Genotoxicity of drinking water disinfection by-products (bromoform and chloroform) by using both *Allium* anaphase-telophase and comet tests

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Abstract Genotoxic effects of bromoform and chloroform, disinfection by-products of the chlorination of drinking water, were examined by using mitotic index (MI), mitotic phase, chromosome aberrations (CAs) and comet assay on root meristematic cells of *Allium cepa*. Different concentrations of bromoform (25, 50, 75 and 100 µg/mL) and chloroform (25, 50, 100 and 200 µg/mL) were introduced to onion tuber roots. Distilled water was used as a negative control and methyl methanesulfonate (MMS-10 µg/mL) as positive control. All obtained data were subjected to statistical analyses by using SPSS 15.0 for Windows software.

For comparison purposes, Duncan multiple range tests by using one-way analysis of variance were employed and $p < 0.05$ was accepted as significant value. Exposure of both chemicals (except 25 µg/mL applications of bromoform) significantly decreased MI. Bromoform and chloroform (except 25 µg/mL applications) increased total CAs in *Allium* anaphase-telophase test. A significant increase in DNA damage was also observed at all concentrations of both bromoform and chloroform examined by comet assay. The damages were higher than that of positive control especially at 75–100 µg/mL for bromoform and 100–200 µg/mL for chloroform.

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Introduction

Disinfection of drinking water using chlorine may lead to the formation of by-products through reaction of the added chlorine with dissolved organic matter. Of all the compounds possibly formed, trihalomethanes (THMs) are the most common and monitored class of drinking water disinfection by-products (DBPs). They are formed when free available chlorine reacts with natural organic matter in water during water disinfection (Goslan et al. 2009; Zidane et al. 2012).

Some scientific studies have linked THMs to increasing risk of both bladder and colorectal cancer (Kargalioglu et al. 2002; Gruau 2004; Monarca et al. 2004). Epidemiologic studies showed that much of the risk for bladder cancer in drinking water was associated with three factors: THM levels, showering/bathing/swimming (i.e., dermal/inhalation exposure) and the role of genotype (Richardson et al. 2007). Other studies also reported the link between these compounds and pregnancy problems (Ohe et al. 2004; Komulainen 2004). The proposed maximum acceptable concentration for THMs in drinking water is 100 µg/L. The World Health Organization has established guidelines for the concentration of bromoform at 100 µg/L and chloroform at 200 µg/L (WHO 2000; Goslan et al. 2009; El-Attafia and Soraya 2010).

Bromoform is found in chlorinated drinking-water as a consequence of the reaction between chlorine added during water treatment and natural organic substances in the presence of bromide ion. It has also been detected in untreated water at lower levels. Bromoform is the major THM produced by chlorination of seawater during desalination. Although bromoform generally does not induce gene mutations in the standard test systems, a few results related to its inducement on sister chromatid exchanges and micronuclei were also reported (Komulainen 2004; Richardson et al. 2007). Recently these THMs were evaluated for genotoxicity in CHO cells and observed that they were refractory to concentrations of 5 mM. The rank order of chronic CHO cell cytotoxicity was bromoform > chlorodibromomethane > chloroform > bromodichloromethane. Unlike chloroform, bromoform is activated by glutathione S transferase-theta (GSTT1-1) in a transgenic strain of *Salmonella*. Bromoform produced tumors in the large intestine of rats, adenomatous polyps and adenocarcinomas. It also induced aberrant crypt foci in the colon of male rats like other brominated THMs (Kargalioglu et al. 2002; Richardson et al. 2007). At equimolar doses bromoform seemed to be less toxic in the female mouse liver than chloroform although the profile of toxicity was rather similar. There was some evidence of genotoxicity of bromoform in mammalian cells both in vitro and in vivo when tested with sister chromatid exchange test. The International Agency for Research on Cancer (IARC 1991) reported that the evidence in animals was considered limited and

bromoform was considered not classifiable as to its carcinogenicity to humans (Komulainen 2004).

Chloroform is a colorless volatile liquid at room temperatures with a sweet taste and has a characteristic but not unpleasant odor, high lipid solubility, an appreciable solubility in water compared to other THMs (Davidson et al. 1982). Chloroform is by far the most prevalent of the THMs measured. Although chloroform is reported to be non mutagenic or genotoxic in some examined test systems both in vivo and in vitro (Monarca et al. 2004), some positive responses have been observed as well (Kargalioglu et al. 2002; Richardson et al. 2007). Unlike some of the THMs, chloroform is not activated by GSTT1-1 to a mutagen in *Salmonella* (Kargalioglu et al. 2002; Richardson et al. 2007). As cited by Komulainen (2004), the International Agency for Research on Cancer has also evaluated the carcinogenicity of chloroform in experimental animals, and classified it in the group of “possibly carcinogenic to humans”, IARC (1991). Chloroform has caused liver tumors in mice (both sexes), kidney tumors in male mice and rats and liver tumors in female rats (Kargalioglu et al. 2002; Richardson et al. 2007). There has been a consistent, tissue-, species-, strain- and sex-specific pattern in the rate of metabolism, cytotoxicity and cell proliferation in the liver and kidneys, the target organs of carcinogenicity. These findings have suggested that the mode of action for tumorigenesis of chloroform has involved cytotoxicity in the liver and kidneys and that the cytotoxicity induced cell proliferation may be a key component in the carcinogenesis in these tissues. Chloroform can undergo both oxidative and reductive biotransformation in tissues (Komulainen 2004).

Thus some other appropriate bioassays are needed to evaluate these compounds on their potential hazard to human and water environment.

Higher plants are recognized as excellent genetic models to detect environmental mutagens and are frequently used in monitoring studies. Among the plant species, *A. cepa* has been used to evaluate DNA damages, such as chromosome aberrations (CAs) and disturbances in the mitotic cycle since 1940s. It has been used to this day to assess a great number of chemical agents, which contributes to its increasing application in environmental monitoring. The *Allium* test is characterized as a low cost test. It is easily handled and has advantages over other short-term tests

that require previous preparations of tested samples, as well as the addition of exogenous metabolic system. The *Allium* test also enables the evaluation of different endpoints. Among the endpoints, CAs have been the most used one to detect genotoxicity along the years. The mitotic index (MI) and some nuclear abnormalities are used to evaluate cytotoxicity and analyze micronucleus to verify mutagenicity of different chemicals. Not only can onions be stored and handled but also its macroscopic and microscopic parameters are observed easily. Moreover, this system is well correlated with the data obtained from the eukaryotic and prokaryotic system (Sabti and Kurelec 1985; Leme and Marin-Morales 2009; Yıldız et al. 2009; Liman et al. 2011).

The comet assay (single cell gel electrophoresis, SCGE) is a technically simple, highly sensitive, fast and economic test which detects in vitro and in vivo genotoxicity in any cell types examined. It has been introduced to detect even small changes in DNA structure, such as repair activities, its packing mode and its entirety besides requiring just few cells for its execution (Yıldız et al. 2009).

The aim of this study was to evaluate the genotoxic effects of bromoform and chloroform on MI, mitotic phase flaws and damage on DNA by employing both *Allium* anaphase-telophase and comet test systems.

Materials and methods

Organisms

A. cepa (2n = 16) onion bulbs, 25–30 mm diameter, without any treatment, were purchased from a local supermarket in Turkey.

Chemicals

Bromoform 99 % (CAS No. 53-23-3), Chloroform 99.2 % (CAS No.22-38-40-48), methyl methanesulfonate (MMS, CAS No.67-27-3), normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), Trizma base, Tris HCl, Triton X-100 and SDS were purchased from Sigma Aldrich (Munich, Germany).

Mitotic index analysis

Prior to initiating the *Allium* anaphase-telophase test, outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. A series of six bulbs were placed in distilled water for 24 h and afterwards the best growing five bulbs, exposed for 24 h to different aqueous concentrations of bromoform solutions (25, 50, 75 and 100 µg/mL respectively), chloroform solutions (25, 50, 100, and 200 µg/mL, respectively) and MMS (10 µg/mL) at room temperature ($\sim 21 \pm 4$ °C), were used. The concentrations used in this study are chosen to match those found in drinking water in Algeria and the proposed maximum contaminant level regulated by WHO (Achour and Moussaoui 1993; WHO 2000; Komulainen 2004; El-Attafia and Soraya 2010). Fixation and staining of the root tip cells were carried out as reported earlier (Liman et al. 2010, 2011). The MI and the frequencies of CAs were carried out according to Saxena et al. (2005). For each test group, five slides (1 root tip/slide) were prepared by squashing root tips with a solution of ethanol (99 %) and glacial acetic acid (3:1). Slides were randomly coded and scored blindly. For MI, the different stages of mitosis were counted in a total of 5,000–6,000 cells (1,000 cells/slide) per concentration, and expressed a percentage. In the CA test, 100 cells in anaphase or telophase were examined for CAs per slide if it is possible. Types of aberrations scored include disturbed anaphase-telophase, chromosome laggards, stickiness and anaphase bridges.

Application of the comet assay (single cell gel electrophoresis)

20 to 30 seedlings were placed in a petri dish kept on ice and spread with 500 µL of ice-cold Tris-MgCl₂ buffer (0.2 M Tris, pH 7.5; 4 mM MgCl₂·6H₂O; 0.5 % w/v Triton X-100). The roots were immediately chopped with a fresh razor blade and isolated root nuclei collected in the buffer. The preparation of microscope slides with isolated nuclei was as previously described (Liman et al. 2011). Each microscope slide was pre-coated with a layer of 1 % NMPA and thoroughly dried at room temperature. Next, 100 µL of 0.8 % LMPA at 37 °C was mixed with 20 µL of the nuclear suspension and dropped on top of the first layer. The slides were allowed to solidify for 2 min on

an ice-cooled tray and were then immersed in ice-cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5 % w/v SDS, pH 12.3) for 1 h. Subsequent to lysing, the slides were placed in a horizontal gel electrophoresis chamber and the DNA was allowed to unwind for 1 h in the electrophoretic buffer, containing 30 mM NaOH and 1.5 mM EDTA at pH 12.3 (Sabti and Kurelec 1985). Electrophoresis was then conducted for 20 min at 25 V (1 V cm^{-1}) in the chamber cooled on ice. Following electrophoresis, the slides were rinsed three times with water and stained with 50 μL ethidium bromide ($20 \mu\text{g mL}^{-1}$) for 5 min, and covered with a cover slip. For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope (Olympus, Tokyo, Japan). Two slides were evaluated per treatment and each treatment was repeated at least twice. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either 0, 1, 2, 3, or 4 so that the total scores of slide could be between 0 and 100 arbitrary units (AU microgel^{-1}) (Koçyiğit et al. 2005; Liman et al. 2011).

Statistical analysis

The data of MI, mitotic phases and comet scores, expressed as percentages, and the levels of significance in the different treatment groups were analyzed. Duncan multiple range tests were performed by using one-way analysis of variance (ANOVA) on SPSS 15.0 version for Windows software.

Results and discussion

The effects of bromoform and chloroform on MI and mitotic phases in the root tips of *A. cepa* after 24 h are shown in Table 1. In the present study MI values were found significantly lower at all concentrations used in the incubations of root both for bromoform except 25 $\mu\text{g/mL}$ and chloroform and MMS compared to the negative control. The decreasing MI was in a dose dependent manner for bromoform ($r = -0.798$, $p < 0.01$). Based on their solubility, chloroform is considered weakly hydrophilic, and bromoform is hydrophobic. The cytotoxicity level can be determined by the decreased rate of the MI. A MI decrease below 22 % of the control causes sub lethal effects on the test organisms while a decrease below 50 % usually has

lethal effects (Chakraborty et al. 2009). As a result of this statement, we can say that bromoform and chloroform exhibit sub lethal effects at the examined concentrations. The inhibition of MI, significant reduction in percentage of prophase and significant induction in percentage of telophase at all concentrations applied both for bromoform and chloroform as well as MMS, indicated that the treatments interfered with the normal sequence of cell division. This could be probably due to the arrest in the cell cycle before metaphase to restore the integrity of DNA (Abu and Duru 2006). The damaged DNA could increase the time they stay at G2 and prophase. This could be because of the consequence of the maintenance of checkpoints for unrepaired DNA. In this case, post replication repairing or the inhibition of cell cycle's specific proteins results in delaying the mitotic effect (Gonzalez-Fernandez and Lopez-Saez 1985; Hidalgo et al. 1989). On the other hand, the control system of the cell division cycle is blocked by some elements of transition called negative regulators or checkpoints (Del Campo et al. 2005).

CAs results are shown in Table 2. Disturbances were observed at all concentrations of bromoform, chloroform and MMS. Bromoform (except 25 $\mu\text{g/mL}$ application) and 100 and 200 $\mu\text{g/mL}$ applications of chloroform significantly increased the frequency of CAs in *A. cepa* anaphase-telophase cells when compared to the negative control. At these concentrations bromoform and chloroform are as potent as the positive control MMS which is a potent alkylating agent (Rank and Nielsen 1997). Total aberrations for both bromoform and chloroform were found in a dose dependent manner ($r = 0.792$, $p < 0.01$ and $r = 0.831$, $p < 0.01$, respectively). While the lowest anomalies of $3.2 \pm 0.44 \%$ were observed at 25 $\mu\text{g/mL}$ for chloroform, the highest one of $7.62 \pm 0.43 \%$ was observed at 10 $\mu\text{g/mL}$ for MMS. Chromosome laggards and anaphase bridges are the most important CAs found in a dose response manner for both chemicals examined. Disturbed anaphase-telophase and chromosome laggards could occur by the effect of bromoform and chloroform on microtubule formations that may arise due to inhibition of tubulin polymerization (Kuriyama and Sakai 1974; Kumari et al. 2009). Chromosome laggards at anaphase may be due to the failure of the chromosomes or acentric chromosome fragments to move to either of the pole and may result in micronucleated cells (Chakraborty et al. 2009). Anaphase bridges, especially at 75 and 100 $\mu\text{g/mL}$ of bromoform and 200 $\mu\text{g/mL}$ of chloroform,

Table 1 The effects of bromoform and chloroform on mitotic index and mitotic phases in the root tips of *A. cepa* after 24 h

Treatments	Doses ($\mu\text{g}/\text{mL}$)	CCN	Mitotic index $\pm$ SD	Mitotic phases (%) $\pm$ standard deviation (SD)*			
				Prophase	Metaphase	Anaphase	Telophase
Control	–	5,109	23.35 $\pm$ 1.1a	77.8 $\pm$ 4.8a	9.8 $\pm$ 4.43ab	6.03 $\pm$ 1.14a	6.36 $\pm$ 1.91a
MMS	10	5,194	7.04 $\pm$ 0.75b	57.09 $\pm$ 6.78b	14.08 $\pm$ 3.2b	9.39 $\pm$ 3.52bc	19.42 $\pm$ 3.96b
Bromoform	25	5,235	23.02 $\pm$ 2.05a	46.58 $\pm$ 5.66c	7.84 $\pm$ 3.62a	5.11 $\pm$ 0.55a	40.46 $\pm$ 4.49c
	50	5,165	13.62 $\pm$ 0.91c	58.37 $\pm$ 5.83b	13.87 $\pm$ 3.86b	11.94 $\pm$ 1.82 cd	15.8 $\pm$ 2.38b
	75	5,138	13.11 $\pm$ 0.89c	47.01 $\pm$ 3.01c	19.48 $\pm$ 1.9c	13.68 $\pm$ 1.53d	19.82 $\pm$ 2.96b
	100	5,259	12.63 $\pm$ 0.39c	49.28 $\pm$ 6.62c	9.37 $\pm$ 2.6ab	6.92 $\pm$ 1.93ab	34.41 $\pm$ 4.19d
Control	–	5,109	23.35 $\pm$ 1.1a	77.8 $\pm$ 4.8a	9.8 $\pm$ 4.43ab	6.03 $\pm$ 1.14a	6.36 $\pm$ 1.91a
MMS	10	5,194	7.04 $\pm$ 0.75b	57.09 $\pm$ 6.78b	14.08 $\pm$ 3.2a	9.39 $\pm$ 3.52b	19.42 $\pm$ 3.96b
Chloroform	25	5,231	15.5 $\pm$ 0.62c	43.47 $\pm$ 6.58c	5.31 $\pm$ 0.61b	6.17 $\pm$ 2a	45.04 $\pm$ 4.74c
	50	5,461	9.6 $\pm$ 1.15d	47.8 $\pm$ 7.16c	5.75 $\pm$ 2.54b	2.5 $\pm$ 1.92c	44.94 $\pm$ 4.48c
	100	5,066	10.37 $\pm$ 1.14d	50.9 $\pm$ 3.05bc	8.66 $\pm$ 2.89ab	7.41 $\pm$ 0.57ab	32.81 $\pm$ 1.32d
	200	5,092	16.55 $\pm$ 1.2c	49.55 $\pm$ 4.61bc	9.02 $\pm$ 1.67ab	5.46 $\pm$ 0.5a	35.96 $\pm$ 4.48d

MMS methyl methan sulfonate; SD standard deviation; CCN counting cell numbers

* Means with the same letter do not differ statistically at the level of 0.05

Table 2 Percentage of chromosome aberrations of bromoform and chloroform obtained for the *A. cepa* anaphase-telophase test after 24 h

Treatments	Doses ($\mu\text{g}/\text{mL}$)	Anaphase-telophase anomalies %					
		CCN	DAT	CL	S	AB	TA $\pm$ SD*
Control	–	500	0.6	–	2.2	1.2	4 $\pm$ 0.7a
MMS	10	474	4.33	0.65	0.83	1.81	7.62 $\pm$ 0.43b
Bromoform	25	500	–	–	1.56	1.87	3.43 $\pm$ 0.26a
	50	500	1.4	0.4	2.6	1.6	6 $\pm$ 1c
	75	500	–	0.6	3.2	2.8	6.6 $\pm$ 0.89bc
	100	500	0.6	1.8	1.8	2.8	7 $\pm$ 0.7bc
Control	–	500	0.6	–	2.2	1.2	4 $\pm$ 0.7ac
MMS	10	474	4.33	0.65	0.83	1.81	7.62 $\pm$ 0.43b
Chloroform	25	500	0.8	0.2	0.8	1.4	3.2 $\pm$ 0.44a
	50	500	1.6	0.8	0.2	1.6	4.2 $\pm$ 0.83ac
	100	500	1.2	1.4	1	1.2	4.8 $\pm$ 0.89c
	200	500	0.8	1.2	2.6	2	6.6 $\pm$ 1.14b

SD standard deviation; MMS methyl methan sulfonate; CCN counting cell numbers; DAT disturbed anaphase-telophase; CL chromosome laggards; S stickiness; AB anaphase bridge; TA total anomalies

* Means with the same letter do not differ statistically at the level of 0.05

indicating structural chromosomal mutations, could happen during the translocation of unequal chromatid exchange, due to dicentric chromosome presence, due to the breakage and fusion of chromosomes and chromatids or due to less active replication enzymes (El-Ghamery et al. 2000; Luo et al. 2004).

The sensitivity of plant assays is very high, in the range 82–100 %. However, the false positive results are only false in the sense that they do not correlate with carcinogenicity data. If a chemical is able to cause damage to the chromosomes in a reliable plant assay, then the chemical should be considered as

Table 3 Detection of DNA damage in nuclei of *A. cepa* root meristems after exposure to bromoform and chloroform using the comet assay

Treatments	Doses ($\mu\text{g}/\text{mL}$)	DNA damage (arbitrary unit $\pm$ SD)*
Negative control	–	$3.5 \pm 0.7\text{a}$
MMS	10	$13.5 \pm 2.12\text{b}$
Bromoform	25	$7.5 \pm 0.7\text{c}$
	50	$10.5 \pm 0.7\text{d}$
	75	$15.5 \pm 0.7\text{b}$
	100	$21 \pm 1.41\text{e}$
Negative control	–	$3.5 \pm 0.7\text{a}$
MMS	10	$13.5 \pm 2.12\text{bc}$
Chloroform	25	$13 \pm 2.82\text{bc}$
	50	$11.5 \pm 2.21\text{b}$
	100	$17 \pm 2.82\text{bc}$
	200	$15 \pm 0.01\text{c}$

MMS methyl methan sulfonate; SD standard deviation

* Means with the same letter do not differ statistically at the level of 0.05

having the potency of damaging the chromosomes of other organisms in the environment (Chakraborty et al. 2009).

The alkaline comet assay (pH 12.3) has been used as a useful method for monitoring genotoxic effects of environmental pollutants in the root nuclei of various plants species which allows the detection of single- and double-strand breaks, incomplete excision-repair sites and cross-links (Yildiz et al. 2009; Liman et al. 2011). Results obtained from the comet assay are summarized in Table 3. Comet assay results showed that DNA damages were significantly higher at different concentrations of bromoform and chloroform compared to the negative control. The values for bromoform concentrations of 75–100 $\mu\text{g}/\text{mL}$ and for chloroform concentrations of 100–200 $\mu\text{g}/\text{mL}$ are higher than for the positive control. This could be due to DNA damage induced by oxidative stress. The secondary genotoxicity causing potential of the agents could be due to the production of reactive oxygen species or lipid peroxidation, or both. It is notable that, for the test compounds employed in this study, secondary genotoxicity is associated with high concentrations approaching the level for cytotoxicity and may explain why they are only weakly positive in certain genotoxicity assays at high concentrations (Luo et al. 2004). Many studies showed a dose-dependent increase

in DNA double strand breaks by chloroform that occurred prior to cytolethality (Davidson et al. 1982; Beddows et al. 2003). It also increased the formation of DNA strand breaks and lipid peroxidation at non-cytotoxic concentrations. This increase mirrored the elevation in lipid carbonyls and suggests that chloroform can induce indirect DNA damage as a consequence of lipid peroxidation (Beddows et al. 2003). Other studies have shown that THMs, including chloroform, also yield CO as a metabolite. Intraperitoneal administration of haloforms (1–4 mmol/kg) to rats led to dose-dependent elevations in blood CO levels. The order of yield of CO was greatest for bromoform > chloroform for the same dose (Davidson et al. 1982). There is indication that brominated DBPs may be more carcinogenic than chlorinated analogs (WHO 2000).

Both *Allium* and comet test results clearly exhibited the dose-dependent genotoxicity of both bromoform and chloroform. However, DNA strand break measurement was found to be more sensitive than the MI in detecting genotoxicity. Further studies on different applications of both preparations should be of interest. The *Allium* test was carried out for in situ monitoring of the presence of genotoxic and cytotoxic substances in drinking water. The *Allium* test was simultaneously adapted for detecting low levels of DNA damage through comet assay (Chakraborty et al. 2009; Liman 2013). The measurement of DNA damage in the nuclei of higher plant tissues is a new area of study with SCGE. This assay could be incorporated into in situ monitoring atmosphere, water and soil: the comet assay allows a fast detection without any need to wait for progressing mitosis (Cotelle and Férard 1999; Poli et al. 1999).

Based on the results we can conclude that the *Allium* anaphase-telophase test can give more comprehensive data when done in combination with comet assay, which is faster, simpler and independent of mitosis. As a result, both bromoform and chloroform had genotoxic effect by increasing CAs and DNA damage.

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