitochondrially active cells into purple formazan crystals and thus can be used to quantify the number of mitochondrially active cells (24). The resulting purple dye from this process can be quantified using a microplate reader as an estimate for the number of live cells present in a sample. Treated cell lines were suspended in solution and then split from a six-well plate evenly into a 96-well plate. Ten microliters of MTT was added to each well and the plate was placed in a 37°C incubator for two hours. After two hours, 80 µL of DMSO (dimethyl sulfoxide) was added to each well, and the plate was left at room temperature for 15 minutes. The plate was read using a Bio-Rad imark microplate reader and Microplate Manager 6 software at 595 nm to get absorbance values. Average absorbance values were measured for each treatment group and to calculate percent viability (proliferation/survival) (**Equation 1**).

$$\% \text{ viability} = (\text{Sample Avg. Abs.} / \text{Control Avg. Abs.}) * 100$$

The caspase assay, procedure and materials were from the G-Biosciences CasPASE Colorimetric Apoptosis Assay (G-Biosciences #BAQ007). Caspase-3 is a key protease in the final stages of the cell apoptotic chain (42). Thus, the caspase activity was used to infer cell apoptosis. 50 µL of caspase assay buffer and 45 µL of caspase lysis buffer were added to each well of a six-well plate. 2.5 ml of generic assay buffer and 20 µL of Triton-X (lysis solution) were also added. After incubating for 30 minutes, this solution was distributed evenly from the six-well plate to a 96-well plate. Next, 5 µL of caspase substrate was added to each well to complete the assay. The plate was then read for average absorbance values using a Bio-Rad imark microplate reader and Microplate Manager 6 at 415 nm. Readings were done every 15 minutes; time between readings was spent incubating in a dark box at room temperature. % change in caspase activity was calculated (**Equation 2**).

$$\% \text{ change in caspase} = \frac{(S_n - S_i)/t - (C_n - C_i)/t}{(C_n - C_i)/t} \times 100$$

was used, where S_n and C_n are the average absorbance readings for treatment and control groups at various times respectively, and S_i and C_i are the average initial absorbance readings for treatment and control groups respectively. t is the time between the current reading and the initial reading right after the completion of the assay procedures.

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