Universitat Autònoma de Barcelona

Departament de Genètica i de Microbiologia

RIESGO GENOTÓXICO DE LOS SUBPRODUCTOS DE LA DESINFECCIÓN DEL AGUA

TESIS DOCTORAL

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2010

RIESGO GENOTÓXICO DE LOS SUBPRODUCTOS DE LA DESINFECCIÓN DEL AGUA

Memoria presentada para optar al grado de Doctor

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Bellaterra (Cerdanyola del Vallès), Julio 2010

VºBº

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A mis padres y hermanos

Agradecimientos

Y ahora la parte más difícil de escribir, los agradecimientos, y no es porque no tenga a quien(es) agradecer, sino que escribiéndola soy aún más consciente de que mi tiempo aquí llega a su fin. Que puedo decirles… son varios años que este laboratorio y sus integrantes (Mutas, Fanconis, 1era planta) han formado parte de mi vida, viéndonos de lunes a viernes, e inclusive sábado y domingo… es que la pasamos muy bien juntos.

Me gustaría agradecer antes que todo a los directores de esta Tesis, el Dr. Ricard Marcos y el Dr. Amadeu Creus, por haberme brindado la oportunidad de poder realizar este trabajo bajo su tutela, gracias por el tiempo, el apoyo, la dedicación y por la confianza depositada en mi persona.

Expresar mis agradecimientos a la Universitat Autònoma de Barcelona, lugar donde he podido llevar acabo el trabajo de tesis doctoral, por haberme otorgado una beca PIF (PIF409-009) la cual me ha permitido dedicarme exclusivamente a este trabajo y además, el poder realizar una estancia de investigación en la University of Illinois at Urbana-Champaign (Illinois, EEUU), y al proyecto del Plan Nacional de Salud (SAF2005-07643-C03-03).

Gracias a todos por los momentos de té y café… esos momentos de relax que después nos permitía continuar trabajando… gracias por los conciertos, las fiestas, las cenas, los viajes, las excursiones (en la que alguna vez casi nos perdimos pero abriendo nuevos senderos, encontramos el camino)… recuerdos que siempre llevaré conmigo... con fotos y vídeos incluidos.

A todos aquellos que me ayudaron en el transcurso de esa etapa de mi vida… enseñándome las técnicas, apoyándome en los buenos y malos días, brindándome su amistad, aquellos que estuvieron en el momento justo cuando necesitaba hablar con alguien… gracias por todo.

A María, a Carol, a Magaly y a Jenny... aquellas con las que inicie hace varios años el doctorado, y por alguna razón están o no conmigo en este momento… muchas gracias chicas por la amistad que nos une… las quiero mucho… a Mar, a Naouale y a Gonzalo, a pesar de conocernos menos tiempo... sé que puedo contar con Uds. y puedo considerarlos mis amigos... la amistad no se mide por el tiempo que las personas se conocen, sino por la afinidad y la compenetración que sucede de manera espontánea.

Mis más sinceros agradecimientos a Glòria Umbert y a Lourdes Alarcón por toda la ayuda brindada y siempre estar dispuestas a ayudarnos… moltes gràcies Glòria por no haberme dejado abandonar los experimentos y por las numerosas conversaciones

que hemos tenido planeando los experimentos, los resultados que salían de ellos y muchas cosas más.

Y como olvidar a Mounaim... uno de los chicos más amables que he conocido en mi estancia en Barcelona... pero al cual siempre asociaré con el partido de Paintball del 10 de abril de 2010, pero tú sabes que te aprecio mucho.

No es justo recordar sólo aquellos amigos que descubrí en mi experiencia en tierras catalanas… también debo agradecer aquellos que a pesar de la distancia, un océano de distancia, me hicieron sentir que podía contar con ellos, gracias por los mensajes, las horas de chat, las llamadas, por saber que estaban a mi lado apoyándome y preocupándose por mí… muchas gracias queridos amigos, espero poder verlos pronto y seguir con la amistad que nos une desde hace mucho tiempo, con algunas personas inclusive desde el tiempo del colegio.

Y agradecer especialmente a mi familia; a mis tías, tíos, primos y primas que se encuentran en este momento a mi lado en Barcelona… gracias por haber hecho que mi estancia en Barcelona sea mucho más acogedora, agradecer a toda mi familia de Perú por todo el apoyo brindado, y por último, debo agradecer y dedicar este trabajo a mis padres y hermanos… gracias a Uds. soy quien soy ahora y este trabajo ha sido posible gracias a su apoyo incondicional… les estaré eternamente agradecida.

Y como ya se ha dicho antes… GRACIAS TOTALES!!!

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RESUMEN

Los subproductos de la desinfección del agua de consumo son compuestos químicos que se generan por la reacción entre el desinfecante usado, cloro en la mayoría de los casos, y la materia orgánica e inorgánica presente en el agua a desinfectar. El perfil de estos subproductos es diferente entre las distintas plantas de tratamiento, debido a varios factores involucrados en su formación como son el tipo de desinfectante usado, el desinfectante secundario, el pH, la temperatura y la materia orgánica e inorgánica presente, entre otros factores.

Además de la exposición a los SPD mediante el agua de consumo, otra forma de exposición la constituye el uso recreacional del agua de las piscinas. En estas aguas se pueden llegar a encontrarse niveles mayores de SPD, en comparación a los del agua de consumo; este incremento en los niveles de SDP se debe a factores como la recirculación del agua, un mayor tiempo de contacto con el desinfectante y la presencia de precursores orgánicos provenientes de los bañistas.

Numerosos estudios epidemiológicos han investigado los efectos adversos que la exposición a estos SPD puede tener en la salud humana, encontrando una posible asociación entre el incremento en el riesgo de cáncer de vejiga y la exposición a estos subproductos.

Por lo tanto, con la finalidad de incrementar la información sobre el potencial genotóxico de algunos SPD, se seleccionaron 11 SPD (bromonitrometano, tricloronitrometano, tribromoacetaldehido, hidrato de cloral, ácido mucobrómico, ácido mucoclórico, nitrosodimetilamina, nitrosodietilamina, ácido yodoacético, ácido bromoacético, ácido cloroacético), los cuales se evaluaron en los ensayos del cometa, de micronúcleos y de linfoma de ratón. En función de los resultados obtenidos, la mayoría de los SPD seleccionados pueden ser considerados como genotóxicos (ensayo del cometa), aunque tan sólo uno ha inducido daño clastogénico/aneugénico (ensayo de micronúcleos) y otro se ha mostrado como mutagénico (ensayo de linfoma de ratón).

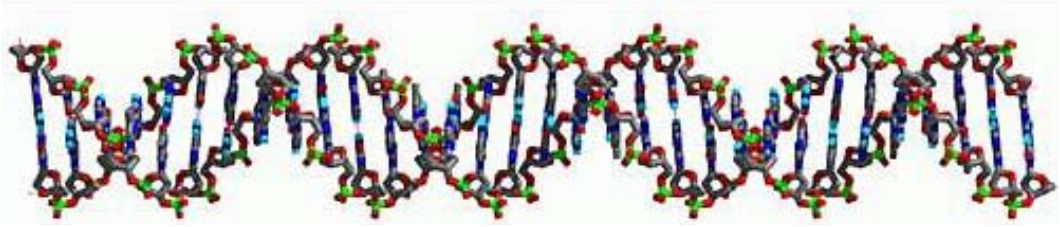
Además, la evaluación del efecto genotóxico del agua de piscina ha corroborado que el proceso de desinfección adicional incrementa su potencial genotóxico, al tiempo que se ha encontrado una posible asociación entre los niveles de ciertos trihalometanos en el aire exhalado de usuarios de piscina y los incrementos en los marcadores de daño genotóxico (frecuencia de micronúcleos).

ABREVIATURAS

BAA:	ácido bromoacético
BCDMH:	bromoclorodimetilhidantoina
BDCM:	bromodiclorometano
BF:	bromoformo
BNM:	bromonitrometano
CAA:	ácido cloroacético
CBPI:	*cytokinesis-block proliferation index,* índice de proliferación celular
CH:	hidrato de cloral
CHO:	células de ovario de hámster chino
CF:	cloroformo
DBCM:	dibromoclorometano
DBPs:	*disinfection by-products*
DNA:	*Deoxyribonucleic Acid,* ácido desoxirribonucleico
DOC:	*dissolved organic carbon,* carbono orgánico disuelto
EPA:	*Environmental Protection Agency*
endo III:	endonucleasa III
FPG:	formamidopirimidina
h:	horas
HAA:	ácidos haloacéticos
HDL:	*high-density lipoprotein,* lipoproteína de alta densidad
HNM:	halonitrometanos
HOBr:	ácido hipobromoso
HOCl:	ácido hipocloroso
IAA:	ácido yodoacético
L:	litros
LDL:	low-density *lipoprotein*, lipoproteína de baja densidad
LMA:	*low melting point agarose,* agarosa de bajo punto de fusión
mA:	miliAmperios
NMA:	*normal melting point agarose,* agarosa de normal punto de fusión
NOM:	*natural organic matter,* materia orgánica natural
MCA:	ácido mucoclórico
MBA:	ácido mucobrómico

MLA:	ensayo de linfoma de ratón
min:	minutos
mg:	miligramos
mL:	mililitros
mM:	milimolar
MMC:	mitomicina C
MN:	micronúcleos
MTT:	azul de tiazolil
MX:	mutágeno X, 3-cloro-4-(diclorometil)-5-hidroxi-2(5H)-furanona
NDEA:	nitrosodietilamina
NDMA:	nitrosodimetilamina
OD:	*odds ratio*
OTM:	*olive tail moment*
RR:	riesgo relativo
RTG:	*relative total growth*
S9:	fracción metabólica microsomal S9
SCGE:	*Single-cell gel electrophoresis,* ensayo del cometa
SPD:	subproductos de la cloración del agua
TBA:	tribromoacetaldehido
T:	temperatura
TCNM:	tricloronitrometano
THM:	trihalometanos
TK6:	línea celular linfoblastoide humana TK6
TTHM:	trihalometanos totales
TOC:	*total organic carbon,* carbón orgánico total
µg:	microgramos
µL:	microlitros
UE:	Unión Europea
UV:	rayos ultravioleta
V:	voltios
WHO:	*World Health Organization*

INTRODUCCIÓN



I. INTRODUCCIÓN

El agua es un elemento esencial para la supervivencia de todas las formas de vida, pero tanto el exceso de agua como su escasez o su calidad deficiente pueden afectar de manera importante la salud y el bienestar de los seres humanos. La disponibilidad de agua de buena calidad para consumo se encuentra entre las principales preocupaciones de la población, a raíz de las graves consecuencias que puede provocar en la salud la presencia de microorganismos patógenos en el agua de consumo.

I.1. Desinfección del agua

La desinfección del agua destinada al consumo humano ha sido la principal medida tomada para proveer protección frente a las diversas enfermedades infecciosas que pueden ser transmitidas por el agua. Desde la introducción de la desinfección del agua potable, se ha observado una reducción muy significativa en la incidencia de personas afectadas en todo el mundo por enfermedades que se transmiten por la ingestión de agua contaminada por microorganismos patógenos. Esta ingestión puede producirse de diversas maneras: directa, mediante la ingestión de agua, indirecta, debido a alimentos o bebidas que han estado en contacto o contienen agua contaminada o accidental, al producirse durante la natación u otras actividades recreativas.

Los desinfectantes más empleados son el cloro libre, las cloraminas, el dióxido de cloro y el ozono. De todos ellos, el cloro es considerado como el desinfectante químico universal porque es el más eficaz en relación a su capacidad desinfectante y a su coste. Además, su potencia oxidante favorece el control del sabor y el olor del agua, restringe el crecimiento de las algas, contribuye a la remoción del hierro y del manganeso, a la destrucción del sulfuro de hidrógeno y, además, tiene una persistencia mayor en los sistemas de distribución.

I.1.1. Introducción histórica

La primera vez que se planteó la hipótesis sobre el papel del agua como medio de transmisión de una enfermedad fue en 1849, año en el que el médico inglés John Snow la relacionó con un brote de cólera. Años más tarde, en 1854, Snow pudo confirmar su teoría con motivo de una epidemia de cólera en Londres, al cerrar el suministro de agua y conseguir frenar la transmisión del cólera; además, también fue

capaz de encontrar la fuente de infección que era un soldado portador del cólera que había regresado de la India, y que vivía cerca de una alcantarilla rota que contaminaba el sistema de distribución de agua (White, 1999).

Uno de los primeros usos del cloro en un proceso continuo de desinfección del agua fue probablemente el que se llevó a cabo en la pequeña ciudad de Middelkerke (Bélgica) en 1902; el proceso se conocía como *Ferrochlor process*, en el cual el cloruro férrico se mezclaba por coagulación con hipoclorito de calcio para formar hidróxido de hierro y ácido hipocloroso.

La cloración continua del agua empezó a utilizarse en los primeros años del siglo XX en Gran Bretaña, dando como resultado una reducción significativa de las muertes producidas por la fiebre tifoidea. Al observar estos resultados, la desinfección mediante el uso de cloro (cloración) comenzó a usarse en Jersey City (New Jersey) en 1908. Esta medida fue después adoptada por otras ciudades y pueblos de Estados Unidos (EEUU), resultando en una eliminación casi completa de las enfermedades transmitidas por el agua tales como el cólera, la fiebre tifoidea, la disentería y la hepatitis A.

Antes del uso del cloro en el tratamiento del agua potable, la tasa de mortalidad anual producida por la fiebre tifoidea era de alrededor de 36 fallecimientos por cada 100.000 personas en los EEUU. A partir de la cloración, la mortalidad empezó a disminuir, llegando a ser de 20 por cada 100.000 personas en 1910, de 3 por cada 100.000 personas en 1935, hasta prácticamente desaparecer a principios de la década de los años cincuenta (Figura 1.1).

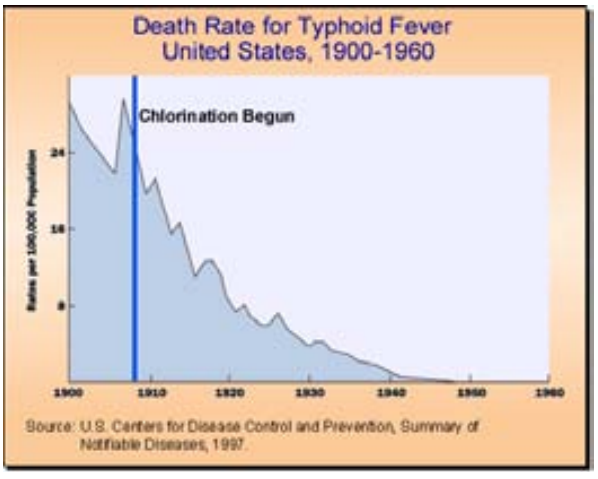


Figura 1.1. Disminución de la tasa de fiebre tifoidea a partir de la desinfección del agua – EEUU. Fuente: Chlorine Chemistry Council.

1.1.2. Importancia de la desinfección del agua en la eliminación de enfermedades transmitidas por la misma

La Organización Mundial de la Salud estima que alrededor de 1.100 millones de personas, a nivel mundial, beben agua no apta para el consumo y que el 88% de las enfermedades diarreicas son atribuibles al agua no apta, así como a la falta de higiene y de sanidad (WHO, 2003); asimismo, se estima que el 80% de todas las enfermedades y más de un tercio de las muertes en los países en desarrollo son causadas por el consumo de agua contaminada. De acuerdo con la información disponible sobre los mayores factores de riesgo para la salud humana; el consumo de agua no apta, la sanidad precaria y la falta de higiene se encuentran en la tercera posición, siendo los dos factores de mayor riesgo la desnutrición y la práctica de relaciones sexuales sin medidas de seguridad.

Las enfermedades transmitidas por el agua son la razón principal de que se desinfecten los sistemas de abastecimiento de agua potable. Estas enfermedades, que son causadas por la ingestión de agentes patógenos que se encuentran en el agua potable o en alimentos contaminados, están consideradas entre las enfermedades que producen los efectos más severos sobre la salud humana. La transmisión de enfermedades infecciosas y parasitarias, confirmada por estudios epidemiológicos y microbiológicos, incluye la hepatitis A y E, la gastroenteritis retroviral y la debida al virus Norwalk, el cólera, la fiebre tifoidea, la enteritis por Campylobacter, la shigelosis, infecciones por *Eschericia coli*, la enteritis por *Yersenia enterocolitica*, la criptosporidiosis, la giardiasis, la disenteria amebiana y la dracontiasis (Galal-Gorchev, 1996a). El impacto que tienen estas enfermedades sobre la salud humana es enorme; todos los años un gran número de personas enferman y mueren debido a ellas (Tabla 1.1.). Además, en promedio, nada menos que la décima parte del tiempo productivo de cada persona se pierde como consecuencia de enfermedades relacionadas con el agua.

Tabla 1.1. Tasas mundiales de morbilidad y mortalidad de las principales enfermedades relacionadas con el agua

	Número / Año	
	Casos de enfermedad	**Defunciones**
Cólera (1993)[a]	297.000	4.971
Fiebre tifoidea	500.000	25.000
Giardiasis	500.000	Pocas
Amebiasis	48.000.000	110.000
Enfermedad diarreica (menos de 5 años)	1.600.000.000	3.200.000
Dracontiasis (enfermedad del gusano de Guinea)	2.600.000[b]	---
Esquistosomiasis	200.000.000[b]	200.000

[a] Las cifras máximas para el cólera en 1991 fueron 595.000 casos y 19.295 defunciones.

[b] Número total de casos en el mundo. (Galal-Gorchev, 1996b).

Entre las enfermedades transmitidas por el agua, el grupo de las enfermedades diarreicas es la causa principal de mortalidad y morbilidad infantil en los países pobres y en desarrollo, pudiendo afectar también a otros grupos de edad. Se estima que del total de defunciones mundiales vinculadas con la diarrea, más del 90% ocurren en niños menores de cinco años. Las enfermedades diarreicas constituyen una de las amenazas más graves para la salud a la que se enfrenta la población infantil de América Latina y el Caribe. Este grupo de enfermedades se cuenta entre las cinco causas principales de muerte en los niños de menos de un año de edad y, en muchos casos, es la primera causa de muerte en los niños de uno a cuatro años (Traverso, 1996).

De acuerdo con la Figura 1.2., en aquellos países en los que la población tiene un menor acceso al agua de consumo segura o apta, hay una mayor cantidad de muertes de niños menores de 5 años; en países en que menos del 30% de la población tiene acceso, se producen al año alrededor de 160 muertes por cada 1.000 habitantes, mientras que en los países en que casi la totalidad de la población tiene acceso, la mortalidad infantil es menor de 15 por cada 1.000 habitantes y año.

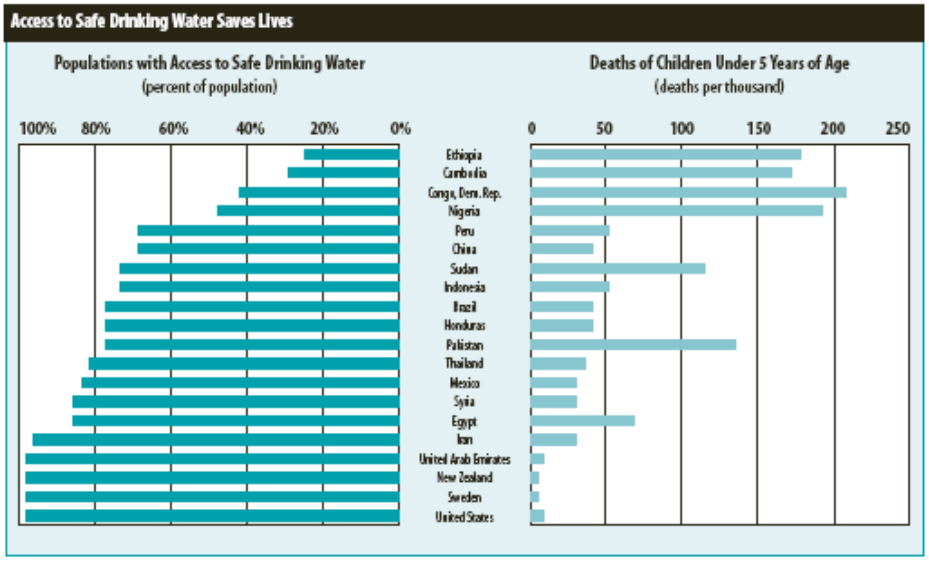


Figura 1.2. Relación entre muertes de niños menores de cinco años y el acceso al agua apta para el consumo. Fuente: Chlorine Chemical Council.

La desinfección del agua, en la mayoría de los casos mediante la adición de cloro, ha supuesto la disminución de los índices de morbilidad y mortalidad de varias enfermedades, especialmente de aquellas cuya transmisión se produce por el agua contaminada o mal desinfectada. En 1991, Esrey et al.analizaron el impacto de mejorar la sanidad y el abastecimiento de agua sobre determinadas enfermedades (diarrea, dracontiasis, esquistotosomiasis), observando que una o más mejoras (calidad del agua, higiene y sanidad) puede reducir substancialmente las tasas de morbilidad de estas enfermedades en un 26%, 77% y 78%, respectivamente. Además, la mejora de la calidad del agua de consumo conlleva una reducción de más de la mitad de la mortalidad infantil (Esrey et al., 1991).

I.2. Subproductos de la desinfección del agua

El agua potable que se produce en las plantas de tratamiento y que es distribuida para consumo humano no puede considerarse como químicamente pura. Aunque fundamentalmente sea H_2O, en ella también se encuentran numerosos compuestos orgánicos e inorgánicos producidos como consecuencia del proceso desinfección. Estos compuestos, que se hallan en mayor o menor cantidad, se encuentran en todas las aguas desinfectadas químicamente que contenían materia orgánica natural.

Actualmente, se llaman subproductos de la desinfección del agua (SPD) *(Disinfection By-Products, DBPs)*, a aquellos compuestos químicos orgánicos e inorgánicos que se forman durante el proceso de desinfección, al reaccionar el desinfectante con los llamados precursores de estos compuestos. La materia orgánica natural presente en el agua y el ión bromuro (u otros iones) se consideran como los precursores orgánicos e inorgánicos, respectivamente.

Cada desinfectante produce una gama de subproductos diferentes, dependiendo de las características del agua, de las condiciones del proceso de desinfección e incluso de la combinación de desinfectantes usados (desinfectante primario y secundario).

Por tanto, aunque la desinfección del agua es una barrera contra los microorganismos que pueden originar enfermedades que en su momento causaron grandes epidemias y que las siguen causando pero en menor medida; también implica la presencia de compuestos potencialmente tóxicos y/o genotóxicos, a los que cualquier persona que consuma agua desinfectada estaría expuesto (Meier et al., 1986; Meier, 1988).

I.2.1. Descubrimiento de los subproductos de la desinfección

A mediados de los años 1970, Rook y Bellar observaron de forma independiente que el cloro reaccionaba con la materia orgánica presente en el agua para formar metanos trihalogenados.

En 1974, Rook descubrió en el agua de consumo de Rotterdam que el cloro libre cuando reaccionaba con la materia orgánica presente en el agua formaba una amplia gama de sustancias, a las cuales identificó como los primeros subproductos de la desinfección: el cloroformo y los otros tres trihalometanos.

En este mismo año, Bellar y colaboradores describieron la presencia de haluros orgánicos en el agua de consumo clorada. Al analizar el agua del río Ohio, que era usada para la producción de agua potable, descubrieron que el cloroformo se hallaba en concentraciones muy bajas, casi ausente en el agua del río, pero que posteriormente se encontraba en concentraciones mucho más altas en el agua que salía de las plantas de tratamiento; es decir, en aquellas aguas que habían sido sometidas al proceso de desinfección (Bellar et al., 1974).

I.2.2. Precursores de los subproductos de la desinfección

Los precursores de los subproductos de la desinfección pueden agruparse en dos grande grupos, los precursores orgánicos y los inorgánicos. La materia orgánica natural (NOM), en particular las sustancias húmicas presentes en el agua no tratada (es decir, en las aguas naturales), constituye el grupo más abundante de precursores orgánicos que reaccionan con el desinfectante en el proceso de desinfección, dando lugar a la formación de los SPD (Bellar et al., 1974; Rook, 1976; Johnson et al., 1982; Christman et al., 1983; Miller and Uden, 1983; Oliver, 1983; Uden and Miller, 1983; Reckhow et al., 1990; Sánchez Jiménez et al., 1993; Singer, 1994; 1999; Nikolaou et al., 2004).

La NOM incluye una variedad de compuestos orgánicos, cuyas concentraciones varían dependiendo de la vegetación próxima a la fuente hídrica, la concentración de algas en el agua y la época del año (Kavanaugh et al., 1980; Singer, 1994). Una fuente importante de NOM la constituyen las sustancias húmicas (ácidos húmicos y fúlvicos) y las sustancias no húmicas, principalmente proteínas y aminoácidos, azúcares y polisacáridos, ligninas, ceras y lípidos (Richardson, 2003). Una característica de las aguas naturales es que el 90% de las sustancias húmicas disueltas son ácidos fúlvicos y el 10% ácidos húmicos.

El otro grupo de precursores, los inorgánicos, está formado por los componentes inorgánicos naturales y antropogénicos presentes en el agua, procedentes de varios minerales y otras sustancias derivadas de fuentes abióticas, como el desgaste geológico. El precursor inorgánico más común es el bromuro, cuya concentración puede variar entre 10 y 100 µg/L en aguas superficiales, pero es menos frecuente en aguas subterráneas. La presencia de bromuro trae consigo la brominación (adición de bromo) de los compuestos orgánicos, incluso durante la cloración. Además del bromuro, otro precursor inorgánico presente en las aguas no tratadas es el yoduro.

I.2.3. Formación de los subproductos de la desinfección

El cloro es el desinfectante de agua potable más comúnmente usado. Al reaccionar con la materia orgánica (sustancias húmicas) presente de forma natural en la mayoría de las fuentes de agua, forma una variedad de subproductos halogenados como son los trihalometanos, los ácidos haloacéticos, los haloacetonitrilos, el hidrato de cloral y la cloropicrina (tricloronitrometano) de la siguiente manera:

$$HOCl + DOC \rightarrow SPD$$

La reacción entre el cloro y las sustancias húmicas (ácidos húmicos y ácidos fúlvicos), es la responsable de la producción de los compuestos organoclorados durante el tratamiento del agua de consumo. Los ácidos húmicos y fúlvicos muestran una alta reactividad con el cloro y constituyen el 50-90% del total del carbono orgánico disuelto (DOC) en las aguas de los ríos y los lagos. Otras fracciones del DOC comprenden los ácidos hidrofílicos (hasta un 30%), los carbohidratos (10%), los ácidos carboxílicos (5%) y las proteínas/aminoácidos (5%). La reactividad de los carbohidratos y los ácidos carboxílicos con el cloro es baja, por lo que se espera que no contribuyan de manera importante a la producción de los compuestos organoclorados (WHO, 2000).

El cloro libre, que corresponde a la suma de ácido hipocloroso (HOCl) y el ión de hipoclorito (OCl), reacciona con los constituyentes del agua mediante tres vías generales: oxidación, adición y sustitución (Johnson and Jensen, 1986). El cloro puede experimentar una reacción de adición si el compuesto orgánico tiene doble enlace. Ahora bien, para muchos compuestos con doble enlace, esta reacción es muy lenta para ser considerada de importancia en el tratamiento del agua. Las reacciones de oxidación con los carbohidratos o los ácidos grasos son también generalmente muy lentas.

Sin embargo, la mayoría de los subproductos de cloración se forman mediante reacciones de oxidación y sustitución. El cloroformo puede producirse mediante una serie de reacciones con grupos funcionales de las sustancias húmicas siendo los mayores grupos funcionales de las sustancias húmicas el acetil, el carboxilo, el fenol, el alcohol, el carbonilo y el metoxil. Es de destacar que las reacciones se dan más rápido a pH altos que a pH bajos. Rook (1977) propuso que las estructuras resorcinol eran las mayores precursoras en el material húmico para la formación de cloroformo.

Muchas aguas tratadas no sólo contienen compuestos clorados, sino también compuestos bromados como el bromoformo. Estos compuestos se forman debido a que el cloro acuoso convierte el bromuro presente en el agua en ácido hipobromoso. Este ácido puede luego reaccionar con la materia orgánica, de la misma manera que el ácido hipocloroso, y formar varios subproductos bromoclorados. Comparado con el ácido hipocloroso, el ácido hipobromoso es un oxidante más débil pero un agente halogenante más fuerte.

La química de la reacciones entre el cloro y los ácidos fúlvicos y húmicos es compleja y no muy bien entendida e, incluso, es imposible poder predecir con exactitud la naturaleza y las cantidades exactas de los productos químicos que pueden

formarse bajo la gran variedad de condiciones que pueden darse en las plantas de tratamiento de agua potable. Así, la formación de los productos químicos durante la desinfección se ha descrito que dependen del tiempo de reacción, del cloro, del tipo y concentración de la materia orgánica presente en las aguas naturales, de la temperatura, del pH de cloración y de la concentración de ión bromuro (Stevens et al., 1989).

I.2.4. Factores que influyen en la formación de los SPD

Existen diversos factores que afectan a la formación y especiación de los SPDs entre los cuales se encuentran la concentración y las propiedades de la NOM, así como la temperatura, la estación del año, el pH, el tiempo de contacto, la concentración de cloro y del cloro residual y la concentración de bromuro.

Teniendo en cuenta que la NOM se considera como el precursor principal en la formación de los SPD; al producirse un incremento en la cantidad de esta sustancia precursora se provocaría, por tanto, un incremento en los productos que se forman (SPD) (Bellar et al., 1974; Rook, 1976; Uden and Miller, 1983; Sánchez Jiménez et al., 1993). La composición de la NOM también juega un papel importante en la formación de los SPD; siendo aquellas aguas ricas en ácidos húmicos (aguas superficiales), en las que se encuentran mayores concentraciones de SPD, comparadas con aquellas con bajos niveles de ácidos húmicos (aguas subterráneas) (Palacios et al., 2000; Nissinen et al., 2002; Villanueva et al., 2003; Nikolaou et al., 2004).

Otro factor que influye en la formación de los SPD es la temperatura ya que, cuando la temperatura se incrementa, las reacciones que tienen lugar son más rápidas y las dosis de cloro que se requieren son mayores, lo que da lugar a un incremento en la formación de SPD. Por tanto, teniendo en cuenta el efecto de la temperatura, se espera que en aquellos meses de calor (verano) se encuentren mayores concentraciones de SPD que en los meses de frío (invierno) (Kavanaugh et al., 1980; LeBel et al., 1997; Williams et al., 1997; Williams et al., 1998; Nissinen et al., 2002; Nikolaou et al., 2004).

Variaciones en el pH del agua provocan la formación preferencial de ciertos SPD; así, aumentos en el pH favorecen incrementos en la formación de trihalometanos y una disminución en la de los ácidos haloacéticos, haloacetonitrilos y halocetonas (Rook, 1976; Kavanaugh et al., 1980; Miller and Uden, 1983; Johnson and Jensen, 1986; Reckhow et al., 1990; Pourmoghaddas and Stevens, 1995).

Se ha observado una fuerte asociación entre la tasa de formación de trihalometanos y la dosis de cloro aplicada (Kavanaugh et al., 1980) resultando que a dosis mayores de cloro, aumentan los niveles de trihalometanos en el agua desinfectada.

Además de la dosis de cloro aplicada, otro factor involucrado es el tiempo de contacto con el desinfectante. La formación de trihalometanos y ácidos haloacéticos se ve incrementada con el tiempo de contacto, lo que implica que estos SPD continúan formándose en el sistema de distribución mientras el cloro libre residual persista. Situación inversa ocurre con los haloacetonitrilos y las haloacetonas, que se forman muy rápidamente al inicio pero luego disminuyen debido a que se hidrolizan (Bellar et al., 1974; Uden and Miller, 1983; Johnson and Jensen, 1986; Singer, 1994; Pourmoghaddas and Stevens, 1995; Yang and Shang, 2004).

La presencia y la concentración del ión bromuro es un factor que influye en la formación de los SPD. Cuando el cloro libre (ácido hipocloroso) se encuentra en presencia del ión bromuro, éste reacciona oxidando rápidamente el ión bromuro a ácido hipobromoso (HOBr), el cual reacciona con la NOM para formar compuestos bromados y bromoclorados y se produce una mayor cantidad de compuestos de bromo (Bellar et al., 1974; Oliver, 1983; Peters et al., 1991; Cowman and Singer, 1995; Pourmoghaddas and Stevens, 1995; Heller-Grossman et al., 1999; Nikolaou et al., 1999; Cancho et al., 2000; Richardson et al., 2003; Yang and Shang, 2004; Hua et al., 2006). El grado de incorporación de bromo en los SPD depende de la concentración de ión bromuro relativa a la cantidad de cloro libre aplicado. A medida que aumenta la concentración de bromo, su incorporación de bromo en los SPD también aumenta. En agua con altas concentraciones de bromo, los subproductos principales que se forman son las especies bromadas, por ejemplo, el bromoformo y el ácido dibromoacético. Hay que señalar que el bromuro presente en el agua a desinfectar puede ser originado por la contaminación humana o por el ingreso de agua salada, además de las fuentes naturales de bromuro.

I.2.5. Beneficios y riesgos de la desinfección del agua

Como ya se ha comentado, la filtración y la desinfección con cloro del agua potable han sido responsables, en gran parte, del 50% de aumento de la expectativa de vida en los países desarrollados durante el siglo XX (Christman, 1998). Por ello, la cloración y la filtración del agua potable se han considerado como el máximo progreso

en salud pública del siglo XX (Calderón, 2000). Sin embargo, como también hemos indicado, este proceso supone la génesis de compuestos químicos no deseados.

Al evaluar los riesgos de la contaminación microbiana respecto a los de la contaminación química del agua, la Organización Mundial de la Salud recomienda que en aquellos lugares en los que las circunstancias requieran la elección entre acatar y/o cumplir las normas de los parámetros microbiológicos o los de los subproductos de la desinfección, siempre deben priorizarse los microbiológicos (WHO, 2004). Así, la contaminación microbiológica se considera como un mayor riesgo para la salud humana que la contaminación química resultante de la cloración (Downs et al., 1999; Havelaar et al., 2000; Ashbolt, 2004).

Los riesgos para la salud relacionados con los desinfectantes y los subproductos de la desinfección son muy pequeños en comparación con los riesgos enormes e inminentes que produciría la falta de desinfección o la desinfección inadecuada del agua potable. Se estima que el riesgo de muerte por patógenos conocidos en aguas superficiales no tratadas es al menos 100 – 1.000 veces mayor que el riesgo de cáncer por los subproductos de la cloración, y que el riesgo de enfermedades producidas por los patógenos parece ser de al menos 10.000 – 1 millón de veces mayor que el riesgo de cáncer por SPD en agua de consumo clorada (Galal-Gorchev, 1996a).

Por lo tanto, para evitar la propagación de enfermedades, el agua de consumo humano siempre tendrá que ser desinfectada (clorada), lo que implica la presencia obligada de SPD en la misma.

A pesar de que se dé prioridad a la contaminación microbiológica frente a la química, esto no quiere indicar que se deba dejar de lado la contaminación química, teniendo en cuenta que podemos estar expuestos a la misma de manera crónica y por un tiempo prolongado (hasta toda la vida). Es por este motivo que se hace necesario lograr la mayor información posible sobre ella: sus componentes, los factores involucrados en su formación, su potencialidad genotóxica, su relación con algunos cánceres, etc.

I.3. Evaluación genotóxica de los SPD

Dado el riesgo atribuido a la presencia de SPD en el agua de consumo, se han llevado a cabo diversos estudios con la finalidad de determinar el potencial genotóxico de los SPD. Algunos de estos estudios han evaluado el riesgo del agua tratada, es decir, evaluando el agua post-tratamiento, abarcando de esta manera todos los posibles SPD que puedan existir en ella; otros estudios, por el contrario, han evaluado

los SPD de manera individual, para poder determinar la potencialidad de cada uno de ellos y conocer cuál es el que causa mayor genotoxicidad.

Este segundo enfoque, en el que los SPD se evalúan de manera individual, es sin duda alguna el más utilizado, dado que permite saber cuáles son los potencialmente más peligrosos para la salud humana. De todos los SPD, los más evaluados desde el punto de vista genotóxico son el grupo de los THM y el denominado mutágeno X (tablas 1.2 y 1.3).

Tabla 1.2. Algunos estudios realizados sobre los trihalometanos

SPD	Estudio	Resultado	Referencia
CF, BF	Ensayo de mutación directa Ara en *S. typhimurium* con y sin S9.	BF se clasificó como mutágeno cuestionable, su mutagenicidad fue anulada en presencia de S9.	Roldán-Arjona and Pueyo, 1993.
CF, BDCM, DBCM, BF	SOS chromotest en *E. coli*, test de Ames y ensayo de MN en larva *Pleurodeles waltl.*	En el SOS chromotest, excepto el CF los compuestos indujeron daño primario en el DNA. En el test de Ames, sólo el BF mostró actividad mutagénica. BDCM y BF efectos clastogénicos según el ensayo de MN.	Le Curieux et al., 1995.
CF, BCDM, DBCM, BF	Administración oral de compuestos. Ensayo de desnaturalización alcalina en células del riñón. Medición de los niveles de testosterona en suero.	Ninguno de los compuestos logró incrementar el número de roturas en el DNA, pero sí disminuyeron las concentraciones de testosterona.	Potter et al., 1996.
BCDM, DBCM, BF	Ensayo de mutagenicidad en *S. typhimurium* y análisis molecular de los revertientes.	La mayoría de las sustituciones inducidas fueron transiciones GC $\rightarrow$ AT.	DeMarini et al., 1997.
DBCM, BDCM, BF	Ensayo de síntesis de DNA no programada en hígado de rata. El BF fue evaluado mediante el ensayo de MN en médula ósea de ratón.	Ningún compuesto mostró evidencia de genotoxicidad *in vivo.*	Stocker et al., 1997.

BF	Inducción de ICH en cultivos de linfocitos humanos. Genotipos *GSTT1-1*$^{+}$ y *GSTT1-1*$^{-}$.	No se observaron diferencias de inducción de ICH entre los genotipos.	Landi et al., 1999a.
BF	Ensayo del cometa en linfocitos humanos, evaluando los genotipos *GSTT1-1*$^{+}$ y *GSTT1-1*$^{-}$.	Inducción de daño (incremento en la longitud de la cola). No se observaron diferencias entre los genotipos.	Landi et al., 1999b.
CF, BDCM, DBCM, BF	Ensayo del cometa en células epiteliales primarias de pulmón humano.	Inductores débiles de daño en el DNA.	Landi et al., 2003.
CF	Test de mutación reversa en varias cepas de *S. typhimurium* y de *E. coli.*	No mutagénico en todas las cepas de *S. typhimurium* evaluadas con y sin S9; mutagénico en *E. coli.*	Araki et al., 2004.
CF, BDCM, DBCM, BF	Ensayo de desnaturalización alcalina en células CCRF-CEM, hepatocitos primarios de ratón *in vitro* y en ratones *in vivo* (vía oral).	Inducción de roturas en el DNA por BDCM, DBCM y BF en células CCRF-CEM. Resultados negativos en los otros sistemas evaluados.	Geter et al., 2004
BDCM	Ensayo del cometa y de MN en cultivos de células de riñón humano y de ratón. *In vivo:* riñón de ratón.	Incremento dependiente de la dosis de la frecuencia de roturas de DNA.	Robbiano et al., 2004.
CF, BDCM, DBCM, BF	Test de Ames en la cepa TA100 con y sin S9.	Resultados negativos en presencia y ausencia de S9.	Kundu et al., 2004a.

Tabla 1.3. Algunos estudios realizados sobre el mutágeno X

Estudio	**Resultado**	**Referencia**
Ensayo de MN en Tradescantia (exposición de inflorescencias).	Relación positiva y significativa, dosis-dependiente, en la inducción de MN.	Helma et al., 1995.
Ensayo del cometa en múltiples órganos de ratón, exposición vía oral.	MX es genotóxico en varios órganos del ratón pero no para el sistema hemapoyético (bazo y médula ósea).	Sasaki et al., 1997.
Test de Ames en la cepa TA100 sin activación metabólica (S9).	Responsable de la mayor parte de la mutagenicidad del agua (de 7 al 67%)	Smeds et al., 1997.

Ensayo del cometa en células de las glándulas digestivas del mejillón.	Repuesta positiva y significativa, dependiente de la dosis en el incremento del porcentaje de DNA en la cola.	Mitchelmore et al., 1998.
Ensayo de elución alcalina en células HL-60, células $LLCPK_1$, células mononucleares de sangre periférica (PBMC), células germinales testiculares de rata y exposiciones *in vivo* en ratones.	Inducción de roturas en el DNA de cadena sencilla, *in vitro.* La presencia de AraC/Hu provoca que las roturas se produzcan a concentraciones más bajas de MX en células proliferativas (HL-60, $LLC\text{-}PK_1$ y algunos PBMC). *In vivo*, incremento moderado de roturas en hígado, riñón y colon de ratones pre-expuestos a AraC/Hu.	Holme et al., 1999.
Ensayo de MN (micrométodo) en células L5178Y y ensayo de síntesis de DNA no programada en cultivos de hepatocitos primarios de ratón.	El MX fue capaz de incrementar la frecuencia de MN y fue genotóxico en hepatocitos de ratón.	Le Curieux et al., 1999.
Ensayo del cometa, de ICH y de aberraciones cromosómicas en células CHO.	Respuesta positiva en el ensayo del cometa, incremento en la frecuencia de ICH y un incremento positivo dosis-dependiente en la frecuencia de aberraciones cromosómicas.	Maki-Paakkanen et al., 2001.
Ensayo de mutagenicidad con células MCL-5, células AHH-1 $TK^{+/-}$ y células h1A1v2.	El MX fue mutagénico en las tres líneas celulares linfoblastoides humanas empleadas.	Woodruff et al., 2001.
Evaluación citotóxica y mutagénica en *S. typhimurium* TA98, TA100, RSJ100, en presencia y ausencia de S9.	Compuesto citotóxico. Mutagénico en la cepa TA98, con y sin S9 y en la TA100 sin S9.	Kargalioglu et al., 2002.
Ensayo del cometa en células CHO.	Incremento significativo de daño genómico.	Plewa et al., 2002.
Ensayo DEL en levaduras (recombinación intra e intercromosómica y aneuploidía).	Gran potencial recombinogénico del MX.	Egorov et al., 2004.
Test de Ames sin S9. Ensayo de mutación *Hprt* y de aberraciones cromosómicas en células CHO.	Positivo en el test de Ames, incrementos ligeros en la frecuencia de mutaciones *Hprt* y en la frecuencia de	Maki-Paakkanen et al., 2004.

	aberraciones cromosómicas.	
Ensayo del cometa en línea celular epitelial de hígado de rata.	El MX indujo un incremento positivo, dependiente de la dosis, en el daño en el DNA en el ensayo del cometa.	Maki-Paakkanen and Hakulinen, 2008.

De acuerdo a los resultados obtenidos en los diferentes estudios de genotoxicidad (tabla 1.2, tabla 1.3, y las revisiones de McDonald and Komulainen, 2005; Richardson et al., 2007), se considera que el CF es un compuesto no genotóxico y no mutagénico; mientras que el resto de THM (BDCM, DBCM, BF) se les considera como compuestos que, aunque generalmente no inducen mutaciones génicas, sí que dan resultados positivos en alguno de los sistemas utilizados. En cambio, el denominado mutagéno X es considerado como un compuesto altamente genotóxico y mutagénico.

I.4. Estudios epidemiológicos

A raíz del descubrimiento de que la desinfección química del agua destinada al consumo humano puede favorecer la formación de subproductos, se han realizado numerosos estudios epidemiológicos para averiguar los efectos que pueden tener sobre la salud humana; estos estudios generalmente se han centrado en la asociación de estos SPD con diferentes tipos de cánceres y en los problemas que se presentan en los recién nacidos.

I.4.1. Cáncer

Son muchos los estudios epidemiológicos que han investigado la posible existencia de una relación causal entre la exposición a los SPD y el riesgo asociado a diferentes cánceres, entre los cuales el más evaluado y con el que se ha encontrado una asociación potencial es con el cáncer de vejiga. Además también se ha descrito una posible asociación con los cánceres de colon y recto. Hay que indicar que la Organización Mundial de la Salud, en su análisis publicado en el año 2000, consideró que la información que aportaban los estudios epidemiológicos realizados hasta aquel momento era insuficiente y no concluyente para establecer una relación de causalidad entre el consumo de agua desinfectada (clorada) y algún efecto adverso para la salud, aunque también concluyeron que existían evidencias que apuntaban a un mayor riesgo

para el cáncer de vejiga en relación a otros cánceres (WHO, 2000). Esta conclusión es la que ha motivado nuevos estudios para tratar de esclarecer esta posible asociación.

El cáncer de vejiga es uno de los más estudiados en relación con la exposición a trihalometanos. En la tabla 1.4 se presentan los resultados obtenidos de varios estudios que han evaluado la exposición a SPD y la incidencia del cáncer de vejiga.

Tabla 1.4. Estudios epidemiológicos que han evaluado el riesgo a cáncer de vejiga por exposición a SPD

Autor	**Resultados**
Wilkins and Comstock, 1981.	Varones expuestos a agua superficial clorada frente a expuestos a agua subterránea con un RR= 1,8 (0,8-4,7) (incremento pequeño y no significativo.
Bean et al., 1982.	No se encontraron diferencias en la incidencia del cáncer de vejiga entre las municipalidades a las que se les distribuía agua superficial o subterránea clorada.
Cantor et al., 1987.	El cáncer de vejiga se asoció significativamente con la duración de la exposición a agua superficial clorada en mujeres y en no fumadores de ambos sexos.
Lynch et al., 1989.	Incrementos de riesgo en exposiciones > a 50 años a agua clorada frente a los no expuestos (ambos sexos) con una OR=2,1 (1,2-3,7).
McGeehin et al., 1993.	Asociación estadísticamente significativas entre el cáncer de vejiga y la duración de la exposición a agua superficial clorada; el riesgo fue similar para ambos sexos y entre fumadores y los no fumadores.
Vena et al., 1993.	El agua del grifo se asoció con un incremento del riesgo de cáncer de vejiga, con una clara relación dosis-respuesta. Asociación del riesgo con el consumo durante más de 65 años; cuartil superior de consumo de agua al día frente a cuartil menor de consumo (varones) con una OR = 3,0 (1,8-5,0).
King and Marrett, 1996.	Se encontraron asociaciones estadísticamente significativas para el cáncer de vejiga y el agua superficial clorada, duración o concentración de niveles de THM y consumo de agua clorada.
Doyle et al., 1997.	No se encontró asociación entre exposición a cloroformo y el cáncer de vejiga.
Freedman et al., 1997.	Débil asociación entre el riesgo de cáncer de vejiga y la duración de la exposición al agua municipal para varones fumadores, como también una relación exposición-respuesta.

Cantor et al., 1998.	Se encontró una asociación positiva y estadísticamente significativa entre el riesgo de cáncer de vejiga y la exposición a agua subterránea o superficial clorada para varones y para fumadores, pero ninguna asociación para los no fumadores (ambos sexos) o para las mujeres.
Koivusalo et al., 1998.	La mutagenicidad del agua de consumo se asoció con un exceso de riesgo pequeño pero estadísticamente significativo entre la exposición y el cáncer de vejiga en los varones y una asociación débil en las mujeres.
Yang et al., 1998.	Residir en municipalidades donde se clora el agua (*vs.* las que no se clora) se asoció de manera estadísticamente significativa con el cáncer de vejiga (ambos sexos).
Villanueva et al., 2001b.	Los niveles de THM en Tenerife y en Asturias corresponden a un incremento del riesgo de cáncer de vejiga del orden del 10% (OR = 1,1), siendo en Manresa y en Barcelona de 1,43 y 1,5, respectivamente; mientras que en Alicante y en Sabadell las OR fueron de 1,5 y 1,66, respectivamente.
Vinceti et al., 2004.	Un incremento pequeño pero estadísticamente significativo en la tasa de mortalidad del cáncer en los varones que consumen agua con altos niveles de THM. En las mujeres, el incremento no es estadísticamente significativo.
Bove et al., 2007.	Altos niveles de consumo de THM incrementan el riesgo de cáncer de vejiga con una OR = 2,34 (1,01-3,66). Los resultados fueron más significativos al analizarse en relación al bromoformo (OR=3,05) y para exposiciones a aguas más lejanas del sistema de distribución (OR=5,85).
Chang et al., 2007.	Se encontró una correlación positiva y significativa entre la concentración de TTHM en el agua potable con el riesgo de muerte debido a cáncer de vejiga (OR = 1,8–2,11).
Villanueva et al., 2007.	Se encontró un riesgo incrementado a cáncer de vejiga asociado cuando se estimó la exposición a SPD mediante la ingestión de agua potable, absorción dérmica e inhalación durante la ducha, el baño y nadar en piscinas. Un riesgo doble para cáncer de vejiga se asoció con la exposición a niveles de SPD de 50 µg/L.

Fuente: Villanueva et al., 2001a; USEPA, 2006.

De acuerdo con lo indicado en la tabla 1.4, varios estudios han encontrado una asociación positiva entre el riesgo de padecer este tipo de cáncer con la exposición a los SPD, aunque en algunos trabajos esta asociación es débil. Toda la información contenida en estos estudios epidemiológicos apoya la posibilidad de la existencia de una relación positiva entre el agua clorada o los SPD y el cáncer de vejiga.

Otros dos cánceres también muy estudiados y asociados con la exposición a los SPD, después del de vejiga, son los de colon y de recto. La tabla 1.5 muestra los resultados obtenidos en diversos trabajos sobre estos dos tipos de tumores.

Tabla 1.5 Estudios epidemiológicos que han evaluado el riesgo a cáncer de colon y de recto por exposición a SPD

Autor	Resultados
Bean et al., 1982.	Municipalidades abastecidas con agua superficial clorada tienen mayores tasas de incidencia de cáncer rectal que aquellas con fuentes subterráneas, en todos los grupos poblacionales. No se encontraron diferencias en la incidencia del cáncer de colon entre municipalidades.
Gottlieb et al., 1982.	Información del certificado de defunción (ocupación, lugar de nacimiento y dirección). Duración de exposición a agua superficial o subterránea (por la compañía de agua). Recto OR=3,18 (1,96-5,19), colon OR=0,90 (0,60-1,37).
Kanarek and Young, 1982.	Se encontró una relación directa dosis-respuesta entre los niveles de THM y la muerte por cáncer de colon.
Zierler et al., 1986.	Menor riesgo de padecer cáncer de colon y recto en aquellas personas cuyas aguas habían sido desinfectadas con cloro frente a aquellas con aguas desinfectadas con cloraminas. Colon OR = 0,89 (0,86-0,93) y recto OR = 0,96 (0,84-1,04).
Young et al., 1987.	Los niveles de THM en el agua potable no están relacionados con un riesgo significativo a cáncer de colon. OR= 0,90 (0,60-1,35).
Doyle et al., 1997.	Se observó un incremento estadísticamente significativo en el riesgo de cáncer de colon para las mujeres expuestas a cloroformo en el agua de consumo, con evidencia de una relación exposición-respuesta. No se encontró asociación entre el cloroformo y el cáncer de recto.
Koivusalo et al., 1997.	Se observó un exceso de riesgo significativo para cáncer de recto en las mujeres. RR=1,38 (1,03-1,85).
Hildesheim et al., 1998.	Riesgo incrementado para el cáncer rectal asociado con la duración de la exposición a agua superficial clorada. El riesgo de cáncer rectal es estadísticamente significativo en exposiciones > 60 años a THM en el agua clorada y en los individuos con una dieta baja en fibras. Los riesgos fueron los mismos para ambos sexos. No se observó ninguna asociación para las mediciones de la exposición a agua con el riesgo de cáncer de colon.
Yang et al., 1998.	Residir en municipalidades donde se clora el agua (*vs.* las que no clora) se asoció estadísticamente con el cáncer rectal (ambos sexos).

King et al., 2000.	El riesgo de cáncer de colon se asoció estadísticamente con la exposición crónica a THM, agua superficial clorada y consumo de agua (varones). No se observaron asociaciones entre las medidas de exposición y el cáncer rectal en ningunos de los dos sexos.
Vinceti et al., 2004.	Se vio un incremento pequeño pero estadísticamente significativo en la tasa de mortalidad de los cánceres de colon y recto para los varones que consumen agua con altos niveles de THM. En las mujeres, se detectó una mayor tasa de mortalidad sin alcanzar significación estadística.
Bove et al., 2007.	Localidades con altos niveles de bromoformo en el agua potable presentan un incremento en el riesgo de cáncer de recto, OR=1,85 (1,25-9,56).
Kuo et al., 2009.	Incremento estadísticamente no significativo en la tasa de riesgo de muerte por cáncer de colon asociado a los niveles de TTHM en agua potable.
Rahman et al., 2010.	Revisión bibliográfica de 13 estudios, evidencia limitada de una posible asociación entre el cáncer colorrectal y la exposición a SPD en agua potable. Colon OR=1,27 (1,08-1,50). Recto OR=1,30 (1,06-1,59).

Fuente: Villanueva et al., 2001a; USEPA, 2006.

Por lo que respecta a los resultados obtenidos en los diferentes estudios que se muestran en la tabla 1.5, éstos indican la existencia de una posible asociación entre la exposición a los SPD y tanto el cáncer de colon como el de recto. Al contrario de lo que ocurre con los estudios epidemiológicos sobre cáncer de vejiga, en los que la mayoría muestran una clara asociación; en este caso, los resultados de recto y colon son variados: asociaciones débilmente significativas, significativas y no significativas para ambos cánceres. Por este motivo, se considera que la relación causal que hay entre los SPD y estos cánceres es sólo algo probable.

Además de los cánceres de vejiga, de colon y de recto, otros tipos de cánceres han sido objeto de evaluación sobre la asociación de su incidencia y la exposición al agua desinfectada y a los subproductos de la desinfección de la misma. Los resultados de estos estudios se presentan de forma resumida en la tabla 1.6.

Tabla 1.6 Estudios epidemiológicos que han evaluado el riesgo a diversos cánceres por exposición a SPD

Autor	Resultados
Wilkins and Comstock, 1981.	Se observó un incremento (estadísticamente no significativo) en las tasas de incidencia de cáncer hepático en mujeres, cuando se comparó los grupos expuestos a agua superficial clorada con los de agua subterránea no clorada.
Bean et al., 1982.	Municipalidades abastecidas con agua superficial clorada tienen mayores tasas de incidencia de cáncer pulmonar que aquellas con fuentes subterráneas, en todos los grupos poblacionales.
Ijsselmuiden et al., 1992.	Asociación positiva entre la exposición al agua clorada y el cáncer de páncreas, OR=2,18 (1,20-3,95).
Doyle et al., 1997.	No se encontró asociación entre la exposición a cloroformo en el agua clorada con los cánceres de riñón, órganos superiores del sistema digestivo, pulmón, ovario, endometrio, mama, melanoma o linfoma no-Hodgkin.
Koivusalo et al., 1997.	Se observó un exceso de riesgo significativo de cáncer de esófago (RR=1,90) y mama (RR=1,1) para las mujeres.
Yang et al., 1998.	Residir en municipalidades donde se clora el agua (*vs.* las que no se clora) se asoció estadísticamente con cáncer de pulmón y riñón (ambos sexos). El riesgo de cáncer de hígado, esófago, estómago, páncreas, próstata, cerebro y mama fue más elevado en las municipalidades que cloraban el agua, pero solamente para varones.
Infante-Rivard et al., 2001.	No se observó un riesgo incrementado para la leucemia linfoblastoide relacionado con la exposición prenatal a niveles promedio de THM. Se observó un incremento pequeño (no significativo) en el riesgo para la exposición acumulada a THM y cloroformo.
Infante-Rivard et al., 2002.	Los resultados son sugerentes, pero imprecisos, al relacionar las variantes del DNA con el riesgo de leucemia linfoblastoide aguda asociada con los SPD del agua de consumo. El número de personas genotipadas (genes *GSTT1* y *CYP2E1*) fue bajo para ser concluyente.
Vinceti et al., 2004.	Se encontró un incremento pequeño (estadísticamente significativo) en la tasa de mortalidad de los cánceres de hígado, páncreas, pulmón, melanoma, mama, próstata, riñón, cerebro, linfoma no-Hodgkin y leucemia linfática en los varones que consumen agua con altos niveles de THM. En las mujeres, la mortalidad también estaba incrementada (sin alcanzar significación estadística). La mortalidad por cáncer de estómago en los

	hombres mostró un aumento, estadísticamente significativo, cuando se relacionó con el consumo de agua con niveles altos de THM.
Do et al., 2005.	No se encontró asociación entre el cáncer de páncreas y la exposición a SPD, cloroformo o bromodiclorometano.
Kasim et al., 2006.	Riesgo incrementado (OR=2,20) de leucemia mieloide crónica en adultos expuestos durante más de 36 años a agua superficial clorada. En los otros subtipos de leucemia se observó que el riesgo disminuía al aumentar el tiempo de exposición.

Fuente:Villanueva et al., 2001a; USEPA, 2006.

Por lo que respecta a los otros cánceres estudiados, aunque en algunos trabajos se ha encontrado alguna asociación, en otros que han evaluado el mismo cáncer los resultados son opuestos. Debido a la poca información disponible sobre este grupo de cánceres (aquellos que no son de vejiga, de colon o de recto), no se puede extraer ninguna conclusión fiable sobre la relación que existe entre su incidencia y los subproductos de la desinfección; por lo tanto, se considera que las evidencias epidemiológicas todavía no son claras y que falta disponer de mayor información que incline hacia algún lado la asociación con estos cánceres.

I.4.2. Otros posibles efectos

Además del cáncer, existen otros aspectos relacionados con la salud sobre los que también se han realizado estudios epidemiológicos. Entre ellos están los que se relacionan con los defectos en recién nacidos de madres expuestas, en donde se pretende evaluar la relación que pueda existir entre la ocurrencia de estos defectos con la exposición a SPD. Este tipo de investigaciones son bastante recientes ya que se iniciaron en la década pasada.

Entre los efectos investigados se encuentran: los abortos espontáneos, bajo peso al nacer, retraso en el crecimiento intrauterino, talla pequeña para la edad gestacional, así como determinadas malformaciones congénitas tales como defectos respiratorios, cardíacos, del tracto urinario, del sistema nervioso central, etc. Esta información se resume en la tabla 1.7.

Tabla 1.7 Estudios epidemiológicos que han evaluado los efectos adversos sobre la reproducción en recién nacidos de madres expuestas a SPD

Autor	Resultados
Shaw et al., 1990.	No se observó asociación entre anomalías congénitas cardíacas y el consumo materno de agua tratada (nivel de TTHM) durante el embarazo.
Aschengrau et al., 1993.	Agua clorada *vs.* agua cloraminada. Muerte fetal tardía OR=2,6 (0,9-7,5). Defectos respiratorios OR=3,2 (1,1,-9,5). Anomalías congénitas mayores OR=1,5 (0,7-2,1).
Bove et al., 1995.	Se encontró un débil incremento, estadísticamente significativo, en el riesgo para niveles altos de TTHM para talla pequeña, defectos del tubo neuronal, del sistema nervioso central, fisura del paladar y defectos cardíacos mayores.
Savitz et al., 1995.	No se encontró asociación entre el riesgo de aborto, parto prematuro o bajo peso al nacer y el origen del agua potable o niveles de THM en agua.
Kanitz et al., 1996.	Agua tratada con hipoclorito de sodio *vs.* agua no tratada. Ictericia neonatal OR=1,1 (0,7-2,8); bajo peso al nacer OR=6,0 (0,6-12,6); circunferencia craneal pequeña OR=3,5 (2,1-8,5).
Dodds et al., 1999.	Agua con niveles altos de TTHM *vs.* niveles bajos de TTHM. Muerte fetal tardía OR= 1,66 (1,09-2,52); tamaño pequeño para edad gestacional OR=1,08 (0,99-1,18); defectos en el tubo neural OR=1,18 (0,67-2,10).
Magnus et al., 1999.	Asociación estadísticamente significativa entre los defectos del tracto urinario (OR=1,99) y la cloración y con el agua con alto contenido de compuestos orgánicos. No se encontraron asociaciones para los otros defectos de nacimiento (del tubo neural, respiratorios, fisura del paladar y cardíacos).
Dodds and King, 2001.	La exposición a bromodiclorometano se asoció a un riesgo incrementado para los defectos del tubo neural (RR=2,5) y un riesgo disminuido para las anomalías cardiacas (RR=0,3).
Jaakkola et al., 2001.	No se encontraron evidencias para una asociación entre la exposición prenatal a agua clorada y el bajo peso al nacer o talla pequeña para edad gestacional.
Waller et al., 2001.	Riesgo incrementado (estadísticamente significativo) entre el consumo de niveles altos de THM y abortos espontáneos, cuando se comparó con el consumo de niveles bajos de THM.
Hwang et al., 2002.	El consumo de agua superficial clorada con niveles altos de NOM incrementa el riesgo de defectos de nacimiento (defectos cardiacos, del sistema respiratorio, del tracto urinario).

Shaw et al., 2003.	No se observó asociación o relación exposición-respuesta entre las malformaciones (defectos del tubo neural, fisura del paladar y algunos defectos cardiacos) y la exposición a los THM.
Dodds et al., 2004.	Se observó una asociación positiva estadísticamente significativa entre la muerte fetal tardía y la exposición a niveles altos de THM, BDCM, y CF.
Infante-Rivard, 2004.	No se encontró asociación entre la exposición a THM y el retraso en el crecimiento intrauterino, excepto para los recién nacidos con genotipo CYP2E1*5 (G1259C).
Yang, 2004.	Se encontró una asociación entre el consumo de agua clorada y el riesgo de parto prematuro.
Porter et al., 2005.	No se encontró asociación consistente entre la exposición a THM y el retraso del crecimiento intrauterino.
Lewis et al., 2006.	Incremento del riesgo de bajo peso al nacer en relación con la exposición a niveles altos de THM durante el segundo trimestre del embarazo (OR=1,50) y durante todo el embarazo (OR=1,23).
Savitz et al., 2006.	No se observó un riesgo incrementado en los abortos en relación con los niveles de THM y de HAA.
Lewis et al., 2007.	No se observó asociación entre nacimientos prematuros y la exposición a TTHM en las últimas semanas antes de dar a luz.
Yang et al., 2007.	No se encontró un riesgo incrementado de bajo peso al nacer, talla pequeña para edad gestacional y partos prematuros, en relación con la exposición a TTHM en el agua de consumo.
Chisholm et al., 2008.	Se observó un pequeño incremento (estadísticamente significativo) en el riesgo de defectos de nacimiento en relación con la exposición a niveles altos de THM.
Hwang et al., 2008.	Se observó que la exposición prenatal a SPD incrementa el riesgo de defectos del septo ventricular, labio leporino y anencefalia.
Nieuwenhuijsen et al., 2008.	No se encontró asociación entre la exposición la THM y las anomalías congénitas (respiratorias, cardiovasculares, urinarias, del tubo neural). Riesgo incrementado para defectos del septo ventricular por exposición a THM y de defectos cardiacos mayores por exposición a bromoformo.

Fuente: Villanueva et al., 2001a; USEPA, 2006; Nieuwenhuijsen et al., 2009.

Los resultados obtenidos en los diferentes estudios anteriormente mencionados muestran diferencias en los resultados para el mismo parámetro, lo que hace que los resultados sean considerados como poco consistentes para poder establecer una asociación entre la exposición a los SPD y el riesgo de anomalías en los recién nacidos.

También se han realizado algunos estudios epidemiológicos en los que se pretende determinar la implicación del agua clorada o de los SPD en la aparición de otras enfermedades o alteraciones fisiológicas en las personas expuestas, tal como se indica en la tabla 1.8.

Tabla 1.8. Estudios epidemiológicos que han evaluado otras enfermedades o alteraciones en relación a la exposición a los SPD

Autor	Resultados
Wilkins and Comstock, 1981.	Se encontró un ligero incremento (aunque significativo) en el riesgo de muerte debido a enfermedades cardíacas arterioescleróticas en los residentes de pueblos abastecidos con a agua superficial clorada comparado con los residentes de pueblos donde no se cloraba el agua.
Zeighami et al., 1990.	Al evaluar los niveles de colesterol de personas de 40 a 70 años en relación a si el agua potable se cloraba o no y al grado de dureza, se encontró que en las mujeres que residían en lugares donde el agua se cloraba y era dura, el nivel de colesterol en suero era mayor en comparación de aquellas que consumían aguas blandas cloradas y no cloradas. Se encontró un riesgo estadísticamente significativo de niveles elevados de colesterol en sangre en mujeres pero no en hombres.
Riley et al., 1995.	No se encontraron evidencias que apoyen la hipótesis de que el incremento de duración de la exposición a agua clorada influenciara los niveles de LDL o HDL, colesterol, triglicéridos o apolipoproteínas.
Fenster et al., 2003.	No se encontró asociación entre el nivel de TTHM y la movilidad y morfología espermática.
Windham et al., 2003.	Las observaciones sugieren que la exposición a THM puede afectar la función ovárica. Todos los THM bromados fueron asociados con ciclos menstruales significativamente cortos.
Luben et al., 2007.	No se encontró asociación entre el nivel de TTHM o HAA y la calidad (concentración y conteo) espermática.

Fuente: USEPA, 2006.

Existen pocos estudios epidemiológicos sobre los SPD que no estén relacionados con la incidencia de cáncer o los efectos en neonatos, por este motivo, no se pueden sacar conclusiones respecto a otros efectos adversos para la salud hasta que no se disponga de información.

En estos estudios epidemiológicos hay que tener en cuenta que muchos de ellos, en especial los primeros, sólo tenían en cuenta como única vía de exposición la

ingestión oral de agua de consumo, sin considerar las vías por exposición dérmica y por inhalación. Se conoce que la exposición a los SPD presentes en el agua de consumo puede darse a través de múltiples rutas y, además, puede variar entre la población por la diferencias en la cantidad y en la manera en que las personas hacen uso de ella. Los usos comunes del agua y sus correspondientes exposiciones incluyen el consumo directo del agua y la preparación de bebidas y comidas (exposición por ingestión), además de bañarse y ducharse (exposición dérmica e inhalación), y el uso de máquinas lavavajillas, lavadores de ropa, y humidificadores (exposición por inhalación). Además, hay que considerar que todavía hay millones de personas que lavan los utensilios y la ropa a mano en las cuales, además de la inhalación, la absorción dérmica también sería unaa exposición a considerar.

Tradicionalmente, cuando se evalúa la exposición y los efectos adversos en la salud asociados a contaminantes en el agua potable, se asume que la mayor, y a veces única, ruta de exposición constituye la vía oral, es decir la ingestión. Sin embargo, teniendo en cuenta que entre los SPD se encuentran productos orgánicos volátiles, la inhalación y la absorción dérmica se convierten en rutas de exposición importantes, pudiendo la absorción dérmica llegar a ser responsable de contribuir con un porcentaje no despreciable del total de la dosis (Brown et al., 1984).

Algunos estudios (Andelman, 1985; McKone, 1987; Little, 1992) concluyeron que la exposición por inhalación asociada a los productos orgánicos volátiles emitidos por el agua potable es una vía importante, y que puede ser hasta 6 veces mayor que la vía por ingestión (Andelman, 1985). Asimismo, Li y Hoang (2000) analizaron la exposición a los THM por inhalación en tres escenarios que contribuían en mayor medida a esta vía de exposición (ducharse, lavar los platos, lavar las frutas y verduras y cocinar), encontraron que ducharse era la actividad que contribuía en mayor medida (90%) a la exposición, debido a la gran cantidad de agua involucrada, a la mayor área de contacto, a la temperatura del agua y a que normalmente, los baños son más pequeños que las cocinas. En la cocina, una de las fuentes de THM se produce al hervir el agua, ya que más del 90% de los THM que contiene son emitidos al aire después de hervirla durante 3-10 min.

Diversas investigaciones han mostrado que el bañarse y ducharse pueden suponer incrementar los niveles de THM en sangre de manera más acusada que lo que sucede después de beber 1 litro de agua. Por ejemplo, Weisel y Jo (1996) compararon los niveles de cloroformo, medido en muestras de aire exhalado, en exposiciones por ingestión, inhalación y absorción dérmica, mostrando que la dosis interna obtenida tras

10 min de ducha o 30 min de baño es mayor que la que se obtiene bebiendo 2 litros de agua. Otro estudio (Backer et al., 2000) evaluó las concentraciones de THM en sangre de personas que habían sido expuestas mediante una de las diferentes vías (10 min de ducha, 10 min de baño o beber 1 litro de agua en 10 min); los resultados mostraron que las personas expuestas a 10 min de ducha fueron las que presentaron los niveles más altos de THM en sangre, mientras que las que bebieron agua presentaron valores más bajos, resultando sólo en un ligero incremento de los niveles en sangre.

Dado la importancia de las diversas rutas de exposición, un grupo de personas que han sido estudiadas en relación a la exposición a los SPD es el de los nadadores; en los que la mayor vía de exposición sería la absorción dérmica debido a la gran superficie de la piel que se encuentra expuesta y por la inhalación del aire que se encuentra en el ambiente (Nieuwenhuijsen et al., 2000). Considerando que el agua de las piscinas se trata de manera similar al agua potable, usando los mismos desinfectantes, aquellos SPD que se encuentran en el agua potable también pueden encontrarse en el agua de las piscinas. Además, teniendo en cuenta que el agua en las piscinas recircula por periodos de tiempo muy largos, los SPD que en ellas se encuentran pueden estar en mayores concentraciones que las que normalmente se cuantifican en el agua potable.

Diversos investigadores han estimado la cantidad de cloroformo absorbido durante el ejercicio de la natación, diferenciando en algunos casos entre las diferentes vías de absorción. De acuerdo con los resultados de Levesque et al. (1994), una hora de natación supone una dosis de cloroformo de 65 µg/kg/día, 141 veces la dosis de resultante de 10 min de baño y 93 veces mayor que la exposición por ingestión de agua; indicando que la absorción dérmica contribuye aproximadamente con el 24% de la captación del cuerpo durante la natación. En otro estudio, Erdinger et al. (2004) indicaron que la absorción dérmica sería responsable de 1/3 de la captación total de cloroformo durante la natación, mientras que los otros 2/3 se deberían a la inhalación de aire contaminado. En cambio, Lindstrom et al. (1997) estimaron que la ruta de exposición dérmica puede llegar a representar el 80% de la concentración de cloroformo en sangre durante la natación.

I.5. Regulaciones

A raíz de la preocupación que ha generado la exposición a los subproductos de la desinfección debido a su posible asociación con un incremento en la incidencia de

ciertos cánceres y a que, al ser compuestos presentes en toda agua desinfectada, representan una exposición crónica a lo largo de la vida, varias organizaciones y países han establecido regulaciones sobre los niveles máximos de contaminantes en el agua destinada para consumo humano.

Por tal motivo, la EPA (*Environmental Protency Agency)* de EEUU, ya en 1979 estableció la primera regulación federal con respecto a los SPD, la *Total Trihalomethane Rule*, en la cual el máximo nivel de contaminante (MCL, *maximun contaminant level)* para los THM totales (la suma de los cuatro THM) se estableció en 0,10 mg/L (100 µg/L). Esta regulación solo se aplicaba a aquellos sistemas de tratamiento de agua que era distribuida a más de 10.000 personas. Posteriormente, en diciembre de 1998, la EPA publicó la *Stage 1 Disinfectants/Disinfection by-products rule* (USEPA, 1998), en la cual el MCL de los TTHM disminuía (80 µg/L) y se regulaba por primera vez los niveles de 5 de los ácidos haloacéticos, el bromato y el clorito y, a diferencia de la ley anterior, se aplicaba para todos los sistemas de distribución que aplicaban desinfectante. La Fase 2 (*Stage 2)* de la ley fue promulgada en enero de 2006 (USEPA, 2006) y la diferencia que existe con la Fase 1 radica en que en ésta, en algunas localidades con el mismo sistema de distribución de agua se podían exceder los límites, siempre que el promedio global de todos los puntos de muestreo no excedieran los valores permitidos. Por el contrario, en el contenido de la Fase 2, los límites no pueden ser superados en ninguna localidad; así, los promedios anuales son respecto a cada localidad.

Al igual que la EPA ha promulgado normativas sobre los valores máximos permisibles con respecto a los subproductos de la desinfección (Tabla 1.9), también lo ha hecho la Organización Mundial de la Salud (Tabla 1.10) (WHO, 2004) y la Unión Europea (UE), directiva 98/83/EC (Tabla 1.11) (EU, 1998). La UE, al igual que la EPA, considera de manera conjunta a los trihalometanos, TTHM; mientras que la Organización Mundial de la Salud, los considera de manera individual. Sin embargo, algunos países miembros de la UE han adoptado regulaciones más estrictas para los niveles de THM (Tabla 1.12); pero en ningún caso sobre los límites de los HAA (Karanfil et al., 2008).

Dos países que han adoptado regulaciones sobre la presencia de los SPD en agua son Japón y China, los cuales además de regular los niveles de TTHM, también regulan de manera individual los niveles de cada THM y de algunos HAA (Tabla 1.13) (Karanfil et al., 2008).

Tabla 1.9. Regulaciones de los niveles de SPD de la EPA

SPD	**MCL (µg/L)**
TTHM	80
5 HAA	60
Bromato	10
Clorito	1000

Fuente: USEPA, 2006.

Tabla 1.10. Regulaciones de los niveles de SPD de la OMS

SPD	**Valor guía (µg/L)**
Cloroformo	200
Bromodiclorometano	60
Dibromoclorometano	100
Bromoformo	100
Ácido dicloroacético	50
Ácido tricloroacético	20
Bromato	10
Clorito	700
Hidrato de cloral	10

Fuente: WHO, 2004.

Tabla 1.11. Regulaciones de los niveles de SPD de la UE

SPD	**Valor estándar (µg/L)**
TTHM	100
Bromato	10

Fuente: EU, 1998.

Tabla 1.12. Regulaciones de los niveles de TTHM de diferentes países

País o región	**Límite (µg/L)**
Austria	30
Bélgica	30
Italia	30
Alemania	50
Luxemburgo	50
Suecia	50
Estados Unidos	80
Taiwán	80
China	100
Escocia	100
España	100
Irlanda	100
Japón	100
Noruega	100
Reino Unido	100
República Checa	100

Fuente: Karanfil et al., 2008.

Tabla 1.13. Regulaciones de los niveles de SPD en Japón y China

SPD	**Nivel (µg/L)**	
	Japón	*China*
Cloroformo	60	60
Bromodiclorometano	30	60
Dibromoclorometano	100	100
Bromoformo	90	100
TTHM	100	100
Ácido cloroacético	20	---
Ácido dicloroacético	40	50
Ácido tricloroacético	200	100
Bromato	10	10
Clorito	---	70

Fuente: Karanfil et al., 2008.

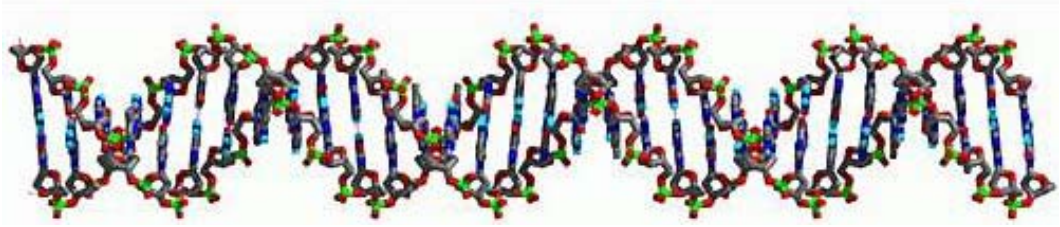
I.6. Perspectivas

En la actualidad se han sido identificados más de 600 SPD en el agua de consumo, los cuales se estima que representan menos del 50% de los posibles SPD. Al resto se les considera como "subproductos perdidos/desconocidos". Teniendo en cuenta el dato anterior puede considerarse que se tiene poca información respecto a lo que realmente nos encontramos expuestos mediante el agua de consumo, por lo cual es necesario conocer más sobre los "nuevos" o "recién descubiertos" subproductos y ampliar la información que se tiene de ellos.

Actualmente, sólo están siendo regulados los niveles de un pequeño porcentaje de SPD, aquellos que se encuentran en mayor concentración en el agua de consumo y de los que se tiene mayor cantidad de información. Por tanto, quedan sin estar regulados la mayoría de ellos. Aunque estos SPD puedan encontrarse en menores concentraciones, la información disponible indica que son más genotóxicos que los SPD regulados.

Hay que tener en cuenta que el campo relacionado a los subproductos de la desinfección del agua es relativamente nuevo; sólo han pasado 35 años desde el descubrimiento de los primeros subproductos en el agua clorada, y el número de SPD identificados va en aumento a medida que los sistemas de detección se van perfeccionando. En la actualidad se buscan nuevas formas de desinfectar el agua, que puede generar nuevos SPD, y se conoce que cada planta potabilizadora posee su propio perfil de SPD debido a los numerosos factores involucrados en el proceso. Así pues, parece conveniente continuar investigando este campo para obtener mayor información sobre los riesgos asociados con la exposición crónica a los SPD.

OBJETIVOS



II. OBJETIVOS

Esta Tesis se enmarca dentro de la línea de investigación desarrollada en el Grupo de Mutagénesis del Departamento de Genética y de Microbiología de la Universitat Autònoma de Barcelona, y tiene por objetivo general evaluar el riesgo genotóxico del agua de consumo tratada con desinfectantes. En este contexto, los objetivos específicos propuestos en el presente trabajo de Tesis Doctoral son los siguientes:

1. Estudiar el potencial genotóxico de 11 subproductos de la desinfección (SPD) del agua de consumo, pertenecientes a cinco grupos químicos distintos.

2. Evaluar su posible acción clastogénica y/o aneugénica (inducción de roturas cromosómicas y/o aneuploidía) mediante el ensayo de micronúcleos con bloqueo de la citocinesis, utilizando la línea celular linfoblastoide humana TK6 y linfocitos humanos de sangre periférica.

3. Evaluar la capacidad de los SPD seleccionados de inducir roturas simples y de doble cadena en el DNA o sitios álcali-lábiles, mediante el ensayo del cometa en células TK6.

4. Estudiar la cinética de reparación del daño inducido por los SPD seleccionados en células TK6.

5. Evaluar la capacidad de los SPD seleccionados de inducir daño oxidativo en el DNA.

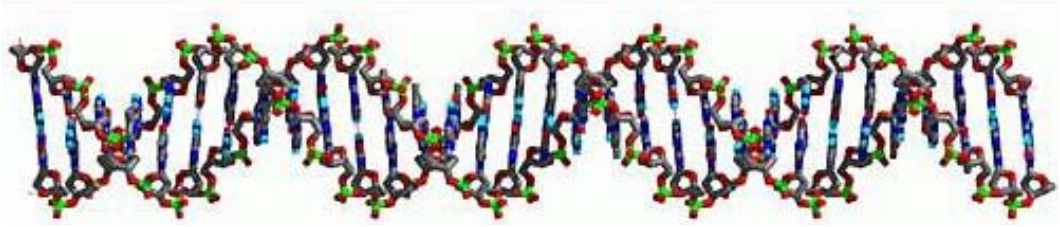
6. Evaluar el potencial mutagénico de los SPD seleccionados mediante el ensayo de linfoma de ratón.

7. Evaluar el potencial genotóxico de diferentes muestras de agua de piscinas, sometidas a distintos procesos de desinfección.

8. Determinar los efectos genotóxicos producidos por los SPD del agua clorada de piscinas en personas que usan dichas instalaciones.

9. Analizar la influencia del elemento halógeno en la capacidad genotóxica de los SPD seleccionados.

MATERIALES Y MÉTODOS



III. MATERIALES Y MÉTODOS

III.1. COMPUESTOS EVALUADOS (Evaluación *in vitro)*

III.1.1. SPD

Los once SPD seleccionados para ser evaluados en los distintos ensayos de genotoxicidad *in vitro* han sido los siguientes:

Grupo	Compuestos
✸ Halonitrometanos:	bromonitrometano y tricloronitrometano.
✸ Haloaldehidos:	tribromoacetaldehido e hidrato de cloral.
✸ Halofuranonas:	ácido mucrobrómico y ácido mucoclórico.
✸ Nitrosaminas:	nitrosodimetilamina y nitrosodietilamina.
✸ Ácidos acéticos:	ácido yodoacético, ácido bromoacético y ácido cloroacético.

En la tabla 3.1 se indican la fórmula química, el peso molecular, el número de CAS y la casa comercial suministradora de cada SPD evaluado.

Tabla 3.1 Información de los compuestos evaluados

Compuesto	**Fórmula química**	**Peso molecular** (uma)	**Nº CAS**	**Casa comercial**
Bromonitrometano (BNM)	CH_2BrNO_2	139,94	563-70-2	Sigma-Aldrich
Tricloronitrometano (TCNM)	CCl_3NO_2	164,38	76-06-2	Riedel-de Haën
Tribromoacetaldehido (TBA)	C_2HBr_3O	280,76	115-17-3	Sigma-Aldrich
Hidrato de cloral (CH)	$C_2H_3Cl_30_2$	165,4	302-17-0	Fluka
Ácido mucobrómico (MBA)	$C_4H_2Br_2O_3$	257,88	488-11-9	Sigma-Aldrich
Ácido mucoclórico (MCA)	$C_4H_2Cl_2O_3$	168,96	87-56-9	Sigma-Aldrich
Nitrosodimetilamina (NDMA)	$(CH_3)_2NNO$	74,08	62-75-9	Sigma-Aldrich
Nitrosodietilamina (NDEA)	$(C_2H_5)_2NNO$	102,14	55-18-5	Sigma-Aldrich
Ácido yodoacético (IAA)	ICH_2COOH	185,95	64-69-7	Sigma Aldrich
Ácido bromoacético (BAA)	$BrCH_2COOH$	138,95	79-08-3	Fluka
Ácido cloroacético (CAA)	$ClCH_2COOH$	94,5	79-11-8	Fluka

Las estructuras químicas de estos SPD están representadas en las figuras 3.1 a 3.11.

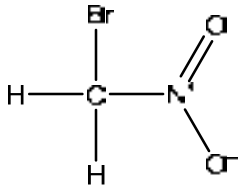


Figura 3.1 Bromonitrometano

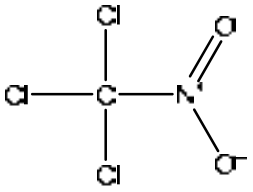


Figura 3.2 Tricloronitrometano

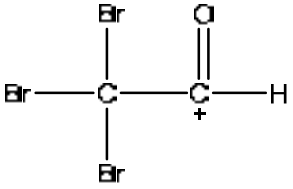


Figura 3.3 Tribromoacetaldehido

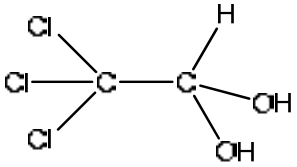


Figura 3.4 Hidrato de cloral

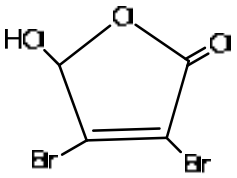


Figura 3.5 Ácido mucobrómico

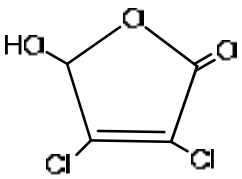


Figura 3.6 Ácido mucoclórico

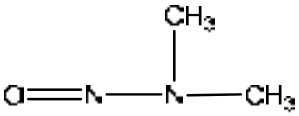


Figura 3.7 Nitrosodimetilamina

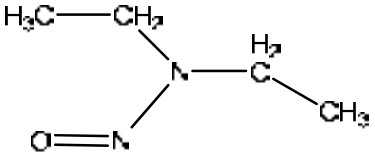


Figura 3.8 Nitrosodietilamina

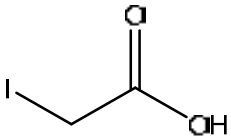


Figura 3.9 Ácido yodoacético

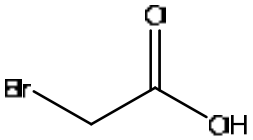


Figura 3.10 Ácido bromoacético

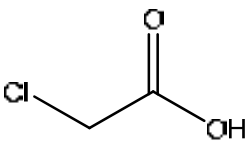


Figura 3.11 Ácido cloroacético

III.1.2. Muestras de agua evaluadas *in vitro*

Se evaluaron 9 muestras de extracto XAD de agua de piscina y agua potable, cuyas características se presentan en la tabla 3.2.

Tabla 3.2 Información de las muestras de extracto XAD de agua de piscina evaluadas

Muestra	**Tipo**	**Lugar**	**TOC (mg/L)**	**T (°C)**	**Total cloro residual (mg/L)**	**Desinfectante**
S3	Agua de grifo	Interior	1,2	14	1,4	Cloro libre
S1	Piscina fría	Interior	18,1	25	3,7	UV y cloro libre
S7	Piscina fría	Interior	5,2	25	1,6	UV y cloro libre
S4	Piscina fría	Interior	124,8	26	2,4	BCDMH
S5	Piscina fría	Interior	12,6	29	3,4	Cloro libre
S6	Piscina caliente	Interior	33,1	36	3,7	Cloro libre
S2	*Jacuzzi*	Interior	12,1	40	2,8	Cloro libre
S8	Piscina fría	Interior	23,7	25	1,7	Cloro libre
S9	Piscina fría	Exterior	33,1	20	1,4	Cloro libre

III.2. MUESTRAS DE SANGRE PERIFÉRICA HUMANA (Evaluación *in vivo)*

Muestras de sangre periférica de una población de 49 personas de edades comprendidas entre 18 y 50 años (67% mujeres, 33% hombres, con una edad media de 30,1 años) se evaluaron como parte de un estudio para determinar los efectos genotóxicos *in vivo* de la exposición a SPD. Dicha exposición consistió en nadar en una piscina con agua clorada durante un tiempo de 40 min. La extracción de las muestras de sangre de cada participante se realizó antes y después haber nadado.

III.3. LÍNEAS CELULARES UTILIZADAS

III.3.1. Línea celular linfoblastoide humana TK6

El sistema celular utilizado para evaluar la genotoxicidad de los SPD *in vitro,* tanto en el ensayo de micronúcleos como en el del cometa, ha sido la línea celular linfoblastoide humana TK6 (Skopek et al., 1978). Estas células crecen en suspensión en medio RPMI 1640 suplementado con 15% suero bovino fetal, 50 U/mL penicilina, 50 µg/mL estreptomicina, 2 mM L-glutamina. Los cultivos celulares se mantienen a una densidad de 0,5-1 x 10^6 células/mL, a condiciones de 37 ºC, 5% CO_2 y 95% humedad.

III.3.2. Línea celular de ovario de hámster chino

Para la evaluación de la genotoxicidad *in vitro* de las muestras extracto de agua, las células empleadas han sido de ovario de hámster chino (*Chinese hamster ovary cells, CHO cells)* concretamente de la línea AS52, clon 11-4-8 (Wagner et al., 1998); dichas células crecen adheridas a la superficie de las placas de cultivo en medio Ham's F12 suplementado con 5% suero bovino fetal, 100 U/mL penicilina, 100 µg/mL sulfato de estreptomicina, 0,25 µg/mL anfotericina B, 2 mM L-glutamina, a condiciones de 37 ºC, 5% CO_2 y 95% humedad.

III.3.3. Línea celular L51758Y

Para el ensayo de linfoma de ratón (*Mouse lymphoma assay, MLA),* se han empleado células L51758Y $Tk^{+/-}$, clon 3.7.2c, las cuales fueron purgadas antes de ser utilizadas en el ensayo para eliminar el exceso de células con genotipo $Tk^{-/-}$, mutantes para el gen de la timidina quinasa. Estas células crecen en suspensión en medio RPMI 1640 suplementado con 10% suero de caballo, 1 mM sodio piruvato, 100 U/mL penicilina, 100 µg/mL estreptomicina, 2,5 µg/mL anfotericina B, 2 mM L-glutamina, a condiciones de 37 ºC, 5% CO_2 y 95% humedad.

III.3.4. Linfocitos humanos de sangre periférica

En el ensayo de micronúcleos *in vitro* se utilizaron muestras de sangre periférica de las personas sanas, las cuales se cultivaron en medio RPMI 1640 suplementado con 15% suero bovino fetal, 50 U/mL penicilina, 50 µg/mL estreptomicina, 2 mM L-glutamina y 1% fitohemaglutinina, e incubaron a 37 ºC durante 72 h.

III.4. ENSAYOS DE GENOTOXICIDAD

III.4.1. Ensayo de micronúcleos con bloqueo de la citocinesis

El ensayo de micronúcleos con bloqueo de la citocinesis se ha utilizado para la evaluación de la genotoxicidad *in vitro* en cultivos de células TK6 y de linfocitos de sangre periférica; y para la evaluación *in vivo,* mediante cultivos de sangre periférica; siendo, el tiempo de incubación de 48 h para los cultivos de células TK6 y de 72 h para los de linfocitos de sangre periférica.

En el caso de las células TK6, se establecieron cultivos celulares de 3 mL con una densidad celular de 0,5 x 10^6 células/mL, las cuales se trataron con 30 µL de una determinada concentración de SPD. Los cultivos de linfocitos de sangre periférica (evaluación *in vivo* e *in vitro*) se establecieron mediante la adición de 0,5 mL de sangre periférica en 4,5 mL de medio RPMI 1640 suplementado.

En los cultivos para la evaluación *in vitro,* los tratamientos se añadieron a las 0 h y a las 24 h en los cultivos de células TK6 y de sangre periférica, respectivamente. Además, se realizaron cultivos para el control negativo (disolvente, agua destilada o dimetil sulfóxido, DMSO) y para el control positivo (mitomicina C, MMC; 0,2 µM y 0,3 µM para cultivos de sangre periférica y de células TK6, respectivamente). Se realizaron dos cultivos (réplicas) por cada tratamiento (SPD/disolvente/MMC).

Una concentración final de 6 µg/mL de citocalasina B (Sigma) en cultivo se adicionó a las 0 h (células TK6) y a las 44 h (sangre periférica). Transcurrido el tiempo de incubación, los cultivos se centrifugaron, permitiendo descartar el sobrenadante y se sometieron a choque hipotónico (5 mL de KCl 0,075M a 4 ºC). Se centrifugan nuevamente, se aspira el sobrenadante y se fijan las células con fijador Carnoy (solución metanol/ácido acético 3:1 v/v) y formaldehido al 37%. Se centrifugan y se realizan dos lavados más con fijador Carnoy. Se resuspende el botón celular en el doble de volumen de sobrenadante, se gotean dos portaobjetos (2 gotas de 20 µL) y se dejan secar a temperatura ambiente. Finalmente, los portaobjetos se tiñen con solución Giemsa al 10% en tampón fosfato (Na_2HPO_4 12 H_2O:KH_2PO_4, proporción 1:1 a pH 6,8) durante 7 minutos.

El análisis de los micronúcleos se realizó utilizando un microscopio Diaplan Leitz de campo claro a un aumento de 1000X. Se analizaron 1.000 células binucleadas por cada tratamiento (500 por réplica) para determinar el número de micronúcleos en células binucleadas, teniendo en cuenta los criterios descritos por Kirsch-Volders et al. (2000). Además, se contaron 500 células (250 por réplica) para determinar la toxicidad mediante el cálculo del índice de proliferación celular con bloqueo de la citocinesis (CBPI), que tiene en cuenta el número de núcleos que posee la célula. El CBPI se calculó teniendo en cuenta la siguiente fórmula (Surrallés et al., 1995):

$$CBPI = \frac{M_I + 2\,M_{II} + 3\,(M_{III} + M_{IV})}{\text{Número total de células}}$$

Siendo M_I, M_{II}, M_{III} y M_{IV} las células con uno, dos, tres y cuatro o más núcleos, respectivamente.

III.4.2. Ensayo del cometa (*Single-cell gel electrophoresis, SCGE)*

El ensayo del cometa se ha utilizado para la evaluación de la genotoxicidad *in vitro* con células TK6 y para la evaluación *in vivo,* utilizando sangre periférica.

Los controles usados en el ensayo del cometa para la evaluación *in vitro* fueron el disolvente (agua destilada/DMSO) como control negativo y el peróxido de hidrógeno (H_2O_2 2 mM) como control positivo.

Debido a que la máxima concentración permisible en los ensayos de genotoxicidad *in vitro* en el ensayo del cometa no ha de provocar una mortalidad celular superior al 30% (Henderson et al., 1998), fue necesario realizar ensayos de viabilidad previos para cada SPD con el fin de determinar dicha concentración. La viabilidad celular se evaluó mediante la tinción diferencial entre células vivas y muertas empleando una mezcla de éster de diacetato de fluoresceína (FDA) y bromuro de etidio (BrEt) en tampón de fosfato sódico (Strauss, 1991). La viabilidad se evaluó en 400 células en un microscopio de fluorescencia con el filtro triple MF que combina filtros con capacidad de excitación a 370, 545 y 495 nm y filtros de barrera de 460, 605 y 520 nm a 400X.

Los tratamientos (*in vitro*) se realizaron en cultivos de células TK6 (a una concentración de 10^6 en 1 mL de medio RPMI 1640), a los cuales se les añade 10 µL de cada tratamiento (SPD/control negativo/control positivo). La exposición al tratamiento se realizó durante 3 h. Al cabo de ese tiempo, se centrifugan los cultivos a 2.500 rpm durante 2 min, se lavan y se resuspenden en PBS. De esta suspensión celular se obtienen las células que se utilizarán en el ensayo del cometa y en el de viabilidad (confirmación de citotoxicidad celular menor del 30%). Para cada compuesto se realizaron dos experimentos independientes.

El protocolo empleado para el ensayo del cometa (*Single-cell gel electrophoresis, SCGE)* ha sido el descrito por Singh et al. (1988) con algunas modificaciones. Se emplearon dos capas de agarosa, la primera formada por 150 µL de agarosa de punto de fusión normal (*Normal Melting Agarose*, NMA) al 0,5% (secada a 65 ºC durante 15 min) y la segunda capa formada por 20 µL de suspensión celular resuspendidas en 75 µL de agarosa de bajo punto de fusión (*Low Melting Agarose,* LMA) al 0,5% a 37 ºC y solidificada a 4 ºC durante 15 min.

Posteriormente, se realiza la lisis celular a 4 ºC durante 2 h (células TK6) o durante toda la noche (sangre periférica) en una solución de lisis recién preparada (2,5 mM NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO (células TK6) o 10% H_2O (sangre periférica), 1% Triton X-100, 1% laurilsarcosinato sódico, pH 10). Transcurrido

el tiempo de lisis celular, se procede con la desnaturalización de las células en una solución tampón de electroforesis (1 mM Na_2EDTA, 300 mM NaOH, pH 13) durante un periodo de 40 min (células TK6) o 20 min (sangre periférica) e inmediatamente después, la electroforesis a 25 V (0,73 V/cm) y 300 mA durante 20 minutos; tanto la desnaturalización como la electroforesis se realizan a 4 ºC. A continuación se realizan dos lavados de 5 minutos con tampón de neutralización (0,4 mM Tris, pH 7,5) y se deshidratan los portaobjetos con etanol absoluto durante 3 min.

Los portaobjetos se tiñen antes del análisis microscópico con 60 µL de bromuro de etidio (0,4 µg/mL). El análisis de los portaobjetos se ha realizado a 400X con el software KOMET 5.5 Image Analysis System (*Kinetic Imaging Ltd, Liverpool, UK)* en un microscopio de fluorescencia Olympus BX50 con una combinación de filtro de excitación de banda amplia de 480-550 nm y filtro de barrera de 590 nm, analizándose 100 células al azar por cada tratamiento (50 de cada réplica). Los parámetros utilizados para la evaluación del daño genético han sido el porcentaje de DNA en la cola *(% DNA tail)* y el momento de la cola *(Olive tail moment).*

Además, de la versión básica alcalina del ensayo del cometa, se realizaron variaciones del mismo para poder analizar la cinética de reparación del daño genotóxico producido y la posibilidad de determinar la existencia de daño oxidativo; ambas variaciones se realizaron en la evaluación *in vitro* empleando células TK6.

En el ensayo del cometa realizado para evaluar la cinética de reparación, sólo una concentración de cada SPD se ha evaluado con diferentes tiempos de recuperación o reparación (0, 45, 90, 135, 180, 225, 270 min y 24 h). Luego del tratamiento, cada cultivo tratado se lava y se resuspende en medio completo a 37 ºC (excepto las muestras del control negativo y del tiempo 0); y se incuban a 37ºC, 5% CO_2 hasta su respectivo tiempo de reparación. Después de cada periodo de reparación, las células se resuspenden en PBS y se continúa con el protocolo del ensayo del cometa.

Para determinar el nivel de bases oxidadas producidas por cada SPD, también se ha evaluado una única concentración de cada SPD. Inmediatamente después de la lisis celular, los portaobjetos se lavan tres veces (5 min, 4 ºC) con una solución de tampón de enzima (40 mM HEPES, 0,1 M KCl, 0,5 mM EDTA, 0,2 mg/mL BSA, pH 8). Luego, alícuotas de tampón, que contienen las enzimas bacterianas endonucleasa III (endo III) o formamidopirimidina (FPG) o sin enzima (grupo control), se colocan sobre la segunda capa de agarosa y se incuban durante 30 min a 37 ºC. Luego de este tratamiento enzimático, se continúa con la electroforesis y los posteriores pasos del ensayo del cometa.

III.4.3. Ensayo del cometa – versión placa multipocillo

Este protocolo se aplicó al estudio de las muestras de agua. Como en los estudios con las células TK6, las concentraciones de los diferentes extractos de agua se seleccionaron en base a una citotoxicidad inferior al 30% (Henderson et al., 1998); empleándose como control negativo el disolvente (dimetilsulfóxido, DMSO) y como control positivo el metanosulfonato de etilo (EMS, 3,8 mM). Cada pocillo representa una determinada concentración de extracto de agua, o uno de los controles usados.

El día anterior al tratamiento, en cada pocillo se establecen cultivos de 2 x 10^4 células CHO en 200 µL de medio F12 completo y se guarda la placa en el incubador. El día del tratamiento, las células se lavan con solución salina equilibrada de Hank (*Hank's balanced saline solution, HBSS)* y se tratan con una determinada concentración de extracto de agua, o de DMSO o EMS durante 4 h a 37 ºC, 5% CO_2. Finalizado el tratamiento, las células se lavan y se someten a una solución de tripsina para poder despegarlas del fondo de los pocillos e individualizarlas, y se inactiva la acción de la tripsina mediante la adición de medio completo.

Una alícuota de 10 µL de suspensión celular se mezcla con 10 µL del colorante vital azul de tripán al 0,05% para evaluar la citotoxicidad. El resto de suspensión celular de cada pocillo se mezcla con agarosa LMA y se coloca sobre un portaobjeto que tiene una capa de agarosa NMA. Para conseguir la lisis de las membranas los portaobjetos se sumergen en solución de lisis (2,5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% laurilsarcosinato sódico, 1% Triton X-100, 10% DMSO, pH 10) a 4 ºC durante toda la noche.

Al concluir el tiempo de lisis, los portaobjetos se colocan en una cubeta de electroforesis con solución de electroforesis (1 mM Na_2EDTA, 300 mM NaOH, pH 13,5) y se desnaturaliza el DNA durante 20 min a 4 ºC y después, se realiza la electroforesis a 25 V, 300 mA (0.72 V/cm) durante 40 min a 4 ºC. Posteriormente, los portaobjetos se lavan tres veces con tampón Tris 400 mM (pH 7,5), se sumergen en agua desionizada fría, se deshidratan con metanol frío durante un periodo de 20 minutos y se secan a 50 ºC durante 5 min.

Para el análisis microscópico, los portaobjetos se hidratan en agua fría durante 30 min y se tiñen con 65 µL de bromuro de etidio (20 µg/mL). Las imágenes se analizan utilizando el software KOMET version 3.1 Image Analysis System (*Kinetic Imaging Ltd. Liverpool, UK)* en un microscopio de fluorescencia Zeiss con una combinación de filtro de excitación BP 546/10 nm y filtro de barrera de 590 nm. Se

analizan 50 células por tratamiento (25 por cada réplica), siendo el parámetro para la evaluación del daño genético el momento de la cola *(SCGE tail moment)*.

III.4.4. Ensayo de linfoma de ratón

Se establecen cultivos de células L5178Y de 10 millones de células en medio RPMI enriquecido con un 10% suero y, para favorecer que alcancen la fase exponencial de crecimiento, se mantienen a 37 ºC, 5% CO_2 durante 30 min. Se adiciona medio RPMI enriquecido sin suero para disminuir la concentración de suero en cultivo. Los cultivos se tratan con 200 µL del agente (SPD/control negativo/control positivo) y se incuban durante 4 h a 37 ºC y a 50 rpm. Siendo los controles empleados, el metanosulfonato de metilo (MMS, 10 µg/mL) como control positivo y el disolvente como control negativo (agua destilada/DMSO).

Los cultivos se lavan dos veces y se resuspenden en medio RPMI suplementado con 20% de suero y se diluyen a 2 x 10^5 células/mL utilizando medio RPMI suplementado con de 20% suero en frascos de cultivos. Los cultivos se mantienen durante dos días, contándose diariamente y subcultivándose a 2 x 10^5 células/mL. Al tercer día, los cultivos se diluyen a 10 células/mL en medio RPMI suplementado con un 20% de suero y se siembran por duplicado en placas de 96 pocillos con 200 µL/pocillo, estas placas corresponden a las de viabilidad. También, se siembran placas para evaluar la resistencia a la 5-trifluorotimidina (TFT), en las que los cultivos se diluyen a 10^4 células/mL en medio RPMI suplementado con 20% de suero y se les adiciona TFT y se siembran por duplicado en placas de 96 pocillos con 200 µL/pocillo. Todas las placas se mantienen a 37 ºC, 5% CO_2 durante 12 días. Transcurridos los 12 días, las placas de viabilidad y de TFT se analizan; en las placas de viabilidad se determina la presencia o ausencia de colonias y en las placas de TFT, mediante la tinción con 2,5 mg/mL de azul de tiazolil (MTT) durante 4 h se puede analizar el tamaño de las colonias (pequeñas o grandes).

III.5. CONCENTRACIONES USADAS

De cada SPD, se ha evaluado una serie de concentraciones en cada uno de los ensayos de genotoxicidad empleados. Las tablas 3.3, 3.4 y 3.5 corresponden a las concentraciones evaluadas de cada SPD en el ensayo de micronúcleos, en el del cometa y en el de linfoma de ratón, respectivamente.

De las concentraciones de la tabla 3.3, se seleccionaron aquellas cuya toxicidad permitiera su análisis; siendo la máxima concentración permitida, aquella que causara una citotoxicidad no mayor del 50%. Se han contado al menos 3 concentraciones subtóxicas (Kirsch-Volders et al., 2000).

Tabla 3.3 Concentraciones evaluadas de los compuestos en el ensayo de micronúcleos en células TK6 y en cultivos de linfocitos de sangre periférica

Compuesto	**Concentraciones (µM)**	
	Células TK6	*Sangre periférica*
Bromonitrometano	0,5; 1; 2; 5; 10; 15	1; 2; 5; 10; 15
Tricloronitrometano	75; 150; 300; 500; 600	----
Tribromacetaldehido	0,05; 0,1; 0,5; 1; 2	0,1; 0,5; 1; 2; 5
Hidrato de cloral	250; 500; 1.000; 2.500; 5.000	----
Ácido mucobrómico	1; 5; 10; 50; 100	0,5; 1; 5; 10; 50
Ácido mucoclórico	0,1; 1; 5; 10; 50	----
Nitrosodimetilamina	500; 1.000; 2.500; 5.000; 10.000	250; 500; 1.000; 2.500; 5.000; 10.000
Nitrosodietilamina	500; 1.000; 2.500; 5.000; 10.000	----

Las concentraciones evaluadas en el ensayo del cometa (Tabla 3.4) se determinaron teniendo en cuenta que la citotoxicidad celular no sea mayor del 30% (Henderson *et al.*, 1998) y como máxima concentración permisible para compuestos no citotóxicos y altamente solubles sea 10 mM o 5 mg/mL (Tice et al., 2000). Para el ensayo de la cinética de reparación se seleccionó una concentración de SPD capaz de inducir un daño significativo y, de esta manera, poder observar cómo va disminuyendo o no el daño inducido. En el ensayo para evaluar el daño oxidativo, la concentración seleccionada además de ser capaz de inducir daño genético, tenía que permitir poder cuantificar un posible daño oxidativo enmascarado, por lo cual estas concentraciones seleccionadas fueron de menor concentración que aquellas empleadas en los ensayos de cinética de reparación.

Tabla 3.4 Concentraciones evaluadas de los SPD en el ensayo del cometa en células TK6

Compuesto	**Concentraciones (µM)**		
	Versión alcalina	*Cinética de reparación*	*Daño oxidativo*
Bromonitrometano	1; 5; 10; 20; 30; 40	30	20
Tricloronitrometano	10; 50; 100; 500; 800; 900	800	400
Tribromoacetaldehido	0,1; 1; 5; 10; 20; 25	10	5
Hidrato de cloral	50; 100; 1.000; 5.000; 10.000	10.000	10.000
Ácido mucobrómico	50; 100; 500; 1.000; 1.500; 2000	50	30
Ácido mucoclórico	50; 100; 500; 1.000; 1.500; 2.000	50	30
Nitrosodimetilamina	50; 100; 1.000; 5.000; 10.000	----	10.000
Nitrosodietilamina	50; 100; 1.000; 5.000; 10.000	----	10.000

Las concentraciones de cada SPD evaluadas en el ensayo del linfoma de ratón (Tabla 3.5) se determinaron en base al valor del crecimiento total relativo, RTG (*relative total growth*) de cada concentración, siendo la máxima permisible aquella con la cual se obtuviera un valor de RTG de 20% o 1 mM, en caso de ausencia de toxicidad (ICH, 2008).

Tabla 3.5 Concentraciones evaluadas de los SPD en el ensayo del linfoma de ratón

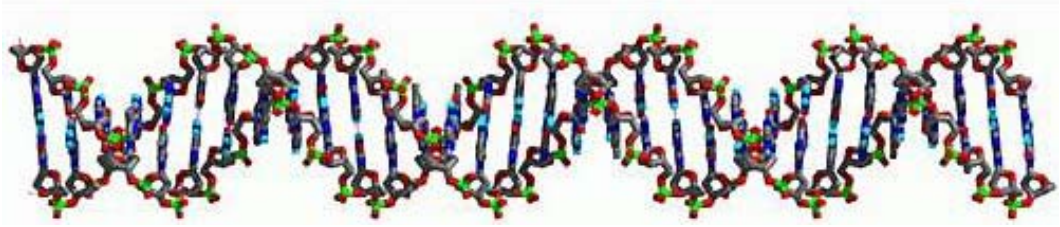
Compuesto	**Concentraciones (µM)**
Bromonitrometano	0,1; 0,5; 1; 2,5; 5; 7; 10; 15;
Tricloronitrometano	1; 5; 10; 15; 20; 30; 40
Tribromacetaldehido	0,01; 0,05; 0,1; 0,25; 0,5; 0,75; 1; 1,5; 2; 3;
Hidrato de cloral	1; 10; 50; 100; 250; 500; 750; 1.000
Ácido mucobrómico	0,05; 0,1; 1; 2; 5; 7;10; 12
Ácido mucoclórico	0,1; 0,5; 1; 2; 5; 7; 10

Las diferentes concentraciones evaluadas de las muestras de extracto de agua se muestran en la tabla 3.6; aquellas concentraciones que produjeron una citotoxicidad mayor del 30% se descartaron para su evaluación genotóxica.

Tabla 3.6 Concentraciones evaluadas de los extracto de muestras de agua en el ensayo del cometa en células CHO

Muestras de extracto de agua	**Concentraciones (X)**
S1	160, 200, 300, 400, 500, 600, 700, 800, 900, 1.000
S2	40, 80, 120, 160, 200, 240, 280, 320
S3	40, 80, 120, 160, 200, 240, 320, 480, 640, 900, 1.000, 1.400, 1.800, 2.200, 2.600, 3.000, 3.600
S4	40, 80, 100, 110, 120, 140, 160, 200, 240, 320
S5	40, 80, 120, 160, 200, 240, 280
S6	40, 80, 120, 160, 200, 240, 280, 320, 360, 400, 440, 480, 640
S7	40, 80, 120, 160, 200, 240, 320, 480, 640, 680, 720, 760, 800, 840
S8	40, 80, 120, 160, 170, 180
S9	40, 80, 120, 160, 200, 240, 320, 480, 640, 680, 720, 760, 800, 840, 880, 920

RESULTADOS



IV. RESULTADOS

Los resultados obtenidos durante el desarrollo del trabajo de Tesis Doctoral están contenidos en los artículos que se incluyen en la memoria y que se especifican a continuación:

- Artículo 1

  Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cell treated in vitro. Environmental Research (2009), 109: 232-238.

- Artículo 2

  Genotoxicity analysis of two hydroxyfuranoes, byproducts of water disinfection, in human cells treated in vitro. Environmental and Molecular Mutagenesis (2009), 50: 413-420.

- Artículo 3

  DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection. Water Research (2010), 44: 2638-2646.

- Artículo 4

  Genotoxic evaluation of the non-halogenated disinfection by-products nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA). Journal of Hazardous Materials (enviado)

- Artículo 5

  Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis –block micronucleus assay. Mutagenesis (en prensa)

- Artículo 6

  Genotoxicity of six disinfection by-products in the mouse lymphoma assay. Environmental Science & Technology (enviado)

- Artículo 7

  Genotoxicity of water concentrates form recreational pools after various disinfection methods. Environmental Science & Technology (2010), 44: 3527-3532.

- Artículo 8

  Genotoxic effects in swimmers exposed to disinfection by-products in indoor swimming pools. Environmental and Health Perspectives (enviado)

ARTÍCULO 1

Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cells treated in vitro

Danae Liviac, Amadeu Creus, Ricard Marcos

Environmental Research (2009), 109: 232-238.

Resumen del artículo 1

Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cells treated in vitro.

La cloración del agua de consumo, además de eliminar los microorganismos patógenos presentes, favorece la formación de compuestos químicos debido a la reacción del cloro (desinfectante) con la materia orgánica e inorgánica natural. Estos compuestos químicos son conocidos como SPD y están siendo investigados por su posible potencial genotóxico y su relación con un incremento en la incidencia de algunos cánceres. Entre estos SPD podemos encontrar los halonitrometanos, grupo de compuestos recientemente identificados y que son estructuralmente parecidos a los halometanos (grupo con mayor prevalencia entre los SPD), pero que poseen un grupo nitro en lugar de un hidrógeno, por lo que pueden presentar una mayor genotoxicidad y mutagenicidad.

Se han evaluado un halonitrometano bromado (BNM, bromonitrometano) y uno clorado (TCNM, tricloronitrometano). Para analizar el potencial genotóxico de cada halonitrometano se utilizó el test de MN y el ensayo del cometa, este último también en sus variantes de cinética de reparación y de análisis de daño oxidativo. Estos ensayos se realizaron empleando la línea linfoblastoide humana TK6. Además, el BNM también fue evaluado en el test de MN en cultivos de linfocitos humanos de sangre periférica.

Los resultados obtenidos en el ensayo del cometa muestran que ambos compuestos inducen daño genotóxico, siendo el BNM más citotóxico y genotóxico que el TCNM; pero, a pesar de que ambos HNM son capaces de producir daño en el DNA, este daño no llega a fijarse y es reparado eficientemente por la célula, siendo el daño producido por el TCNM reparado de forma más rápida. Además, ambos compuestos producen un alto nivel de daño oxidativo, siendo mayor el producido por el BNM. En ambos casos, las purinas son las bases más dañadas por la acción oxidante de los dos halonitrometanos. A pesar de los resultados obtenidos en el ensayo del cometa, ambos compuestos no lograron incrementar la frecuencia de MN ni las células TK6 ni en los cultivos de linfocitos humanos.

Por lo tanto, se puede concluir que ambos compuestos son claramente genotóxicos; siendo el compuesto bromado más genotóxico que el clorado y que ambos inducen un alto nivel de daño oxidativo. Sin embargo, el daño inducido no es capaz de convertirse en un daño genético fijado.

Environmental Research 109 (2009) 232–238

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Genotoxicity analysis of two halonitromethanes, a novel group of disinfection by-products (DBPs), in human cells treated in vitro

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ARTICLE INFO

Article history:
Received 24 July 2008
Received in revised form
12 December 2008
Accepted 19 December 2008
Available online 6 February 2009

Keywords:
Disinfection by-products
Halonitromethanes
Genotoxicity
Comet assay
Micronucleus assay

ABSTRACT

Halonitromethanes (HNMs) constitute an emerging class of disinfection by-products (DBPs) produced when chlorine and/or ozone are used for water treatment. The HNMs are structurally similar to halomethanes, but have a nitro-group in place of hydrogen bonded to the central carbon atom. Since little information exists on the genotoxic potential of HNMs, a study has been carried out with two HNM compounds, namely trichloronitromethane (TCNM) and bromonitromethane (BNM) by using human cells. Primary damage induction has been measured with the Comet assay, which is used to determine both the repair kinetics of the induced damage and the proportion of induced oxidative damage. In addition, the fixed DNA damage has been evaluated by using the micronucleus (MN) assay. The results obtained indicate that both compounds are genotoxic, inducing high levels of DNA breaks in the Comet assay, and that this DNA damage repairs well over time. In addition, oxidized bases constitute a high proportion of DNA-induced damage (50–75%). Contrarily, no positive effects were observed in the frequency of micronucleus, which measures both clastogenic and aneugenic effects, neither using TK6 cells nor peripheral blood lymphocytes. This lack of fixed genetic damage would minimize the potential mutagenic risk associated with HNMs exposure.

1. Introduction

Chlorination, the most widely used method of disinfecting water, leads to the formation of numerous mutagenic and carcinogenic by-products (Meier et al., 1986; Cantor, 1997; Plewa et al., 2002; Richardson et al., 2007), with important long-term human health implications (Arbuckle et al., 2002; Komulainen, 2004).

Disinfection by-products (DBPs) originate from the reaction of chlorine with organic and inorganic water constituents and the main determinants in their formation are the quality of water sources, different water parameters, and the concentration of chlorine applied. Although hundreds of different DBPs are formed in reaction with chlorine, most of them are produced at very low concentrations (Richardson, 1998), which reduce their potential health risk. In addition, if water contains bromide ions, brominated by-products are also generated during chlorination (IARC, 1991).

The first described class of DBPs was trihalomethanes, identified in water by Rook (1974). Of the various trihalomethanes described, chloroform has been the most intensively evaluated for carcinogenicity, and the International Agency for Research on Cancer has classified this compound as a possible carcinogenic to humans (Group 2B) (IARC, 1999; Komulainen, 2004). Nevertheless, studies in animals reveal that chloroform can cause an increased incidence of tumors only at dose levels that result in cytotoxicity, and that the dose–response curves for both cytotoxic and cell proliferation responses are nonlinear (US EPA, 2001).

One emerging class of DBPs is halonitromethanes (HNMs) (Weinberg et al., 2002), which are soluble low-molecular-weight compounds produced when chlorine and/or ozone are used for water treatment. The HNMs are structurally similar to halomethanes, but have a nitro-group in place of hydrogen bonded to the central carbon atom. Although trichloronitromethane (TCNM) is the HNM more commonly found, special attention must be focused to the brominated forms, i.e. bromonitromethane (BNM), since brominated DBPs are more reactive than the chlorinated forms (Woo et al., 2002). In this context, the aim of the present study is to characterize the genotoxicity induced by these two compounds in human cells.

For this objective, we have used the single-cell gel electrophoresis (SCGE) assay or Comet assay that is a rapid, simple, and sensitive technique for measuring DNA damage (Fairbairn et al.,

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0013-9351/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2008.12.009

1995). This assay is sensitive enough to detect DNA damage frequencies less than 1 in 10^7 bases (Lacoste et al., 2006) and with this assay, effects such as DNA single-strand breaks, incomplete excision repair sites, and alkali-labile sites can be detected by analyzing the amount of DNA that migrates out of immobilized cell nuclei that are subjected to electrophoresis. In addition, the Comet assay also permits to discriminate the specific oxidative damage induced in DNA, by using enzymes that detect the oxidized bases. Thus, the bacterial repair enzymes formamidopyrimidine DNA glycosilase (fpg) and endonuclease III (endo III) break the DNA at sites with oxidized forms of purines and oxidized form of pyrimidines, respectively (Collins, 2005). In addition, the assay also permits to determine how rapid the repair of such lesions occurs. This repair process is easily measured by means of repair kinetics experiments, which measure the remaining unrepaired DNA over time (Collins et al., 1995, Collins, 2004; Lacoste et al., 2007).

As a complement of the Comet assay, which permits to evaluate primary DNA damage induction, we have used the micronucleus (MN) assay. This assay permits to determine fixed damage induced by double-strand breaks or by aneuploidy (Fenech et al., 1999; Decordier and Kirsch-Volders, 2006), the two genotoxic effects with great relevance in carcinogenesis. Thus, a recent study has shown the usefulness of the micronucleus assay as a surrogate biomarker of cancer risk (Bonassi et al., 2007).

The combined use of the indicated approaches allows us to get information about the characteristics of the induced DNA damage in human cells by the selected HNMs (TCNM and BNM).

2. Materials and methods

2.1. TK6 and lymphocyte cultures

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lymphocyte cultures were set up by adding 0.5 mL of heparinised whole blood in 4.5 mL RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Chemicals

Bromonitromethane (BNM, $BrCH_2NO_2$) (CAS 563-70-2, 90% purity) was purchased from Sigma-Aldrich (St Louis, MO) and trichloronitromethane (TCNM, CCl_3NO_2) (CAS 76-06-2, 97.5% purity) from Riedel-de-Haën (Seelze, Germany). Endonuclease III (endo III) and formamidopyrimidine glycosylase (fpg) enzymes were kindly provided by Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway).

2.3. Comet assay

TK6 cell cultures were centrifuged at 500*g* for 2 min and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 mL). Each disinfection by-product was dissolved in distilled water, 5 or 6 concentrations of each DBP were evaluated. Aliquots of 10 μL of each solution were added to the cultures for 3 h at 37 °C. An aliquot of 10 μL H_2O_2 (2000 μM) was used as a positive control and 10 μL of vehicle (distilled H_2O) was used as a negative control. Cell viability was evaluated as soon as possible, with a mixture of FDA and EtBr (Strauss, 1991). Two hundred cells were scored for viability in each treatment. More than 70% of the cells were viable in all selected treatments, which agree with the usual testing conditions required in the Comet assay (Henderson et al., 1998). The Comet assay was performed as previously described by Singh et al. (1988), with minor modifications. Approximately 40,000 cells in 20 μL were carefully resuspended in 75 μL of 0.5% LMA, layered onto microscope slides pre-coated with 150 μL of 0.5% NMA (dried at 65 °C) and covered with a cover slip and kept at 4 °C until solidification. Then, the coverslips were removed and cells were lysed for 2 h at 4 °C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly cold electrophoresis solution. Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. After electrophoresis, slides were neutralized with two 5-min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min and stored in the dark at room temperature until scoring. Just before the microscopic analysis, slides were stained with 60 μL of EtBr (0.4 μg/mL). The images were examined at 400 × magnification with a Komet 5.5 Image Analysis System (Kinetic Imaging Ltd., Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. Two different experiments were carried out per compound, each experiment with two different replicates. One hundred randomly selected cells (50 cells from each of the two replicate slides) were analyzed per experiment. The Olive tail moment (OTM) and the percentage of DNA in the tail were used as measures of DNA damage and computed by the Komet version 5.5 Software.

2.4. DNA damage repair analysis

To determine how DNA damage repairs over time, a repair kinetics study was carried out. In this study, only 1 concentration per disinfection by-product was evaluated (30 μM BNM and 500 μM TCNM). Different times of recovery or repair were used (0, 45, 90, 135, 180, 225, and 270 min, as well as 24 h). After treatment, each treated culture was washed with RPMI 1640 medium and resuspended in complete medium preheated to 37 °C (except the negative control and time 0 samples); after that, cultures were incubated at 37 °C in a 5% CO_2 incubator until its specific time of repair. After each recovery period, cell samples were resuspended in PBS and follow the same steps like the alkaline Comet assay. For the evaluation of repair kinetics, DNA repair capacity was calculated using the average value of the Olive tail moment and from the formula "percent DNA repair capacity = [(DNA damage immediately after treatment–DNA damage at the time *t* of repair)/(DNA damage immediately after treatment–DNA damage of untreated cells)] × 100". Two different experiments were carried out with two replicates for each. One hundred cells were scored per experiment (50 per replicate).

2.5. Detection of induced oxidative damage

To determine the level of oxidized bases induced by treatments, 1 concentration per each disinfection by-product was evaluated (15 μM BNM and 250 μM TCNM). After cell lysis, slides were washed 3 times (5 min, 4 °C) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0). Then, aliquots of 100 μL of buffer, containing bacterial enzymes endo III or fpg (enzyme concentration 1/1000) or no enzyme (as control group), were dropped onto the agarose, and incubated for 30 min at 37 °C. After enzyme treatments, cell samples were processed as in the standard alkaline Comet assay procedure. As in the other Comet studies, two different experiments were carried out with two replicates for each. One hundred cells were scored per experiment (50 per replicate).

2.6. Micronucleus test

3 mL of a TK6 culture (500,000 cells/mL) was set up in complete medium. Aliquots of 30 μL of each DBP concentration were added to the cultures. All treatments were added at the beginning of the incubation, and cultures were kept for 48 h at 37 °C in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 h after initiation, 50 μL of each DBP concentration was added to a 5-mL culture. Mitomycin and distilled water were used as positive and negative controls, respectively. For each DBP concentration and control, 2 different cultures were made, and for each culture 2 slides were prepared.

The CBMN test was carried out using the standard technique proposed by Fenech (1993). Cyt-B, at a final concentration of 6 μg/mL, was added to each culture (at the beginning and 44 h after the start, for the TK6 and lymphocyte cultures, respectively). After the incubation period, cells were harvested. Cultures were centrifuged at 150*g* for 8 min, then the supernatant was taken off and the cells were subjected to a hypotonic treatment (5 mL KCl 0.075 M, 4 °C 7 min), and another centrifugation was carried out. Cells were fixed with methanol/acetic acid (3:1 vol) at least 3 times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped on clean microscope slides (2 drops of 20 μL each one). After drying, cells were stained with Giemsa solution for 7 min.

All slides were coded prior to scoring, which was carried out by the same person using a Leitz–Leica light microscope at 1000 × magnification under oil immersion. The criterion for scoring MN was that described by Kirsch-Volders et al. (2000). One thousand binucleated cells were scored and classified, according to the number of micronuclei, to calculate the induction of micronuclei. In addition, cells were scored to evaluate the cytokinesis-block proliferation index (CBPI), calculated according to Surrallés et al. (1995). For each culture, 1 or more slides were scored for the induction of micronuclei and CBPI; the final reported results represent the pooled data from the two cultures.

2.7. Statistical analysis

According to the current recommendations for the Comet in vitro studies (Tice et al., 2000), the culture was used as an experimental unit. All assays were repeated on a least two separate occasions. Homogeneity of variances between concentration levels was determined using Levene's test. Independent tests using Dunnett's correction for multiple-test adjustment were performed to compare each level of concentration with the negative control when the overall *F*-test was significant. Result was considered statistically significant at $p<0.05$. All the analyses were performed using SAS proc MIXED v9.1 (SAS Institute, Cary, NC). For the micronucleus test, data for binucleated cells with micronucleus were compared for each treatment using the one-tailed Fisher's exact test. The Chi-square test was used for the analysis of CBPI among treatments (Kirsch-Volders et al., 2000).

3. Results

A preliminary screening on the toxicity of two HNMs studied showed important differences in toxicity, BNM being more cytotoxic than TCNM in the TK6 cell line. Thus, the highest concentration of TCNM inducing more than 80% of viable cells was 900 μM, while only 40 μM of BNM was able to induce such effects (Fig. 1).

TCNM was clearly genotoxic in TK6 cells, inducing a direct concentration–response effect. Fig. 2 shows the genetic damage induced by this compound, when both the Olive tail moment and the percentage of DNA in the tail are used. Similar results were obtained with BNM (Fig. 3), although this compound showed a very high DNA damaging effect, inducing more damage than the positive control used (2000 μM H_2O_2). BNM could be considered as highly genotoxic and TCNM as relatively weak genotoxic.

The DNA damage induced by TCNM is repaired over time according to the kinetics indicated in Fig. 4. The slope of the curve shows two phases with a first period showing a fast repair that reduces about 50% of the induced damage in about 90 min. In the second stage, the slope of the curve is lower with about a 95% of the total damage removed at the end of the experiment (24 h).

DNA damage induced by BNM is repaired faster than that induced by TCNM. The repair kinetics for BNM is shown in Fig. 5, where the percentage of repaired damage over time is indicated. About 70% of the induced damage is repaired in 90 min and about 90% of the total damage is removed in 150 min. These results indicate that, although BNM induces more DNA damage than TCNM, such damage is more easily repaired.

Fig. 6 shows the levels of oxidized bases induced, as detected by the treatment with fpg and endo III enzymes. Both agents induced more purine than pyrimidine damage, since the damage detected when fpg is used, is always higher than when endo III is used. Thus, fpg treatment increased about 50% the level of DNA damage detected by the treatment with TCNM alone, and increased by about 75% the amount of DNA damaged induced by BNM alone. This means that BNM induces more oxidative damage than TCNM.

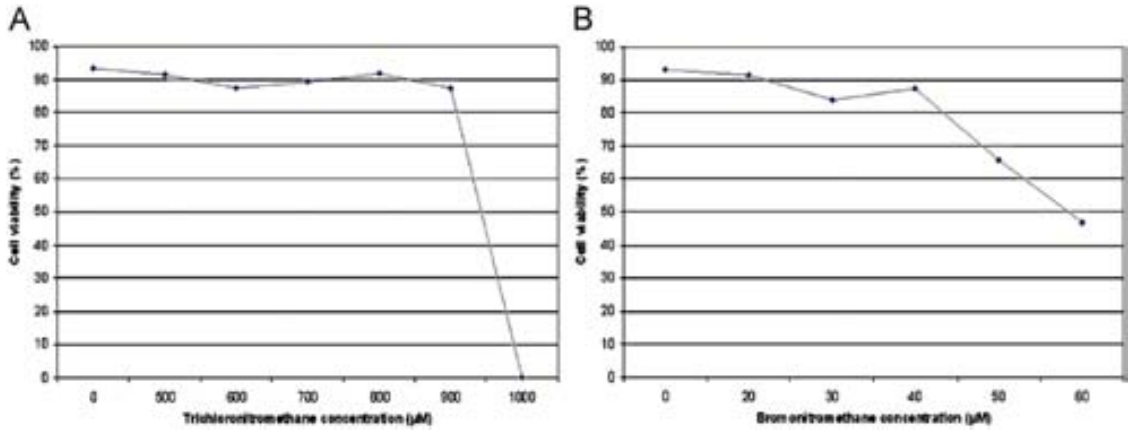

Fig. 1. Viability studies of trichloronitromethane (A) and bromonitromethane (B) on TK6 cells. Viability was measured by using the FDA/EtBr stain.

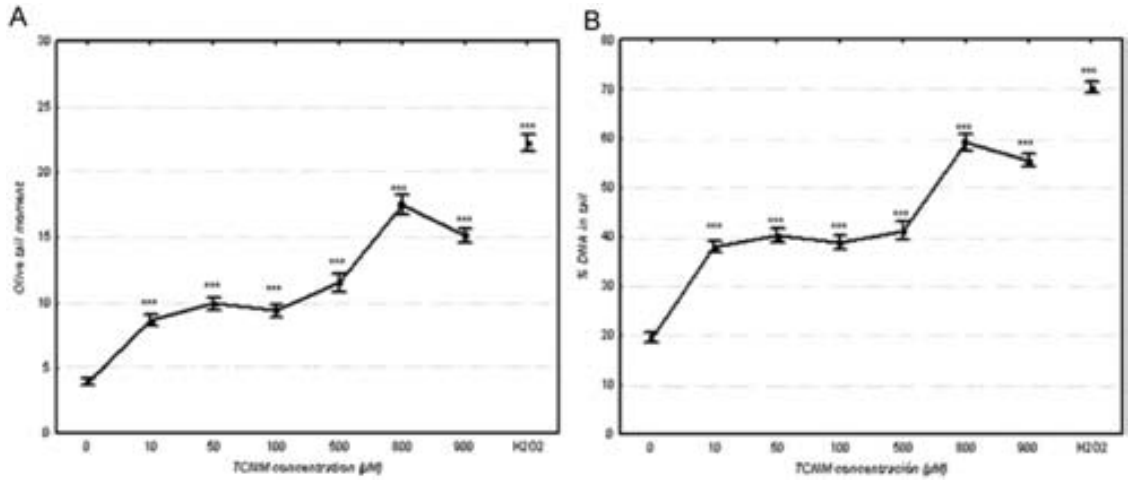

Fig. 2. Genotoxicity of trichloronitromethane (TCNM) in the Comet assay after 3 h of treatment. Genetic damage is measured as the OTM (A) and the percentage of DNA in tail (B). Statistical significance: $^{***}p<0.001$. Data represent the average of two experiments; bars, SE. H_2O_2, positive control.

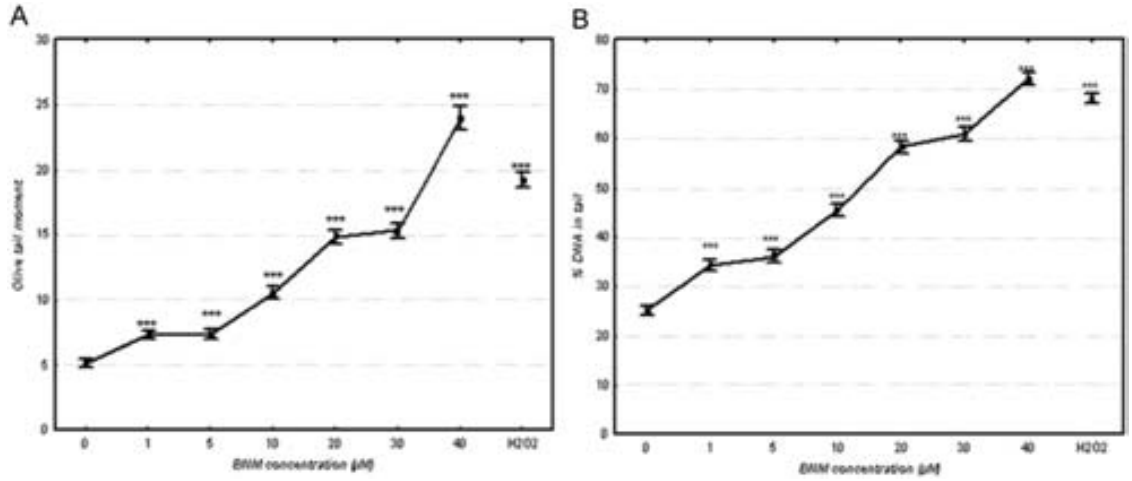

Fig. 3. Genotoxicity of bromonitromethane (BNM) in the Comet assay after 3 h of treatment. Genetic damage is measured as the OTM (A) and the percentage of DNA in tail (B). Statistical significance: $^{***}p<0.001$. Data represent the average of two experiments; bars, SE.

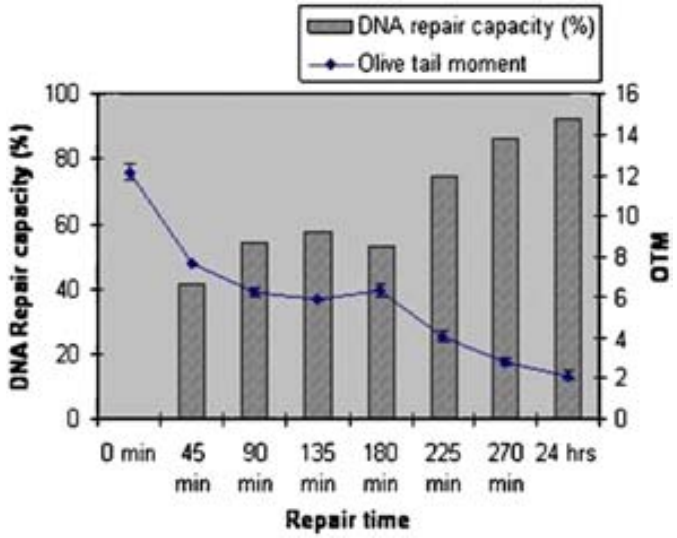

Fig. 4. Olive tail moment in TK6 cells treated with trichloronitromethane (500 μM) for 3 h and then maintained in fresh medium for 0, 45, 90, 135, 180, 225, 270 min, and 24 h. Data represent the average of two experiments; bars, SE; H_2O_2, positive control.

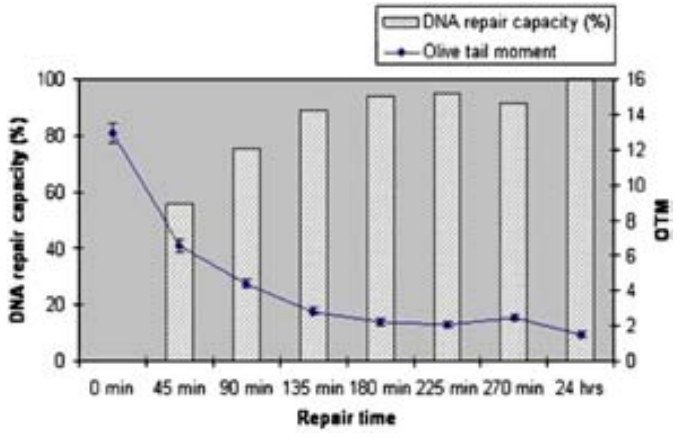

Fig. 5. Olive tail moment in TK6 cells treated with bromonitromethane (30 μM) for 3 h and then with fresh medium for 0, 45, 90, 135, 180, 225, 270 min, and 24 h. Data represent the average of two experiments; bars, SE.

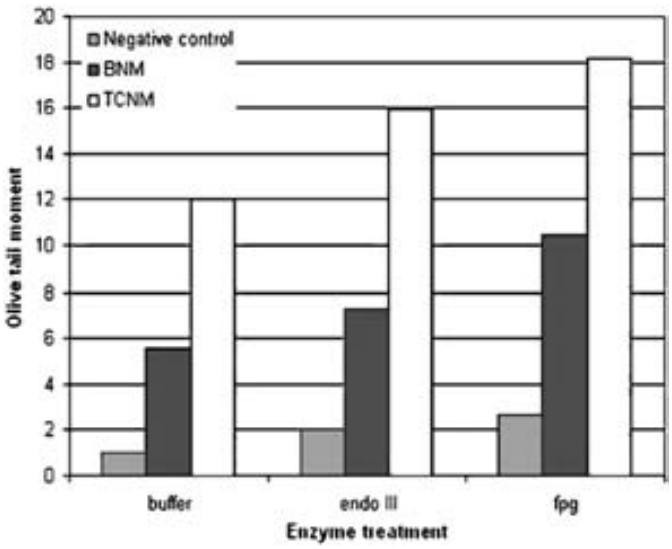

Fig. 6. Effect of the enzyme (endo III and fpg) treatments in TK6 cells previously treated with bromonitromethane (20 μM) or trichloronitromethane (400 μM). Data represent the average of two experiments.

In spite of the high ability of the tested HNMs to interact with DNA and induce genetic damage, as shown in the Comet experiments, both agents were unable to induce significant increases in the frequency of micronucleated cells. Thus, Table 1 shows the results observed after the treatments with TCNM. The total number of micronuclei scored per 1000 binucleated cells, as well as the number of binucleated cells showing at least 1 micronucleus, did not show any significant increase with respect to the control values. Nevertheless, this compound induced significant increases in cytotoxicity as indicated by the CBPI; the concentration of 500 μM being completely toxic.

Similar results were observed after treatments with BNM. Although none of the 6 concentrations assayed induced increases in the basal frequency of micronuclei, at the highest concentration tested (15 μM), high cytotoxicity was induced and less than 1000 binucleated cell were collected. It must be indicated that, in both experiments, the concurrent treatments carried out with MMC (positive control) induced clear and significant increases in the frequency of binucleated cells with micronuclei.

To conclude that both HNMs did not induce micronuclei, and to confirm that the previous negative results were not a particular

Table 1
Micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with trichloronitromethane and bromonitromethane.

Concentration (μM)	Distribution of MN in BN cells					Total MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
TCNM												
0	977	20	3	0	0	26	23	55	218	43	184	2.34
75	988	12	0	0	0	12	12	80	274	32	114	2.13***
150	984	14	2	0	0	18	16	98	329	16	57	1.95***
300	984	15	1	0	0	17	16	171	275	9	45	1.77***
500	Cytotoxic											
MMC	917	71	11	1	0	96	83***	195	290	4	11	1.64***
BNM												
0	979	18	2	0	1	26	21	56	217	23	204	2.34
0.5	971	24	3	2	0	36	29	52	183	22	243	2.43
1	970	26	3	1	0	35	30	38	214	27	221	2.42
2	976	21	3	0	0	27	24	47	197	24	232	2.42
5	976	22	2	0	0	26	24	98	238	15	149	2.13***
10	980	18	1	1	0	23	20	253	188	7	52	1.61***
15[a]	540	18	2	0	0	22	20	400	92	0	8	1.22***
MMC	904	85	10	0	1[b]	109	96***	135	333	14	18	1.79***

***Statistically significant from control ($p \leqslant 0.001$),
[a] Only 560 binucleated cells were detected.

Table 2
Induction of micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values in cultured lymphocyte treated with bromonitromethane.

Concentration (μM)	Distribution of MN in BN cells					MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
0	998	1	1	0	0	3	2	276	205	11	8	1.49
1	998	1	1	0	0	3	2	295	176	14	15	1.47
2	994	5	1	0	0	7	6	269	212	8	11	1.50
5	998	2	0	0	0	2	2	265	220	7	8	1.50
10	996	4	0	0	0	4	4	297	192	5	6	1.43
15	994	6	0	0	0	6	6	336	153	8	3	1.35***
MMC	979	20	1	0	0	22	21***	429	71	0	0	1.14***

***Statistically significant from the control ($p \leqslant 0.001$).

characteristic of the TK6 cell line used, an additional experiment with human lymphocytes treated with BNM, the most genotoxic HNM in the Comet assay, was carried out. Results are indicated in Table 2. As it can be observed, none of the 5 concentrations assayed was able to induce increases in the frequency of binucleated cells with micronuclei. As in the previous experiments with TK6 cells, MMC treatments induced clear and significant increases in the frequency of binucleated cells with micronuclei. These findings indicate the inability of the tested HNMs to induce micronuclei in mammalian cells.

4. Discussion

An increasing interest exists in the study of genotoxic risk related with the new emerging unregulated DBPs (Richardson et al., 2007). Obviously, the main aim is to determine future carcinogenic risk estimations resulting from these exposures. Thus, it is not only necessary to know their potential genotoxicity, but also the way in which these compounds exert their genotoxic action. In this sense, the present study adds new information about how two DBPs belonging to the new class of the halonitromethanes act as genotoxicants.

In general, few data exist on the genotoxicity of DBPs and, in particular, on the HNMs. Thus, genotoxicity of TCNM and BNM has been previously evaluated in bacterial tests. TCNM, also known as chloropicrin, was initially evaluated in Salmonella resulting in genotoxicity only when assayed with the post-mitochondrial supernatant fraction (S9) of rat liver homogenate (Giller et al., 1995).

Further studies demonstrated that P450 was not activating this agent and only glutathione addition was necessary to obtain the ultimate genotoxicant (Schneider et al., 1999). This role of GSH has been confirmed by using a Salmonella strain expressing glutathione transferase theta (GSTT1) (Kundu et al., 2004a b). Nevertheless, all these studies have classified TCNM as a weak mutagen, mainly inducing base substitutions at GC sites, because the hisG46 allele of TA100 reverts almost exclusively by such mutations.

Genotoxicity of halonitromethanes using the Comet assay has also been reported in Chinese hamster ovary cells, including TCNM (Plewa et al., 2004). In these studies TCNM was clearly

genotoxic, getting a fourth place in a range of 9 HNM evaluated. It must be pointed out that all the HNM tested were more potent DNA damaging agents than potassium bromate and ethyl methane sulfonate, used as positive controls. The results of these studies are in concordance with our findings in the Comet test where TCNM acted as a potent genotoxicant in TK6 cells.

With respect to brominated HNM, namely BNM, genotoxicity studies have also been carried out in Salmonella with a final classification of weak mutagen. Nevertheless, and unlike TCNM, BNM was more genotoxic without S9, than with the presence of the metabolic fraction. In addition, the induced mutations were presumptively at AT as well as GC sites, as evidenced by its ability to revert TA104, which also can revert by 3-, 6-, or 9-base deletions (Kundu et al., 2004a b). BNM was more genotoxic in the pre-incubation assay than in the plate-incorporation assay, presumably due to the lower toxicity generally afforded by the plate incorporation assay (Kundu et al., 2004a b). In spite of the low genotoxicity observed in Salmonella, BNM was as genotoxic as TCNM in the Comet assay. Thus, it was placed in the fifth position in a range of 9 compounds This contrasts with our results in the Comet test with TK6 cells showing a higher genotoxicity of BNM, which would agree with the general view indicating that bromide HMs are more genotoxic than chloride ones (Richardson et al., 2007). It must be indicated that from the 9 compounds tested for genotoxicity, the first 3 ranked were brominated species; dibromonitromethane being the most genotoxic (Plewa et al., 2004).

Our studies show that an important component of genotoxic effects is the induction of oxidative damage, induced in both purine and pyrimidine bases. This damage is easily repaired, as observed in the studies on repair kinetics of the damage induced by both TCNM and BNM. Although there is a general lack of information about the mammalian metabolism of the HNMs, the data of bacteria indicate that reduction is an important step, as indicated by the effect of GSH. In contrast with the HM, the HNMs are not activated by GST1 since minimal differences were observed between a strain expressing GSTT1 and its control strain (Kundu et al., 2004a b). However, this does not exclude GSH conjugation, as observed for TCNM in Salmonella (Schneider et al., 1999) and mammals (Sparks et al., 1997), with the GST reaction facilitating a dehalogenation step to form the mutagenic DCNM. Thus, the reduction process involved in the HNMs metabolism would lead to the induction of ROS, causing the DNA damage observed in the Comet assay (Valko et al., 2005).

The genetic damage detected in the Comet assay is a primary damage that can be easily repaired, as demonstrated in this study. Nevertheless, and in terms of risk, it is more interesting to determine the proportion of fixed DNA damage, generated after DNA lesions are resolved. In this context, the micronucleus assay showed special relevance in terms of risk assessment (Bonassi et al., 2007). Surprisingly, and in spite of the high genotoxic potential exerted by the 2 HNMs studied in the Comet assay, both compounds were unable to increase the frequency of binucleated cells with micronuclei in TK6 cells. Given the relevance of these data, results were confirmed by using peripheral blood lymphocytes from a healthy donor, and also a lack of micronuclei induction was obtained. Thus, we can confirm the inability of the tested HNMs to induce fixed DNA damage.

As a summary of our study testing the genotoxicity of TCNM and BNM, we conclude that both compounds are highly genotoxic in the Comet assay, showing the brominated compounds more genotoxic potential than the chlorinated ones. A high proportion of the genetic damage induced is oxidative and it is easily repaired, as indicated by the repair kinetics. Nevertheless, this primary genetic damage is not translated to fixed genetic damage, as detected in the micronucleus assay. These results open an interesting discussion on the risk associated with the use/consumption of water containing such chemicals. Nevertheless, it must be taken into consideration the complex interactions that can take place among the different components present in water, information that cannot be obtained from the analysis of single compounds.

Acknowledgments

D. Liviac is supported by a postgraduate fellowship from the Universitat Autònoma de Barcelona. This investigation has been supported in part by the Spanish Ministries of Education and Science (SAF2005–07643-CO3–03) and Environment (A036/2007/3–14.4), and by the Generalitat de Catalunya (CIRIT, 2005SGR-00136).

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ARTÍCULO 2

Genotoxicity analysis of two hydroxyfuranones, byproducts of water disinfection, in human cells treated in vitro

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Environmental and Molecular Mutagenesis (2009), 50: 413-420.

Resumen del artículo 2

Genotoxicity analysis of two hydroxyfuranones, byproducts of water disinfection, in human cells treated in vitro.

Los SPD son compuestos químicos que se generan por la reacción entre el desinfectante y la materia orgánica e inorgánica presente en el agua a desinfectar. Estos SPD pueden variar entre las diferentes plantas de tratamiento debido a factores como el tipo de desinfectante usado, el pH y la temperatura, entre otros. Entre los SPD podemos encontrar a las hidroxifuranonas, grupo de gran relevancia debido a la presencia en el mismo del SPD conocido como mutágeno X (MX), compuesto al que se le atribuye ser responsable de al menos la tercera parte de la mutagenicidad del agua desinfectada.

En este estudio se ha evaluado una hidroxifuranona bromada (ácido mucrobrómico) y una clorada (ácido mucoclórico). Para evaluar el potencial genotóxico de cada hidroxifuranona se utilizó el test de MN y el ensayo del cometa, este último en sus variantes de cinética de reparación y de análisis de daño oxidativo. Estos ensayos se realizaron empleando la línea celular linfoblastoide humana TK6. Además, el MBA también fue evaluado en el test de MN en cultivos de linfocitos humanos de sangre periférica.

Los resultados obtenidos en el ensayo del cometa muestran que ambas hidroxifuranonas inducen daño genotóxico, siendo el MBA igual de citotóxico pero más genotóxico que el MCA y en ambos casos el daño genotóxico producido fue incluso mayor que el producido por el control positivo. En los experimentos de cinética de reparación se ha observado que las células no son capaces de reparar todo el daño en el DNA inducido por el MCA, permitiendo que una parte del daño pueda llegar a fijarse; en cambio, el daño producido por el MBA es reparado totalmente. Además, ambos compuestos fueron capaces de inducir un alto nivel de daño oxidativo, evidenciado mediante el tratamiento con las enzimas bacterianas endo III y FPG, siendo mayor aquel producido por el MCA. En ambos casos, las bases más dañadas fueron aquellas detectadas con la enzima endo III, las pirimidinas. A pesar de los resultados obtenidos en el ensayo del cometa, el MBA no fue capaz de incrementar la frecuencia de MN en las células TK6 ni en los cultivos de linfocitos humanos; sin embargo, el MCA si que pudo incrementar la frecuencia de MN en las células TK6.

Por lo tanto, se puede concluir que ambas hidroxifuranonas son compuestos altamente genotóxicos; siendo el compuesto bromado más genotóxico que el clorado y

que ambos inducen un alto nivel de daño oxidativo. No obstante, sólo el daño genotóxico inducido por el MCA es capaz de convertirse en un daño genético fijado.

Environmental and Molecular Mutagenesis 50:413–420 (2009)

Research Article

Genotoxicity Analysis of Two Hydroxyfuranones, Byproducts of Water Disinfection, in Human Cells Treated In Vitro

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In general, water for human consumption is chemically disinfected, usually by adding chlorine. As well as producing safe drinking water however, the chlorine treatment, also results in a number of disinfection byproducts (DBPs). One important class of these DBPs is made up of hydroxyfuranones (HFs). In this article, we report the results of a recent investigation to assess the genotoxicity of two HFs, namely mucobromic acid (MBA) and mucochloric acid (MCA), in cultured human cells. The comet assay is used to measure the induction of primary DNA damage and to determine the DNA repair kinetics and the ability of the tested compounds to cause oxidative damage. In addition, the micronucleus (MN) assay is applied to evaluate chromosome damage. The results of the comet assay reveal that both HFs are clearly genotoxic leading to high levels of DNA breaks, and that MBA is more effective than MCA. According to the comet results, the DNA damage induced by MBA repairs well over time, but not the one induced by MCA. Furthermore, HFs produce high levels of oxidized bases. In contrast, the results from the MN assay, which measures the induction of clastogenic and/or aneugenic effects, are mainly negative for the two HFs tested, although MCA is able to increase significantly the frequency of micronuclei in binucleated TK cells, at the concentration of 10 μM. Environ. Mol. Mutagen. 50:413–420, 2009.

Key words: disinfection byproducts; hydroxyfuranones; genotoxicity; comet assay; micronucleus assay

INTRODUCTION

Chemical treatment is the most common means of disinfecting drinking water, but a number of different disinfection byproducts (DBPs) are formed during the chlorination process, which could be considered important environmental hazardous chemicals because of the long-term implications for human health [Arbuckle et al., 2002; Komulainen, 2004]. DBPs are produced during the chlorination process in the treatment plants by the reaction of chlorine with the organic and inorganic matter present in raw water. Hundreds of different DBPs may be formed, depending on the amount of organic matter and the dose of chlorine used. In addition, other factors can contribute to the generation of different kinds of DBPs, such as the use of secondary disinfectants, pH, and temperature [Miller and Uden, 1983; Reckhow et al., 1990; Zhang et al., 2000; Nissinen et al., 2002; Nikolaou et al., 2004; Hua et al., 2006]. If the raw water contains bromide or iodide ions, brominated or iodinated byproducts are formed [Richardson et al., 2007].

The first group of DBPs described was the trihalomethanes (THMs) [Rook, 1974]. To date, more than 600 DBPs have been reported in the literature [Richardson, 1998], but only a small amount have been evaluated for genotoxic or mutagenic potential and possible adverse health effects. It is worth remembering that more than

Grant sponsor: Spanish Ministry of Education and Science; Grant number: SAF2005-07643-CO3-03; Grant sponsor: Spanish Ministry of Environment; Grant number: A036/2007/3-14.4; Grant sponsor: Generalitat de Catalunya (CIRIT); Grant number: 2005SGR-00136; Grant sponsor: Universitat Autònoma de Barcelona.

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Received 25 September 2008; provisionally accepted 9 January 2009; and in final form 12 January 2009

DOI 10.1002/em.20479

Published online 26 March 2009 in Wiley InterScience (www.interscience.wiley.com).

60% of the chlorinated DBPs are considered unknown organic halogens, the so-called unidentified DBPs.

Among DBPs, the THMs (chloroform, dichlorobromomethane, chlorodibromomethane, and bromoform) are the most studied, being also the main group detected in drinking water. Another group that has deserved much attention is the one formed by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, known as Mutagen X or MX, and their structural analogues. MX is one of the most potent direct-acting mutagens ever tested in the Ames strain TA100, and it is responsible for at least one-third of the total mutagenicity detected in chlorine-disinfected drinking water [Meier et al., 1987; Fekadu et al., 1994].

In the group of chlorohydroxyfuranones (CHFs), there are also other compounds, but with less mutagenic potency than MX. One of them is the mucochloric acid (MCA, 3,4-dichloro-5-hydroxy-2(5H)-furanone), which has been identified in drinking water at approximately the same concentration of MX [Kronberg and Franzen, 1993; Smeds et al., 1997]. Because of the positive results of CHFs in several mutagenicity assays and their similar structure to the MX, they have raised concern about their potential health hazards. Thus, all CHFs, including the MCA, have been incorporated by scientists of the U.S. Environmental Protection Agency in the list of priority DBPs for a Nationwide Occurrence Study [Krasner et al., 2006]. One HF that has not been identified in drinking water is the mucobromic acid (MBA, 3,4-dibromo-5-hydroxy-2(5H)-furanone), brominated analogue of MCA, but it is believed to be present especially in surface water with high amounts of bromide. Therefore, the aim of this study was to evaluate the genotoxic potential of two HFs, namely MBA and MCA, in cultured human cells.

These HFs have been tested using two genotoxicity assays. One is the single-cell gel electrophoresis (SCGE) assay or comet test, which is a very simple, rapid, and sensitive technique for measuring DNA damage [Fairbairn et al., 1995]. It can detect DNA damage frequencies of less than 1 in 10^7 bases [Lacoste et al., 2006], DNA single and double-strand breaks, incomplete excision repair, and alkali-labile sites.

With the comet assay, oxidative DNA damage can also be analyzed, by using enzymes detecting this kind of damage, such as formamidopyrimidine DNA glycosilase (fpg) and endonuclease III (endo III). These repair enzymes break the DNA at sites with oxidized purines and pyrimidines, respectively [Collins, 2005]. Furthermore, by measuring the remaining unrepaired DNA over time by means of repair kinetics experiments, it can be determined whether the cells can repair the induced lesions and how rapid the repair process is [Collins et al., 1995; Collins, 2004; Lacoste et al., 2007].

The second assay used is the micronucleus (MN) test. With this assay, other types of effects are evaluated corresponding to the chromosome damage induced by double-strand breaks or by aneuploidy [Fenech et al., 1999; Decordier and Kirsch-Volders, 2006]. Both genotoxic effects are of great relevance in carcinogenesis. It should be mentioned that a recent study has shown the usefulness of the MN assay as a surrogate biomarker of cancer risk [Bonassi et al., 2007].

The combined use of the indicated experimental approaches enables us to obtain new information on the characteristics of the DNA damage induced in human cells by the selected HFs (MBA and MCA).

MATERIALS AND METHODS

TK6 and Lymphocyte Cultures

The human lymphoblastoid TK6 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This cell line has been used extensively for mutagenicity and other genotoxicity studies, including both the comet and the MN assays [McNamee et al., 2000; Guillamet et al., 2004; Ferrara et al., 2006; Mishima et al., 2008].

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood, from one young nonsmoking healthy male donor, in 4.5 mL RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Chemicals

Mucobromic acid (MBA, $C_4H_2Br_2O_3$) (CAS 488-11-9, 99% purity) and mucochloric acid (MCA, $C_4H_2Cl_2O_3$) (CAS 87-56-9, 99% purity), as well as mitomycin C (MMC) and hydrogen peroxide (H_2O_2) used for positive controls were purchased from Sigma-Aldrich (St Louis, MO).

Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway) kindly provided endonuclease III (endo III) and formamidopyrimidine glycosylase (fpg).

Comet Assay

TK6 cell cultures were centrifuged at 500*g* for 2 min, and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 mL). Each DBP was dissolved in distilled water, and five or six concentrations of each DBP were tested. Aliquots of 10 μL of each solution were added to the cultures for 3 hr at 37°C. An aliquot of 10 μL H_2O_2 (2,000 μM) was included as positive control, and 10 μL of vehicle (dimethyl sulfoxide, DMSO) was used as negative control. Cell viability was evaluated as quickly as possible, with a mix of fluorescein diacetate (FDA) and ethidium bromide (EtBr) [Strauss, 1991]. Two hundred cells were scored for viability in each treatment. More than 70% of cells were viable in the applied treatments, which agree with the conditions required in the comet assay [Henderson et al., 1998]. The assay was performed as previously described by Singh et al. [1988] with minor modifications. Approximately 40,000 cells in 20 μL were carefully resuspended in 75 μL of 0.5% low-melting-point agarose (LMA), layered onto microscope slides precoated with 150 μL of 0.5% normal-melting-point agarose (NMA) (dried at 65°C), and covered with a coverslip and kept at 4°C until solidification. Then, coverslips were removed and cells were lysed for 2 hr at 4°C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile

sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly cold electrophoresis solution. Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. Next, slides were neutralized with two 5-min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, the slides were stained with 60 μL of EtBr (0.4 μg/mL). The images were examined at 400× magnification with a Komet 5.5 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. One hundred randomly selected cells (50 from each of the two replicate slides) were analyzed per sample. The percentage of DNA in the tail was used to evaluate DNA damage and computed using the Komet version 5.5 software.

DNA Damage Repair Analysis

To determine how DNA damage is repaired over time, we conducted a repair kinetics study [Collins et al., 1997]. In this study, only one concentration per DBP was evaluated (50 μM MBA and 50 μM MCA), and different times of recovery or repair were used (0, 45, 90, 135, 180, 225, and 270 min). After treatment, each treated culture was washed with RPMI 1640 medium and resuspended in complete medium preheated to 37°C (except the negative control and time 0 samples); after that, cultures were incubated at 37°C in a 5% CO_2 incubator until its specific time of repair. After each recovery period, cell samples were resuspended in PBS and follow the same steps like in the alkaline comet assay. For the evaluation of the repair kinetics, DNA repair capacity was calculated using the average value of the Olive tail moment and from the formula: percent DNA repair capacity = [(DNA damage immediately after treatment − DNA damage at the time t of repair)/(DNA damage immediately after treatment − DNA damage of untreated cells)] × 100.

Detection of Induced Oxidative Damage

To determine the induction of oxidized bases, one concentration per each DBP was evaluated (30 μM MBA and 30 μM MCA). After cell lysis, slides were washed three times (5 min, 4°C) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0). Then, aliquots of 100 μL of buffer, containing the bacterial enzymes endo III or fpg (enzyme concentration 1/1,000) or no enzyme (control), were dropped onto the agarose and incubated for 30 min at 37°C. After enzyme treatments, cell samples were processed as in the standard alkaline comet assay.

Micronucleus Test

Three milliliters of a TK6 culture (500,000 cells/mL) was set up in complete medium. Aliquots of 30 μL of each DBP concentration were added to the cultures. All treatments were added at the beginning of the incubation, and cultures were kept for 48 hr at 37°C in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 hr after the initiation, 50 μL of each MBA concentration was added to a 5-mL culture. Mitomycin C (MMC) and DMSO were used as positive and negative controls, respectively. For each DBP concentration and control, two replicates were made. Lymphocyte cultures lasted for 72 hr.

The CBMN test was carried out using the standard technique proposed by Fenech [1993]. Cyt-B, at a final concentration of 6 μg/mL, was added to each culture (at the beginning and 44 hr after the start, for the TK6 and lymphocyte cultures, respectively). After incubation, cells were harvested. Cultures were centrifuged at 150g for 8 min; then, the supernatant was removed and the cells were subjected to hypotonic treatment (5 mL KCl 0.075 M, 4°C 7 min), and another centrifugation was carried out. Cells were fixed with methanol/acetic acid (3:1 vol.) at least three times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped onto clean microscope slides (two drops of 20 μL each one). After drying, cells were stained with a Giemsa solution for 7 min.

All slides were coded before scoring, which was carried out by the same person using a Leitz-Leica light microscope at 1,000× magnification, under oil immersion. The criterion for scoring MN was that described by Kirsch-Volders et al. [2000]. One thousand binucleated cells were scored and classified, according to the number of MN, to calculate the induction of MN. In addition, 500 cells were scored to calculate the cytokinesis-block proliferation index (CBPI), according to the following formula: CBPI = $(M_I + 2\,M_{II} + 3(M_{III} + M_{IV})/N$, where M_I to M_{IV} represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored [Surrallés et al., 1995]. The induction of MN and the calculation of CBPI were done for each replicate, and the values presented correspond to the pooled data.

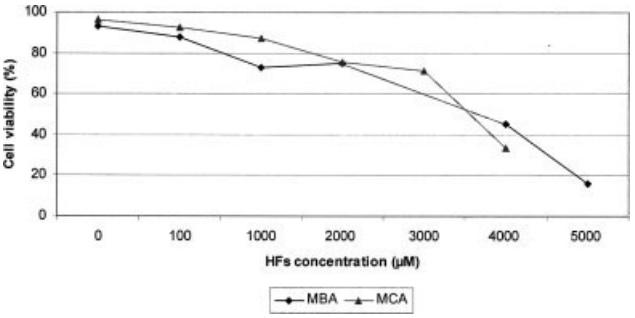

Fig. 1. Viability results after treatment of TK6 cells with MBA and MCA. Viability was measured by using the FDA/EtBr stain.

Statistical Analysis

For the comet assay, a general linear model was used to determine the statistical significance of the results. The homogeneity of variances between concentration levels was determined using Levene's test. Independent tests using Dunnett's correction for multiple test adjustment were performed to compare each level of concentration to the negative control when the overall F-test was significant. A result was considered statistically significant at $P < 0.05$. All the analyses were performed using SAS proc MIXED v9.1 (SAS Institute, Cary, NC). For the MN test, data for the binucleated cells with MN were compared for each treatment using the one-tailed Fisher's exact test. The χ^2 test was used for the analysis of CBPI among treatments.

RESULTS

According to the FDA/EtBr viability assay, both compounds (MBA and MCA) were cytotoxic in TK6 cells. The cytotoxicity of the two chemicals is similar in the lower concentrations but, at the highest one (4,000 μM), MCA appears to be slightly more cytotoxic (Fig. 1).

The results obtained in the comet assay (Fig. 2) clearly indicate the genotoxic potential of MBA. This brominated furanone induces a direct concentration-response effect, and all concentrations tested were able to produce even more damage than that produced by the positive control (H_2O_2 2,000 μM). The genotoxic potential of MCA is similar (Fig. 2), also showing a direct concentration

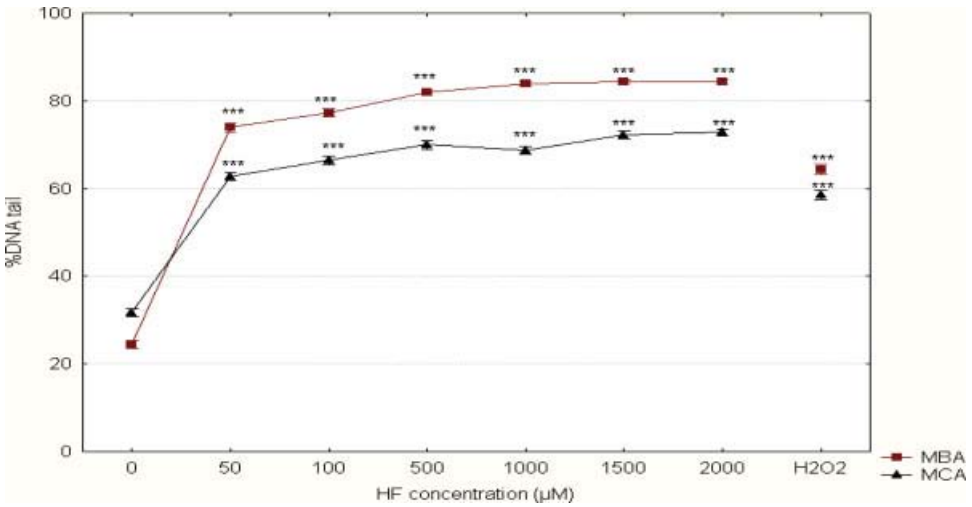

Fig. 2. Genotoxicity of MBA and mucochloric acid MCA in the comet assay after 3 hr of treatment. Genetic damage was measured as the percentage of DNA in tail. Statistical significance: ***$P < 0.001$. Data represent the average of two experiments; bars, SE. H_2O_2, positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

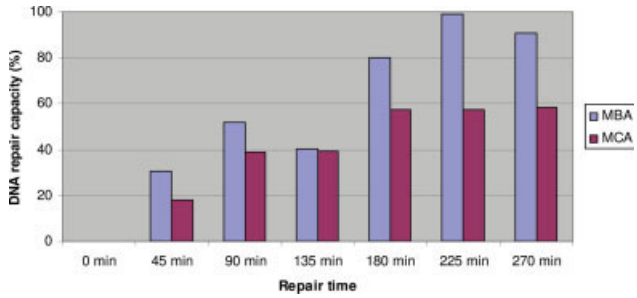

Fig. 3. DNA repair ability in TK6 cells treated with MBA (50 μM) and MCA (50 μM) for 3 hr and then maintained in fresh medium for 0, 45, 90, 135, 180, 225, and 270 min. Data represent the average of two experiments; bars, SE. H_2O_2, positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

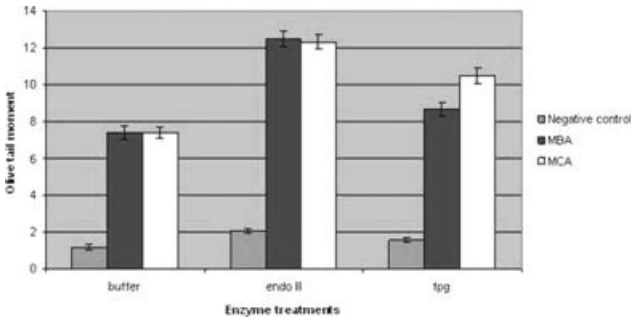

Fig. 4. Effect of the enzyme (endo III and fpg) treatments in TK6 cells previously treated with MBA (30 μM) or MCA (30 μM). Data represent the average of two experiments.

effect. We conclude that both compounds are highly genotoxic, the brominated one being more effective in the comet assay.

The repair kinetic experiments reveal that the DNA damage induced by MBA is repaired over time (Fig. 3). About 50% of the initial damage is repaired at 90 min, 80% at 180 min, and after 225 min almost all damage had been removed, showing that the cells repair the primary genetic damage caused by MBA, but not very fast. The repair kinetics and repair capacity after MCA treatment are somewhat different (see also Fig. 3); the cells being unable to remove all the damage produced by MCA. At the beginning, the curve representing the percentage of DNA repair capacity vs. the repair time shows a slight slope. About 20% of the damage had been repaired at the first 45 min and 40% is repaired between 90 and 135 min. Afterward, some further damage is repaired but only about 60% of the initial damage induced by MCA is removed. Some unrepaired damage could probably be fixed.

The levels of oxidized bases induced by MBA and MCA, which can be detected by the treatment with fpg and endo III enzymes, are shown in Figure 4. For both furanones, the damage detected with endo III treatment was higher than that detected with fpg treatment, which indicates that they induce more damage in pyrimidines than in purines. In both agents, the endo III treatment increased by 65% the level of DNA damage induced by each HF alone. Nevertheless, when both agents were challenged with fpg, a higher oxidative damage was observed with MCA (about more than 40% the level of DNA damage induced by MCA); thus, MCA was able to induce more oxidative damage than MBA.

In the comet assay, both furanones demonstrated their high ability to interact with DNA and to cause primary DNA damage. In contrast, only MCA was able to increase

TABLE I. Micronuclei (MN), Binucleated Cells with MN (BNMN), and CBPI Values Observed in TK6 Cells Treated with Mucobromic Acid (MBA) and Mucochloric Acid (MCA)

	Distribution of MN in BN cells							Distribution of cells according to the number of nuclei				
Concentration (μM)	0	1	2	3	>3	Total MN	BNMN (‰)	1	2	3	4	CBPI
MBA												
0	973	22	5	0	0	32	27	55	214	10	221	2.35
1	979	20	0	0	1[a]	25	21	40	209	23	228	2.42
5	977	22	1	0	0	24	23	47	279	16	158	2.25**
10	968	32	0	0	0	32	32	128	318	15	39	1.85***
50	Cytotoxic							Cytotoxic				
100	Cytotoxic							Cytotoxic				
MMC	913	80	6	1	0	95	87***	106	355	16	23	1.87***
MCA												
0	981	18	1	0	0	20	19	44	243	25	188	2.34
0.1	985	13	1	1	0	18	15	61	257	19	163	2.24
1	985	15	0	0	0	15	15	65	252	20	163	2.24*
5	994	6	0	0	0	6	6	88	306	5	101	2.04***
10	966	28	5	0	1[a]	43	34*	215	233	10	42	1.67***
50	Cytotoxic							Cytotoxic				
MMC (0.3 μM)	895	93	9	2	1[b]	121	105***	179	306	10	5	1.67***

Statistically significant from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
[a]BN cells with 5 MN.
[b]BN cells with 4 MN.

the frequency of MN. The number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei, and the CBPI after the treatment with furanones are summarized in Table I. As observed, MBA was unable to induce MN; it only produced a decrease in the CBPI values, being completely cytotoxic at the higher concentrations assayed (50 and 100 μM).

The chlorinated furanone MCA was able to induce a significant increase in the frequency of MN, but only at the highest concentration tested (10 μM). Besides, it induced significant increases in cytotoxicity, as indicated by the CBPI values.

An additional experiment was carried out with human lymphocytes treated with MBA to confirm that the negative results obtained for MN were not due to the type of cell used. The results of this experiment are shown in Table II and confirm the inability of MBA to induce MN in mammalian cells. As expected, in all MN experiments performed, the MMC treatments (positive control) lead to significant positive increases in the MN frequency.

DISCUSSION

The use of chlorine to disinfect water for human consumption resulted in a significant decrease in the number of deaths produced by waterborne pathogen diseases. Besides producing safe drinking water, chlorine disinfection also generates DBPs and their study is considered of high priority. Currently, there is a growing interest to investigate the effect of exposure to these byproducts. Several epidemiological studies relate such exposure to an increase in the incidence of bladder cancer [Chang et al., 2007; Villanueva et al., 2007]. Considering the crucial role of mutagenic events in the carcinogenic process, it is important to determine the genotoxic activity of DBPs and to clarify the type of genetic damage produced. In this context, this study adds new information on how both DBPs tested act as genotoxicants.

In relation to the genotoxicity data available for the two HFs evaluated, the amount of information on MCA is higher than that of MBA because MCA had been quantified in drinking water and forms part of CHFs (this group is of special relevance because among its compounds can be found the mutagen X, which has great genotoxic potency).

MCA is structurally related to MX and, for this reason, it has been subjected to more evaluations. The difference between MCA and MX is in the C-4; whereas MX has a dichloromethyl group, MCA has chlorine. This change influences significantly the genotoxic and mutagenic potential, MCA being approximately four orders of magnitude less mutagenic than MX [Fekadu et al., 1994].

MCA had been evaluated alone and as a CHF mixture [Mäki-Paakkanen et al., 2004]. It is a strong mutagen in bacterial gene mutation assays, and several findings suggest that it is a SOS-dependent mutagen. Its molecular mutational spectrum is completely different from the

TABLE II. Induction of Micronuclei (MN), Binucleated Cells with MN (BNMN), and CBPI Values in Cultured Lymphocytes Treated with Mucobromic Acid (MBA)

	Distribution of MN in BN cells							Distribution of cells according to the number of nuclei				
Concentration (μM)	0	1	2	3	>3	Total MN	BNMN (‰)	1	2	3	4	CBPI
0	995	5	0	0	0	5	5	320	173	1	6	1.37
0.5	992	7	1	0	0	9	8	298	185	9	8	1.44
1	995	5	0	0	0	5	5	337	159	2	2	1.33
5	995	5	0	0	0	5	5	319	172	4	5	1.38
10	991	7	2	0	0	11	10	297	191	5	7	1.43
50	989	11	0	0	0	11	11	330	164	3	3	1.35
MMC (0.3 μM)	970	30	0	0	0	30	30***	382	116	2	0	1.24***

Statistically significant from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

other CHFs, inducing primarily G:C → A:T transitions with a 4:1 preference of the second position of the CCC codon [Hyttinen et al., 1995; Knasmüller et al., 1996]. Fekadu et al. [1994] demonstrated that MCA is a direct-acting mutagen because the addition of a metabolic activation system (rat S-9 mix or bovine serum albumin) in DNA repair assays with *E. coli* resulted in almost a complete loss of its DNA damaging activity. MCA had also been evaluated for its aneugenic/clastogenic potential in three MN studies; one with plants and the others with mammalian cells. The first one [Helma et al., 1995] showed a dose-dependent increase of MN in pollen mother cells of *Tradescantia* when MCA was applied directly to the inflorescence, but not when applied on the stems. The other studies [Le Curiex et al., 1999; UNEP, 2003] were performed on mouse lymphoma cells, L5178Y and V79 Chinese hamster lung cells, respectively. This showed that MCA can induce a significant genotoxic effect and an increase of the frequency of MN at the highest concentration (25 μM) in L5178Y, and from 23.7 μM in V79 cells. These results are in accordance with our MN results, showing the capacity of MCA to increase the frequency of MN (at 10 μM), and would confirm the ability of this HF to induce chromosome damage.

In the other assay (comet test), MCA demonstrated a high capacity to induce DNA damage in TK6 cells. These findings agree with the results reported by Mäki-Paakkanen et al. [2001] in CHO cells. In both studies, MCA showed a dose-dependent effect, from 5 μg/mL (29.6 μM) in CHO cells, and from 50 μM in TK6 cells (the lowest concentration tested). According to these results, and from other studies that demonstrated the ability of MCA to induce sister chromatid exchanges, chromosome aberrations, and duodenal nuclear anomalies of B6C3F1 mice [Daniel et al., 1991; Mäki Paakkanen et al., 2001], it can be stated that MCA is a genotoxic agent with clastogenic activity.

With respect to the brominated hydroxyfuranone MBA, as far as we know this is the first study evaluating its genotoxicity in mammalian cells. In in vitro studies, MBA was able to form adducts with adenosine and guanosine [Kronberg et al., 1996]. MBA was also positive in the Ames test without metabolic activation, being almost twice as mutagenic as MCA [LaLonde and Leo, 1994]. This brominated DBP had been recently identified in drinking water in a U.S. National Occurrence Study [Krasner et al., 2006], especially in water with high amount of bromide. Our results from the two complementary assays performed demonstrate the high capacity of MBA to induce DNA damage, although it is unable to increase to a significant extent the frequency of MN. Therefore, MBA can be considered to have a high genotoxic potential. When comparing the results from the two HFs, the brominated one appears to be more genotoxic, in agreement with other studies that compare the effects of brominated vs. chlorinated compounds [LaLonde and Leo, 1994; Kargalioglu et al., 2002; Plewa et al., 2002, 2004; Myllykangas et al., 2003].

Our results show that an important component of the genotoxic effect produced by both HFs is the oxidative damage. In the case of MCA, this was even more relevant than the direct effect produced by the chemical itself. Both HFs can induce oxidized forms of pyrimidines and purines, the proportion of oxidized pyrimidines being far superior. The possibility of quantifying this damage, only revealed by endo III and fpg treatments, shows the high capacity of MCA and MBA to induce oxidative damage, with MCA being more effective. There is also a difference between the repair kinetics of the damage induced by both compounds. In the case of MBA, it is easily repaired but for MCA the repair is not complete, because only about 60% of the induced damage is removed and the rest is possibly fixed.

The kind of genetic damage detected in the comet assay is primary DNA damage, which can be repaired fast; but the more important damage in terms of risk is the fixed DNA damage. This damage can be adequately evaluated by means of the MN test. Only MCA was

effective in both assays and, regardless of the clearly positive results of MBA in the Comet test, it was unable to increase the frequency of MN.

We conclude that the two HFs tested, MBA and MCA, are highly effective in the comet assay, the brominated compound being the most genotoxic. As indicated by the repair kinetic studies, the damage induced by MBA is easily repaired but, in the case of MCA, only about 60% of DNA damage is repaired. In addition, both chemicals are capable of inducing high levels of oxidative damage. Finally, it can be indicated that although MBA is not effective in producing chromosome damage, as detected as MN in binucleated cells, MCA increases the MN frequency. These results are of interest when dealing with the potential risk associated with the consumption of water containing such chemicals. Nevertheless, taking into account that several HFs are able to induce point mutations in *Salmonella*, studies are required to determine whether these compounds may also produce point mutations in mammalian cells.

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Accepted by—
J. Fuscoe

ARTÍCULO 3

DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection

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Water Research (2010), 44: 2638-2646.

Resumen del artículo 3

DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection.

Los SPD son compuestos químicos que se encuentran en toda agua desinfectada químicamente, como sucede en el agua destinada para consumo humano. Hasta el momento, se han identificado un gran número de SPD, constituyendo los haloacetaldehídos el tercer grupo en prevalencia en aguas cloradas, habiéndose también identificado en grandes cantidades en aguas desinfectadas con ozono.

En este trabajo se ha realizado la evaluación de dos acetaldehídos halogenados, uno bromado (tribromoacetaldehido) y uno clorado (hidrato de cloral). Para poder analizar el potencial genotóxico de cada compuesto se utilizaron el test de MN con bloqueo de la citocinesis y el ensayo del cometa, este último también en sus variantes de cinética de reparación y de análisis de daño oxidativo. Los dos ensayos se realizaron empleando la línea celular linfoblastoide humana TK6. Además, el TBA también fue evaluado en el test de MN en cultivos de linfocitos humanos de sangre periférica.

De acuerdo con los resultados obtenidos en el ensayo del cometa, ambos compuestos pueden considerarse como agentes genotóxicos, siendo el TBA más citotóxico y genotóxico que el compuesto clorado (CH); pero, aunque ambos compuestos son capaces de producir daño en el DNA, este daño no llega a fijarse y es reparado por la célula, siendo el daño originado por el CH el que se repara de forma más rápida. Con respecto al análisis de daño oxidativo, ambos compuestos son capaces de producirlo; pero, mientras el TBA produce más daño en purinas, el CH lo hace en las pirimidinas. A pesar de los resultados obtenidos en el ensayo del cometa, ambos acetaldehídos fueron incapaces de incrementar la frecuencia de MN en células binucleadas tanto en cultivos de células TK6 como en cultivos de linfocitos humanos.

En resumen, se puede concluir que los dos compuestos evaluados (TBA y CH) son genotóxicos; siendo el compuesto bromado mucho más genotóxico que el clorado y que ambos son capaces de inducir daño oxidativo, siendo un tipo de daño diferente para cada uno. Además, el daño producido en el DNA por ambos compuestos es rápidamente reparado por las células, evitando de esta manera que pueda llegar a fijarse y evidenciarse como MN.

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DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form
20 January 2010
Accepted 25 January 2010
Available online 10 February 2010

Keywords:
Disinfection byproducts
Halogenated acetaldehydes
Genotoxicity
Comet assay
Micronucleus test

ABSTRACT

Drinking water contains disinfection byproducts, generated by the interaction of chlorine (or other disinfecting chemicals) with organic matter, anthropogenic contaminants, and bromide/iodide naturally present in most source waters. One class of these chemicals is the halogenated acetaldehydes (HAs), identified in high quantities when ozone is used as primary or secondary disinfectant. In this study, an analysis of the genotoxic potential of two HAs, namely tribromoacetaldehyde (TBA) and chloral hydrate (CH) has been conducted in human cells (TK6 cultured cells and peripheral blood lymphocytes). The comet assay was used to 1) measure the induction of single and double-strand DNA breaks, 2) evaluate the capacity of inducing oxidative DNA damage, and 3) determine the DNA repair kinetics of the induced primary genetic damage. In addition, chromosome damage, as a measure of fixed damage, was evaluated by means of the micronucleus test. The results of the comet assay show that both compounds are clearly genotoxic, inducing high levels of DNA breaks, TBA being more effective than CH. According to the comet results, both HAs produce high levels of oxidized bases, and the induced DNA damage is rapidly repaired over time. Contrarily, the results obtained in the micronucleus test, which measures the capacity of genotoxic agents to induce clastogenic and aneugenic effects, are negative for the two HAs tested, either using TK6 cells or human peripheral blood lymphocytes. This would indicate that the primary damage induced by the two HAs is not fixed as chromosome damage, possibly due to an efficient repair or the death of damaged cells, which is an important point in terms of risk assessment of DBPs exposure.

1. Introduction

Chlorination is the most widely used method of disinfecting water for human consumption. This procedure is effective for killing harmful waterborne pathogens; however, it also leads to the formation of a number of genotoxic compounds in drinking water. The presence of these chemicals, commonly known as disinfection byproducts (DBPs), raised a concern about the possible adverse effects associated with long-term consumption of chlorinated water (Komulainen, 2004). DBPs originate from the reaction of chlorine with organic and inorganic matter present in raw water. Each water treatment plant has a unique profile in terms of types and concentrations of DBPs, depending on the quality of source water and the nature and the dose of disinfectant applied. Other factors that can significantly contribute to the generation of different

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0043-1354/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.watres.2010.01.026

kinds of DBPs are the use of secondary disinfectants, as well as pH and temperature (Miller and Uden, 1983; Reckhow et al., 1990; Nissinen et al., 2002; Nikolaou et al., 2004; Hua et al., 2006). If raw water contains elevated levels of bromide or iodide, then brominated or iodinated byproducts are also formed (Richardson et al., 2007).

The first described class of DBPs was trihalomethanes (THMs), identified by Rook (1974). It is of concern that, although more than 600 DBPs have been already reported in the literature (Richardson, 1998), only a small number has been quantified and evaluated for their genotoxic or mutagenic potential, as well as for their possible adverse health effects. It must be recalled that more than 60% of the chlorinated DBPs are considered unknown organic halogens, the so-called unidentified DBPs.

The THMs (chloroform, bromoform, bromodichloromethane, and chlorodibromomethane) have been studied intensively for genotoxicity over the past 30 years since they constitute the major group of DBPs detected in drinking water (IARC, 1999; Richardson et al., 2007). The group of halofuranones has also been studied because one of its constituents namely 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5*H*)-furanone, known as mutagen X or MX, is one of the most potent direct-acting mutagens in the Ames test and it is responsible for a significant portion of the mutagenic activity detected in chlorine-disinfected drinking water (Meier et al., 1987; Holme et al., 1999). MX has been classified in group 2B (as possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC) because it is a potent genotoxicant in a wide variety of genetic assays *in vitro* and it is capable of inducing rat tumors in multiple organs (IARC, 2004).

After THMs and haloacetic acids, chloral hydrate (CH, trichloracetaldehyde) is the next prevalent DBP present in chlorinated drinking water (Williams et al., 1997). CH has been classified in group 3 by the IARC, and it is the most common halogenated acetaldehyde (HA) detected in almost all chlorine-disinfected drinking waters (Koudjonou and LeBel, 2008). It must be pointed out that, in systems that use ozone as primary disinfectant, CH has been detected in higher levels than in chlorine-disinfected waters. In addition to CH, other HAs such as brominated acetaldehydes were also identified in treated water containing high concentrations of bromide (Koudjonou and LeBel, 2006, 2008). Tribromoacetaldehyde (TBA) is a brominated HA that has been included by scientists of the U.S. EPA in the list of priority DBPs for a Nationwide DBP Occurrence Study (Weinberg et al., 2002). Thus, the aim of the current study was to increase our knowledge on the genotoxicity of CH and TBA using cultured human cells.

For this objective, two different genotoxicity assays have been used. One is the single-cell gel electrophoresis (SCGE) assay or comet assay, which is a rapid, relatively simple and sensitive technique for measuring primary DNA damage (Fairbairn et al., 1995). The comet assay is able to detect DNA damage frequencies less than 1 in 10^7 bases (Lacoste et al., 2006). In addition to DNA single and double-strand breaks, the comet assay is also able to detect incomplete excision repair and alkali-labile sites (Collins, 2004).

Moreover, the comet assay also allows the analysis of the oxidative DNA damage induced in the presence of the bacterial repair enzymes formamidopyrimidine DNA glycosilase (FPG) and endonuclease III (endo III). FPG and endo III detect and break the DNA mainly at sites with oxidized forms of purines and pyrimidines, respectively (Collins, 2005; Azqueta et al., 2009). Furthermore, with the comet assay it is feasible to carry out repair kinetics experiments that measure the remaining unrepaired DNA over time. This approach allows determining the capacity of cells to repair the induced DNA damage, as well as the efficiency and the kinetics of the repair process (Collins et al., 1995; Collins, 2004; Lacoste et al., 2007). In this study the comet assay was carried out on TK6 cells.

The second assay used is the cytochalasin-B micronucleus (CBMN) which allows the evaluation of chromosome breaks and aneuploidy (Fenech et al., 1999; Decordier and Kirsch-Volders, 2006). Such endpoints are of relevance in carcinogenesis, as it has been demonstrated that the CBMN assay is a reliable biomarker of cancer risk (Bonassi et al., 2007). The CBMN assay was performed on TK6 cells (TBA and CH) and human peripheral blood lymphocytes (TBA).

We consider that the combined use of the described experimental approaches enable us to characterize the genotoxicity of the two selected HAs in human cells.

2. Materials and methods

2.1. TK6 and lymphocyte cultures

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Lymphocyte cultures were set up by adding 0.5 ml of heparinised whole human blood in 4.5 ml RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Blood sample was from one healthy, non-smoking male, aged 28 years old. After the informed consent was obtained, blood was collected under protocols approved by the Ethic Committees of our institution. Approximately 10 ml of blood was collected, by venipuncture, into syringes containing sodium heparin as anticoagulant. Blood was taken the same day of the initiation of the experiment between 9:00 and 9:30 a.m. to minimize possible confounding effects of dietary factors.

2.2. Chemicals

Tribromoacetaldehyde (TBA, C_2HBr_3O; CAS 115-17-3, 97% purity) was obtained from Sigma–Aldrich (St Louis, MO) and chloral hydrate, also known as trichloroacetaldehyde hydrate (CH, $C_2H_3Cl_3O_2$; CAS 302-17-0, 98% purity) was from Fluka Chemie GmbH (Germany). Endonuclease III (endoIII) and formamidopyrimidine glycosylase (FPG) enzymes were kindly provided by Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway).

2.3. Alkaline comet assay

TK6 cell cultures were centrifuged at 500 g for 2 min and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 ml).

Each disinfection byproduct was dissolved in distilled water and 5 or 6 concentrations of each DBP were evaluated. Aliquots of 10 μl of each solution were added to the cultures for 3 h at 37 °C. These amounts represent a final volume in culture of 1% of the total. An aliquot of 10 μl H_2O_2 (2 mM) was used as positive control and 10 μl of vehicle (distilled H_2O) was used as negative control. In all cases, treatments attain a final volume in culture of 1%. Cell viability was evaluated immediately after treatment with a mixture of the stains fluorescein diacetate (FDA) and ethidium bromide (EtBr). FDA permits to detect only living cells (stained green), while EtBr detects only dead cells (stained red) (Strauss, 1991). Any treatment exceeding 30% toxicity was disregarded (Henderson et al., 1998). The comet assay was performed as previously described by Singh et al. (1988) with minor modifications. Approximately 4×10^4 cells in 20 μl were carefully resuspended in 75 μl of 0.5% low melting agarose (LMA), layered onto microscope slides pre-coated with 150 μl of 0.5% normal melting agarose (NMA) (dried at 65 °C) and covered with a coverslip and kept at 4 °C until solidification. Then, coverslips were removed and cells were lysed for 2 h at 4 °C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly prepared cold electrophoresis solution. Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. After electrophoresis, slides were neutralized with two 5-min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min and stored in the dark at room temperature until scoring. Just before the microscopic analysis, slides were stained with 60 μl of ethidium bromide (0.4 μg/ml). The images were examined at 400× magnification with a Komet 5.5 image analysis system (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. Two different experiments were carried out per compound, each experiment with two different replicates. One hundred randomly selected cells (50 cells from each of the two replicate slides) were analyzed per sample. Ghost cells were not included in the scoring. The percentage of DNA in the tail was used as measure of DNA damage and computed using the Komet version 5.5 software.

2.4. DNA damage repair analysis

To determine the efficiency of DNA repair over time, a repair kinetics study was carried out. In this study, only one concentration per disinfection byproduct was evaluated (10 μM TBA and 10 mM CH). Different repair times were selected (0, 45, 90, 135, 180, 225 and 270 min, and 24 h). After treatment, each treated culture was washed with RPMI 1640 medium and resuspended in complete medium preheated at 37 °C (except the negative control and time 0 samples); after that, cultures were incubated at 37 °C in a 5% CO_2 incubator until the repair time concluded. After each recovery period, cell samples were resuspended in PBS and followed the same steps described for the alkaline comet assay. Two independent experiments were carried out with two replicates for each. One hundred cells were scored per experiment (50 per replicate).

2.5. Detection of induced oxidative damage

To determine the level of oxidized bases induced by treatments, one concentration per each disinfection byproduct was evaluated (5 μM TBA and 10 mM CH). After cell lysis, slides were washed three times (5 min, 4 °C) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0). Then, aliquots of 100 μl of buffer, containing the bacterial enzymes endo III or FPG (enzyme concentration 1/1000) or no enzyme (as control group), were dropped onto the agarose, and incubated for 30 min at 37 °C. After enzyme treatment, cell samples were processed as for the standard alkaline comet assay procedure. One hundred cells were scored per each of two experiments (50 per replicate).

2.6. Micronucleus test

A volume of 3 ml of TK6 culture (0.5×10^6 cells/ml) was set up in complete medium. Aliquots of 30 μl of each DBP concentration, representing 1% of the total volume, were added to the cultures. All treatments were added at the beginning of the incubation, and cultures were kept for 48 h at 37 °C in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 h after initiation, 50 μl of each DBP concentration, representing 1% of the total volume, was added to 5-ml culture. Mitomycin C (MMC, 0.3 μM) and distilled water were used as positive and negative controls, respectively. For each DBP concentration and control, 2 independent cultures were prepared, and for each culture 2 slides were prepared.

The Cytokinesis-blocked micronucleus (CBMN) test was carried out using the standard technique proposed by Fenech (1993). Cytochalasin-B (Cyt-B), at a final concentration of 6 μg/ml, was added to each culture (at time 0 and 44 h after phytohaemagglutinin (PHA) stimulation, for the TK6 and lymphocyte cultures, respectively). After incubation, the cells were harvested. Cultures were centrifuged at 150 g for 8 min, then the supernatant was removed and the cells were subjected to a hypotonic treatment (5 ml KCl 0.075 M, 4 °C 7 min) after which another centrifugation was carried out. Cells were fixed with methanol/acetic acid (3:1 vol) at least 3 times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped on clean microscope slides (2 drops of 20 μl each one). After drying, cells were stained with Giemsa solution for 7 min.

All slides were coded by a person outside the study and the scoring was carried out by a unique person using a Leitz-Leica light microscope at 1000× magnification under oil immersion. The criterion for scoring MN was that described by the HUMN project (Fenech et al., 2003). One thousand binucleated cells were scored and classified, according to the number of micronuclei. In addition, cells were scored to evaluate the cytokinesis-block proliferation index (CBPI), which was calculated according to Surrallés et al. (1995). For each culture, 1 or more slides were scored for the induction of micronuclei and CBPI; the final reported results correspond to the pooled data from the two cultures.

2.7. Statistical analysis

The slide was used as the experimental unit. The percentage of DNA in tail was the parameter assessed for the comet assay.

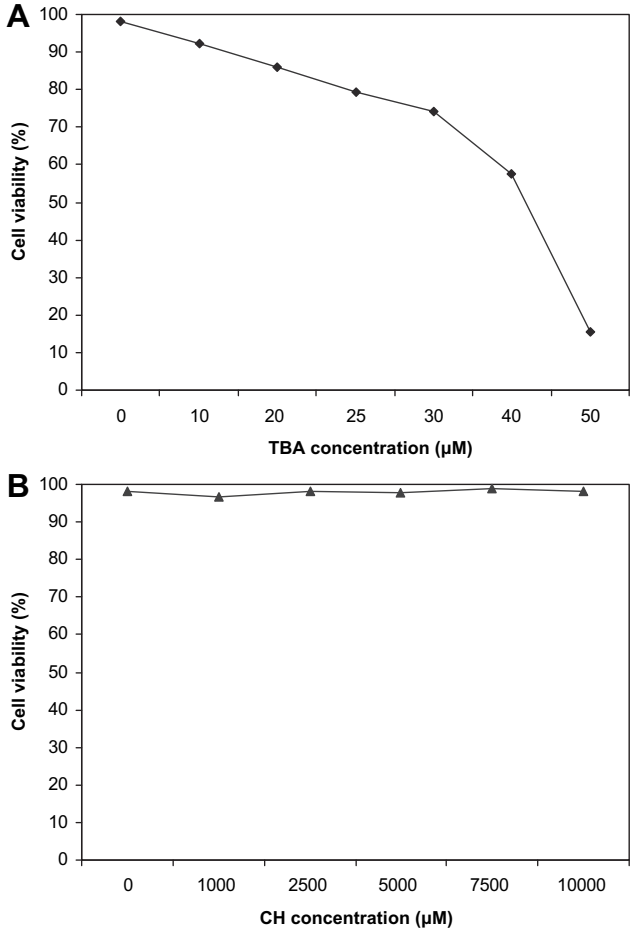

Fig. 1 – Viability results after treatment of TK6 cells with TBA (A) and CH (B). Viability was measured by using the FDA/EtBr stain.

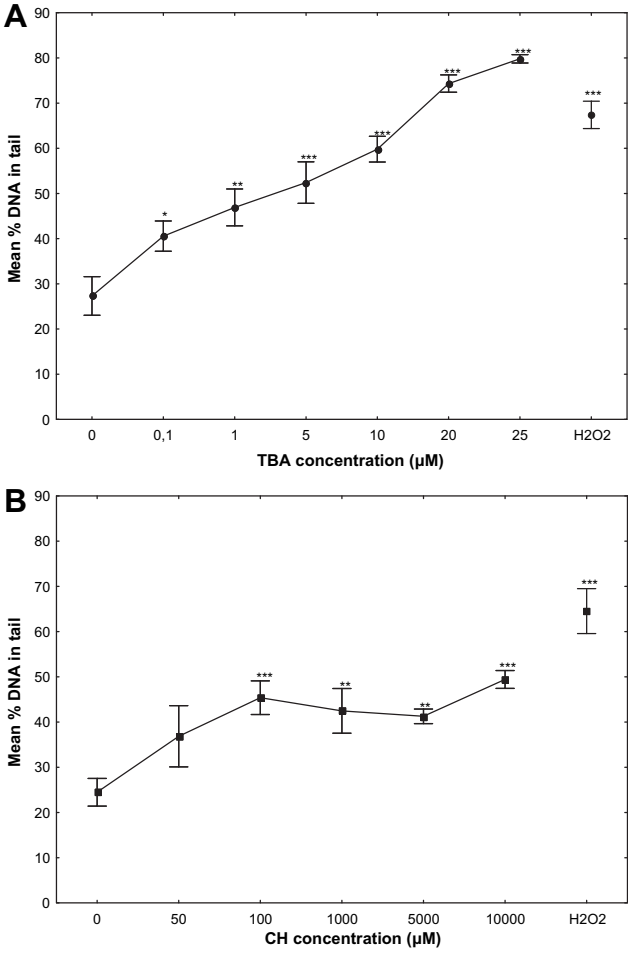

Fig. 2 – Genotoxicity of TBA (A) and CH (B) in the comet assay in TK6 cells after 3 h of treatment. Genetic damage is measured as the percentage of DNA in tail. Statistical significance: $^{***}p < 0.001$. Data represent the average of two experiments; bars, SE. H_2O_2, positive control.

The mean %DNA in tail for each slide was determined, and, the data was compared by one-way ANOVA. Independent tests using Dunnett's correction for multiple-test adjustment were performed to compare each level of concentration to the negative control when the overall *F*-test was significant. A result was considered statistically significant at $p < 0.05$. For the micronucleus test, data for CBMN were compared between each treatment and negative controls using the one-tailed Fisher's exact test. The Chi-square test was used for the analysis of CBPI among treatments.

3. Results

As indicated in Fig. 1, the two studied HAs showed great differences in toxicity, TBA being much more cytotoxic than CH in TK6 cells. According to the FDA/EtBr viability assay, TBA was clearly cytotoxic inducing a direct concentration-response effect, being the cell viability lower than 70% at 35 μM. On the other hand, CH did not show the same pattern since all tested concentrations were not cytotoxic. This included the highest concentration recommended (10 mM) in *in vitro* experiments.

Fig. 2 shows the results obtained in the comet assay indicating that TBA was genotoxic, inducing a direct concentration-response effect. TBA was able to induce more damage than the positive control (H_2O_2 2 mM) at the concentrations of 20 μM and 25 μM. CH also showed a direct

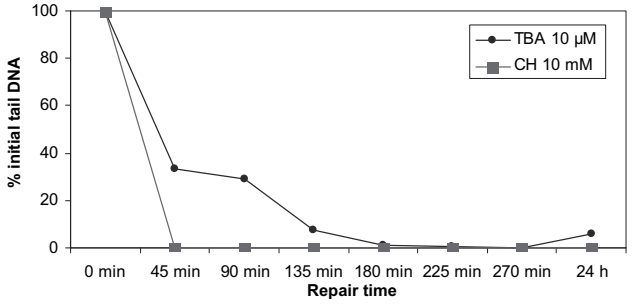

Fig. 3 – DNA repair kinetics in TK6 cells treated with TBA (10 μM) and CH (10 mM) for 3 h and then maintained in fresh medium for 0, 45, 90, 135, 180, 225, 270 min and 24 h. Data represent the average of two experiments; bars, SE.

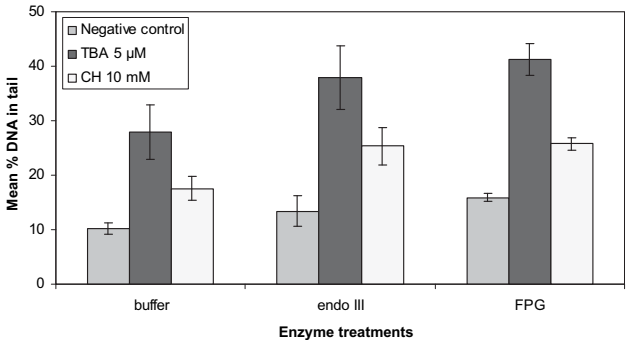

Fig. 4 – Effect of the enzyme (endo III and FPG) treatments in TK6 cells, previously treated with TBA (5 μM) and CH (10 mM). Data represent the average of three experiments; bars, SE.

concentration-response effect. The effects were lower than TBA at higher concentrations.

Fig. 3 shows that DNA damage induced by both HAs is repaired over time, although with a different repair kinetics. It was observed that DNA damage induced by CH was repaired faster than the damage induced by TBA; thus, the induced damage was repaired after 45 min, which reveals the high capacity of the cells to repair the primary genetic damage induced by CH. With regards to TBA the kinetic displays two phases. The first one showing rapid repair, with more than 60% of the initial induced DNA damage repaired after 45 min. In the second step, the slope of the curve was lower with almost all induced damage removed after 180 min.

Fig. 4 shows the levels of oxidized bases induced by one selected concentration of TBA and CH, as detected by using FPG and endo III enzymes. It is observed that enzymatic treatments significantly increase the level of DNA damage, indicating the induction of oxidative damage on both, purine and pyrimidine bases, at similar rates. The oxidative damage induction was observed for both selected compounds.

In contrast to the high ability of both HAs to interact with DNA and induce DNA primary damage, as showed in the comet experiments, both agents were unable to induce significant increases in the frequency of micronuclei. Table 1 shows the number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei and the CBPI values, after treatment with the halogenated acetaldehydes. Neither the number of binucleated cells showing at least one micronucleus nor the frequency of micronuclei scored per 1000 binucleated cells showed any significant increase with respect to the control values. As observed, TBA and CH treatments were cytotoxic at the highest concentrations assayed of 2 μM and 5 mM, respectively. It must be noted that significant reductions in CBPI were observed for CH treatments at concentrations equal or superior to 500 μM. In both experiments, the concurrent treatments carried out with MMC induced significant increases in the frequency of binucleated cells with micronuclei.

Table 2 shows, as occurred with the TK6 cells, that none of the concentrations assayed could induce increases in the frequency of binucleated cells with micronuclei, confirming the inability of TBA to induce MN in the studied mammalian cells. Significant decreases in the CBPI were observed in three out of the five concentrations assayed. As expected, MMC

Table 1 – Total micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with TBA and CH.

Concentration (μM)	Distribution of MN in BN cells					Total MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
TBA												
0	983	16	1	0	0	18	17	69	149	35	247	2.43
0.05	980	20	0	0	0	20	20	49	199	26	226	2.41
0.1	973	24	3	0	0	30	27	31	154	28	287	2.57
0.5	978	21	1	0	0	23	22	44	162	35	259	2.50
1	972	27	1	0	0	29	28	69	149	35	247	2.43
2	Cytotoxic							Cytotoxic				
MMC	924	62	13	1	0	91	76**	95	345	12	48	1.93***
CH												
0	977	22	1	0	0	24	23	38	211	33	218	2.43
250	973	24	2	1	0	31	27	36	254	20	181	2.50
500	983	17	0	0	0	17	17	45	254	20	181	2.32***
1000	987	13	0	0	0	13	13	75	322	6	97	2.06***
2500	986	13	1	0	0	15	14	379	121	0	0	1.24***
5000	Cytotoxic							Cytotoxic				
MMC	931	60	7	0	2[a]	82	69***	147	333	5	15	1.75***

***Statistically significant from control ($p < 0.001$).
a BN cells with 4 MN.

Table 2 – Induction of total micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values in cultured lymphocytes treated with TBA.

Concentration (μM)	Distribution of MN in BN cells					MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
0	992	8	0	0	0	8	8	310	178	5	7	1.40
0.1	997	3	0	0	0	3	3	344	150	0	6	1.32*
0.5	992	8	0	0	0	8	8	283	200	8	9	1.47
1	996	3	1	0	0	5	4	324	139	2	5	1.31*
2	993	6	1	0	0	8	7	284	211	3	2	1.44
5	995	5	0	0	0	5	5	361	130	3	6	1.30**
MMC	950	49	1	0	0	51	50***	422	77	0	1	1.16***

Statistically significant from the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

treatments induced clear and significant increases in the frequency of MN in the experiments with lymphocytes.

4. Discussion

The finding that water disinfection generates DBPs has lead to an increasing interest about their possible undesirable health effects. The main efforts have been focused to evaluate the effects that their exposure could have to humans, especially the potential risk for cancer and reproductive effects associated with DBPs. Several epidemiologic studies have shown an association between DBP levels and increases in the incidence of urinary bladder cancer (Lynch et al., 1989; McGeehin et al., 1993; King and Marret, 1996; Cantor et al., 1998; Vinceti et al., 2004; Chang et al., 2007; Villanueva et al., 2007). Besides data from epidemiologic studies, it is necessary to identify which DBPs are genotoxic, the extent of the genotoxicity and the mode of action. In this context, this study provides new information on the mode of action for the two selected DBPs.

In relation to the genotoxicity data available for the two halogenated acetaldehydes evaluated in the current work, some information are available on CH but no data exist on TBA. It must be recalled that, besides its occurrence in drinking water, CH is used as a sedative and hypnotic drug in pediatric medicine (Istaphanous and Loepke, 2009). Although medical pediatric exposure is greater than that from drinking water consumption, it is acute and selective; thus, it is considered that more than 80% of the total CH exposure is from drinking water where concentrations as high as 22.5 μg/l have been reported (WHO, 2005). Positive mutagenicity data for CH have been reported in the *Salmonella* mutation test, indicating that it could be able to induce point mutations (Richardson et al., 2007). In addition, sister-chromatid exchanges, chromosomal aberrations and aneuploidy in both *in vitro* and *in vivo* systems have been showed after CH exposure (IARC, 2004). Kiffe et al. (2003) reported lack of toxicity by CH in CHO cell line. The cell viability for all the concentrations tested (up to 5 mg/ml) was higher than 98%. These viability data are in accordance with our results. However, the comet assay failed to detect significant increases in the induction of primary DNA damage, which differ from our findings where CH produces a significant increase in DNA damage.

In terms of cytogenetic assessment, a number of studies reporting positives and negatives are described in the literature. CH was able to increase the frequency of MN in mouse spermatocytes (Russo and Levis, 1992), as well as MN and chromosome loss in germ cells of male mice (Allen et al., 1994; Nutley et al., 1996). In addition, significant increases in the frequencies of MN and SCE in blood peripheral lymphocytes have reported in infants treated with CH (Ikbal et al., 2004). In *in vitro* experiments, CH induced MN in cultured human fibroblasts (Bonatti et al., 1992) and lymphocytes (Vian et al., 1995). By contrast, negative results were obtained in cultured mouse lymphoma cells (Harrington-Brock et al., 1998). Summing up the comet and MN data, CH could be considered as a weak genotoxin because it must be present in high concentrations to produce detectable genotoxic damage (Moore and Harrington-Brock, 2000).

With regard to the brominated acetaldehyde TBA, as far as we know this is the first study evaluating its genotoxicity. TBA can be detected especially in disinfected water that contains high amount of bromide (from natural or anthropogenic sources). Our results show that this halogenated acetaldehyde has a high genotoxic potential, being more genotoxic than the positive control used, as seen in the comet assay, and more cytotoxic and genotoxic than its chlorinated form (CH). These findings are in good agreement with the results from previous studies comparing the effects of brominated *vs.* chlorinated compounds (Kargalioglu et al., 2002; Plewa et al., 2002, 2004; Myllykangas et al., 2003; Liviac et al., 2009).

Our results show that an important component of the genotoxic effect produced by both HAs is the oxidative damage. This finding reveals the high capacity of this chlorinated HA to produce a masked damage, which was only possible to quantify when endo III and FPG treatments were used. Oxidative stress is also involved in the genotoxicity and mutagenicity of iodoacetic acid, another DBP, as it has been discovered using the antioxidants catalase and butylated hydroxyanisole (BHA) (Cemeli et al., 2006).

It must be pointed out that both HAs produced a kind of primary DNA damage that is rapidly and efficiently repaired, as observed in the repair kinetic experiments. Particularly

efficient is the repair of the damage induced by CH, which agrees with the kinetics of oxidative damage, which is easily recognized and repaired. DNA damage generated by oxidative stress it is well known to be rapid and efficiently repaired as reviewed by Collins (2009).

A more relevant damage in terms of risk is the fixed DNA damage that corresponds to the non-repaired or misrepaired DNA damage. An assay that permits to detect fixed damage is the micronucleus test. Thus, regardless the positive results induced by the HAs in the comet assay, the failure to induce increases in the frequency of MN, suggests that the tested HAs do not generate fixed damage visualized as chromosome lesions (aneuploidy and/or clastogenicity), in both TK6 cells and peripheral human blood lymphocytes. The lack of concordance between the results in the MN test and in the comet assay has also been observed in other studies (Van Goethem et al., 1997; Maffei et al., 2005; Marabini et al., 2007; Liviac et al., 2009), and this confirms the different mechanisms underlying the genotoxic effects detected by these two assays.

5. Conclusions

We conclude that the two selected HAs (TBA and CH) are genotoxic according to the results of the comet assay, the brominated compound being more cytotoxic and genotoxic than the chlorinated one. As indicated by the repair kinetic studies, the induced DNA damage is efficiently repaired, mainly the one produced by CH. Also, both HAs are capable of inducing high levels of oxidative DNA damage. In contrast, both compounds are unable to produce fixed genetic damage, as detected by their inability to increase the frequency of micronucleated cells. These negative findings in the MN assay have a particular relevance in terms of risk assessment exposure to HAs. Finally, our results are of interest because they contribute with new data to the understanding on how particular DBPs act as genotoxicants and maintain open the discussion on the risk associated to DBPs exposure.

Acknowledgments

Danae Liviac is supported by a postgraduate fellowship from the Universitat Autònoma de Barcelona. This investigation was supported in part by the Spanish Ministries of Education and Science (SAF2005-07643-C03-03) and Environment (A036/2007/3-14.4), and by the Generalitat de Catalunya (2009SGR-725).

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ARTÍCULO 4

Genotoxic evaluation of non-halogenated disinfection by-products nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA)

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Journal of Hazardous Materials (enviado)

Resumen del artículo 4

Genotoxic evaluation of the non-halogenated disinfection by-products nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA).

La desinfección del agua de consumo conlleva a la reducción de organismos patógenos presentes en ella, pero también la formación de subproductos de la desinfección. Estos SPD son compuestos químicos que se originan como resultado de la reacción entre el desinfectante, el cloro en la mayoría de los casos y la materia orgánica e inorgánica presente en el agua a desinfectar. Entre estos SPD se hallan las N-nitrosaminas, un grupo de compuestos que recientemente se ha asociado al proceso de desinfección, que han sido investigados debido a su posible potencial carcinogénico y que son producidos (fundamentalmente) en aquellas plantas de tratamiento que usan como desinfectante la monocloramina.

En este trabajo se ha evaluado el potencial genotóxico de dos nitrosaminas, cuyas estructuras químicas se diferencian entre si debido a la presencia en una de ellas de dos grupos metilo (nitrosodimetilamina) y en la otra de dos grupos etilo (nitrosoetilamina). Para analizar la capacidad genotóxica de cada compuesto se utilizó el test de MN con bloqueo de la citocinesis y el ensayo del cometa. El ensayo del cometa se realizó incorporando fracción microsomal S9 y empleando la variante para el análisis del daño oxidativo. Ambos ensayos, test de MN y ensayo del cometa, se realizaron utilizando la línea celular linfoblastoide humana TK6. Además, la NDMA también fue evaluada en el test de MN en cultivos de linfocitos de sangre periférica humana.

Los resultados obtenidos en el ensayo del cometa muestran que ambos compuestos son ligeramente genotóxicos, pero sólo a las máximas concentraciones evaluadas y en presencia de la fracción microsomal S9, siendo la NDEA más efectiva que la NDMA. Respecto al análisis del daño oxidativo, sólo la NDEA es capaz de producir daño oxidativo, principalmente en las bases purínicas. De acuerdo con los resultados observados en el test de MN, ninguna de las nitrosaminas es capaz de incrementar la frecuencia de MN en células TK6 ni en cultivos de linfocitos humanos.

En resumen, se puede concluir que ambos compuestos evaluados (NDMA y NDEA) son ligeramente genotóxicos y que la NDEA, además de ser más genotóxica, es la única capaz de inducir daño oxidativo, pero a la máxima concentración permitida para la evaluación *in vitro.* Además, ninguno de los compuestos induce daño que pueda ser evidenciado como MN.

El contenido de este artículo se encuentra en el anexo 1.

ARTÍCULO 5

Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay

Danae Liviac, Amadeu Creus, Ricard Marcos

Mutagenesis (en prensa)

Resumen del artículo 5

Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay.

En 1974, se descubrió la presencia de compuestos químicos en el agua desinfectada, los cuales eran generados por el proceso de desinfección de la misma; estos compuestos químicos son los conocidos como subproductos de la desinfección del agua (SPD). De acuerdo a las condiciones del agua a desinfectar y del proceso de desinfección, el perfil de los SPD que se pueden generar puede variar entre las plantas de tratamiento. Hasta la actualidad, se han identificado más de 600 SPD entre los que se encuentran el grupo de los ácidos haloacéticos, los cuales representan el segundo grupo de mayor prevalencia.

En este trabajo se ha evaluado el potencial genotóxico de tres ácidos haloacéticos que corresponden a los monohaloacéticos, que sólo tienen un halógeno en su estructura. Los ácidos monohaloacéticos evaluados han sido uno yodado (ácido yodoacético), uno bromado (ácido bromoacético) y uno clorado (ácido cloroacético). Para evaluar la capacidad genotóxica de cada compuesto se utilizó el test de MN con bloqueo de la citocinesis empleando la línea celular linfoblastoide humana TK6.

Los resultados obtenidos en el ensayo de MN muestran que ninguno de los compuestos evaluados es capaz de incrementar significativamente la frecuencia de MN en células binucleadas en los cultivos de células TK6; siendo el más citotóxico de todos ellos el ácido yodoacético y el menos citotóxico el ácido cloroacético.

Por lo tanto, se puede concluir que los tres compuestos evaluados (IAA, BAA, CAA) no son capaces de producir un daño genético que pueda fijarse y evidenciarse como MN.

El contenido de este artículo se encuentra en el anexo 2.

ARTÍCULO 6

Genotoxicity of six disinfection by-products in the mouse lymphoma assay.

Danae Liviac, Amadeu Creus, Ricard Marcos

Environmental Science & Technology (enviado)

Resumen del artículo 6

Genotoxicity of six disinfection by-products in the mouse lymphoma assay.

La desinfección del agua de consumo que se realiza en las plantas de tratamiento, además de conseguir producir agua apta para consumo humano, también genera la formación de subproductos químicos. Estos subproductos de la desinfección se forman como producto de la reacción entre el desinfectante y la materia orgánica e inorgánica presente en el agua a desinfectar. El perfil de SPD de cada planta de tratamiento depende de factores como el tipo y la dosis de desinfectante usado, la temperatura, el pH, la materia orgánica e inorgánica.

En este trabajo se han evaluado el potencial genotóxico de seis subproductos de la desinfección, dos halonitrometanos (bromonitrometano y tricloronitrometano), dos haloaldehídos (tribromoacetaldehído e hidrato de cloral) y dos halofuranonas (ácido mucobrómico y ácido mucoclórico). Para evaluar la capacidad genotóxica de cada SDP seleccionado se utilizó el ensayo de linfoma de ratón empleando células L5178Y $TK^{+/-}$-3.7.2.c

Los resultados obtenidos en el ensayo de linfoma de ratón muestran que el único compuesto capaz de incrementar la frecuencia de colonias mutantes es el ácido mucobrómico (MBA), produciendo una mayor proporción de colonias pequeñas, lo que indicaría que la naturaleza de las mutaciones es de tipo cromosómico. Este incremento significativo en la frecuencia de mutación sólo fue obtenido en la máxima concentración evaluada, cuyo %RTG se encuentra muy cercano a los valores mínimos permitidos.

Por lo tanto, se puede concluir que de los seis SPD seleccionados, sólo el MBA puede ser considerado un compuesto mutagénico debido a su capacidad de incrementar la frecuencia de mutación en el ensayo de linfoma de ratón.

El contenido de este artículo se encuentra en el anexo 3.

ARTÍCULO 7

Genotoxicity of water concentrates from recreational pools after various disinfection methods

Danae Liviac, Elizabeth D. Wagner, William A. Mitch, Matthew J. Altonji, Michael J. Plewa

Environmental Science & Technolology (2010), 44: 3527-3532.

Resumen del artículo 7

Genotoxicity of water concentrates from recreational pools after various disinfection methods.

El agua de las piscinas y de las bañeras de hidromasaje *(jacuzzis),* a diferencia del agua potable, tiene que recircular durante periodos largos de tiempo, por lo que se somete a procesos adicionales de desinfección para poder prevenir posibles rebrotes de enfermedades infecciosas; además, el tiempo de contacto con el desinfectante es de entre semanas a meses. En esta agua se puede encontrar nuevos precursores de la formación de los SPD como son el sudor, la piel y los productos cosméticos utilizados por los bañistas.

En este trabajo se han evaluado diferentes muestras de extracto orgánico de agua procedentes de diferentes piscinas que tenían una misma fuente de agua potable, la cual también ha sido evaluada. Para analizar el potencial genotóxico se utilizó el ensayo del cometa en su versión multipocillo empleando células de ovario de hámster chino (*CHO).*

Los resultados obtenidos demuestran que todas las muestras de agua de piscina inducen mayor daño en el DNA genómico que la muestra de agua potable que las abastece. A pesar de que algunas muestras de agua procedentes de piscina presentaban valores totales de cloro residual similares a los del agua potable, éstas eran más genotóxicas, debido posiblemente a un mayor tiempo de contacto con el desinfectante. Otro factor que aumentaría la genotoxicidad de las muestras estaría relacionado con la materia orgánica presente en las piscinas y que es generada por los usuarios. Además, el tipo de desinfectante empleado también alteraría el potencial genotóxico del agua, siendo las muestras procedentes de una misma piscina desinfectada con un agente bromado las más genotóxicas. Cuando se compararon los resultados de dos muestras de una piscina recogidas en diferentes tiempos del año, se observó que las condiciones de iluminación (exposición a la luz solar) reducen la potencia genotóxica de la muestra evaluada.

El contenido de este artículo se encuentra en el anexo 4.

ARTÍCULO 8

Genotoxic effects in swimmers exposed to disinfection by-products in indoor swimming pools

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Environmental and Health Perspectives (enviado)

Resumen del artículo 8

Genotoxic effects in swimmers exposed to disinfection by-products in indoor swimming pools.

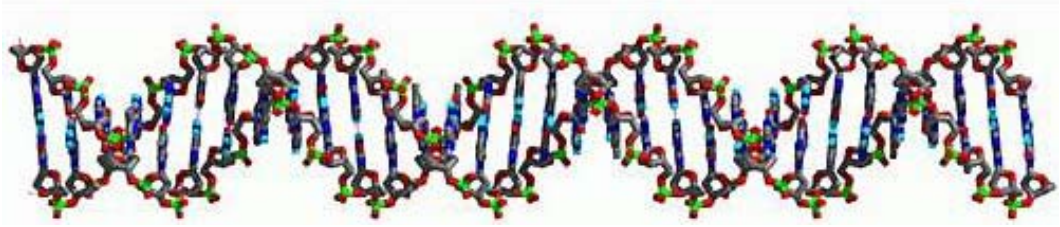
Además del agua potable, otra fuente de exposición a los SPD es el agua clorada de las piscinas; pero, en este caso, las principales vías de exposición son la absorción dérmica debido a la gran superficie de piel que está expuesta y la inhalación de los SPD que se encuentran en el aire del recinto.

En este estudio se han analizado muestras de sangre, orina y aire exhalado de 49 personas, antes y después de una exposición al agua clorada de una piscina durante un periodo de 40 min. Nuestra contribución a dicho estudio consistió en analizar las muestras de sangre empleando el ensayo de micronúcleos y el ensayo del cometa, y las muestras de orina mediante el ensayo de micronúcleos.

Los resultados obtenidos demuestran que la exposición al agua clorada de la piscina durante 40 min no es capaz de producir ningún incremento significativo en la frecuencia de MN ni en los cultivos de linfocitos, ni en las células uroteliales, ni en el nivel de mutagenicidad de la orina. Sin embargo, en el ensayo del cometa se observó una disminución significativa del daño en el DNA posterior a la exposición. Al analizarse conjuntamente las diferencias en la frecuencia de MN pre- y post-exposición con los niveles de THM en el aire exhalado, se encontró que la frecuencia de MN en linfocitos se incrementaba en relación a los niveles de bromodiclorometano y de bromoformo en el aire exhalado. No se observó ninguna asociación entre los niveles individuales o en conjunto de los THM en aire exhalado y los niveles de daño en el DNA cuantificados por el ensayo del cometa o con la frecuencia de MN en células uroteliales; pero sí, con el de mutagenicidad en orina después de nadar, en relación con los niveles de bromoformo exhalado. Además, se encontraron posibles asociaciones entre determinados polimorfismos de genes responsables de la metabolización de los SPD con las respuestas en los biomarcadores de genotoxicidad (MN en sangre y en orina).

El contenido de este artículo se encuentra en el anexo 5.

DISCUSIÓN



IV. DISCUSIÓN

Desde su descubrimiento, hace 35 años, los subproductos de la desinfección (SPD) del agua de consumo han sido objeto de diversos estudios encaminados a investigar distintos aspectos como: (a) la estructura química, (b) su proceso de formación y los factores que lo puedan alterar, (c) la capacidad mutagénica, genotóxica y carcinogénica de los SPD y (d) la relación que puede existir entre su exposición y el incremento en el riesgo de ciertas enfermedades, en especial con el cáncer de vejiga, de colon y de recto. Hay que señalar que, en los últimos años, los estudios epidemiológicos se están centrando en relacionar la exposición a los SPD y los posibles defectos en recién nacidos.

Entre los SPD más estudiados se encuentran los trihalometanos (THM) que constituyen un grupo químico formado por el cloroformo, el diclorobromometano, el clorodibromometano y el bromoformo. Los THM representan el grupo de SPD que se encuentra en mayor proporción en el agua desinfectada, y sus niveles máximos en el agua de consumo vienen regulados por diversas normativas tanto nacionales como internacionales. Otros subproductos muy estudiados son los ácidos haloacéticos, los cuales representan el segundo grupo en cantidad en el agua de consumo y, al igual que sucede con los THM, para algunos de ellos sus niveles máximos están regulados. Otro SPD muy estudiado es el 3-cloro-4-diclorometil-5-hidroxi-2(5H)-furanona, más conocido como mutágeno X o MX, debido a su alta capacidad mutagénica observada en el test de Ames en *Salmonella*. El resto de subproductos también han sido objeto de diversos estudios, en especial aquellos que han podido ser fácilmente cuantificados en el agua, aunque aún falta mucha información sobre la mayoría de los SPD.

La necesidad de conocer con mayor exactitud la presencia de SPD en el agua de consumo ha llevado a mejorar las técnicas de detección, lo que ha permitido poder detectar e identificar nuevos SPD. Este hecho es básico para poder hacer estimas del riesgo que puede suponer el consumo de agua desinfectada. Si tenemos en cuenta que, dependiendo de las condiciones del agua y del proceso de desinfección, cada planta de tratamiento puede producir agua potable con una particular composición (cantidad y tipo) de subproductos diferentes, los SPD que se puede detectar en las diferentes muestras de agua puede variar. Además de por ingestión, se puede estar expuesto a los SPD por otras rutas entre las que destacan la ducha y el baño en piscinas. Si consideramos que el agua que se encuentra en las piscinas recircula durante periodos muy largos de tiempo, para mantenerla apta es necesario aplicar

dosis extras del desinfectante, lo que supone la presencia de niveles mayores de SPD que los que podemos encontrar en el agua del grifo. Además, en las piscinas se pueden encontrar nuevos precursores de SPD provenientes de diversas fuentes como el sudor, el cabello, la piel y los productos cosméticos usados por los bañistas. Así, la existencia de múltiples variables involucradas en la formación de los SPD conlleva a que el perfil de SPD a los que están expuestas las personas sea diferente entre países e incluso entre ciudades.

Las condiciones climáticas de cada zona hacen que los criterios de desinfección puedan ser más o menos estrictos. Así, entre los distintos países de la Unión Europea, España se encuentra en el segundo lugar –después de Portugal- en lo que a niveles de trihalometanos se refiere. Cuando se sectoriza España, se observa que es en la franja mediterránea donde se encuentran los niveles más altos, debido a la peor calidad de sus aguas superficiales (Villanueva et al., 2001b). Si consideramos que, de acuerdo con estos autores, el riesgo de cáncer de vejiga atribuible a los SPD puede ser de un 20%, parece normal considerar el estudio del riesgo de los SPD como prioritario, empezando por el análisis del riesgo genotóxico asociado con su exposición.

En este contexto y, debido a la necesidad existente de ampliar la información sobre los compuestos de los que existen pocos datos, se procedió a evaluar 11 SPD; los cuales se agrupan en 5 grupos químicos diferentes: los halonitrometanos (bromonitrometano y tricloronitrometano), los haloaldehídos (tribromoacetaldehído e hidrato de cloral), las halofuranonas (ácido mucobrómico y ácido mucoclórico), las nitrosaminas (nitrosodimetilamina y nitrosodietilamina) y los ácidos acéticos (ácidos yodoacético, ácido bromoacético y ácido cloroacético).

La evaluación del potencial genotóxico se ha llevado a cabo utilizando dos ensayos: cometa y micronúcleos, con diversas variaciones. Los resultados del ensayo del cometa en células TK6 demostró que todos los subproductos evaluados *in vitro* pueden ser considerados como genotóxicos, debido a la capacidad que poseen para inducir daño genético primario en el DNA (**artículo 1, artículo 2, artículo 3 y artículo 4**). Hay que señalar que esta capacidad de producir roturas en el DNA no es la misma en todos los compuestos; así, algunos de ellos pueden ser clasificados como altamente genotóxicos mientras que otros sólo muestran ser ligeramente genotóxicos. Además, hay que tener en cuenta que las concentraciones evaluadas para cada subproducto han sido diferentes, siendo la máxima concentración evaluada en tres SPD (CH, NDMA y NDEA) de 10.000 µM (10 mM), que se corresponde con la concentración

máxima permisible para compuestos no citotóxicos y altamente solubles (Tice et al., 2000).

Al evaluar estos mismos compuestos mediante el ensayo de micronúcleos con bloqueo de la citocinesis en células TK6, no se observaron incrementos significativos en la frecuencia de MN en células binucleadas (**artículo 1, artículo 2, artículo 3, artículo 4, artículo 5**), con excepción de los resultados obtenidos con MCA, que fue el único SPD capaz de inducir MN, pero sólo a la máxima concentración evaluada (**artículo 2**). Estos resultados difieren significativamente de los observados cuando se utiliza el ensayo del cometa, y se explicarían de acuerdo con el tipo de efecto que mide cada ensayo, donde el ensayo del cometa mide daño primario en el DNA mientras que el ensayo de micronúcleos mide daño fijado.

Para comprobar que los resultados negativos obtenidos mediante el ensayo de MN, no eran debidos a una característica particular de la línea celular TK6, algunos de los SPD se reevaluaron utilizando cultivos de linfocitos humanos. Estos experimentos complementarios confirmaron la incapacidad de los compuestos evaluados de inducir actividad clastogénica y/o aneugénica.

Además de ser evaluados mediante el ensayo del cometa y el de micronúcleos, 6 de los SPD seleccionados (BNM, TCNM, MBA, MCA, CH, TBA) también fueron evaluados en el ensayo de linfoma de ratón empleando células L5178Y $Tk^{+/-}$. Los resultados obtenidos indican que tan sólo uno de los compuestos, el MBA, fue capaz de producir efectos genotóxicos medibles con el ensayo MLA y sólo a la máxima concentración evaluada.

El evaluar dos compuestos de cada uno de los grupos seleccionados (o tres en el caso de los ácidos haloacéticos) nos ha permitido analizar la influencia del elemento halógeno en la capacidad citotóxica y genotóxica de los SPD, pudiendo determinar cuál de ellos (cloro, bromo, yodo) influye más en dichas capacidades.

Además de la exposición diaria al beber y/o cocinar utilizando agua clorada, la exposición también ocurre a nivel recreativo cuando las personas se encuentran expuestas al agua de piscinas o *jacuzzis,* donde el agua utilizada también está desinfectada químicamente y además, para asegurar una desinfección de mayor duración, es necesario tratarla repetidamente. Teniendo en cuenta los posibles riesgos asociados con este tipo de exposición, también se ha realizado la determinación de los efectos genotóxicos producidos por la exposición al agua de piscina en personas que utilizan dichas instalaciones (**artículo 8**). Finalmente, también se ha realizado la evaluación del potencial genotóxico de diferentes muestras de agua de piscinas

(**artículo 7**) sometidas a diferentes procesos de desinfección para poder compararlas entre sí y determinar que tipo de proceso produce un mayor nivel de SPD con potencialidad genotóxica.

Entre los grupos de SPD más recientemente descubiertos se encuentran los halonitrometanos (HNM), que son compuestos que sólo se diferencian estructuralmente de los halometanos por presentar un grupo nitro en lugar de un hidrógeno unido al átomo de carbono central. Esta simple variación les confiere una mayor capacidad mutagénica (Kundu et al., 2004b) aunque, en general, existe muy poca información sobre el potencial genotóxico/mutagénico de estos subproductos. Este grupo de SPD se generan cuando los desinfectantes que se emplean para la desinfección del agua son tanto el cloro, como el ozono y las cloraminas; sin embargo, la combinación ozonización-cloración es la que produce una mayor cantidad de HNM (Hu et al., 2010a; 2010b). Una particularidad que tienen los HNM es que casi la totalidad de sus componentes (8 de 9) se encuentran incluidos en la lista de la EPA de SPD que requieren una alta prioridad de investigación (Weinberg et al., 2002).

En nuestro trabajo (**artículo 1**), evaluamos 2 de los 9 HNM existentes, en particular evaluamos el bromonitrometano (BNM) y el tricloronitrometano (TCNM), este ultimo también conocido como cloropicrina. Ambos subproductos generaron resultados positivos en tratamientos de 3 h en el ensayo del cometa; sin embargo, mientras que el BNM consiguió resultados positivos a partir de concentraciones de 1 µM, el TCNM lo hizo a partir de 10 µM. A pesar de que la concentración mínima genotóxica fue tan sólo 10 veces mayor, la máxima concentración que se pudo evaluar con el BNM fue de 40 µM, mientras que para el TCNM fue de 900 µM, lo que indicaría la gran diferencia que existe entre citotoxicidad y genotoxicidad para ambos compuestos.

Los resultados obtenidos en el ensayo del cometa concuerdan con los únicos resultados encontrados en la literatura para los halonitrometanos en el ensayo del cometa, empleando otro tipo de línea celular, las células CHO (Plewa et al., 2004a). Así, ambos HNM son compuestos claramente genotóxicos siendo más genotóxico el BNM, que es a su vez más citotóxico.

Además de estos resultados con el ensayo del cometa que indican claramente que ambos compuestos son genotóxicos, ambos son también mutagénicos de acuerdo con los resultados observados en el test de reversión de Ames (Giller et al., 1995; Schneider et al., 1999; Kundu et al., 2004a; Kundu et al., 2004b) donde ambos compuestos (TCNM y BNM) inducen sustituciones de bases en sitios GC y el BNM también en sitios AT. Kundu et al. (2004a) evaluaron conjuntamente los HNM y los

halometanos para poder así comparar la influencia de la estructura química en la capacidad citotóxica, concluyendo que la presencia del grupo nitro (NO2) en los HNM les confiere una toxicidad 10 veces mayor en relación a sus homólogos halometanos.

Estudios *in vivo* llevados a cabo en nuestro grupo de investigación (Garcia-Quispes et al., 2009) empleando el ensayo SMART en *Drosophila melanogaster* han mostrado que ambos compuestos no son capaces de causar daño genético detectable con dicho ensayo, que se basa en la pérdida de heterogosidad en genes marcadores para el fenotipo de las quetas alares. Estos resultados negativos, aunque difieren de lo observado en el ensayo del cometa, concordarían con los encontrados en el ensayo de micronúcleos para ambos HNM (**artículo 1**) en las células TK6 y para el BNM, eb cultivo de linfocitos humanos.

Aparte de los estudios mencionados, no existe otra información sobre la genotoxicidad/mutagenicidad del BNM u otros HNM, salvo para el TCNM. Este halonitrometano monoclorado, que se empleó hace tiempo como insecticida y fungicida, es capaz de inducir intercambios entre cromátidas hermanas y posee capacidad genotóxica en *E. coli,* pero no es capaz de incrementar la frecuencia de MN ni en *Pleurodeles walti* ni en células de médula ósea de ratón (Giller et al., 1995).

Otro grupo de SPD analizado como parte del trabajo de tesis doctoral corresponde a las hidroxifuranonas (**artículo 2**). Este grupo forma parte de la fracción orgánica de los SPD y entres sus componentes, se encuentra el denominado mutágeno X (MX, 3-cloro-4-(diclorometil)-5-hidroxi-2(5H)-furanona), al que se le atribuye la mayor parte de la capacidad mutagénica del agua de consumo (Meier et al., 1987; Kronberg and Vartiainen, 1988) y está clasificado en el grupo 2B según la IARC.

Además del MX, en este grupo también se pueden encontrar sus formas abiertas y cerradas (dependiented del pH), su isómero geométrico, la forma reducida y oxidada, sus análogos bromados (BMX) y otras hidroxifuranonas, como son el ácido mucoclórico (MCA, 3,4-dicloro-5-hidroxi-2-(5H)-furanona) y el ácido mucobrómico (MBA, 3,4-dibromo-5-hidroxi-2-(5H)-furanona), que son las dos hidroxifuranonas evaluadas en nuestro trabajo. Todo el grupo de hidroxifuranonas está incluido en la lista de la EPA de los SPD sobre los que existe prioridad de investigación. El MBA, análogo bromado del MCA, a pesar de que todavía no se ha identificado en el agua de consumo, se presume que debe estar presente en ella, especialmente en aguas con altos niveles de bromo.

Por lo que respecta a la información disponible sobre las hidroxifuranonas evaluadas, existe muy poca información sobre el MBA, en comparación con la que hay

sobre el MCA, debido principalmente a que el MCA ya ha sido identificado en el agua de consumo y tiene un parecido estructural con el MX. La única diferencia entre ambos compuestos radica en el C-4, dado que el MX posee un grupo diclorometil en esta posición, mientras que el MCA posee un átomo de cloro. Este simple cambio en el C-4 provoca que el MCA sea aproximadamente 4 veces menos mutagénico que el MX (Fekadu et al., 1994).

Los resultados obtenidos al evaluar el potencial genotóxico del MBA y del MCA en el ensayo del cometa han demostrado que ambos compuestos tienen una gran capacidad para inducir daño genotóxico en las células TK6, siendo el MBA igual de citotóxico pero más genotóxico que el MCA y, en ambos casos, el daño genotóxico producido fue incluso mayor que el producido por el control positivo (H_2O_2). Mientras que para el MBA esta es la primera vez que evalúa su genotoxicidad en células de mamíferos, el MCA ya ha sido evaluado con anterioridad. Así, los resultados obtenidos por Mäki-Paakkanen et al. (2001) indican que el MCA es capaz de producir roturas en el DNA (ensayo del cometa con células CHO) con un efecto dependiente de la dosis a partir de 5 µg/mL (29,6 µM). Estos resultados son similares a los encontrados por nosotros en células TK6, donde los resultados positivos los hemos encontrado a partir de 50 µM (la menor concentración evaluada). Los otros estudios *in vitro* sobre el MBA han demostrado que este compuesto es capaz de formar aductos con la adenina y la guanina (Kronberg et al., 1996), dando respuestas positivas en el test de Ames, llegando a ser incluso dos veces más mutagénico que el MCA (LaLonde and Leo, 1994).

Aunque el MBA ha sido incapaz de incrementar la frecuencia de micronúcleos en células binucleadas (células TK6 y cultivos de linfocitos humanos), hay que resaltar que el ácido mucoclórico (MCA) ha sido el único SPD evaluado capaz de producir incrementos significativos en la frecuencia de micronúcleos (**artículo 2**). Los resultados de otros estudios empleando el ensayo de micronúcleos confirman la capacidad clastogénica y/o aneugénica del MCA, tanto en células de mamíferos como en plantas (Helma et al., 1995; Le Curieux et al., 1999; UNEP, 2003). Al igual que en nuestro resultados con las células TK6 (10 µM), el MCA fue capaz de incrementar la frecuencia de imicronúcleos en células de linfoma de ratón L5178Y (Le Curieux et al., 1999) y en células de pulmón de hámster chino V79 (UNEP, 2003) tan sólo a las máximas concentraciones evaluadas (25 µM y 23,7 µM). Esto confirmaría la capacidad de esta hidroxifuranona para inducir un tipo de daño en el DNA diferente al generado

por los otros SPD evaluados, ya que este daño es capaz de fijarse como daño cromosómico.

El MCA es un compuesto considerado como un mutágeno de acción directa en los ensayos de mutación génica en bacterias y un mutágeno dependiente de la respuesta SOS. Su espectro mutacional es diferente al de las otras clorohidroxifuranonas, induciendo principalmente transiciones G:C →A:T con una preferencia de 4:1 por la segunda posición del codon CCC (Hyttinen et al., 1995; Knasmuller et al., 1996). Fekadu et al. (1994) demostraron que el MCA es un mutágeno de acción directa al evaluarlo en diferentes cepas de *E. coli* en presencia/ausencia de fracción metabólica S9 o BSA (albúmina sérica bovina) y observar que con la fracción microsomal se producía una pérdida casi una completa de su actividad mutagénica. Otros efectos demostrados del MCA son que es capaz de formar aductos *in vitro* con ciertas bases nitrogenadas (adenina, citosina y guanina) (Kronberg et al., 1996), es un inductor débil de intercambio de cromátidas hermanas, y produce una respuesta positiva, dependiente de la dosis, en la inducción de aberraciones cromosómicas (Maki-Paakkanen et al., 2001).

Por lo que respecta a los haloaldehídos, cabe señalar que el CH es el compuesto más importante de este grupo en términos de su abundancia en agua tratada. Así, se ha identificado en casi todas las aguas de consumo desinfectadas con cloro (Koudjonou and LeBel, 2006), y sus niveles se ven incrementados si se emplea ozono como desinfectante primario. Así, después de los THM y los HAA, el CH es el SPD que se encuentra en mayor concentración en las aguas cloradas. En aguas con elevados niveles de bromo, también se han podido identificar acetaldehídos bromados, entre los cuales se encuentra el tribomoacetaldehído, que es un SPD que se encuentra en la lista de compuestos de la EPA prioritarios para ser investigados.

Sobre el CH existe bastante literatura dado que, además de ser un SPD, también se ha utilizado como sedativo e hipnótico en pediatría (Istaphanous and Loepke, 2009) pero este tipo de exposición, a pesar de ser mayor que la del agua de consumo, es aguda y selectiva y representa menos del 20% de la exposición total al CH. Este compuesto se ha clasificado como un mutágeno de acción directa, capaz de inducir sustituciones de bases en bacterias, capaz de incrementar la frecuencia de micronúcleos en células germinales de ratón macho (Russo and Levis, 1992; Allen et al., 1994; Nutley et al., 1996), de incrementar la frecuencia de micronúcleos y de intercambios entre cromátidas hermanas en linfocitos humanos de niños tratados con CH como sedativo (Ikbal et al., 2004) y de incrementar la frecuencia de micronúcleos

en cultivos de fibroblastos humanos (Bonatti et al., 1992). Sin embargo, también se han obtenido resultados negativos en células de linfoma de ratón (Harrington-Brock et al., 1998) y en cultivos de linfocitos humanos aislados (Vian et al., 1995). Por lo que hace referencia al ensayo del cometa, Kiffe et al. (2003) trabajando con células CHO observaron una falta casi total de toxicidad y de genotoxicidad, ya que la viabilidad celular de las concentraciones evaluadas (hasta 5mg/mL, aproximadamente 30 mM) era mayor del 98%, sin inducción de daño en el DNA. Aunque nuestros resultados de viabilidad concuerdan con los descritos por Kiffe et al., ya que hemos obtenido viabilidades muy altas, no es así para la genotoxicidad, ya que en nuestro caso el CH induce daño en el DNA cuantificable en el ensayo del cometa, a partir de una concentración de 50 µM (0,05 mM).

Sobre el acetaldehído bromado TBA, nuestro trabajo (**artículo 3**) es el primero en el que se evalúa su genotoxicidad. Este SPD ha mostrado ser un compuesto altamente genotóxico, produciendo niveles de daño incluso mayores que los obtenidos en el control positivo (H_2O_2), siendo más citotóxico y genotóxico que su forma clorada (CH).

El segundo grupo de SPD en función de su prevalencia es el de los ácidos haloacéticos (HAA), los cuales pueden detectarse en aguas desinfectadas con cloro, cloraminas, dióxido de cloro y ozono, pero cuyos niveles se ven incrementados en aguas de consumo cloradas. Recientemente se ha identificado una nueva clase de HAA entre los SPD, los ácidos yodoacéticos. Estos nuevos SPD, a diferencia del resto de HAA, poseen yodo en su estructura y pueden encontrarse con una alta prevalencia en las aguas de consumo tratadas con cloraminas.

En nuestro trabajo (**artículo 5**) se han evaluado tres ácidos haloacéticos con un solo átomo de halógeno en su estructura, que constituyen los llamados ácidos monohaloacéticos (IAA, BAA, CAA). Estos tres SPD han mostrado ser citotóxicos y significativamente mutagénicos en *S. typhimurium* (Kargalioglu et al., 2002; Plewa et al., 2004b; Cemeli et al., 2006), siendo el IAA el que posee mayor potencial mutagénico y el CAA, el menor; además, estos compuestos son capaces de incrementar significativamente la frecuencia de aberraciones cromosómicas en células CHO (Hilliard et al., 1998). En los estudios que han evaluado su capacidad de inducir roturas en el DNA (ensayo del cometa) en células CHO (Plewa et al., 2002; 2004b; Richardson et al., 2008; Komaki et al., 2009), los tres monoHAA mostraron ser claramente genotóxicos, con el mismo orden de genotoxicidad que el observado en los estudios con bacterias. Si embargo, y a pesar de los resultados positivos descritos, en

nuestro trabajo ninguno de los tres HAA evaluados fue capaz de incrementar la frecuencia de micronúcleos en cultivos de células TK6. A pesar de esto, en los estudios de citotoxicidad se obtuvo una respuesta según el orden descrito previamente.

A diferencia del resto de grupos evaluados, las nitrosaminas son SPD que no contienen halógenos en su estructura. Este grupo se caracteriza por poseer actividad alquilante y por tener un grupo nitro en su estructura, pudiendo ser compuestos alifáticos o con estructuras cíclicas. Las nitrosaminas, además de generarse durante la desinfección del agua, también se producen en otros procesos, como puede ser la combustión y, por lo tanto, son contaminantes ambientales ampliamente distribuidos. Se les considera como un grupo importante de mutagénos y carcinógenos ambientales no halogenados, que requieren ser metabolizados para ejercer su potencialidad genotóxica. Una característica de las nitrosaminas es que se siguen generando durante el sistema de distribución de agua, por lo que sus niveles serían mayores en el agua que surge del grifo que los existentes en la planta de tratamiento subestimándose, por tanto, la exposición real de las personas cuando se tienen en cuenta los valores obtenidos durante el proceso de desinfección (Richardson et al., 2007).

Las nitrosaminas evaluadas en este estudio han sido la NDMA y la NDEA (**artículo 4**). La NDMA es un compuesto volátil que, además de ser un SPD, puede encontrarse en la comida y puede causar cáncer en una amplia variedad de especies animales, siendo los órganos diana el hígado, el riñón y el pulmón. En función de las evidencias epidemiológicas disponibles, la NDMA es considerada por la IARC como un cancerígeno probable en humanos. Este compuesto ha mostrado ser capaz de inducir mutaciones génicas y cromosómicas, incrementar la frecuencia de intercambios entre cromátidas hermanas, así como inducir un ligero incremento de micronúcleos en reticulocitos de médula ósea de mamíferos (Wolf et al., 2003).Además, ha demostrado que es capaz de inducir daño primario en el DNA, detectado por el ensayo del cometa, en células HepG2 y en cultivos de hepatocitos primarios (Uhl et al., 1999; Valentin-Severin et al., 2003; Arranz et al., 2007) pero sólo en concentraciones muy altas; lo que concuerda con lo obtenido por nosotros en células TK6 en presencia de fracción S9.

En relación al ensayo de micronúcleos, nuestros resultados sugieren que el NDMA no es capaz de inducir un incremento significativo en su frecuencia ni en células TK6 ni en linfocitos humanos. Hay que comentar que otros investigadores han sido capaces de obtener incrementos significativos de micronúcleos, pero en células HepG2 y con concentraciones muy altas del NDMA (Valentin-Severin et al. (2003). Esta

discrepancia en los resultados del ensayo de micronúcleos puede estar relacionada con las diferencias de actividad metabólica entre las células empleadas. Así, mientras que las células TK6 y los linfocitos humanos tienen una capacidad metabólica limitada, las células HepG2, que son de una línea celular hepática, mantienen una importante capacidad metabólica.

En relación a la otra nitrosoamina evaluada, la NDEA, se ha descrito resultados discordantes en relación a su capacidad de inducir efectos clastogénico/aneugénico (en el test de MN), pero en nuestro caso no ha sido capaz de inducir tales efectos en las células TK6. Sin embargo, fue capaz de causar incrementos significativos en los niveles de daño detectados con el ensayo del cometa, aunque sólo a las máximas concentraciones evaluadas (5 mM y 10 mM) y en presencia de la fracción microsomal S9.

Para determinar si los SPD evaluados eran capaces de inducir, además del daño genético primario (roturas), otro tipo de daño como es el daño oxidativo, se llevó a cabo una variante del ensayo del cometa, usando enzimas específicas que reconocen y cortan el DNA en sitios con bases oxidadas. De esta manera, el daño oxidativo en el DNA se hace visible en el ensayo del cometa. Una de estas enzimas de reparación es la endonucleasa III (endo III), de origen bacteriano, que actúa como una glicosilasa reconociendo las pirimidinas oxidadas en el DNA y las elimina, dejando un sito abásico. Gracias a su actividad endonucleasa estos sitios abásicos se convierten en roturas de simple hebra en el DNA. Otra enzima bacteriana es la formamidopirimidina glicosilasa (FPG), que de un modo similar reconoce las purinas oxidadas en el DNA y las convierte en roturas de simple hebra (Collins, 2009).

De acuerdo con nuestros resultados (**artículo 1, artículo 2, artículo 3, artículo 4**), todos los SPD evaluados han sido capaces de inducir daño oxidativo en el DNA. Así, un daño que había permanecido oculto en los distintos tratamientos, sólo fue posible detectarlo y cuantificarlo utilizando los tratamientos enzimáticos con endo III y FPG. De los SPD evaluados, 6 de ellos (BNM, TCNM, MCA, MBA, TBA, CH) inducen incrementos significativos de daño oxidativo tanto en purinas como en pirimidinas, mientras que los otros dos (NDMA y NDEA) sólo lo producen en purinas. Si embargo, a pesar de inducir daño oxidativo en ambos tipos de bases, tienden a tener preferencia por un tipo de base; así, mientras que BNM, TCNM y TBA producen mayores niveles de purinas oxidadas (detectadas por la enzima FPG), MCA, MBA y CH tienen preferencia por las pirimidinas (enzima endo III). Además, se observa que algunos de los SPD evaluados (BNM, TCNM, MBA, MCA, NDEA) producen elevados niveles de daño

oxidativo, llegando a ser mayores que los otros tipos de daño (aquellos detectados sin el tratamiento enzimático).

Otra ventaja del ensayo de cometa es que no tan sólo detecta los niveles de daño genético inducido, sino que permite detectar la capacidad que tiene la célula de reparar dicho daño. Esta variante del ensayo de cometa es lo que se conoce como cinética de reparación. De acuerdo con los resultados obtenidos en los experimentos de cinética de reparación (**artículo 1, artículo 2, artículo 3, artículo 4**), todos los SPD, a excepción del MCA, producían daños en el DNA genómico que eran reparados de manera total, aunque con diferentes velocidad de reparación. El MCA fue el único SPD cuyo daño no pudo ser reparado totalmente por las células, ya que sólo se reparó un 60% del daño inducido.

Los experimentos de cinética de reparación, conjuntamente con el ensayo de micronúcleos, evidencian la capacidad de los compuestos para producir daño en el DNA que es capaz de fijarse. Este daño fijado es el resultado de la falta de reparación o de una reparación incorrecta, y es el daño que tiene particular relevancia en el proceso de carcinogénesis (Bonassi et al., 2007). Si analizamos los resultados de los ensayos de micronúcleos y de la cinética de reparación, de manera conjunta, podemos observar que existe una concordancia entre ambos. Así, cuando un compuesto es incapaz de inducir incrementos significativos en el ensayo de micronúcleos, se hace evidente en los experimentos de cinética de reparación que el daño primario producido por el compuesto se repara casi en su totalidad. Por el contrario, si el SPD es capaz de inducir un incremento en la frecuencia de micronúcleos, entonces no todo el daño primario inducido en el DNA genómico (analizado en el ensayo del cometa) es reparado. Esta correlación entre los resultados del ensayo de micronúcleos y los de cinética de reparación se observan en los 11 SPD evaluados *in vitro* (**artículo 1, artículo 2, artículo 3, artículo 4, artículo 5)**, así como también en un artículo reciente de Komaki et al., 2009.

Teniendo en cuenta los resultados obtenidos en el ensayo del cometa, se puede considerar que el BNM, el TCNM, el MBA, el MCA, el TBA y el CH son SPD altamente genotóxicos, llegando algunos a inducir más daño primario en el DNA que el inducido por el control positivo utilizado. Por el contrario, la NDMA y la NDEA se pueden considerar genotóxicos débiles, pero sólo si se les evalúa en presencia de la fracción microsomal S9. Si tenemos en cuenta la máxima concentración evaluada en el ensayo del cometa, el TBA es el compuesto más citotóxico de todos, siendo los menos citotóxicos el CH, la NDMA y la NDEA. Si consideramos la concentración mínima

efectiva, para así poder determinar cuál de los SPD evaluados es el más genotóxico, entonces el TBA y el BNM son los más genotóxicos (1 µM), seguidos del TCNM (10 µM), el MBA y el MCA (50 µM), el CH (100 µM), la NDEA (5 mM) y, por último, el menos genotóxico de todos es la NDMA (10 mM). Este orden de genotoxicidad podría verse alterado si se hubieran evaluado concentraciones más bajas de MBA y MCA, dado que ambos compuestos ya dieron respuesta genotóxica en las concentraciones mas bajas evaluadas.

En cuanto a los resultados del ensayo de micronúcleos, hay que resaltar que el MCA fue el único compuesto capaz de inducir efectos clastogénicos/aneugénicos. Analizando las máximas concentraciones evaluadas y contadas, de los 11 SPD evaluados en células TK6 en tratamientos de 24 h, el TBA mostró ser el compuesto más citotóxico (1 µM), seguido del IAA (5 µM), siendo los menos citotóxicos la NDMA y la NDEA (10 mM).

Además del ensayo del cometa y del test de micronúcleos, también se ha utilizado el ensayo de linfoma de ratón (MLA) en la evaluación de los SPD. Este ensayo es capaz de cuantificar las alteraciones genéticas inducidas que afectan a la expresión del gen de la timidina quinasa *(TK)* en células linfoblastoides de ratón (L5178Y $Tk^{+/-}$ clon 3.7.2c.). Dicho ensayo está considerado como el más sensible de los ensayos de mutación génica en células de mamíferos *in vitro* (Applegate et al., 1990). (Applegate et al., 1990). El ensayo de linfoma de ratón es capaz de detectar un amplio rango de alteraciones genéticas (mutaciones puntuales, cambios cromosómicos, recombinación mitótica, aneuploidías, etc.) que pueden estar involucradas en la etiología del cáncer y de otras enfermedades humanas. La línea celular que se emplea es heterocigota para el locus autosómico (cromosoma 11) de la timidina quinasa *(TK);* presentando, además, ambos alelos del gen *Trp53* mutados, por lo que es deficiente en reparación, lo que da como resultado una mayor inestabilidad genómica y un aumento de su susceptibilidad a acumular mutaciones. Debido a la mutación en el gen *P53*, las células L5178Y no entran en apoptosis o en parada del ciclo celular como consecuencia de la exposición a los agentes genotóxicos favoreciendo, por tanto, la acumulación de mutaciones y un aumento de su supervivencia respecto a una línea con dicho gen funcional. Estas características genéticas de la cepa utilizada permiten aumentar la sensibilidad del ensayo. La cuantificación de los efectos inducidos por los agentes genotóxicos se realiza valorando los incrementos en la frecuencia de colonias mutantes $Tk^{-/-}$, pudiendo ser estas colonias pequeñas o grandes, dependiendo del tipo de daño genético producido.

En relación a los resultados obtenidos en el ensayo de linfoma de ratón (MLA) (**artículo 6**), el único compuesto capaz de incrementar la frecuencia mutagénica fue el MBA, el resto de SPD evaluados (el BNM, el TCNM, el MCA, el TBA, el CH) no dieron resultados positivos. De los 6 SPD evaluados en el MLA, sólo uno de ellos (el CH) ha sido evaluado con anterioridad en el mismo ensayo. Así, Harrington-Brock et al. (1998), al evaluar el CH en el MLA, concluyeron que este compuesto podía ser considerado como mutágeno débil, debido a que había sido capaz de incrementar la frecuencia de células mutantes, pero sólo a las concentraciones más altas. Este resultado positivo no concuerda con los obtenidos en nuestro trabajo pero, al analizar las concentraciones a las cuales el CH había inducido mutaciones, son muy altas en comparación con la máxima concentración utilizada en nuestro estudio, que fue de 1mM. Esta es la máxima concentración permisible de evaluar de acuerdo a la normativa de la ICH (2008). Al comparar nuestra concentración máxima (1 mM = 165,4 µg/mL) con la correspondiente al estudio de Harrington-Brock et al. (1998), nuestro valor es menos de la mitad de su concentración mínima evaluada (350 µg/mL), concentración que generó resultados negativos. Por lo tanto, los resultados negativos por nosotros concordarían con los resultados previamente descritos por Harrington-Brock et al. Respecto a sus resultados positivos, las directrices actuales nos hacen ser muy cautos a la hora de considerar como positivos aquellos resultados obtenidos con concentraciones muy citotóxicas.

En nuestro estudio, el MBA ha sido el único compuesto capaz de inducir efectos genotóxicos cuantificados por el ensayo de linfoma de ratón. Este compuesto pertenece a la familia de las hidroxifuranonas, del cual también forma parte el mutágeno X. Al evaluar el MBA en el ensayo del cometa, todas las concentraciones utilizadas (50 – 2.000 µM) causaron niveles significativos de daño genotóxico, siendo más potente que el control positivo (H_2O_2) realizado concurrentemente; pero, a pesar de estos resultados claramente positivos, no fue capaz de incrementar la frecuencia de micronúcleos ni en las células TK6 ni en los cultivos de linfocitos humanos. Sin embargo, de acuerdo con los resultados del ensayo de linfoma de ratón, el MBA es un compuesto mutagénico capaz de incrementar la frecuencia de colonias mutantes, tanto pequeñas como grandes, pero produciendo una mayor proporción de colonias pequeñas, lo que indicaría que la naturaleza de las mutaciones detectadas en el MLA es de tipo cromosómico (alteraciones numéricas o estructurales) y no mutaciones puntuales, efecto que se observa como colonias grandes. Hay que considerar que solamente la máxima concentración evaluada (10 µM) fue capaz de producir resultados

positivos, cuyo valor de % RTG se encuentra muy cercano a los valores mínimos permitidos (%RTG = 20%) (ICH, 2008), pudiendo ser que parte de la respuesta mutagénica esté asociada a la citotoxicidad. Aunque también se evaluó también una concentración mayor (12 µM) de MBA, que indujo una frecuencia de mutaciones superior a la obtenida con 10 µM, debido a que su %RTG no se consideró relevante ya que lo más probable es que el aumento de la frecuencia de mutación sea consecuencia de la citotoxicidad. Hay que destacar que el valor de %RTG = 20% está establecido como nivel máximo de citotoxicidad, para evitar el exceso de falsos positivos o resultados positivos no relevantes biológicamente; por lo tanto, si la respuesta es positiva con %RTG $<$ de 20%, el agente es considerado como no mutagénico.

Al igual que se observó en los estudios anteriores (ensayos de MN y del cometa) en relación a la citotoxicidad de los SDP, en el ensayo MLA también se ha observado que los compuestos bromados son más citotóxicos que los clorados, a excepción de las hidroxifuranonas.

La falta de concordancia entre los resultados obtenidos con el ensayo del cometa, el test de micronúcleos y el ensayo de linfoma de ratón se debe a que estos tres ensayos miden diferentes tipos de daño. Así, el ensayo del cometa detecta el daño primario en el DNA, y que se produce rápidamente tras la exposición al compuesto, mientras que en el ensayo de micronúcleos, al tratarse de un ensayo que dura más tiempo (48 h *vs.* 3 h del cometa), el daño reflejado por los micronúcleos es aquel que no logra ser reparado y se fija (daño irreversible), y en el ensayo de linfoma de ratón (4 h de tratamiento) el daño genotóxico se detecta tanto como mutaciones puntuales como aberraciones cromosómicas estructurales y numéricas. La falta de concordancia entre los resultados del ensayo de micronúcleos y los del ensayo del cometa también se ha observado en otros estudios (Van Goethem et al., 1997; Maffei et al., 2005; Marabini et al., 2007), al igual que la falta de concordancia entre los ensayos del cometa, de micronúcleos y linfoma de ratón (Koyama et al., 2006; Michael McClain et al., 2006; Ogura et al., 2008).

Al realizar comparaciones entre los miembros de cada grupo químico, se observa que los compuestos bromados son más citotóxicos y genotóxicos que los clorados; asimismo, los compuestos yodados son más citotóxicos que los bromados. Estas diferencias en la capacidad tóxica, mutagénica y/o carcinogénica que se observan entre compuestos que se diferencian por tener yodo, bromo o cloro en su estructura química han sido halladas reiteradamente en otros estudios (Roldan-Arjona and Pueyo, 1993; LaLonde and Leo, 1994; Giller et al., 1997; LaLonde et al., 1997;

Muller-Pillet et al., 2000; Kargalioglu et al., 2002; Plewa et al., 2002; Myllykangas et al., 2003; Kundu et al., 2004a; Kundu et al., 2004b; Plewa et al., 2004a; Richardson et al., 2008; Komaki et al., 2009), que ponen de manifiesto un mayor grado de inducción de daño por parte de las estructuras bromadas, y mayor aún por las yodadas, sea cual esa el grupo químico analizado.

LaLonde y Leo (1994), al evaluar el efecto de la sustitución de cloro por bromo sobre la capacidad mutagénica de las halofuranonas mediante el test de Ames (*S. typhimurium,* TA100), observaron que cuando el halógeno presente es el bromo (MBA), el compuesto es 1,9 veces más mutagénico que si el compuesto tuviera un cloro (MCA) y estas diferencias en la mutagenicidad se hacen aún mayores si en el carbono 5 del compuesto en lugar de un hidroxilo se encuentra un hidrógeno, logrando que la relación de mutagenicidad del compuesto bromado con el clorado sea de 11.

Otro estudio de un grupo de SPD sobre el papel de los halógenos, fue el realizado por Kundu et al., (2004a; b). En este caso, los compuestos escogidos fueron los HNM, que habían sido recientemente reconocidos como SPD. Los nueve compuestos que forman parte de los HNM se evaluaron en diferentes cepas de *S. typhimurium* (Kundu et al., 2004b), pero sólo en la cepa TA100 se evaluaron de manera simultánea con los halometanos (Kundu et al., 2004a). Los ensayos se realizaron en ausencia y presencia de fracción microsomal S9 y los resultados muestran que algunos HNM fueron capaces de inducir una respuesta mutagénica positiva, algunos en ausencia y otros en presencia de la fracción S9. Ambos trabajos concluyen que los HNM bromados son más citotóxicos y mutagénicos que los clorados y que los compuestos dihalogenados que contienen bromo son los más potentes del grupo.

De forma natural, las fuentes hídricas usadas para la obtención de agua potable pueden contener bromo, dando lugar a SPD tanto clorados como bromados tras el proceso de desinfección y, a medida que se incrementa la concentración de bromo en las fuentes hídricas, se producen mayores cantidades de subproductos bromados o clorobromados. Este aumento de la cantidad de bromo se da normalmente en localidades costeras en las cuales ocurre una filtración de agua salada en las aguas superficiales o subterráneas. Esto también puede suceder en lugares del interior, en las que las fuentes hídricas se pueden ver contaminadas por depósitos naturales de sales de océanos antiguos o salmueras de yacimientos petrolíferos. Teniendo en cuenta la mayor capacidad mutagénica de los compuestos bromados, los habitantes de estas

zonas se verían expuestos a mayores concentraciones de estos compuestos incrementando su riesgo genotóxico, en comparación a otras zonas.

Además de los estudios *in vitro* (micronúcleos, cometa y linfoma de ratón) realizados para evaluar el potencial genotóxico de los SPD específicos seleccionados, también hemos realizado un estudio con diferentes muestras de agua de piscinas, las cuales habían sido sometidas a distintos procesos de desinfección o presentaban características distintas (niveles de TOC, temperatura, nivel de cloro residual, etc).En los últimos años se ha llevado a cabo diversos estudios con mezclas complejas de SPD, provenientes de piscinas o de las fuentes de agua de consumo para evaluar su potencial genotóxico/mutagénico. Estos estudios suelen emplear muestras de extractos de aguas, donde se encuentran concentrados los SPD de varios litros de agua. Los resultados obtenidos en los diferentes estudios son variados. Así, Albaladejo Vicente et al. (1995) y Granados et al. (2004), al evaluar concentrados de muestras de agua del sistema de distribución de Madrid empleando el test de reversión en *S. typhymurium* y en *E. coli*, respectivamente, obtuvieron resultados negativos de mutación génica empleando varias cepas bacterianas y en ausencia/presencia de fracción microsomal S9.

Sin embargo, en otros estudios llevados a cabo en Italia se obtuvieron resultados diferentes en el análisis de extractos de aguas de consumo tratadas con diferentes desinfectantes, entre ellos el NaClO, resultando que tanto la muestra de la fuente natural como las desinfectadas dieron respuestas positivas pero sólo en ausencia de S9 (Monarca et al., 1998). En otro estudio más reciente, en el cual se empleó una batería de ensayos *in vitro,* se observó que las muestras de aguas desinfectadas con NaClO (desinfectante más usado) y con desinfectantes alternativos (ClO_2 y ácido peracético) mostraron actividad mutagénica en varios de los ensayos utilizados (Mutatox en *Vibrio fischeri*, SOS cromotest en *E. coli*, ensayos de conversión génica, mutación puntual y mutabilidad del DNA mitocondrial en *S. cerevisiae*), pero los resultados fueron negativos en el test de Ames (Guzzella et al., 2004).

Otros estudios con muestras de agua empleando el ensayo de micronúcleos con células HepG2 (Buschini et al., 2004) y con linfocitos humanos aislados (Maffei et al., 2005), no evidenciaron incrementos de daño genotóxico. Sin embargo, utilizando el ensayo del cometa, Buschini et al. (2004) encontraron que se producían incrementos significativos en el daño en el DNA, tanto en linfocitos humanos como en células HepG2. Por otra parte, Maffei et al. (2005) al evaluar extractos de agua obtenidos en diferentes meses para poder observar el efecto de la variación estacional en la

capacidad genotóxica del agua, empleando linfocitos humanos y el ensayo del cometa, observaron que se producían incrementos en el daño del DNA con todos los desinfectados usados (NaClO, ClO_2, ácido peracético), observándose diferencias entre los diferentes meses de muestreo debido a la variación estacional en la composición del agua a desinfectar.

Otro estudio ha analizado la capacidad genotóxica de muestras de agua en diferentes momentos de su ciclo de producción/distribución: antes de ser distribuida y en dos puntos situados a distancias diferentes del sistema de distribución. Los resultados muestran la presencia de compuestos mutagénicos en todas las muestras de agua desinfectada, lo que indicaría que el proceso de desinfección en las plantas de tratamiento provoca la formación de sustancias mutagénicas y que estas permanecen en la red de distribución hasta en puntos muy distantes del sitio de tratamiento (Guzzella et al., 2006). Otros estudios fueron realizados por el mismo grupo (Marabini et al., 2007; Maffei et al., 2009), también analizaron muestras de agua de la fuente natural (sin desinfectar) en dos plantas de tratamiento, empleando para dicha evaluación el ensayo de micronúcleos y el ensayo del cometa en cultivos de linfocitos humanos y en células HepG2. Los resultados obtenidos indican que las muestras antes de ser desinfectadas son más citotóxicas que las muestras desinfectadas, pero estas últimas son más genotóxicas (ensayo del cometa) en los cultivos de linfocitos humanos. Sin embargo, en las células HepG2 sólo las muestras desinfectadas de una planta de tratamiento indujeron daño genotóxico. La comparación de estos resultados sugiere que las diferencias observadas son debidas a que la mayor parte de los efectos genotóxicos son inducidos por compuestos de acción directa, los cuales son destoxificados al ser evaluados en las células HepG2. En relación al ensayo de micronúcleos, ninguna de las muestras de agua fue capaz de inducir incrementos significativos en su frecuencia, ni en los cultivos de linfocitos ni en células HepG2.

Cuando se realizan estudios sobre la evaluación del potencial genotóxico del agua de consumo, estos suelen hacerse con muestras de extractos de aguas desinfectadas en diferentes plantas de tratamientos y del sistema de distribución; es decir, agua cuyo uso está relacionado con el consumo en el hogar. Son pocos los estudios que han evaluado la genotoxicidad de muestras de agua de uso no doméstico, como es el agua de piscina. Para contribuir a llenar esta laguna, en uno de nuestros estudios (**artículo 5**) se han evaluado varias muestras de extracto orgánico de aguas procedentes de diferentes piscinas que tenían una misma fuente de agua potable, la cual también fue evaluada. El análisis del potencial genotóxico de estas muestras fue

realizado utilizando el ensayo del cometa en su versión multipocillo, empleando células de ovario de hámster chino (CHO). Debido a que las muestras de agua procedían de diferentes piscinas, con parámetros químicos similares o diferentes, fue posible realizar comparaciones entre ellas y, así, determinar el efecto de dichos parámetros en la genotoxicidad. Los resultados muestran que todas las muestras de agua de piscina, sin importar el desinfectante empleado o el resto de parámetros químicos, fueron capaces de inducir daño en el DNA genómico, siendo significativamente más genotóxicas que las muestras de agua original de las que se abastecen las distintas piscinas. Esta clara diferencia se debería a la presencia de una mayor cantidad de compuestos genotóxicos en el agua de piscina, los cuales se originarían por diversos factores como el tiempo de contacto con el cloro y la presencia de precursores de los SPD, entre otros.

A pesar de que el número de muestras de agua de piscina con el mismo tipo de desinfectante o parámetros químicos ha sido reducido, lo cual no permite establecer asociaciones estadísticamente significativas, hemos podido apreciar el efecto de algunos factores sobre su potencialidad genotóxica. Así, el tiempo de contacto con el cloro (1 semana en el agua de consumo *vs.* meses en el agua de piscina) es un factor que incrementa de manera significativa la capacidad genotóxica cuando se comparan muestras con niveles de cloro similares. Este factor ya ha sido postulado por distintos autores, demostrando que la persistencia del cloro y el mayor tiempo de contacto favorece la formación de determinados SPD en el agua (Uden and Miller, 1983; Johnson and Jensen, 1986; Singer, 1994; Pourmoghaddas and Stevens, 1995; Yang and Shang, 2004). Un segundo factor que incrementa la genotoxicidad de las muestras de agua de piscina es la presencia de precursores externos como son los restos de piel, cabello, sudor y los productos cosméticos de los bañistas, que favorecen la generación de nuevos SPD en el agua de piscina. Por lo que respecta al tipo de desinfectante aplicado para mantener en condiciones óptimas el agua de piscina, la muestra procedente de una piscina que empleaba BDCMH como desinfectante fue la más genotóxica. Este desinfectante reacciona con la materia orgánica generando ácido hipobromoso (HOBr), lo que favorece la formación de SPD bromados, que serían los responsables de la elevada genotoxicidad de estas muestras de agua.

Los resultados positivos de genotoxicidad observados en nuestro estudio con muestras de agua de piscina concuerdan con los de otro estudio en el que se evaluó este tipo de agua mediante el ensayo del cometa empleando células HepG2 (Glauner et al., 2005). En este caso, el agua de piscina fue fraccionada en tres partes (< 0,2 KDa, 0,2 - 1 KDa, > 1 KDa); siendo la fracción de menor peso molecular (< 0,2 KDa) y

la intermedia las que indujeron incrementos significativos en los niveles de daño genómico, presentando mayor genotoxicidad la fracción de menor peso molecular.

Además de la evaluación *in vitro* de diferentes muestras de agua de piscinas, también hemos realizado un pequeño estudio de biomonitorización con un grupo de personas expuestas a los SPD del agua clorada de piscina. La finalidad de dicho estudio fue determinar si dicha exposición era capaz de incrementar los niveles basales de daño genético, medidos antes de la actividad en la piscina.

Como ya se ha comentado, la formación de SPD en el agua de piscina se ve favorecida por tres factores principales: a) la recirculación del agua, b) la cloración continua y por último, c) el material orgánico procedente de los bañistas. Estos factores permiten presuponer que el agua clorada de las piscinas contenga mayores concentraciones de SPD que el agua de consumo. Se ha comprobado que las cremas de protección solar, la piel, el cabello, el sudor de los bañistas y otros factores de menor relevancia contribuyen a incrementar los niveles de TOC del agua de piscina (Kim et al., 2002); y, según Chu y Nieuwenhuijsen (2002), las concentraciones de TOC y de THM se correlacionan directamente con el número de bañistas.

Al igual que sucede con el agua de consumo, los trihalometanos son los SPD predominantes en el agua de piscina y, al ser compuestos con una alta volatilidad, en especial el cloroformo, pueden encontrarse en concentraciones importantes en el aire del ambiente de la piscina. Cuando se evalúa la exposición a los SPD, las vías de exposición dérmica e inhalación usualmente no son tenidas en cuenta; sin embargo, constituyen las rutas más importantes en la exposición humana a los SPD volátiles en la piscina, siendo la ingestión (ingestión accidental del agua) una vía de menor relevancia (Whitaker et al., 2003; Lee et al., 2009). Backer et al. (2000) determinaron las concentraciones de THM en sangre de voluntarios que previamente habían sido expuestos a SPD mediante tres vías alternativas: a) 10 min de ducha, b) 10 min de baño y c) beber 1 litro de agua en 10 min. Los resultados obtenidos indicaron que las concentraciones de THM en la sangre de las personas expuestas a 10 min de ducha o de baño eran significativamente mayores que las encontradas en las personas que había bebido 1 litro de agua. Esto se debe a que la mayor parte de la dosis ingerida por vía oral es excretada o metabolizada antes de pasar a la sangre. Otro estudio en el que se evaluó la exposición a cloroformo en mujeres embarazadas expuestas a situaciones similares (beber agua potable, ducharse, bañarse y nadar) concluyó también que la absorción de cloroformo era mayor al nadar que al ducharse y al

bañarse, siendo la ingestión la vía que suponía una menor exposición (Whitaker et al., 2003).

A pesar de que la exposición a los SPD del agua de piscina no sea crónica ni de tanta duración como la exposición a los SPD del agua de consumo, este tipo de exposición debe de tenerse en cuenta debido a los altos niveles de SPD en dicho tipo de exposición. La cantidad de personas que se encuentran expuestas a los SPD del agua clorada de piscina varía de acuerdo con la época del año, siendo los meses de calor (verano) donde se hace más uso de las piscinas. Si tenemos en cuenta que la temperatura y la luz solar son factores que favorecen la formación de SPD, entonces los niveles de exposición serían mayores en esta época del año. Además de lo comentado, existe un grupo de personas cuya exposición es a lo largo de todo el año, ya que van a la piscina tanto en los meses de calor como en los de frío, utilizando piscinas descubiertas y cubiertas, respectivamente. Este grupo está formado por los nadadores (aficionados y de élite), cuya exposición sería crónica durante los años de entrenamiento y podría representar cierto riesgo.

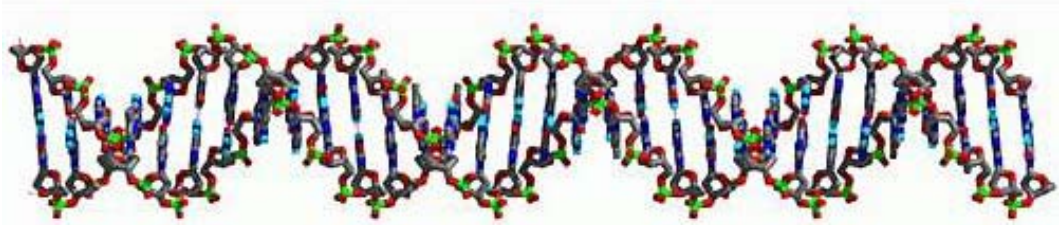
Caro y Gallego (2007) analizaron la exposición a THM en piscinas cubiertas, tanto en nadadores como en trabajadores de la piscina, encontrando que la absorción de THM en los nadadores después de nadar 1 h era mayor que la de los trabajadores después de 4 h de trabajo. Esta diferencia entres ambos grupos se debe a que, mientras que la única ruta de exposición de los trabajadores es la inhalación, en los nadadores la absorción dérmica y una posible ingestión accidental del agua, también contribuyen a la exposición. Hay que señalar que el estrés físico (intensidad del ejercicio) incrementa la ventilación pulmonar y, por tanto, la tasa de inhalación, por lo cual aumenta la absorción de THM se ve incrementada.

En nuestro trabajo hemos evaluado los niveles de exposición en muestras de aire exhalado y hemos cuantificado los efectos genotóxicos en muestras de sangre y de orina de las personas expuestas (**artículo 8**). El daño genético se evaluó mediante los ensayos del cometa y de micronúcleos en muestras de sangre, mientras que en muestras de orina se llevo a cabo el ensayo de micronúcleos. Hay que resaltar que este es el primer estudio realizado sobre la genotoxicidad de la exposición a los SDP en nadadores de piscina. Los resultados indican que la exposición al agua clorada de la piscina durante 40 min no es capaz de producir ningún incremento en la frecuencia de micronúcleos, ni en linfocitos, ni en células uroteliales, ni en el nivel de mutagenicidad de la orina; pero en cambio se observó una disminución en el daño detectado mediante el ensayo del cometa. Sin embargo, al analizar la frecuencia de micronúcleos

en relación a las concentraciones de THM en el aire exhalado, se observó un incremento estadísticamente significativo en la frecuencia de micronúcleos para los niveles del BDCM y BF. La misma situación se encontró para los valores de mutagenicidad de la orina, pero sólo con los niveles de BF en aire exhalado. No se encontró ninguna asociación entre los niveles de los diferentes THM en aire exhalado y los niveles de daño en el DNA, cuantificados mediante el ensayo del cometa o con la frecuencia de micronúcleos en células uroteliales. Al evaluar el papel de determinados polimorfismos de genes relacionados con la metabolización de los SPD (*GSTZ1, GSTT2, CYP2E1, APEX1*), se encontraron posibles asociaciones con las respuestas en los biomarcadores de genotoxicidad (MN en sangre y orina); sin embargo, la muestra estudiada fue demasiado pequeña para poder extraer conclusiones consistentes. Al ser éste el primer estudio realizado para determinar el efecto genotóxico de la exposición al agua de piscinas en nadadores, tampoco existen datos previos con los cuales comparar; pero, teniendo en cuenta que el número de personas incluidas en el estudio es relativamente pequeño, se necesitaría ampliarlo para poder extraer conclusiones definitivas sobre el posible riesgo genotóxico asociado con la exposición a SPD en piscinas.

Teniendo en cuenta nuestros resultados de genotoxicidad, tanto *in vitro* como *in vivo,* y los encontrados en la literatura relativos a los SPD y al agua de consumo, consideramos que hay evidencias suficientes que demuestran que la desinfección del agua destinada al consumo humano supone la generación de subproductos con potencialidad genotóxica. Entre estos subproductos podemos encontrar compuestos capaces de inducir diferentes tipos de daño en el DNA, desde daño primario que se produce rápidamente tras la exposición, a daños de tipo cromosómico (aneugénico y/o clastogénico) fijado en la célula, pasando por mutaciones puntuales. Por lo que respecta al agua de piscina, la desinfección extra a la que es sometida, al igual que la presencia de nuevos precursores y el tiempo más prologando de contacto con los desinfectantes provoca que su genotoxicidad sea mayor a la del agua de consumo. Por lo tanto, debido al riesgo potencial que para la salud humana suponen estos SPD, en base a los resultados obtenidos en los diversos estudios realizados (ensayo del cometa, ensayo de MN, ensayo de MLA, evaluación de la exposición al agua de piscina *in vitro* e *in vivo)*, es necesario llevar a cabo más estudios para esclarecer aún más sus efectos, que pueden verse incrementados en aquellas zonas con niveles altos de SPD en el agua y con niveles de subproductos bromados.

CONCLUSIONES



VI. CONCLUSIONES

Tomando en consideración los objetivos propuestos y los resultados presentados en los diferentes artículos que forman parte de este trabajo de Tesis Doctoral, se pueden formular las siguientes conclusiones:

1. Todos los SPD seleccionados, al ser evaluados con el ensayo del cometa *in vitro*, han evidenciado una importante capacidad de inducir daño en el DNA, por lo que se les puede considerar como agentes genotóxicos. Seis de los SDP evaluados pueden ser considerados como compuestos fuertemente genotóxicos, mientras que las dos nitrosaminas evaluadas (NDMA y NDEA) sólo se pueden considerar como débilmente genotóxicas.

2. El ácido mucoclórico (MCA) es el único compuesto, de los once evaluados en el ensayo de MN, que ha mostrado poseer acción clastogénica y/o aneugénica al lograr incrementar de manera significativa la frecuencia de micronúcleos en células binucleadas.

3. Sólo el ácido mucobrómico (MBA), entre los seis SPD evaluados en el ensayo de linfoma de ratón, ha producido efectos genotóxicos. El incremento de la frecuencia de colonias pequeñas respecto a las grandes sugiere que el efecto producido es de tipo cromosómico. Sin embargo, dados los valores de %RTG a los que se obtiene dicha respuesta, es posible que parte de este efecto mutagénico sea consecuencia de la citotoxicidad inducida.

4. Las células TK6 son capaces de reparar de manera eficiente el daño genético inducido por los SPD evaluados, con la excepción de las lesiones producidas por el MCA, cuyo daño no llega a ser reparado totalmente. Este daño puede llegar a fijarse, evidenciándose como micronúcleos.

5. Los post-tratamientos con las enzimas endonucleasa III y formamidopirimidina han permitido evidenciar que todos los SPD evaluados tienen la capacidad de inducir daño oxidativo en el DNA. Aunque la mayoría de los SPD inducen daño oxidativo, tanto en purinas como en pirimidinas, algunos SPD muestran preferencia por algún tipo de base.

6. Se ha corroborado que el proceso de desinfección adicional al que se encuentra sometida el agua de piscina incrementa significativamente su potencial genotóxico. Éste puede verse aumentado debido a diversos factores como los niveles de TOC y el tiempo de contacto con el cloro, factores que favorecen la formación adicional de SPD en el agua de piscina.

7. El estudio relativo a la exposición *in vivo* al agua clorada de piscina evidenció una posible asociación entre los niveles de BDCM y de BF en el aire exhalado y los incrementos en la frecuencia de micronúcleos en linfocitos de sangre periférica.

8. La evaluación de distintos grupos de SPD con diversas estructuras químicas ha permitido confirmar que los compuestos bromados son más citotóxicos y genotóxicos que sus homólogos clorados, y que los compuestos yodados son los que presentan una mayor citotoxicidad. Por lo tanto, el orden de riesgo genotóxico sería el siguiente: compuestos yodados > bromados >> clorados.

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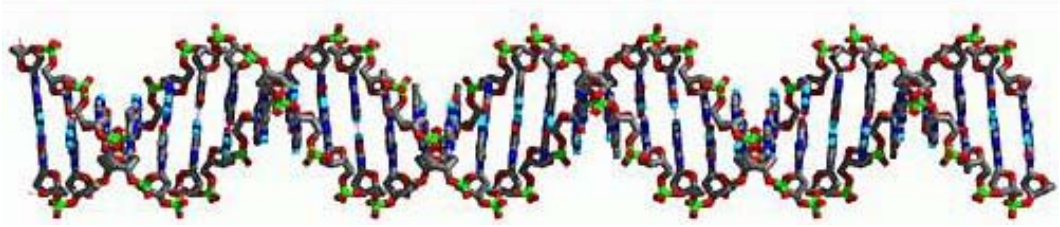

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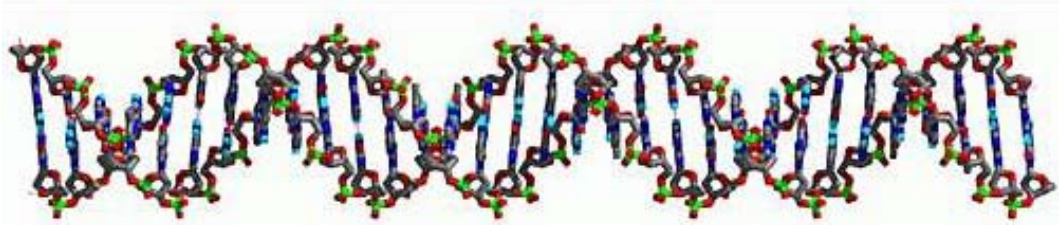
ANEXOS



VIII. ANEXOS

ANEXO 1

Genotoxic evaluation of non-halogenated disinfection by-products nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA)

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Journal of Hazardous Materials (enviado)

Genotoxic evaluation of the non-halogenated disinfection by-products nitrosodimethylamine and nitrosodiethylamine

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ABSTRACT

Disinfection by-products (DBPs) are chemicals that are produced as a result of chlorine being added to water for disinfection. As well as the halogenated DBPs, N-nitrosamines have recently been identified as DBPs, especially when amines and ammonia ions are present in raw water. In this work, the genotoxicity of two nitrosamines, namely nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA), has been studied in cultured human cells. To evaluate their genotoxic potential two assays were used, the comet assay and the micronucleus test. The comet assay measures the induction of single and double-strand breaks, and also reveals the induced oxidative DNA damage by using endoIII and FPG enzymes. Chromosomal damage was evaluated by means of the cytokinesis-blocked micronucleus test. The results of the comet assay show that both compounds are slightly genotoxic but only at high concentrations, NDEA being more effective than NDMA. Enzyme treatments revealed that only NDEA was able to produce increased levels of oxidized bases, mainly in purine sites. The results obtained in the micronucleus assay, which measures the capacity of the tested agents to induce clastogenic and/or aneugenic effects, are negative for both of the nitrosamines evaluated, either using TK6 cells or human peripheral blood lymphocytes.

Keywords: Disinfection by-products; Nitrosamines; Genotoxicity; Comet assay; Micronucleus test.

1. Introduction

A significant development relating human health has been the discovery that disinfecting water drastically decreased human deaths caused by water-borne diseases. According to Freese and Nozaic [1], chlorine disinfection of water has been credited with saving a vast number of lives worldwide on a daily basis. However, besides eliminating water pathogens, the chlorination process also leads to the formation of several compounds with genotoxic potential. These chemicals, known as disinfection by-products (DBPs), originate from the reaction of chlorine with organic or inorganic natural matter present in raw water. An important aspect to take into account is that each water treatment plant has its unique profile of DBPs, depending on factors such as the quality of raw water, the type and amount of disinfectants applied the use of secondary disinfectants, and the pH and temperature conditions, among others [2-6].

In 1974, Rook [7] described trihalomethanes (THMs) as the first class of DBPs identified in treated water, and Bellar et al. [8] determined the increasing levels of chloroform, the most frequent THM present in disinfected water. Regarding DBPs, it is of considerable concern that although more than 600 DBPs have already been reported [9], only a small number have in fact been quantified or evaluated for their genotoxic or mutagenic potential, and for their possible adverse health effects.

One class of non-halogenated DBPs is made up of nitrosamines, which have been recently identified as by-products in chlorinated drinking water [10, 11], as well as in chlorinated pools [12]. N-nitrosamines are alkylating agents characterized by the presence of the *N*-nitroso group and may be aliphatic or ring structures. They are considered an important class of environmental non-halogenated mutagens and carcinogens and, according to the weight-of-evidence characterization, they have been included in the group B2 by the U.S.EPA [13] (http://www.epa.gov/iris/subst/0045.htm). This means that, on the basis of the induction of tumors at different sites in both rodents and non-rodent mammals exposed to nitrosamines by various routes, these compounds are probably carcinogenic in humans. One important feature of nitrosamines is that their carcinogenic properties decrease as the length of the aliphatic chain increases. The single exception is nitrosodiethylamine (NDEA), which has a higher carcinogenic potency than nitrosodimethylamine (NDMA) [14]. It must be pointed out that, while these compounds were only recently identified as by-products in chlorinated drinking water, considerable toxicological research on nitrosamines over 30 years has yielded important discoveries regarding carcinogenesis. Currently, several jurisdictions have implemented regulations that require widespread measurements of NDMA and NDA in both raw and drinking water.

NDMA is formed in the chlorination process when raw water contains dimethylamine and other secondary amines, and its concentration is closely related to the ratio of chlorine, ammonia ions and dimethylamine [10, 15]. NDMA and other nitrosamines can be continuously formed in the water distribution systems and, in this way, higher levels can be detected in the distribution system than in the treatment plant. Thus, nitrosamine measures at the treatment plant may underestimate the actual human exposure [16]. According to the U.S. EPA, the maximum admissible levels of NDMA and NDEA in water were established at very low concentrations as 7 and 2 ng/L, respectively.

In this context, the aim of the present work is to provide more information on the genotoxic activity of NDMA and NDEA, which can be considered as emerging contaminants, in cultured human cells. To reach this objective, two different genotoxicity assays have been used. One is the single-cell gel electrophoresis (SCGE) assay or comet assay, which is a rapid, relatively simple and sensitive technique for measuring primary DNA damage [17]. Besides detecting single and double DNA strand breaks, this technique can also detect incomplete excision repair and alkali-labile sites. It must be emphasized that due to its high sensitivity it is able to detect DNA damage in frequencies of less than 1 damaged base in 10^7 bases [18].

In addition, the comet assay also allows the analysis of oxidative DNA damage, when the bacterial repair enzymes formamidopyrimidine DNA glycosilase (FPG) and endonuclease III (endoIII) are used. FPG and endoIII detect and break the DNA at sites with oxidized forms of purines and pyrimidines, respectively [19].

The other assay used is the micronucleus (MN) test. This assay enables other kinds of damage to be detected, such as chromosome breaks and aneuploidy [20, 21], both events being of great relevance in carcinogenesis. In addition, as has been demonstrated in a recent study, the MN assay can be used as a surrogate biomarker of cancer risk [22].

The application of the two selected experimental approaches enables us to report new information on the DNA damage induced in human cells by the two nitrosamines tested (NDMA and NDEA).

2. Materials and Methods

2.1. TK6 and lymphocyte cultures

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. Lymphocyte cultures were set up by adding 0.5 mL of heparinised whole blood in 4.5 mL RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. Cultures were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 in air.

2.2 Chemicals

Nitrosodimethylamine (NDMA, $(CH_3)_2NNO$, CAS 62-75-9) and nitrosodiethylamine (NDEA, $(C_2H_5)_2NNO$, CAS 55-18-5) were obtained from Sigma-Aldrich (St Louis, MO, USA). Endonuclease III (endoIII) and formamidopyrimidine glycosylase (FPG) enzymes were kindly provided by Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway).

2.3. S9 fraction

The metabolic activation fraction used was S9 from male Sprague-Dawley rats induced with Aracolor 1254 (ICN Biomedicals Inc., Aurora, OH, USA). The freshly prepared S9 mix was prepared according to Pérez-Machado et al. [23].

2.4. Alkaline comet assay

TK6 cell cultures were centrifuged at 500 g for 2 min and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 mL). Each disinfection by-product was dissolved in distilled water and 5 concentrations of each DBP were evaluated with and without S9 mix, simultaneously. Aliquots of 10 µL of H_2O_2 (positive control without S9 mix) or benzo(α)pyrene (B(α)P, positive control with S9 mix) or H_2O (negative control), were added to the cultures for 3 h at 37 ºC. An aliquot of 100 µL of S9 mix was added at the beginning in those cultures with metabolic activation. Cell viability was evaluated immediately after treatment, with a mixture of fluorescein diacetate (FDA) and ethidium bromide (EtBr) [24]. Two hundred cells were scored for viability in each treatment; and those concentrations inducing more than 70% of viable cells were used to carry out the comet assay [25]. The assay was performed as previously described by Singh et al. [26] with minor modifications. Approximately 40,000 cells in 20 µL were carefully resuspended in 75 µL of 0.5% low-melting-point agarose (LMA), layered onto microscope slides pre-coated with 150 µL of 0.5% normal- melting-point agarose (NMA) (dried at 65 ºC), and covered with a coverslip and kept at 4 ºC until solidification. Then, coverslips were removed and cells were lysed for 2 h at 4 ºC in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly cold electrophoresis solution.

Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. After electrophoresis, slides were neutralized with two 5-min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, the slides were stained with 60 µL of EtBr (0.4 µg/mL). The images were examined at 400X magnification with a Komet 5.5 image analysis system (Kinetic Imaging Ltd, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope, equipped with a 480-550 nm wide band excitation filter and a 590 nm barrier filter. Two different experiments were carried out per compound, each experiment with two different replicates. One hundred randomly selected cells (50 from each of the two replicate slides) were analyzed per sample. The percentage of DNA in the tail was used to measure DNA damage and data were computed using the Komet version 5.5 software.

2.5. Detection of induced oxidative damage

To determine the level of oxidized bases induced by the treatments, one concentration (10 mM) per each DBP was evaluated. After cell lysis, slides were washed three times (5 min, 4 ºC) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0). Then, aliquots of 100 µL of buffer, containing the bacterial enzymes endoIII or FPG (enzyme concentration 1/1,000) or no enzyme (control) were dropped onto the agarose and incubated for 30 min at 37 ºC. After enzyme treatments, cell samples were processed as in the standard version of the alkaline comet assay. Two different experiments were performed with two replicates for each. One hundred cells were scored per experiment (50 per replicate).

2.6. Micronucleus test

Three milliliters of a TK6 culture (500,000 cells/mL) were set up in complete medium. Aliquots of 30 µL of each DBP concentration were added to the cultures. All treatments were done at the beginning of the incubation, and cultures were kept for 48 h at 37 ºC in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 h after initiation, 50 µL of each DBP concentration was added to 5 mL culture. Mitomycin C (MMC) (0.2 µM for lymphocyte cultures and 0.3 µM for TK6 cell cultures) and distilled water were used as positive and negative controls, respectively. For each DBP concentration and control, two independent cultures were prepared, and for each culture two slides were prepared.

The cytokinesis-blocked micronucleus (CBMN) test was conducted using the standard technique proposed by Fenech [27]. Cytochalasin B (Cyt-B), at a final concentration of 6 µg/mL, was added to each culture (at time 0 and 44 h after PHA stimulation, for the TK6 and lymphocyte cultures, respectively). After incubation, cells were harvested. Cultures were centrifuged at 150 g for 8 min; then, the supernatant was removed and the cells were subjected to hypotonic treatment (5 mL KCl 0.075 M, 4 ºC 7 min), after which another centrifugation was carried out. Cells were fixed with methanol/acetic acid (3:1 vol.) at least 3 times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped onto clean microscope slides (two drops of 20 µL each one). After drying, cells were stained with a Giemsa solution for 7 min.

All slides were coded before scoring, which was carried out by the same person using a Leitz-Leica light microscope at 1,000X magnification, under oil immersion. The criterion for scoring MN was that described by Kirsch-Volders et al. [28]. One thousand binucleated cells were scored and classified, according to the number of MN, to calculate the induction of MN. In addition, cells were scored to determine the cytokinesis-block proliferation index (CBPI), according to the formula proposed by Surrallés et al. [29]. For each culture, one or more slides were scored for the induction of MN and CBPI and the values presented correspond to the pooled data from the two cultures.

2.7. Statistical analysis

The slide was used as the experimental unit. The percentage of DNA in the tail (% DNA tail) was the parameter assessed in the comet assay. The mean % DNA tail for each slide was determined and the data were compared by means of the one-way analysis of variance (ANOVA). Independent tests using Dunnett's correction for multiple test adjustment were performed to compare each level of concentration to the negative control when the overall *F*-test was significant. A result was considered statistically significant at $p < 0.05$. For the MN test, data for the binucleated cells with MN were compared for each treatment using the one-tailed Fisher's exact test. The Chi-square test was used for the analysis of CBPI between treatments.

3. Results

According to the FDA/EtBr viability assay (Fig. 1), neither of the nitrosamines was cytotoxic at all, and the highest concentration recommended (10 mM) for *in vitro* experiments did not reduce the cell viability.

When the genotoxicity of NDMA and NDEA was evaluated in the comet assay, neither compound was effective in inducing primary DNA damage. Thus, the two selected nitrosamines were re-evaluated by using the S9 metabolic fraction and the treatments with or without S9 mix were conducted simultaneously. The results obtained for NDMA (Fig. 2) showed that only the highest concentration tested (10 mM) using S9 mix, was genotoxic. NDEA was slightly more genotoxic since two concentrations (5 and 10 mM) induced significant increases in DNA damage (Fig. 3). These data suggest that the presence of two ethyl groups in the nitrosamine is more genotoxic than the presence of two methyl groups.

The levels of oxidized bases induced by the selected concentration of each nitrosamine (10 mM) were determined by using the treatments with FPG and endoIII enzymes (Fig. 4). Both compounds were unable to induce oxidized pyrimidines; NDMA only induced slight damage in purines, whereas the proportion of this kind of damage caused by NDEA was much higher. The oxidative damage induced by NDMA only represents the 5% of the overall damage produced by this chemical, while the oxidative damage produced by NDEA was more than 360% of the overall damage.

The number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei and the CBPI values after treatment with the nitrosamines are summarized in Table 1. The results of the MN test show that both agents were unable to induce significant increases in the frequency of micronuclei. Neither the number of binucleated cells showing at least one MN, nor the frequency of MN scored per 1,000 binucleated cells showed any significant increase with respect to the control. Only NDEA was able to produce a decrease in the CBPI values at the highest concentration (10 mM), being more cytotoxic than MMC (positive control).

To be sure that the negative MN results obtained with both nitrosamines were due to their inability to induce fixed genetic damage and not related to the particular characteristics of TK6 cells, an additional experiment was carried out with peripheral blood human lymphocytes to test NDMA (Table 2). As occurs with TK6 cells, none of the evaluated concentrations increases the frequency of MN in binucleated cells. Only increases in the CBPI values, as occurred with the TK6 cells, were observed in the study with lymphocytes. These results confirm the inability of NDMA to induce MN in mammalian cells. In all the MN experiments with TK6 cells and human lymphocytes, the positive control MMC induced clear and significant increases in the frequency of MN.

4. Discussion

The discovery that disinfection procedures generate DBPs in treated water has led to a strong and growing interest in their undesirable health effects, mainly focused on those aspects concerning the potential risk of cancer and adverse reproductive effects. Several epidemiologic studies have shown a possible association between DBP levels and increases in the incidence of cancer of the bladder, colon

and rectum [30-38]; but, besides these data from epidemiologic studies, it is necessary to determine whether DBPs act as genotoxicants and exactly what type of genetic damage they produce.

N-nitrosamines are well-known environmental toxins that can be metabolized into genotoxic agents. Among them, both NDMA and NDEA exert their mutagenic activity after cytochrome P450-dependent α-oxidation; but, depending on which P450 genes are expressed, the mutagenic response may range from 0.0027 to 4.15 revertants/(nmol p-mol) in a bacterial strain expressing CYP3A4 and CYP2A6, respectively [16]. The reactive intermediates of nitrosamine metabolism also have the ability to alkylate nucleophilic sites of DNA producing alkali-labile adducts, which can lead to the formation of abasic sites.

NDMA is the volatile N-nitrosamine most commonly encountered in food samples and it is considered as a potent liver, lung and kidney carcinogen. Its genotoxic effects had been studied in both *in vitro* and *in vivo* assays, inducing gene and chromosome mutations, sister-chromatid exchanges, unscheduled DNA synthesis, as well as a weak induction of bone marrow MN in peripheral mammalian reticulocytes [39]. With respect to its ability to induce primary DNA damage, as detected in the comet assay, some studies have reported genotoxic effects for DMNA, but only at high concentrations in human hepatoma cell lines and primary hepatocytes cultures [40-42], which would agree with our data. With respect to the results obtained in the MN test, our findings have shown that NDMA is not able to induce significant increases in the frequency of MN in binucleated cells. These results are different from those reported by Valentin-Severin et al. [41] in HepG2 cells, where they observed significant increases in the number of MN, but at very high concentrations. They are also different from those reported by the U.S. EPA, who classified NDMA as inductor of bone marrow micronuclei *in vivo*. These discrepancies are probably due to the different cell lines used in the various studies. Thus, TK6 cells and peripheral blood lymphocytes have a limited metabolic activity and an external metabolic source (S9) is required for testing non direct mutagens, although this never completely mimics the *in vivo* situation. On the contrary, HepG2 is a hepatic cell line that retains an important metabolic activity.

With regard to NDEA, it results from the chlorination of water containing diethylamine in the presence of ammonia ions [14]. The EPA has classified the results of NDEA in the mammalian micronucleus test as inconclusive, since the *in vitro* data reveal discordant results or very weak responses. A possible explanation may be the volatility of its mutagenic metabolites [43]. Our results show that only the two higher concentrations (5 mM and 10 mM) cause significant increases of DNA damage in the comet assay, but only when the S9 mix was added to the cultures. As occurred with NDMA, NDEA was ineffective to increase the frequency of MN in binucleated cells.

To investigate if these nitrosamines are able to produce oxidative DNA damage, we evaluated the effectiveness of NDMA and NDEA in inducing this kind of damage by using endoIII and FPG treatments. Reactive oxygen species may partially contribute to the genotoxic effect of NDMA, as observed in P450 2E1-expressing cells [42, 44]. Our results show that only the FPG treatment enhances the DNA damage produced by NDMA and NDEA, indicating that only oxidized purines are induced. Although this effect is

slightly enhanced in NDMA treatments, it is markedly increased after NDEA treatments, where the proportion of oxidative damage was even higher than the direct damage produced.

5. Conclusions

Our results indicate that although both selected nitrosamines are weakly genotoxic, according to the results obtained in the comet assay, they are unable to induce clastogenic and/or aneugenic effects, either in TK6 cells or human peripheral blood lymphocytes. Concerning their activity to induce oxidative damage, enzyme treatments revealed that only NDEA was able to produce increased levels of oxidized bases, mainly in purine sites. Nevertheless, and taking into account the very high concentrations needed to produce DNA damage, our data suggest a low, if existent, genotoxic risk associated with the presence of these compounds in drinking water.

Acknowledgments

Danae Liviac is supported by a postgraduate fellowship from the Universitat Autònoma de Barcelona. This investigation was sponsored in part by the Spanish Ministries of Education and Science (SAF2005-07643-C03-03) and Environment (A036/2007/3-14.4), and by the Generalitat de Catalunya (2009SGR-725).

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Legends to the figures

Fig.1. Viability results after treatment of TK6 cells with NDMA (A) and NDEA (B). Viability was measured by using the FDA/EtBr stain.

Fig. 2. Genotoxicity of NDMA in the comet assay after 3 h of treatment. Genetic damage is measured as the percentage of DNA in tail. Statistical significance: **p<0.01, ***p<0.001. Data represent the average of two experiments; bars, SE. H_2O_2 and B(α)P positive controls.

Fig. 3. Genotoxicity of NDEA in the comet assay after 3 h of treatment. Genetic damage is measured as the percentage of DNA in tail. Statistical significance: **p<0.01, ***p<0.001. Data represent the average of two experiments; bars, SE. H_2O_2 and B(α)P positive controls.

Fig. 4. Effect of the enzyme (endo III and FPG) treatments in TK6 cells previously treated with 10000 μM of NDMA and NDEA). Data represent the average of three experiments; bars, SE.

Table 1.

Micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with nitrosodimethylamine and nitrosodiethylamine.

Concentration (mM)	Distribution of MN in BN cells					Total MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
NDMA												
0	971	23	2	1	3[a]	42	29	63	222	35	180	2.30
0.5	957	39	3	1	0	48	43	47	175	51	227	2.46
1.0	972	25	3	0	0	31	28	49	218	44	189	2.37
2.5	966	26	7	0	1[b]	45	34	28	179	60	233	2.53
5.0	965	28	4	3	0	45	35	27	191	61	184	2.34
10	976	21	3	0	0	27	24	60	210	46	184	2.34
MMC	891	84	22	3	0	137	109***	95	337	13	55	1.95***
NDEA												
0	976	20	4	0	0	28	24	56	204	37	203	2.37
0.5	980	18	1	1	0	23	20	42	180	40	238	2.47
1.0	986	12	2	0	0	16	14	38	188	44	230	2.47
2.5	977	21	2	0	0	25	23	50	203	42	205	2.39
5.0	982	17	0	0	1	21	18	52	241	39	168	2.31
10	985	13	2	0	0	17	15	187	266	5	42	1.72***
MMC	930	62	6	2	0	80	70**	136	315	13	36	1.83***

*** statistically significant from control (p □0.001)

[a] BN cells with4 MN; [b] BN cells with 5 MN

Table 2.

Induction of micronuclei (MN), binucleated cells with MN (BNMN) and CBPI values in cultured lymphocyte treated with nitrosodimethylamine.

Concentration (M)	Distribution of MN in BN cells					MN	BNMN	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
0	984	16	0	0	0	16	16	316	273	4	7	1.39
0.5	999	1	0	0	0	1	1	248	227	14	11	1.55
1.0	993	6	0	1	0	9	7	232	244	10	14	1.58
2.5	993	7	0	0	0	7	7	243	226	14	17	1.58
5.0	997	2	1	0	0	4	3	238	238	12	12	1.57
10	990	9	1	0	0	11	10	275	195	12	18	1.51
MMC	940	58	2	0	0	62	60***	384	116	0	0	1.23***

statistically significant from the control (*** $p \leq 0.001$)

Figure 1.

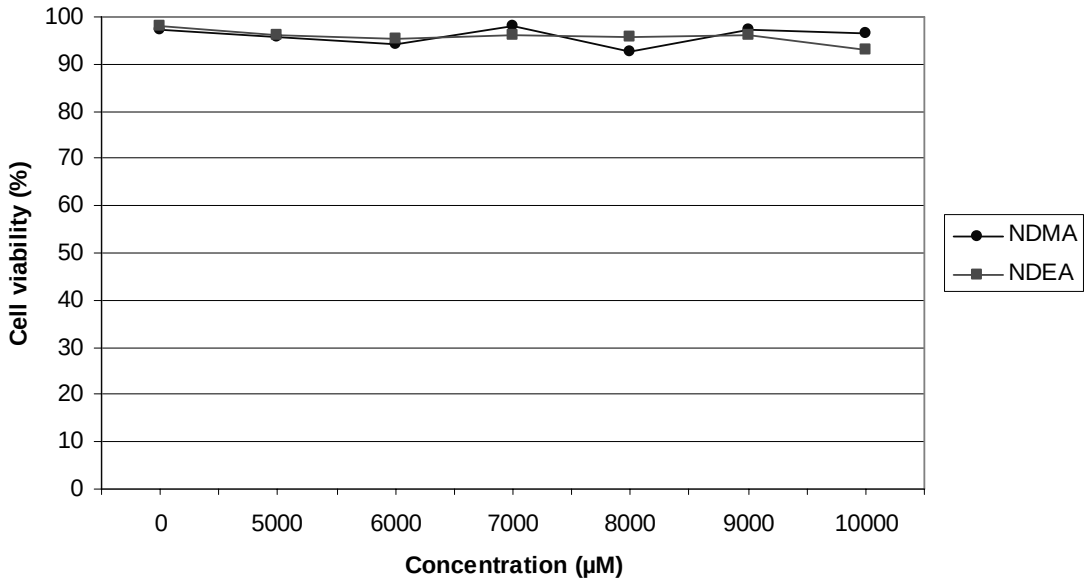


Figure 2.

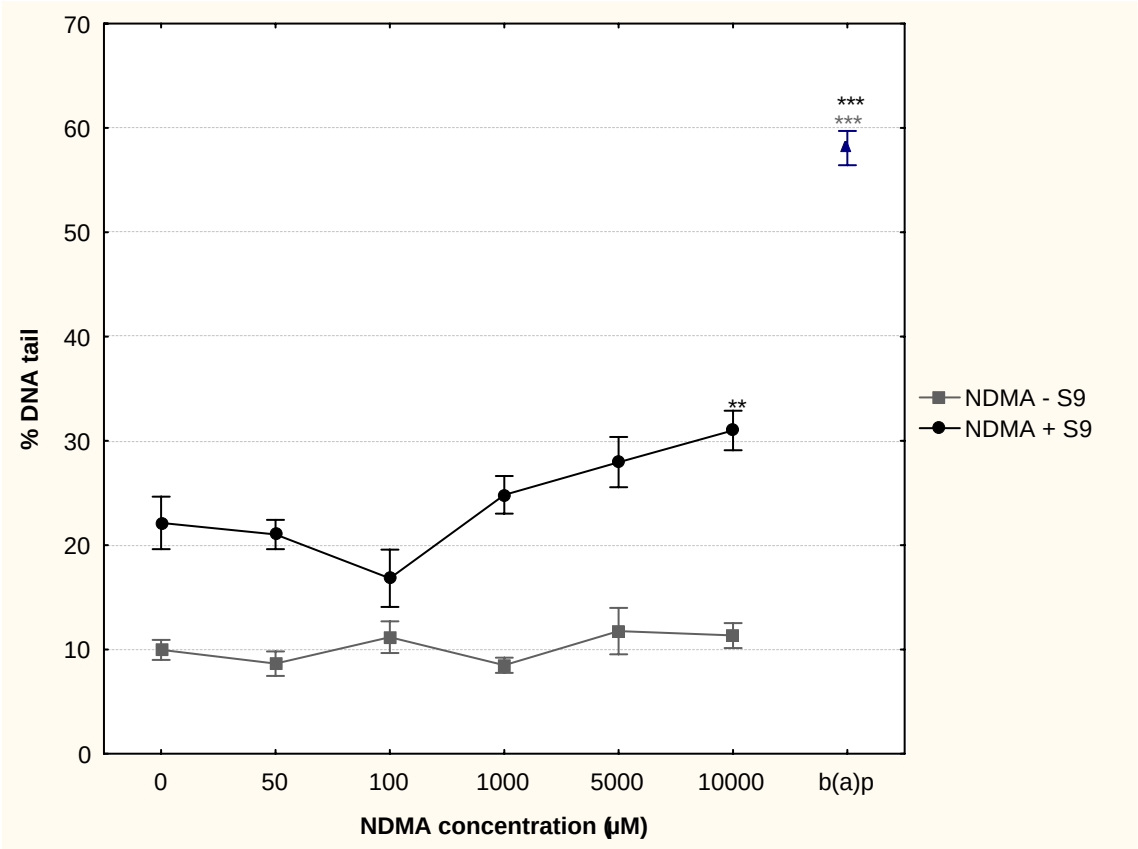


Figure 3.

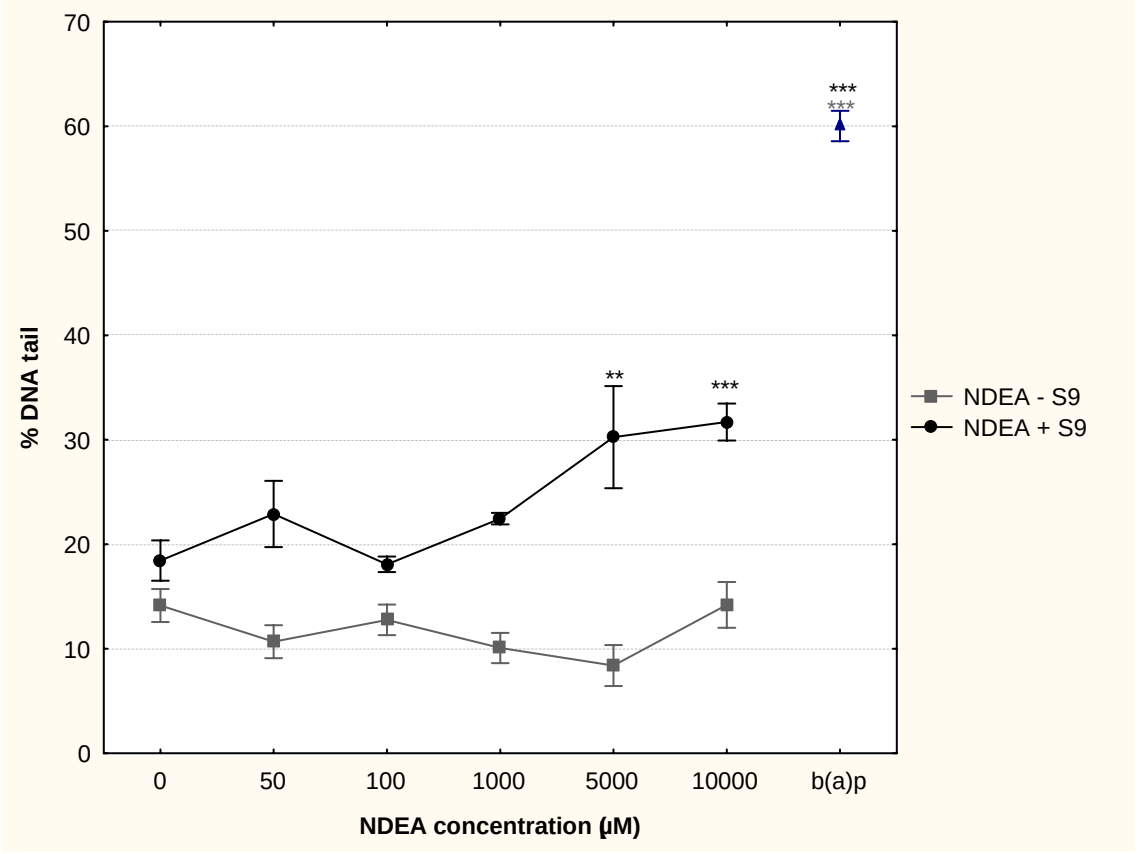
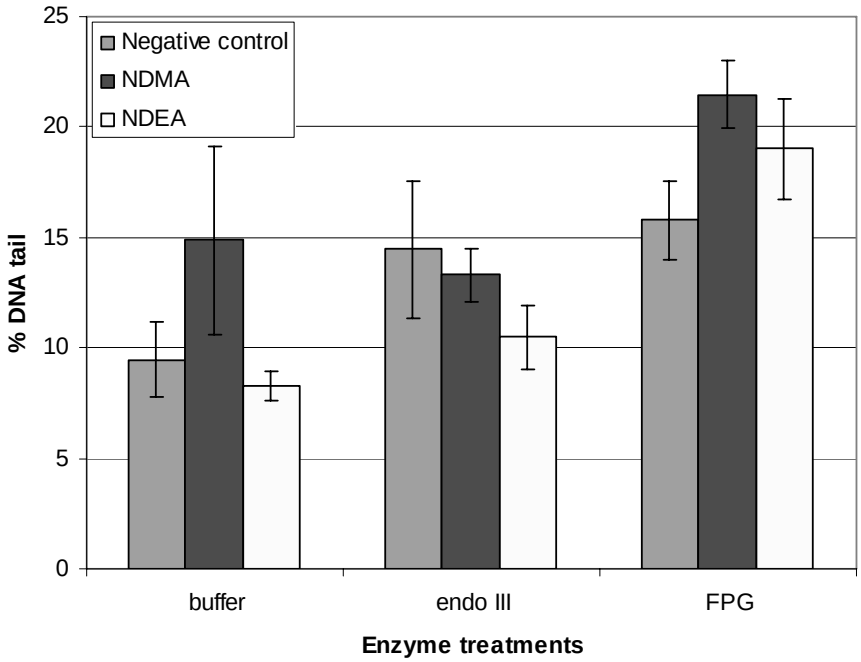


Figure 4.



ANEXO 2

Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay

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Mutagenesis (en prensa)

MUTAGE	geq034	ML
Journal Name	Art. No.	CE Code

Mutagenesis pp. 1–5, 2010 doi:10.1093/mutage/geq034

Genotoxicity testing of three monohaloacetic acids in TK6 cells using the cytokinesis-block micronucleus assay

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Received on March 8, 2010; revised on May 14, 2010; accepted on May 25, 2010

Chemical disinfection of water generates harmful chemical compounds, known as disinfection by-products (DBPs). One class of DBPs is constituted by haloacetic acids (HAAs), the second major group in prevalence (after trihalomethanes) detected in finished drinking water. In this article, we report the results obtained in the evaluation of the chromosome damage induced by three monohaloacetic acids, namely iodoacetic acid (IAA), bromoacetic acid (BAA) and chloroacetic acid (CAA). To evaluate the induction of chromosome damage, we used the cytokinesis-block micronucleus test that measures the ability of genotoxic agents to induce both clastogenic and/or aneugenic effects. No previous data exist on the effects of these compounds on human chromosomes. We tested five doses of each HAA, in addition to the negative and positive controls. The highest dose tested for each HAA was that immediately lower than the dose producing total cytotoxicity. Our results show that none of the three HAAs tested was able to increase significantly the frequency of micronucleus in binucleated TK6 cells, the rank order in decreasing cytotoxicity was IAA > BAA >> CAA.

Introduction

Chemical disinfection of drinking water is considered as one of the major achievements in public health of 20th century. Its introduction as worldwide water treatment allowed controlling waterborne infectious diseases like cholera, typhoid, hepatitis and dysentery. In 1900, before disinfecting water, the death rate from typhoid in the USA was 36 per 100 000 population dropping to 20 per 100 000 in 1910, 3 per 100 000 in 1935 and by 1960, only 20 people were recorded as having died from typhoid throughout the entire USA. Now, typhoid is virtually disappeared in the USA and other developed countries (1,2).

It has been 35 years since the discovery that water chlorination generates a series of chemical compounds, known as disinfection by-products (DBPs) (3,4). These DBPs are formed when the disinfectants (powerful oxidants) react with natural organic matter, anthropogenic contaminants and iodide and bromide present in the source water. An important aspect to be taken into account is that each water treatment plant has its own spectrum and distribution of DBPs, depending on many factors including the quality of raw water, the type and dose of disinfectant applied, the use of secondary disinfectants, the pH and temperature conditions and the water source, among others (5–9). To date, >600 DBPs have already been identified (10), but only a small number have in fact been assessed for their genotoxic or mutagenic potential and for their eventual adverse health effects.

The first class of DBPs identified in treated water was the trihalomethanes (THMs), which constitute the most prevalent. After the THMs, the second major group is the haloacetic acids (HAAs); together, they count for ~25% of the halogenated DBPs (11). HAAs can be formed by disinfection with chlorine, chlorine dioxide, chloramines and ozone, but they are generally formed at the highest levels in chlorination treatments (12). Of all the HAAs, only five of them [bromoacetic acid (BAA), dibromoacetic acid, chloroacetic acid (CAA), dichloroacetic acid and trichloroacetic acid (TCA)] are currently regulated by the US EPA, with a maximum contaminant level for the sum of the five regulated HAAs of 60 μg/l (13).

Richardson *et al.* (12) reviewed the data available about occurrence, genotoxicity and carcinogenicity of regulated and emerging unregulated DBPs, including HAAs. Of the five regulated HAAs, only DCA and TCA are classified by the IARC in group 2B and group 3, respectively (14). Among the HAAs, we can find the monohaloacetic acids (monoHAAs) that have only one halogen in their chemical structure; they are namely CAA, BAA and IAA. All the monoHAAs are mutagenic in *Salmonella* and can induce direct DNA damage (comet assay) in CHO cells (15–19). This primary-induced genetic damage is repaired over time, but with different rates of DNA repair for each monoHAA (20). IAA resulted to be more cytotoxic, genotoxic and mutagenic than their brominated and chlorinated analogs. Concerning their carcinogenic potential, CAA gave no evidence of carcinogenicity in rodent 2-year bioassay after either gavage or drinking water exposure and no carcinogenicity data are available for the other two monoHAAs (12). In addition, no data exist on their ability to induce fixed chromosome damage.

In this context, the objective of the present work is to provide information on the genotoxic activity of these three monoHAAs (CAA, BAA and IAA) to induce chromosome damage in cultured human cells. To reach this objective, the micronucleus (MN) test using the cytokinesis-block technique was the selected assay. This assay enables to detect chromosome fragments (clastogenicity) or whole chromosome loss (aneuploidy) during nuclear division (21,22), both genotoxic effects being of great relevance in carcinogenesis. The MN test is extensively used as a pertinent tool for evaluating the possible effects of the exposure to genotoxic agents. In addition, recently Bonassi *et al.* (23) had provided preliminary evidence indicating that MN frequency is a predictive biomarker of cancer risk in humans.

The use of the selected genotoxicity assay enables us to contribute with new information on the characteristics of the DNA damage induced in human cells by the three HAAs tested (IAA, BAA and CAA).

Materials and methods

TK6 cultures

TK6 cells were cultured in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The doubling time for TK6 cells ranged from 16 to 20 h. This cell line has been used extensively for mutagenicity and other genotoxicity studies, including the MN assays (24,25).

Chemicals

IAA (ICH_2COOH, CAS 64-69-7, ≥99% purity), BAA ($BrCH_2COOH$, CAS 79-08-3, ≥99% purity) and CAA ($ClCH_2COOH$, CAS 79-11-8, ≥99% purity) were kindly provided by Professor M. J. Plewa (Department of Crop Sciences, University of Illinois at Urbana-Champaign, USA). Dimethyl sulfoxide (DMSO) was used as solvent for IAA and distilled water for BAA and CAA.

MN test

A volume of 3 ml of a TK6 culture (0.5×10^6 cells/ml) was set-up in complete medium. Aliquots of 30 μl of each DBP concentration, representing 1% of the total volume, were added to the cultures. All treatments were added at the beginning of the incubation, and cultures were kept for 48 h at 37°C in a 5% CO_2 atmosphere. Mitomycin C (MMC, 0.3 μM) and distilled water or DMSO were used as positive and negative controls, respectively. For each DBP concentration and controls, two independent cultures were made, and for each culture, two slides were prepared. Three independent experiments were done for each DBP.

The cytokinesis-blocked micronucleus assay was conducted following the standard technique proposed by Fenech (26). Cytochalasin B (Cyt-B), at a final concentration of 6 μg/ml, was added to each culture at the beginning. After the incubation period, cells were harvested. The cultures were centrifuged at 150 g for 8 min, then the supernatant was removed and the cells were subjected to a hypotonic treatment (5 ml KCl 0.075 M, 4°C, 7 min), and another centrifugation was carried out. Cells were fixed with methanol:acetic acid (3:1 vol) at least three times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped on clean microscope slides (two drops of 20 μl each one). After drying, cells were stained with Giemsa solution for 5 min.

All slides were coded before scoring by a person outside the study and the scoring was carried out by a unique well-trained person using a Leitz-Leica light microscope at ×1000 magnification, under oil immersion. The criterion for scoring MN was that described by the HUMN project (27). One thousand binucleated cells (500 cells per each culture) were scored and classified, according to the number of MN, to calculate the induction of MN. In addition, 500 cells (250 cells per each culture) were scored to determine the cytokinesis-block proliferation index (CBPI), which was calculated in accordance with the formula proposed by Surrallés *et al*. (28). For each culture, one or more slides were scored to determine the induction of MN and CBPI and the values reported correspond to the pooled data from the two cultures.

Statistical analysis

The analysis of the binucleated cells with MN was performed for each treatment using the one-tailed Fisher's exact test. The Chi-square test was used for the analysis of CBPI among each treatment. Statistical decisions were made with a significance level of 0.05.

Results

Three independent experiments were carried out with each HAA, the first experiment covering a wide range of doses. From this first experiment, we select the highest dose to be scored for genotoxicity as that immediately lower than the dose-inducing total cytotoxicity.

The number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei and the CBPI values after treatment with the monoHAAs tested are summarised in Tables I–III. The results of the MN assay show that the three monoHAAs were unable to induce significant increases in the frequency of MN. Neither the number of binucleated cells showing at least one MN nor the frequency of total MN scored per 1000 binucleated cells showed any significant increase with respect to the negative control.

The concentrations evaluated of each monoHAA in the MN test show that IAA was the most cytotoxic, the highest concentration scored being of 5 μM. The next most cytotoxic HAA was BAA (10 μM) and the lowest cytotoxic HAA was CAA (500–750 μM). IAA was 2-fold more cytotoxic and 100-fold more cytotoxic than BAA and CAA, respectively; and BAA was 50-fold higher cytotoxic than CAA. The rank order of cytotoxicity was IAA > BAA >> CAA.

A reduction in the CBPI has been observed at several concentrations for the three monoHAAs tested and, although generally the CBPI value diminishes when the concentration increases, our data do not allow definitively confirming a dose-dependent exposure.

In two of the three experiments carried out with CAA (Table III), the highest concentration that could be scored was 500 μM but, in the third experiment, it was possible to score a higher concentration (750 μM). The high CBPI value (2.84) observed at this concentration allowed that the few surviving cells could divide and finally be counted. Nevertheless, it was only possible to score 339 binucleated cells at the concentration of 750 μM.

It must be indicated that, in all experiments, the concurrent treatments carried out with MMC (positive control) induced clear and significant increases in the frequency of binucleated cells with MN.

Discussion

An enormous research effort to determine the possible adverse effects of DBPs on human health began since the discovery of their ubiquitous presence in treated water. These investigations are mainly focused to clarify if the presence of determined levels of DBPs is directly related to a significant increase incidence of cancer and adverse reproductive effects. From such studies, several epidemiologic evidences suggest a possible association between DBP levels and increases in the incidence of cancer of the bladder, colon and rectum (29–37). The US EPA had estimated that between 2 and 17% of all observed cases of urinary bladder cancer could be attributed to chlorinated DBPs (38). Besides epidemiological data, it must be recalled that many DBPs are considered cytotoxic, genotoxic and carcinogenic and they represent an important class of environmental hazardous chemicals. Therefore it is necessary to elucidate if the emerging unregulated DBPs could act as genotoxicants and what kind of genetic damage produce.

HAAs are the second major class of DBPs identified in drinking water and are formed in different quantities, depending on the type of disinfectant used. The presence of bromide or iodide from natural and/or anthropogenic sources can favor the formation of bromine- or iodine-containing HAA (9,39). The iodo-acids were recently recognised as DBPs with a high occurrence in finished drinking water treated by chloramination.

Regarding the available data for the selected three monohalogenated acetic acids, all of them have shown to be cytotoxic and mutagenic in *Salmonella typhimurium* (15,17,18), showing that IAA was the most mutagenic of the HAAs analysed (17) and that BAA was 150-fold more mutagenic than CAA (15). Nevertheless, Giller *et al*. (40) reported positive results only for BAA in the SOS chromotest with *Escherichia coli* and negative results in the Ames fluctuation test for both BAA and CAA, the only two monoHAAs tested.

In addition to the positive results found in bacteria, the three monohalogenated acetic acids (IAA, BAA and CAA) induced DNA strand breaks in CHO cells, as measured by the comet assay. In this assay, BAA was found to be a potent genotoxic

Table I. MN, binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with IAA

Concentration (μM)	Distribution of MN in BN cells					Total MN	BNMN per 1000 BN cells	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
Experiment 1												
0	974	23	2	1	0	30	26	34	230	23	213	2.40
0.25	981	16	3	0	0	22	19	49	212	30	209	2.38
0.5	984	16	0	0	0	16	16	39	199	27	235	2.45
1	986	11	3	0	0	17	14	46	209	41	204	2.40
2	990	9	1	0	0	11	10	45	242	24	189	2.34
5	985	13	1	0	1[a]	19	15	62	256	13	169	2.24***
10	Cytotoxic											
MMC	944	53	3	0	0	59	56***	53	366	16	65	2.06***
Experiment 2												
0	964	21	2	1	1[a]	44	36	30	212	68	190	2.46
0.25	968	28	3	1	0	37	32	42	186	51	221	2.46
0.5	958	41	1	0	0	43	42	41	206	62	191	2.42
1	966	31	3	0	0	37	34	47	208	65	180	2.40
2	952	44	4	0	0	52	48	62	212	50	176	2.33**
5	974	25	1	0	0	27	26	45	216	29	210	2.39
MMC	943	52	5	0	0	62	57*	68	349	21	62	2.03***
Experiment 3												
0	973	24	3	0	0	30	27	27	239	22	212	2.41
0.25	968	32	0	0	0	32	32	38	194	25	243	2.46*
0.5	975	22	2	1	0	29	25	18	198	29	255	2.53**
1	960	38	2	0	0	42	40	38	204	27	231	2.44
2	963	34	1	1	0	39	36	44	231	18	207	2.36
5	971	26	3	0	0	32	29	41	219	16	224	2.40
MMC	951	41	6	1	1[b]	61	49**	38	377	12	73	2.09***

Statistically significant from control (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).
[a]BN cells with four MN.
[b]BN cells with five MN.

Table II. MN, binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with BAA

Concentration (μM)	Distribution of MN in BN cells					Total MN	BNMN per 1000 BN cells	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
Experiment 1												
0	986	14	0	0	0	14	14	24	207	40	229	2.49
0.5	989	10	1	0	0	12	11	27	164	47	26	2.56*
1	987	11	1	1	0	16	13	22	185	36	257	2.54
2	982	18	0	0	0	18	18	17	183	56	244	2.57
5	983	17	0	0	0	17	17	31	157	45	267	2.56**
10	982	18	0	0	0	18	18	19	124	41	316	2.68***
20	Cytotoxic											
MMC	974	24	2	0	0	28	26*	29	377	19	75	2.13***
Experiment 2												
0	966	34	0	0	0	34	34	73	213	72	142	2.28
0.5	966	30	4	0	0	38	34	73	214	71	142	2.28
1	977	21	2	0	0	25	23	70	255	53	122	2.21*
2	971	26	2	1	0	33	29	69	214	84	133	2.30
5	970	28	2	0	0	32	30	73	254	66	107	2.20*
10	974	24	1	1	0	29	26	65	203	58	174	2.33
MMC	908	79	11	2	0	107	92***	73	365	23	39	1.98***
Experiment 3												
0	983	15	2	0	0	19	17	59	208	35	198	2.35
0.5	989	11	0	0	0	11	11	56	201	29	214	2.37
1	985	15	0	0	0	15	15	50	194	39	217	2.41
2	987	12	1	0	0	14	13	59	191	55	195	2.38
5	978	20	1	1	0	25	22	46	154	53	247	2.51***
10	993	7	0	0	0	7	7	39	243	34	184	2.36*
MMC	920	74	6	0	0	86	80***	100	368	15	17	1.86***

Statistically significant from control (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

agent, even more cytotoxic (31-fold) and genotoxic (14-fold) than mutagen X in CHO cells (16).

According to the information available for HAAs, it was demonstrated that HAAs containing iodine atoms were more cytotoxic and genotoxic than their brominated analogs, and that brominated acetic acids were more cytotoxic and genotoxic than their corresponding chlorine-substituted acids. Our cytotoxicity data also shows the same pattern

Table III. MN, binucleated cells with MN (BNMN) and CBPI values observed in TK6 cells treated with CAA

Concentration (μM)	Distribution of MN in BN cells					Total MN	BNMN per 1000 BN cells	Distribution of cells according to the number of nuclei				CBPI
	0	1	2	3	>3			1	2	3	4	
Experiment 1												
0	981	19	0	0	0	19	19	52	254	69	125	2.28
100	979	18	3	0	0	24	21	42	198	72	188	2.44***
250	974	25	1	0	0	27	26	41	160	64	235	2.52***
500	985	13	2	0	0	17	15	32	184	81	203	2.50***
750	Cytotoxic											
1000	Cytotoxic											
MMC	891	94	14	1	0	125	109***	85	367	19	29	1.93***
Experiment 2												
0	964	35	1	0	0	37	36	56	196	87	161	2.38
10	983	17	0	0	0	17	17	56	223	64	157	2.33
50	962	31	7	0	0	45	38	59	187	72	182	2.39
100	972	25	3	0	0	31	28	58	198	85	159	2.37
250	968	25	7	0	0	39	32	48	165	106	181	2.48*
500	956	39	4	1	0	50	44	60	132	39	269	2.50***
750	Cytotoxic											
MMC	947	47	6	0	0	59	53*	74	349	21	56	2.01***
Experiment 3												
0	983	15	1	0	1[a]	21	17	17	93	49	341	2.75
50	979	18	3	0	0	24	21	16	87	41	356	2.76
100	977	19	3	1	0	28	23	15	93	51	341	2.75
250	975	23	2	0	0	27	25	11	134	65	290	2.69**
500	965[b]	19	0	0	0	19	19	14	100	59	327	2.74
750	329[c]	8	2	0	0	12	10	7	67	48	378	2.84**
MMC	951	45	3	1	0	54	49***	40	368	15	77	2.10***

Statistically significant from control (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).
[a]BN cells with four MN.
[b]Only 984 binucleated cells were detected
[c]Only 339 binucleated cells were detected.

and agree with those previously reported by Plewa *et al.* (17).

Instead of the clearly positive results reported for these DBPs in the comet assay (15–17,19,20), our results in the MN test showed that none of the three HAAs tested were able to induce significant increases in the frequency of MN in binucleated cells. It must be emphasised that the type of genetic damage detected by both assays is different. The comet assay detects primary DNA damage, which could be repaired fast; while the MN test demonstrated fixed DNA damage. Recently, Komaki *et al.* (20) determined the kinetics of DNA repair of CHO cells treated with IAA, BAA and CAA, revealing that cells treated with BAA showed the lowest rate of DNA repair compared to that of CAA or IAA. In spite of this difference in repair kinetics, all the induced DNA damage was repaired over time. This fast repair of the DNA damage induced by the HAAs would agree with the previous results of our group with other DBPs as halonitromethanes (41), hydroxyfuranones (42) and halogenated acetaldehydes (43). Therefore, our negative data in the MN test can be explained taking into account the repair kinetics demonstrated by Komaki *et al.* (20). Thus, the primary DNA damage induced by the three HAAs cannot be fixed because it is properly repaired over time and, consequently, the initial damage is not translated to MN. These negative results obtained in the MN assay agree with those obtained in the erythrocytes of newt larvae treated *in vivo* with BAA and CAA (40).

Regarding the mechanistic aspects involved in the genotoxicity of the selected HAAs, Cemeli *et al.* (18) demonstrated that oxidative stress is involved in the induction of genotoxicity and mutagenicity by IAA. Thus, when the antioxidants catalase and butylated hydroxyanisole were concurrently incubated with IAA in *S.typhimurium* and CHO cells, both antioxidants were effective in reducing the mutagenicity of IAA in *Salmonella* and the genomic DNA damage in CHO cells. When the comet assay was combined with the use of endonuclease III or formamidopyrimidine glycosylase, it was shown that other DBPs as halonitromethanes (41), hydroxyfuranones (42) and halogenated acetaldehydes (43) also induce large proportions of oxidative damage. These repair enzymes break the DNA at sites with oxidised purines and pyrimidines, respectively. Furthermore, a recent study evaluating the gene expression of BAA treatments in normal non-transformed human cells has shown that the majority of altered transcript profiles correspond to genes involved in DNA repair and cell cycle regulation (44).

It must be indicated that from the point of view of the HAAs structure-activity relationships, the cytotoxicity and genotoxicity are inversely related to the number of halogens per molecule. This means that the monoHAAs are more cytotoxic and genotoxic than their di- and tri-halogenated analogs (40). Thus, the HAAs evaluated in the present study are considered to be the most cytotoxic and genotoxic HAAs.

As a summary of our study, we conclude that the three selected monoHAAs are cytotoxic in TK6 cells, the rank order of cytotoxicity being IAA > BAA > CAA. As indicated by the negative MN results, neither compound caused a significant increase in the frequency of micronucleated cells in TK6 cultures. This lack of effect could be explained in terms of an efficient repair of the initial primary DNA damage induced that prevent its translation to fixed genetic damage.

Funding

A postgraduate fellowship (PIF) from the Universitat Autònoma de Barcelona to D.L.; Spanish Ministries of Education and

Science (SAF2005-07643-C03-03); Environment and Rural and Marine Affairs (A036/2007/3-14.4); Generalitat de Catalunya (2009SGR-725).

Acknowledgements

Conflict of interest statement: None declared.

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ANEXO 3

Genotoxicity of six disinfection by-products in the mouse lymphoma assay.

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Environmental Science & Technology (enviado)

Genotoxicity of six disinfection by-products in the mouse lymphoma assay

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ABSTRACT

All drinking water had been disinfected prior their distribution for human consumption, this water treatment process generates disinfection by-products, formed by the interaction of the disinfectant with organic matter, anthropogenic contaminants, and inorganic (bromide/iodide) matter naturally present in source water. To investigate the mutagenicity potential of several disinfection by-products at the thymidine kinase (*Tk)* gene, a reporter gene for mutation induction, we used the mouse lymphoma assay (MLA). The MLA quantifies a wide genetic alteration that affect the expression of this gene in L5178Y/$Tk^{+/-}$-3.7.2C like point mutations, larger scale chromosomal changes, recombination. In this study, we selected six DBP (bromonitromethane, trichloronitromethane, tribromoacetaldehyde, chloral hydrate, mucobromic acid and mucochloric acid) to be evaluated. The results show, after 4 h of treatment, that only mucobromic acid increase the frequency of mutant colonies, with higher proportion of small colonies. On the other hand, the others five DBP are not mutagenic.

KEYWORDS: disinfection by-products, thymidine kinase mutation assay, mouse lymphoma cells.

INTRODUCTION

Chlorination is the most widely used method of disinfecting water for human consumption. This procedure is effective of killing harmful waterborne pathogens; however, it also leads to the formation of chemical byproducts that are generated by the reaction between the disinfectant (chlorine) and the natural organic and inorganic matter present in raw water. Some of these chemicals compounds, commonly known as disinfection by-products (DBPs) are considered as genotoxic, mutagenic and carcinogenic compounds, raising an alarm about the human health adverse effects that their exposure could have [1, 2]. The profile of DBP, in terms of type and concentration, of each water treatment plant is unique; depending on several factors such as type and amount of primary disinfectant used, pH, temperature, quality of raw water, use of secondary disinfectant [3-8]. If raw water contains bromide or iodide ions, brominated and iodided by-products are also formed and in high concentrations than the chlorinated ones.

In 1974, Rook identified the first group of DBPs, the trihalomethanes (THMs) [9]; also in that year, Bellar et al.[10] discovered higher concentrations of chloroform in finished disinfected water, concentrations of chloroform was almost undetected in water prior the chemical treatment. To date, more than 600 DBP have been reported for the major disinfectants currently used (chlorine, ozone, dioxide chlorine, chloramines) [11], but that only correspond for less than the half of DBPs, the rest of them are considered as “undiscovered/unidentified DBPs”. Only a small number of the identified DBP has been evaluated for their genotoxic and mutagenic potential, as well as for their possible adverse health effects, and only few of them are currently regulated.

THMs (chloroform, bromodichloromethane, dibromochloromethane and bromoform) are the major group of DBPs detected in drinking water, and also, the most studied; besides the THM, the groups of haloacetic acids (HAAs) and chlorohydroxyfuranones (CHFs) had deserved much attention too, HAAs for being the second most prevalent group and, CHFs for having in their group the most potent direct acting mutagen ever tested in the Ames strain TA100 and responsible for at least one-third of the total mutagenicity detected in chlorine-disinfected drinking water, the 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, also known as Mutagen X or MX [12, 13].

Improvement in technology for chemical characterization of drinking water’s constituents had provided the tools for discovery of newly disinfection by-products, groups of DBP in minor quantities. These novel DBPs were identified in treatment plants when a secondary disinfectant is used; some of them are similar to those that are regulated, whereas others are unique. The halonitromethanes (HNMs) are structurally similar to halomethanes, but they have a nitro group, instead of hydrogen, bonded to the central carbon, almost all HNM (8 of 9) are listed in EPA’s high priority target DBPs [14].

Recently, Richardson et al. [15] reviewed the data available about occurrence, genotoxicity, and carcinogenicity of 85 disinfection by-products in drinking water, 11 of which are regulated by the U.S. and the other compounds are considered as emerging DBPs. They concluded that there are needed additional studies to characterize the mode of action for some of the regulated DBPs; and much more studies for the emerging unregulated DBPs, there are few carcinogenicity studies but a growing database of genotoxicity data.

In this context, the aim of the present study is to evaluate the mutagenicity of six DBPs, using the *Tk* gene of mouse lymphoma cells. The L5178Y $Tk^{+/-}$-3.7.2C mouse lymphoma assay (MLA) is the most widely used of the various *in vitro* mammalian cell gene-mutation assays [16]. This assay detects most of the mutational events know to be associated with the etiology of cancer and other human diseases, including point mutations and a number of different types of chromosomal mutations. The thymidine kinase gene is locate on chromosome 11 and present in two copies and due of its autosomal location, the *Tk* gene can recover mutations resulting from a large genetic damage and so, MLA can detect not only intragenetic events, such point mutations, but also loss of heterozygosity (LOH) [17]. LOH implies chromosome loss by any of several mechanisms, incluiding non-disjunction, deletion or recombination (such as mitotic recombination or gene conversion)[18]. So, due of these features the MLA is preferred by a number of international regulatory agencies, including the United States Food and Drug Administration and the United States Environmental Protection Agency, as the *in vitro* mammalian mutation assay in the genotoxicology screening battery [19].

To add more information on the mutagenic action of disinfection by-products, in the present work we show the results obtained in a study on mutation induction by six different DBP by using the Tk microwell mutation assay.

EXPERIMENTAL SECTION

Chemicals. Bromonitromethane (BNM, CH_2BrNO_2) (CAS 563-70-2), tribromoacetaldehyde (TBA, C_2HBr_3O) (CAS 115-17-3), mucobromic acid (MBA, $C_4H_2Br_2O_3$) (CAS 488-11-9) and mucochloric acid (MCA, $C_4H_2Cl_2O_3$) (CAS 87-56-9) were purchased from Sigma-Aldrich (St. Louis, MO), trichloronitromethane (TCNM, CCl_3NO_2) (CAS 76-06-2) was purchased from Rielden-de Haën (Seelze, Germany), and chloral hydrate (CH, $C_2H_3Cl_3O_2$) (CAS 302-17-0) was purchased from Fluka (Chemie GmbH, Germany). All the solutions were prepared just prior use, using an appropriate solvent, and representing a final volumen in culture of 1% of the total. Methyl methanesulfonate (MMS, Sigma-Aldrich) was used as positive control at final concentration of 10 µg/mL.

RPMI 1640 medium, horse serum, L-glutamine, penicillin, streptomycin, sodium pyruvate and amphotericin B were purchased from PAA Laboratories (Pasching, Austria). Trifluorothymidine was purchased from Sigma-Aldrich.

Cells. L5178Y/*Tk*$^{+/-}$-3.7.2C mouse lymphoma cells were utilized for the mutation assay. They were kindly provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France) and cultured to prepare master stocks, which were maintained in liquid nitrogen at a density 2 x 10^6 cells/mL, in a culture medium containing 10% dimethyl sulfoxide (DMSO). They were confirmed as free of mycoplasma by PCR.

L5178Y cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate and 2.5 µg/mL amphotericin B. The serum concentration was lowered during the treatment to 5% and raised to 20% when cells were dispensed into microwells. The cells were routinely diluted at 2 x 10^5 cells/mL each day to prevent overgrowth. Cell density was determined with a cell counter. The cultures were maintained in a humidified incubator with 5 % CO_2 in air at 37 ºC.

Cell cleaning. To prepare working stocks for gene-mutation experiments, cultures were purges of excess of *TK*$^{-/-}$ mutants as previously described [20]. The purged cultures were checked for low background *TK*$^{-/-}$ mutants and stored in liquid nitrogen.

Cell treatment with DBP. Preliminary experiments were conducted to determined solubility and cytotoxicity of the test chemicals. Cytotoxicity was determined by the relative total growth (RTG) following 4h treatments at concentrations up to 1 mM or 500 µg/mL [21]. The RTG measurement takes into account cell loss after treatment, reduction in growth rate over the expression period, and any reduction in cloning efficiency on the day of selection for mutants [22]. The recommended highest concentration was one with an RTG of 20% [21].

Each DBP working solution (100x) were prepared just prior to use by dissolving the compound in distilled water or DMSO. A total of 10^7 cells per culture were grown in R10 media (10% horse serum) and placed in a series of sterile centrifuge. Solvent, test chemical (at least 4 concentrations) or positive control was added. In all cases, including the untreated cells and positive controls, the cells were incubated with gentle shaking (50 rpm) for 4h at 37 ºC. After treatment, the cells were centrifuged, washed twice with fresh medium, and then resuspended in growth medium at a density of 2 x 10^5 cells/mL for a period of 2 days to allow expression of induced mutation (Tk-deficient phenotype).

Tk microwell mutation assay. The cells were counted and the densities were adjusted with fresh growth medium at approximately 1and 2 day following exposure. Then the cells were seeded in duplicate plates per culture to calculate viability and mutation frequency (MF) on day 2.

For the determination of plating efficiency, cultures were adjusted to to 10^4 cells/mL. A portion of each culture was removed and diluted to 10 cells/mL. Each culture was then dispensed at 0.2 mL/well on two 96-microwell plate. For mutant enumeration, trifluorothymidine (TFT) was added to cultures (10^4 cells/mL) at a final concentration of 4 μg/mL. Each TFT-treated culture was dispensed at 0.2 ml/well on two 96-well plates.

Colonies were scored by eye using qualitative judgement. To assist the scoring of TFT mutation plates, thiazolyl blue tetrazolium bromide solution from Sigma-Aldrich (MTT, 2.5 mg/mL) was added to each well and the plates were incubated for 4h., during which cell colonies are stained black. Colony size was estimated in a similar to that described by Honma et al. [23]: a small colony was defined as a colony having a size ≤ one-fourth of the well diameter.

Cell-count data were used, in conjunction with viability data on day 2 to generate the RTG values corrected for post-treatment toxicity, which provides an indication of post-treatment toxicity in comparison with the vehicle controls. The RTG values were used to decide on the acceptability of the toxicity at each dose level. All plates were incubated at 37 ºC in a humidified incubator with 5 % CO_2 in air. After 11-13 days of incubation, colonies were counted.

Data analyses. The IWTG mouse lymphoma workgroup states that biological relevance should be a major factor in a data evaluation. Therefore, it recommends a biologically relevant approach to evaluate MLA data, which requires that the induced mutant frequency (IMF) exceed some value based on the global background mutant frequency (MF) [24]. This value is the global evaluation factor (GEF), which is considered to be 126 for the microwell version. The IMF is obtained bye MF – SMF = IMF, where MF is one of the test culture mutant frequencies and SMF is the spontaneous mutant frequency.

The response of a test agent in an experiment is positive when the IMF for any treatment meets or exceeds the GEF, and when also a positive trend test is obtained. For example, if the negative/vehicle control MF in a microwell experiment is 60 x 10^6, then one of the test cultures must have an MF of at least 60+126 (GEF) = 186 x 10^6 in order to trigger the application of statistics.

The statistical approach used was the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the difference in the MF between the control and treated cultures. The level of statistical significance was set at 5 %.

IWGT considers that only that agent those response positive for both the GEF and the statistical trend analyses are biologically relevant.

RESULTS

The relative total growth (RTG), *Tk* mutant frequency (MF), percent platting efficiently and IMF values obtained after treatment of mouse lymphoma cells with the six selected DBP compound are displayed in Tables 1-3. Each compound was tested in two independent experiments. Positive control with methyl methanesulfonate (10 µg/mL) were run in parallel and showed clear and significantly increased mutant frequency.

Higher concentrations in each DBP were assayed to assess toxicity, but results are not presented when percentage of RTG is lower than 20%, which is the lowest acceptable value. The limit of 20% RTG is widely accepted as the maximum level of cytotoxicity for mutagenicity test, to avoid biologically irrelevant effects that might occur in severely stressed cells [25].

Table 1 shows the results obtained in MLA experiment with treatment of bromonitromethane (BNM) and trichloronitromethane (TCNM). BNM was tested in concentration up to 15 µM and this top concentration induced a pronounced drop in RTG to 42%. TCNM was slightly less cytotoxic and, its highest dose evaluated was 30 µM. Both halonitromethanes were unable to increase the mutant frequency in mouse lymphoma cells, according to the global evaluation factor (GEF), so they can be considered as not mutagenic in this assay.

Results of the experiments of MLA with treatment of two halogenated acetaldehyde are presented in table 2. TBA, the brominated halogenated acetaldehyde, was able to be tested up to 3 µM with a percentage of RTG to 37% in one experiment; in other hand, the highest concentration tested of chloral hydrate was 1 mM, the highest concentration able to test in MLA according to the ICH [21]. The RTG values obtained in experiments with CH show that even thought this DBP was tested in the highest concentration permitted, it can not reduce the percentage of RTG as done by TBA. As in the halonitromethane, the brominated DBP was more toxic than the chlorinated one. In both DBP, the induced mutation frequency (IMF) observed after treatment was lower than the GEF, so they can be considered as not mutagenic.

Like other DBP tested, MCA shows negative results in its ability to induce mutant colonies, its IMF in each treatment (in both experiments) were not meet or exceed the GEF value (252 and 243, for first and second experiment, respectively). But, when a one-way ANOVA followed by Dunnett's test to evaluate the

significance of the difference in MF between the control and the treated cultures, the two highest concentrations in one experiment showed to be statistically significant different.

Results of the last DBP evaluated, mucobromic acid (MBA) are presented in Table 3. This brominated hydroxifuranone showed acceptable toxicity levels at concentrations up to 10 μM and a pronounced drop in %RTG values between 20-30%. The two experiments produced a significantly increased value for mutation frequency at the highest dose evaluated (10 μM). When the ratio between small and large colonies is taken into account, higher proportions of small colonies appeared at increased concentration of mucobromic acid. The induced mutation frequency (IMF) observed after treatment with 10 μM mucobromic, in both experiments, were higher than the GEF. Higher concentration of MBA were tested and also shows to be positive for the GEF and the statistical analysis but this concentration induced a %RTG value lower than 20 %.

To better visualize the overall results obtained in the mutagenicity testing of the six DBP agents investigated in this study, Fig 1-3 show graphically the results with the positive compounds.

DISCUSSION

The finding that water disinfection besides producing safe drinking water, that resulted in a significant decrease in the number of deaths produced by waterborne pathogen disease; also generates chemical products, known as disinfection by-products, has lead to an increasing interest about the possible undesirable health effects of their exposure. This growing interest is related to the fact that chlorinated drinking water can be considered as an important environmental hazardous chemical due of its human long-time exposure. Several epidemiologic studies had investigated the relationship between such exposure and different kinds of cancer or reproductive health outcomes (congenital anomalies, stillbirth, spontaneous abortion, birth weight, prematurity and semen quality); founding an association between DBP levels and increases in the incidence of urinary bladder cancer [26-30] but, an unclear relationship with the reproductive health outcomes [31].

In relation to the genotoxicity data available for the six selected DBP (two halonitromethanes, two halogenated acetaldehydes and two hydroxyfuranones) evaluated in the current work, the amount of information on each one ranged from some data to only one data available; being CH, the DBP with more information available, some data exist on MCA, only few data on BNM and TCNM and only one data on MBA and TBA. This one genotoxicity data for MBA and TBA is the previously reported by us [32, 33] in the comet assay and in the micronucleus test on TK6 cell cultures.

We evaluated three different classes of DBP, in each class selected a brominated and a chlorinated compounds. BNM and TCNM formed part of the halonitromethanes (HNMs), an emerging class of DBP that are produced when chlorine and/or ozone are used for water treatment. CH is the most common halogenated acetaldehyde (HA) detected in almost all chlorine-disinfected drinking water [34] and, is, after trihalomethanes and haloacetic acids, the next prevalent DBP present in chlorinated drinking water [35]; whereas the brominated HA, like the brominated DBP, can be detected especially in disinfected water that contains high amount of bromide (from natural or anthropogenic sources). MCA, one of compounds selected of the third class of DBP selected (hydroxyfuranones), is structurally related to MX (one of the most potent direct-acting mutagen ever tested in Ames test) and, for this reason, it has been subjected to many evaluations whereas, MBA had been recently identified in drinking water in a U.S. National Occurrence Study [36].

When mouse lymphoma cells were exposed to the six DBP selected for 4 h, almost all of them were cytotoxic but, only one was mutagenic, the MBA, at the highest concentration evaluated (10 μM). Only one of these DBP selected (CH) was previously evaluated in mouse thymidine mutation assay[37] and it was classified as weakly mutagen due of its ability of increase mutant frequency at higher concentrations. Our results of this chlorinated HA show that it can not induce any increase in mutant frequency at any of the concentrations evaluated. The reason of this discordance between results is due of the concentrations evaluated, while we only evaluated concentrations up to 1 mM (165.4 μg/mL), Harrington-Brock et al. [37] tested concentrations almost 10 times higher, concentrations up to 1600 μg/mL (9.67 mM); and when the results of similar concentrations (1mM *vs.* 350 μg/mL, their lowest dose tested) were compared, it showed that the results of this previous study are in concordance with our findings where CH is not mutagenic in L5178Y cells.

Previous published results have shown that this chlorinated HA is a direct-acting mutagen that could able to induce point mutations, to increase the frequency of MN in mouse spermatocytes, as well as MN and SCE in blood peripheral lymphocytes in infants treated with CH, MN in cultured human fibroblasts, increase of the induction of primary DNA damage (comet assay) in TK6 cells [33, 38-42]. But contrast, negative results were also obtained in cultured CHO cells (comet assay) and TK6 cell (MN) [33, 43]. With regard to the brominated acetaldehyde TBA, this DBP was considered as highly genotoxic due of its high potential of induce primary DNA damage (comet assay) in TK6 cell cultures [33], damage that can not be fixed.

Treatment of the cells cultures with HNM (BNM and TCNM) shows that both compounds can not induced the frequency of mutant colonies even in concentrations with %RTG values of 35-45%, so they were classified as not mutagenic in the MLA. As been recently discover as DBP, only few studies had been evaluated them; being consider as highly genotoxic compounds that induced high levels of DNA strands

breaks in CHO cells and TK6 cells [44, 45], damage that can be easily repair by the cell. Besides these positive results, negative results were obtained in TK6 cells and *Drosophila melanogaster* using the micronucleus test and in the *Drosophila* wing-spot test, respectively [45, 46].

The third and last class of DBP evaluated was the hydroxyfuranones. One compound of this class, the MBA, was the only one able to increase the frequency of mutant colonies, this increase was detected at the highest dose evaluated (10 μM) with %RTG between 20 – 30 %. The fact that this concentration had a % RTG value very close to the lowest acceptable value, suggest that maybe a part of the mutagenic response is related to cytotoxicity. Also, a higher concentration was assayed, being more mutagenic than 10 μM but a % RTG lower than 20 % (data not shown). With respect to this positive compound, there's few studies that evaluate it; in vitro studies had showed that MBA was able to form adducts with adenosine, positive results in positive in the Ames test without metabolic activation, had a high capacity to induced DNA damage in TK6 cells but, was unable to increase to a significant extent the frequency of MN neither in TK6 cells or cultures of human lymphocytes [32, 47, 48]. While the chlorinated hydroxifuranone (MCA) even though of its negative results in MLA, shows to be a direct-acting mutagen, an ability to induce chromosome damage (seen en MN) in mammalian cells and in plants, an a high capacity to induce DNA damage (comet assay) in mammalian cells [32, 49-51].

The lack of concordance between results in the MN test, the comet assay and in the MLA assay has also been observed in other studies [52-54] and it could be due of the different mechanisms underlying the genotoxic effects detected by these three assays; and also by the different p53 status of the cell lines used [55-57].

In conclusion, our results show that the six DBP evaluated are cytotoxic but, only MBA could be considered as mutagenic (positive for the GEF and statistical analysis). The %RTG values of the positive concentration is close to the lowest acceptable value, so it maybe that some part of the mutagenic response is due of the cytotoxic. Finally, our results are of interest because they contribute with new data to the understanding on how particular DBPs act as genoetoxicants and maintain open the discussion on the risk associated to DBP exposure.

Acknowledgments

Danae Liviac is supported by a postgraduate fellowship from the Universitat Autònoma de Barcelona. This investigation was supported in part by the Spanish Ministries of Education and Science (SAF2005-07643-00-03) and Environment (A036/2007/3-14.4), and by the Generalitat de Catalunya (2009SGR-725).

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Table 1.
Toxicity and mutagenicity of bromonitromethane (BNM) and trichloronitromethane (TCNM) in mouse lymphoma cells

Concentration (µM)	Percent plating efficency	Mutant frequency (MF, x10^{-6})	Relative total growth	MF (S/L)	IMF(MF-SMF)
BNM (1)					
0	91	132	100	113/19	--
0.1	96	153	98	122/31	21
0.5	115	115	127	84/31	-17
1	90	130	100	85/45	-2
2.5	100	127	100	106/21	-5
5	98	140	88	117/23	8
7	94	156	72	104/52	24
10	85	229	42	155/74	97
MMS (10 µg/mL)	69	588***	51	522/66	456
BNM (2)					
0	96	157	100	119/38	---
0.1	106	166	108	137/29	9
0.5	95	175	102	152/23	18
1	102	173	104	161/12	16
2.5	108	126	99	107/19	-31
5	118	168	100	157/11	11
7	96	231	74	214/17	74
10	100	162	75	150/12	5
15	118	175	42	159/16	18
MMS (10 µg/mL)	73	471***	49	421/50	314
TCNM (1)					
0	98	93	100	72/21	--
1	85	107	87	87/20	14
5	88	90	78	74/16	-3
10	96	108	71	90/18	15
15	100	114	61	92/22	21
20	96	111	46	88/23	18
30	106	184	27	157/27	91
MMS (10 µg/mL)	76	544***	50	524/20	451
TCN (2)					
0	127	97	100	76/21	--
1	159	64	122	48/16	-33
5	139	91	80	55/36	-6
10	130	87	59	78/9	-10
15	124	91	45	79/12	-6
20	121	122	36	87/35	25
MMS (10 µg/mL)	93	452***	43	423/29	355

***$P \leq 0.001$ (significant different from negative control).

Table 2.
Toxicity and mutagenicity of tribromoacetaldehyde (TBA) and chloral hydrate (CH) in mouse lymphoma cells

Concentration (µM)	Percent plating efficency	Mutant frequency ($x10^{-6}$)	Relative total growth	MF (S/L)	IMF(MF-SMF)
TBA (1)					
0	91	103	100	75/28	--
0.01	102	77	123	38/39	-26
0.05	102	155	97	86/69	52
0.1	95	131	99	81/50	28
0.5	63	121	64	76/45	18
0.75	108	149	109	70/79	46
1	110	146	101	88/58	43
1.5	74	145	61	86/59	42
2	76	203	56	139/64	100
MMS (10 µg/mL)	77	732***	66	501/231	629
TBA (2)					
0	113	89	100	81/8	--
0.01	124	86	103	67/19	-3
0.1	95	97	70	82/15	8
0.25	121	80	98	71/9	-9
0.5	110	52	88	47/5	-37
1	118	96	79	69/27	-7
2	118	88	62	68/20	-1
3	108	206	37	167/39	117
MMS (10 µg/mL)	106	456***	76	402/54	367
CH (1)					
0	69	141	100	66/75	--
1	110	106	165	61/45	-35
10	84	176	99	90/86	35
50	80	180	114	98/82	39
100	69	169	101	101/68	28
250	87	135	120	78/57	-6
500	68	186	92	112/74	45
750	60	173	84	130/43	32
1000	76	137	109	110/27	-4
MMS (10 µg/mL)	50	729***	56	642/87	588
CH (2)					
0	80	107	100	75/32	--
1	87	174	115	125/49	67
10	68	181	91	125/56	74
50	84	147	101	119/28	40
100	67	174	87	131/43	67
250	76	145	101	107/38	38
500	57	181	66	111/70	74
750	57	187	75	147/40	80
1000	68	98	87	69/29	-9
MMS (10 µg/mL)	55	621***	50	484/137	514

***$P \leq 0.001$ (significant different from negative control).

Table 3.
Toxicity and mutagenicity of mucobromic acid (MBA) and mucochloric acid (MCA) in mouse lymphoma cells

Concentration (µM)	**Percent plating efficency**	**Mutant frequency ($x10^{-6}$)**	**Relative total growth**	**MF (S/L)**	**IMF(MF-SMF)**
MBA (1)					
0	91	117	100	35/82	--
0.05	106	89	94	45/44	-28
0.1	71	110	82	47/63	-7
1	88	136	92	56/80	19
2	71	150	70	61/89	33
5	70	138	58	81/57	21
7	95	190	65	92/98	73
10	71	396***	32	229/167	279
MMS (10 µg/mL)	93	618***	72	434/184	501
MBA (2)					
0	85	107	100	90/17	--
0.05	75	130	98	111/19	23
0.1	91	139	112	113/26	32
1	85	126	102	106/20	19
2	91	158	100	142/16	51
5	64	213	64	190/23	106
7	69	233	54	180/53	126
10	44	439***	21	347/92	332
MMS (10 µg/mL)	46	534***	36	463/59	427
MCA (1)					
0	85	126	100	72/54	--
0.1	63	189	64	106/83	63
0.5	70	87	75	55/29	-39
1	70	138	76	73/65	12
2	73	168	83	104/64	42
5	62	197	55	150/47	71
7	87	225	62	150/75	99
MMS (10 µg/mL)	85	458***	72	374/84	332
MCA(2)					
0	108	117	100	114/3	--
0.1	106	142	98	133/9	25
0.5	77	118	71	103/15	1
1	100	98	94	89/9	-19
2	81	182	69	167/15	65
5	91	154	68	154/0	37
7	83	233**	47	210/23	116
10	78	263**	22	251/12	146
MMS (10 µg/mL)	75	463***	58	429/34	346

**$P \leq 0.05$; ***$P \leq 0.001$ (significant different from negative control).

Legends to the figures

Figure.1. Mutagenicity of BNM and TCNM on L5178Y/ *Tk*$^{+/-}$ mouse lymphoma cells. Results are taken from two different experiments.

Figure 2. Mutagenicity of TBA and CH on L5178Y/ *Tk*$^{+/-}$ mouse lymphoma cells. Results are taken from two different experiments.

Figure 3. Mutagenicity of MBA and MCA on L5178Y/ *Tk*$^{+/-}$ mouse lymphoma cells. Results are taken from two different experiments.

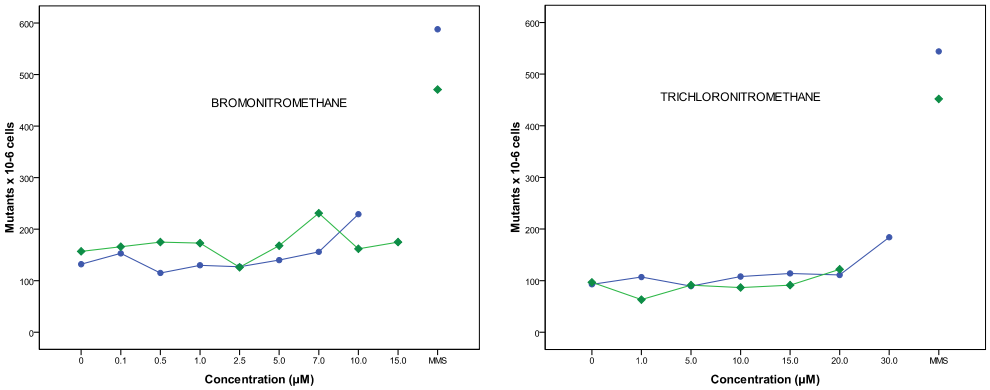

Figure 1.

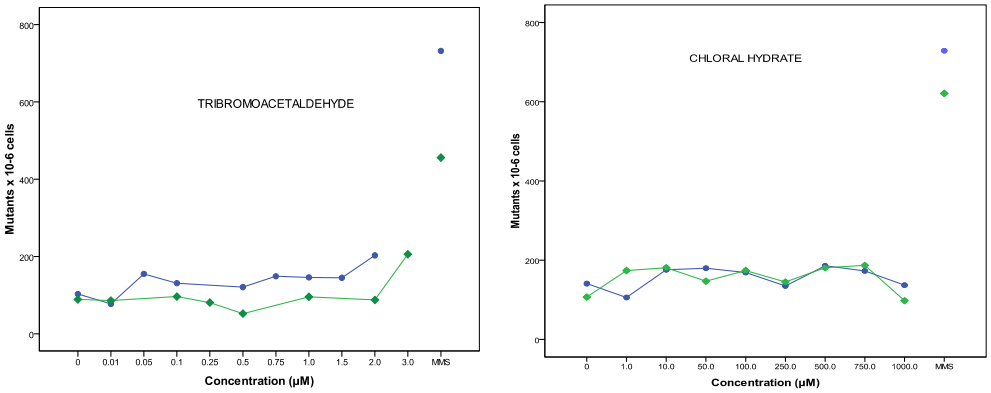

Figure 2.

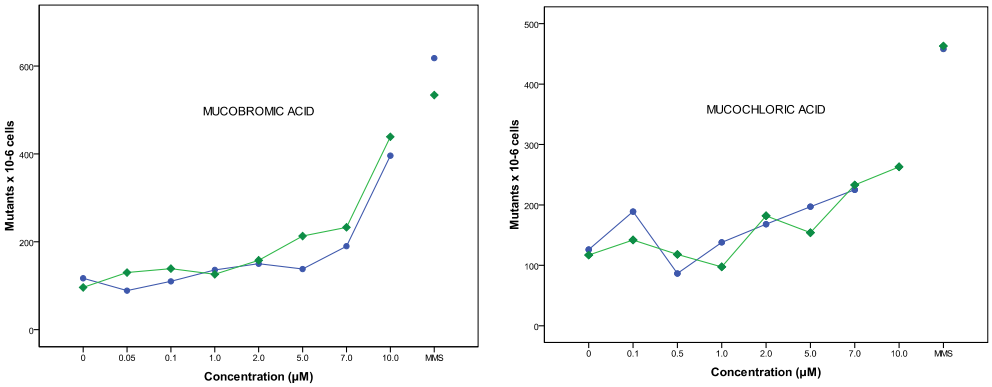

Figure 3.

ANEXO 4

Genotoxicity of water concentrates from recreational pools after various disinfection methods

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Environmental Science & Technology (2010), 44 : 3527-3532.

Environ. Sci. Technol. **2010,** *44,* 3527–3532

Genotoxicity of Water Concentrates from Recreational Pools after Various Disinfection Methods

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Received November 26, 2009. Revised manuscript received February 28, 2010. Accepted March 13, 2010.

Swimming and hot tub bathing are popular exercises and diversions. Disinfection of recreational pools is essential to prevent outbreaks of infectious disease. Recent research demonstrated an association between the application of disinfectants to recreational pools and adverse health outcomes. These pool waters represent extreme cases of disinfection that differ from disinfecting drinking waters. Pool waters are continuously exposed to disinfectants over average residence times extending to months. Disinfection byproduct (DBP) precursors include natural humic substances plus inputs from bathers through urine, sweat, hair, skin, and consumer products including cosmetics and sunscreens. This study presents a systematic mammalian cell genotoxicity analysis to evaluate different recreational waters derived from a common tap water source. The data demonstrated that all disinfected recreational pool water samples induced more genomic DNA damage than the source tap water. The type of disinfectant and illumination conditions altered the genotoxicity of the water. Accordingly, care should be taken in the disinfectant employed to treat recreational pool waters. The genotoxicity data suggest that brominating agents should be avoided. Combining chlorine with UV may be beneficial as compared to chlorination alone. During the recycling of pool water the organic carbon could be removed prior to disinfection. Behavior modification by swimmers may be critical in reducing the genotoxicity of pool water. Actions such as showering before entering the water and informing patrons about the potential harm from urinating in a pool could reduce the precursors of toxic DBPs.

Introduction

The second most popular exercise in the United States is swimming with approximately 339 million visits each year to swimming pools, water parks, and interactive fountains (*1*). Hot tub bathing is also popular. Disinfection of these recreational pools is essential to prevent outbreaks of infectious disease (*2*). However, recent research has highlighted an association between the application of disinfectants to recreational pools and adverse health outcomes (*3*−*8*). Respiratory ailments, including asthma, are promoted in disinfected pools (*3, 8, 9*). Epidemiological studies found that drinking chlorinated water, or swimming in chlorinated recreational waters was associated with an increased risk of bladder cancer, the sixth most prevalent cancer in the United States (*10, 11*).

These negative outcomes are believed to be associated with disinfection byproducts (DBPs) formed from reactions of the disinfectant with organic matter, and bromide/iodide in the source water (*12*). Nearly 700 DBPs have been characterized in disinfected drinking waters (*12*). Disinfected recreational waters may be more complex for two reasons. First, these waters represent extreme cases of disinfection, featuring elevated disinfectant exposures. Within public swimming pools in the United States, a free chlorine residual of 1−3 mg/L as Cl_2 is maintained throughout average residence times extending to months. Second, these waters exhibit elevated precursor concentrations. In addition to humic substance precursors in water, bathers contribute precursors from urine, sweat, hair, skin, and consumer products such as cosmetics and sunscreens (*8*). These latter classes of precursors are often nitrogen-rich. Of particular concern may be nitrogenous disinfection byproducts (N-DBPs), including organic chloramines, halonitroalkanes, halonitriles (*13*), nitrosamines, and nitramines (*14*). Organic chloramines are associated with respiratory ailments (*9*). Elevated genotoxicity and cytotoxicity are associated with many classes of N-DBPs (*15*) including halonitromethanes (*16, 17*), halonitriles (*18*), and haloamides (*19*). Exposure to nitrosamines is associated with bladder cancer (*20*−*22*).

As a focus for toxicity studies, disinfected recreational waters are particularly intriguing. Trihalomethanes and haloacetic acids (regulated DBPs in drinking water) have been used to evaluate DBP exposure in pools (*8, 23*). Trihalomethanes and haloacetic acids exhibit a weak bladder cancer specificity and potency (*10, 24*), and their concentrations do not differ significantly between hot tubs, or indoor or outdoor swimming pools (*25*−*27*).

Recent research characterized the concentrations of nitrosamines and nitramines in disinfected recreational waters (*28*). Within indoor pools, concentrations of *N*-nitrosodimethylamine (NDMA) and *N*-nitrodimethylamine (DMNA) were ∼30 and ∼80 ng/L, respectively. Concentrations increased to ∼300 and ∼200 ng/L, respectively, within indoor hot tubs, presumably due to the increase in temperature. NDMA concentrations in outdoor pools (∼6 ng/L) were significantly lower perhaps as a result of sunlight photolysis. DMNA concentrations were similar between outdoor and indoor pools.

While previous research evaluated concentrations of specific DBPs in recreational waters and, in some cases, attempted to correlate these with adverse health outcomes, these efforts have been hampered by the wide array of potentially toxic constituents in these waters. Rather than attempting to link specific byproducts to genotoxicity, we sought to compare the overall genotoxicity of recreational waters, derived from a single source, that were treated with different disinfectants under different conditions (e.g., temperature and solar exposure). In this work we compared the genotoxicity of organic matter extracted from samples

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10.1021/es903593w
Published on Web 04/09/2010

TABLE 1. Characteristics of Pool Samples

sample number[a]	type	location	TOC[b] (mg/L)	temp. (°C)	total chlorine residual (mg/L as Cl_2)	disinfectant
S3	tap water	indoor	1.2[c]	14	1.4	free chlorine
S1	cold pool	indoor	18.1	25	3.7	UV and free chlorine
S7	cold pool	indoor	5.2	25	1.6	UV and free chlorine
S4	cold pool	indoor	124.8	26	2.4	BCDMH[d]
S5	cold pool	indoor	12.6	29	3.4	free chlorine
S6	warm pool	indoor	33.1	36	3.7	free chlorine
S2	hot tub	indoor	12.1	40	2.8	free chlorine
S8	cold pool	indoor	23.7	25	1.7	free chlorine
S9	cold pool	outdoor	33.1	20	1.4	free chlorine

[a] Sample numbers used in experimental blind. [b] Total organic carbon. [c] Original sample disqualified, this result obtained from a sample collected on a different day. [d] Bromochlorodimethyl-hydantoin.

collected from seven public pools, and the tap water supply serving as the common source. These results may help to identify recreational water conditions that are undesirable.

Experimental Section

Sampling Design. Our goal was to generate robust statistical power for the genotoxicity data determined for each water sample. Accordingly, we sought to thoroughly evaluate the source tap water and each pool extract rather than analyze a large number of pools resulting in lower statistical power. In general to guarantee the required statistical power, we analyzed nine concentrations of each water sample with six SCGE microgels per concentration and a negative and positive control. With this approach we were able to detect the most dominant disinfectant or environmental conditions affecting pool genotoxicity. Future work with a wider number of pool samples would be needed to determine the importance of other correlations between reaction conditions and genotoxicity.

Sample Preparation. Pool water samples (8−10 L) were collected in fluorinated high-density polyethylene containers and the temperatures were recorded. Total residual chlorine was analyzed by the DPD colorimetric method (*29*). Total organic carbon (TOC) was analyzed using a Shimadzu TOC analyzer. TOC was employed to estimate the organic content of the pool waters. Accordingly, the water samples were not filtered. Water sample characteristics are provided in Table 1.

To analyze for genotoxic effects, sample extraction, and concentration were necessary. Due to incomplete recovery, extraction necessarily introduces some fractionation of water constituents. Because the constituents responsible for genotoxicity are unknown, we could not select an extraction method that would optimize the recovery of targeted chemical classes. Instead, we sought to mimic total bather exposure. Since disinfectants may contribute to genotoxicity, we did not quench disinfectant residuals. Solid phase extraction was not used to avoid artifacts from disinfectant reactions with solid phase resins. The solution pH was maintained to that of pool water. Among nonwater-soluble solvents, methylene chloride (log K_{ow} = 1.25) and methyl tert-butyl ether (MtBE; log K_{ow} = 1.15) have been used for extraction of DBPs and exhibit similar polarities. We selected MtBE as a compound more similar to the hydrocarbons constituting cellular lipid bilayers.

For each water sample, a total of 8−10 L was extracted into Fisher HPLC grade MtBE at Yale University within one day of sample collection; the disinfectant residual was not quenched prior to sample extraction. Aliquots (1 L) were extracted 3× by separatory funnel into 100 mL MtBE per extraction. MtBE extracts were combined, dried with ACS grade magnesium sulfate (JT Baker), and concentrated by rotary evaporation to 5 mL, transferred by Pasteur pipet to a glass vial, blown down to dryness under N_2, and suspended in 1 mL of Acros HPLC grade ethyl acetate. Concentrated extracts were shipped to the University of Illinois, and stored in Supelco micro reaction vessels sealed with caps lined with PTFE liners, at −22 °C under dark conditions. The samples were further concentrated such that the organics in 10 L of original water were concentrated into 100 μL of ethyl acetate. For each genotoxicity experiment, 5 μL of concentrated sample in ethyl acetate was transferred to a microfuge tube and mixed with 1 μL of dimethylsulfoxide (DMSO). The ethyl acetate was removed by passing dry N_2 at 42 °C over the sample for 1 min. The sample in DMSO was diluted in F12 cell culture medium. Concentrations were calculated as a concentration factor or as liter equivalents of the original water volume. For L-equivalent measures, the volume of sample extract introduced into a treatment well was multiplied by the concentration factor achieved by the extraction/concentration procedure.

Biological and Chemical Reagents, Chinese Hamster Ovary Cells. General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT) or from Fisher Scientific Co. (Itasca, IL). Chinese hamster ovary (CHO) cells are widely used in toxicology. The transgenic CHO cell line AS52 (*30*) was derived from the parental CHO K1-BH4 line; clone 11−4−8 was isolated from AS52 and expresses normal morphology, cell contact inhibition, and a stable chromosome complement with a consistent cell doubling time (*31*). Cells were grown in Hams F12 medium plus 5% FBS at 37 °C in a humidified atmosphere of 5% CO_2.

Single Cell Gel Electrophoresis (SCGE) Assay. SCGE (Comet assay) is a molecular genetic assay that quantitatively measures the level of genomic DNA damage induced in individual nuclei of cells (*32*−*34*). SCGE is a good measure of carcinogenic potential (*35*). We employed a microplate-based comet assay (*36*). The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 μL of F12 + 5% FBS and incubated. The next day the cells were washed with Hank's balanced salt solution (HBSS) and treated with a series of concentrations of a pool water sample in F12 medium without FBS in a total volume of 25 μL for 4 h at 37 °C, 5% CO_2. The microplate wells were covered with sterile AlumnaSeal. With each experiment a negative control, a positive control (3.8 mM ethylmethanesulfonate) and nine concentrations of a specific water sample were conducted concurrently. After treatment, the cells were washed, harvested, and incorporated into agarose microgels. For each treatment group, acute cytotoxicity was measured using the trypan blue method; the microgels were only analyzed if they were prepared from suspensions that expressed >70% cell viability. The microgels were processed to remove cell

membranes, electrophoresed, and analyzed with a fluorescent microscope equipped with a computerized image system (Komet Version 3.1, Kinetic Imaging Ltd., Liverpool, UK). The digitalized data were transferred to a spreadsheet and statistically analyzed. Complete information on this assay was published (*33, 36*) and is presented in the Supporting Information.

The tail moment metric of the SCGE assay that measures genomic DNA damage is not normally distributed and violates the requirements for analysis by parametric statistics. The median tail moment value for each microgel was determined and the data were averaged. Averaged median values express a normal distribution according to the central limit theorem (*37*). The averaged median tail moment values obtained from repeated experiments were used with a one-way analysis of variance test. If a significant F value of $P \leq 0.05$ was obtained, a Holm−Sidak multiple comparison versus the control group analysis was conducted (power ≥ 0.8 at $\alpha = 0.05$). Concentration−response curves were prepared for the samples, the data were regressed and SCGE genotoxic potency values were calculated. The SCGE genotoxic potency value is the midpoint of the curve within the concentration range that expressed above 70% cell viability (*36*).

Safety. Manipulations of toxic, genotoxic, and/or carcinogenic chemicals were conducted in certified stage-2 containment biological/chemical safety hoods.

Results and Discussion

Description of Water Samples. Rather than attempting to link specific byproducts to genotoxicity, the objective of this research was to compare the overall genotoxicity of recreational waters from a single tap water source that were treated with different disinfectants under different conditions. Most of the pools were a subset of a previous nitrosamine and nitramine survey (*28*). This subset included indoor pools disinfected with free chlorine, bromochlorodimethylhydantoin (BCDMH), or a combination of ultraviolet light and free chlorine. We also sampled a cold pool, a warm pool, and a hot tub from the same indoor facility, and collected indoor and outdoor samples from a pool featuring a retractable dome. A unique feature of this survey is that the makeup water for all of the pools was supplied from the same tap water source, which was also evaluated. For all samples, we determined TOC (1.2−124.8 mg/L), temperature, and total chlorine residual (Table 1).

Genotoxicity of Water Samples. Figure 1 illustrates the concentration−response curve for the SCGE genotoxicity for pool water sample S1, an indoor swimming pool disinfected with free chlorine and ultraviolet light. The concentration range is expressed both as a concentration factor and as liter-equivalents compared to the original water sample. The liter-equivalent is the volume of original water sample that would have contained the mass of organic matter with which the cells were treated. An increase in genomic DNA damage was detected in nuclei from treated cells (Figure 1B, upper right panel) as compared to the concurrent negative control (Figure 1B, lower right panel). SCGE data were analyzed in a concentration range that exhibited no acute cytotoxicity (Figure 1A). Figure 2 presents the genotoxicity concentration−response curves for all of the samples. Genotoxic potency values and the statistical analyses are presented in Table 2. To compare the genotoxicity of the individual water samples we calculated a genotoxicity index value as the reciprocal of the SCGE genotoxic potency ×1000 (Figure 3).

The data reveal a wide diversity in the levels of genotoxic response. All of the recreational pool water samples were significantly more genotoxic than the source tap water common to all pools. The lowest genotoxic response was expressed by the organics isolated from the tap water (Sample S3) at a concentration fold of 2200×, which is equivalent to the organic material isolated from 55 mL of water. The higher genotoxicity of the recreational pools compared to the tap water source could reflect prolonged disinfectant contact times. Although the total chlorine residual in the tap water

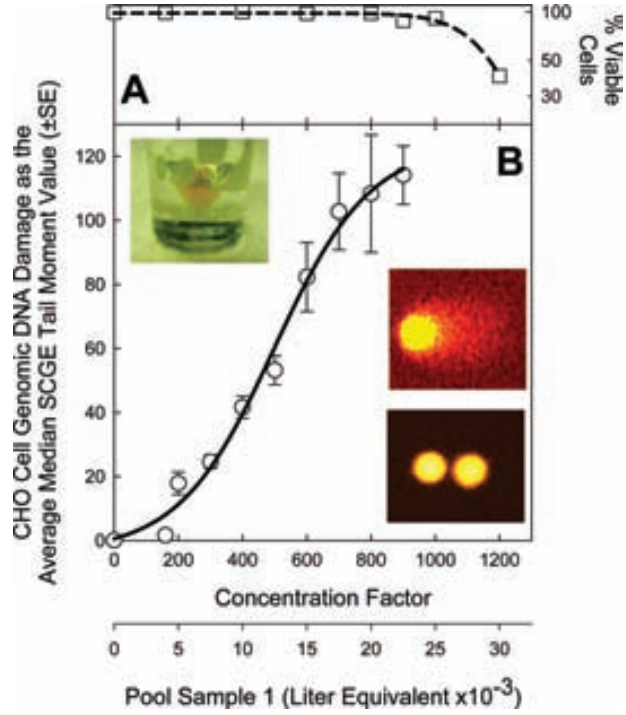

FIGURE 1. The CHO cell acute cytotoxicity concentration−response curve for pool water sample S1 is illustrated in 1A. The concentration−response curve for the SCGE genotoxicity for pool water sample S1 is illustrated in 1B. The ethyl acetate organic concentrate is illustrated in the upper left panel of 1B. Two CHO cell nuclei from the negative control are presented in the lower right panel and a nucleus from a treated cell with a medium level of DNA damage is depicted in the upper right panel of 1B. The concentration range of pool sample 1 is expressed either as a fold concentration factor or as liter-equivalents compared to the original water sample. The liter-equivalent is the volume of original water sample that contains the mass of organic matter with which the cells were treated.

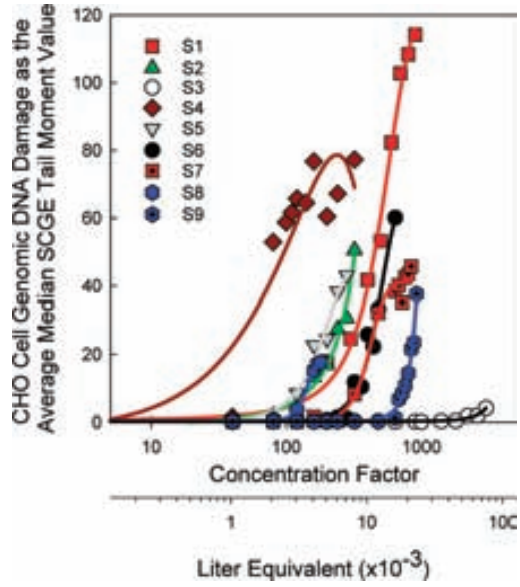

FIGURE 2. Comparison of the CHO cell acute genotoxicity concentration−response curves for the recreational pool water samples and the tap water control (S3).

TABLE 2. Induction of Genomic DNA Damage in CHO Cells by Tap Water and Recreational Water Samples

	range analyzed		lowest genotox. value[a]		SCGE genotoxicity potency[b]		R^{2c}	ANOVA test statistic[d]
sample number	conc. fold[e]	mL equiv.[f]	conc. fold	mL equiv.	conc. fold	mL equiv.		
S3	40 − 3600×	1 − 90	2200×	55	2532×	63.3	0.93	$F_{16, 43} = 5.59$; $P < 0.001$
S1	160 − 1000×	4 − 25	400×	10	486×	12.2	0.98	$F_{10, 43} = 23.8$; $P < 0.001$
S7	40 − 840×	1 − 21	480×	12	421×	10.5	0.98	$F_{14, 45} = 22.5$; $P < 0.001$
S4	40 − 320×	1 − 8	40×	1	71×	1.8	0.93	$F_{10, 45} = 20.0$; $P < 0.001$
S5	40 − 280×	1 − 7	160×	4	173×	4.3	0.95	$F_{7, 46} = 39.3$; $P < 0.001$
S6	40 − 640×	1 − 16	440×	11	461×	11.5	0.96	$F_{13, 46} = 15.1$; $P < 0.001$
S2	40 − 320×	1 − 8	160×	4	252×	6.3	0.96	$F_{8, 39} = 32.7$; $P < 0.001$
S8	40 − 180×	1 − 4.5	160×	4	138×	3.8	0.98	$F_{6, 43} = 17.1$; $P < 0.001$
S9	40 − 920×	1 − 23	760×	19	834×	20.8	0.98	$F_{16, 43} = 14.6$; $P < 0.001$

[a] The lowest genotoxic value in the concentration−response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [b] The SCGE genotoxic potency is the value that was calculated, using regression analysis, at the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated CHO cells. [c] R^2 is the coefficient of determination for the regression analysis upon which the genotoxic potency value was calculated. [d] The degrees of freedom for the between groups and residual associated with the calculated *F*-test result and the resulting probability value. [e] The concentration fold is the factor by which the sample was concentrated. [f] The mL equivalent is the number of mL of the original water sample that contains the mass of organic material extracted.

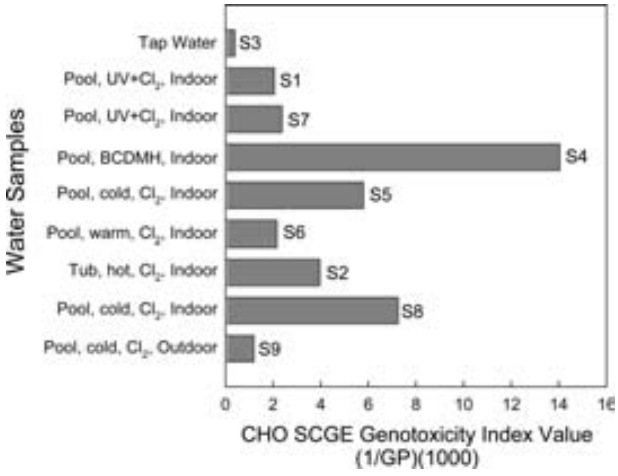

FIGURE 3. Comparison of the CHO genotoxicity index values for the recreational pool water samples and the tap water control.

(1.4 mg/L as Cl_2; Table 1) was comparable to those of pool samples S7, S8, and S9, the disinfectant contact time with the tap water was on the order of 1 week, while in recreational pools residence times are on the order of months. A second factor in the elevated genotoxicity of recreational waters could reflect different organic matter precursors contributed by swimmers. Compared to carbon-rich humic substance precursors in many drinking water supplies, precursors contributed by urine, sweat, hair, skin, and consumer products are often nitrogen-rich.

Important differences were observed among pools based upon disinfectant type and environmental conditions. The most genotoxic sample was S4 (Figure 3; Table 2). This indoor swimming pool was the only one in this study disinfected with bromochlorodimethylhydantoin (BCDMH). Based on lowest genotoxic response, this pool was 4 times more potent than any other waters with a concentration factor of 40× or a 1 mL-equivalent. BCDMH reacts slowly with water releasing hypobromous (HOBr), hypochlorous acid (HOCl) and the nitrogen- containing dimethylhydantoin (*38*). Both HOCl and HOBr disinfect the water with a release of Cl- or Br- as products. More HOBr is produced from the reaction of Br- and HOCl. Reaction of HOCl, HOBr and organic precursors, including nitrogen-rich precursors in the swimming pool may lead to the formation of brominated and chlorinated DBPs as well as N-DBPs. Previous studies have demonstrated that brominated and N-DBPs are far more cytotoxic and genotoxic than their chlorinated analogues (*12, 15*). Dimethylhydantoin was not listed as a genotoxin by the California EPA (http://www.cdpr.ca.gov/docs/risk/toxsums/pdfs/2080.pdf); too little information was available to make a determination for BCDMH. BCDMH or dimethylhydantoin may have accumulated within the pool, as this pool featured an extremely high TOC (125 mg/L). The MtBE extraction procedure likely captured these organics as white crystals precipitated out during the rotary evaporative concentration of the extract. Although BCDMH may have been extracted, we chose the liquid−liquid extraction procedure with MtBE without quenching of the disinfectant as a model of the total genotoxicity of the water experienced by bathers.

Illumination conditions also affected genotoxicity. Samples were taken from a single recreational pool that was enclosed during cold weather (S8) and opened during warm weather (S9). Under indoor conditions (S8) the lowest genotoxic response was 160× (4 mL-equivalent). Under outdoor conditions (S9) the genotoxicity was reduced to 760× or 19 mL-equivalent (Table 2, Figure 3). Despite similar TOC and total chlorine residual, the genotoxic potency of the pool was 5× lower when there was solar exposure, and potentially, increased volatilization.

UV disinfection of indoor pools modified genotoxicity. Despite featuring comparable levels of TOC and total chlorine residual, the genotoxic potency of the pools disinfected with free chlorine alone (S5 and S8) were ∼3× higher than the pool disinfected with the free chlorine and UV combination (S1 and S7). Solar and UV radiation may photolyze DBPs. For example, nitrosamine concentrations were ∼5× lower in outdoor than in indoor pools (*28*).

The sample size of pools with the same disinfectant and environmental conditions was insufficient to develop statistically significant associations between genotoxicity and total residual chlorine, temperature, or TOC. Although samples S1 and S7, collected at different times from the same pool exhibited comparable genotoxicity, sample S1 featured TOC and total residual chlorine concentrations more than 2× higher than sample S7. Samples were collected from a cold pool (S5), a warm pool (S6), and a hot tub (S2) at the same facility. The sample from the warm pool exhibited lower genotoxicity, despite possessing the highest TOC and total residual chlorine concentrations. Analysis of a larger number of pools featuring different temperatures and TOC values may reveal correlations between temperature, TOC and genotoxicity.

There are few studies on the genotoxicity of concentrates derived from recreational pool waters. Organics from a chlorinated swimming pool were extracted using XAD-2 resin and eluted with three different solvents. All three extracts were directly mutagenic in *Salmonella typhimurium* TA100 demonstrating a diversity of mutagens in the organic material (*39*). The mutagenic responses (5−10 mL-equivalents) were similar to those in this study.

In another study chlorinated indoor pool water was fractionated and examined for genotoxicity in Hep-G2 cells (*40*). Increased genomic DNA damage was observed for the <0.2 kDa fraction and for the highest concentration of the 0.2−1 kDa fraction. No genotoxicity was observed in the >1 kDa fraction. These results are intriguing because in disinfected drinking water most of the unknown total organic halogen (TOX) resides in the molecular size range of 0.5−10 kDa (*41*) and genotoxicity was found in drinking water fractions up to 5 kDa (*42*).

A recent study from a single pool in Barcelona, Spain, found that the amounts of DBPs and the mutagenic potency of the water (*S. typhimurium*) were similar to those of drinking water (*43*). Evaluation of swimmers showed that swimming produced mutagenic urine assayed with *S. typhimurium* and micronuclei in exfoliated urothelial cells (*44*). This genotoxicity was associated with the levels of brominated trihalomethanes in the pool water, supporting a possible role of brominated DBPs for the increased risk of bladder cancer among swimmers (*11*).

Our study represents the first systematic genotoxicity analysis with mammalian cells to evaluate different recreational waters derived from a common tap water source. The data demonstrated that all disinfected recreational pool water samples were more genotoxic than the source tap water. As trihalomethane concentrations are similar between tap waters and pool waters (*25*−*27*), the practice of using trihalomethanes to monitor exposure in epidemiological studies may need re-examination. The type of disinfectant and illumination conditions altered the genotoxicity of the water. Accordingly, care should be taken in the disinfectant employed to treat recreational pool water. The genotoxicity data suggest that brominating agents should be avoided as disinfectants of recreational pool water. Combining UV treatment with chlorine may be beneficial when compared to chlorination alone.

Our experimental design focused on sampling pools supplied by the same tap water, and exposed to a wide variety of disinfectant and environmental (e.g., sunlight, temperature) conditions. While this design was suited to distinguishing some of the most important determinants in pool water genotoxicity, the sample size within pool types was insufficient to draw firm conclusions regarding the importance of other factors, including precursor loading (as measured by TOC) and temperature. It is possible that a reduction in TOC may reduce the level of toxic byproducts in pool waters. Some TOC reduction may be achieved by reducing cosmetics and personal care products, some of which can be converted into mutagens after chlorination (*45*). During the recycling of the pool water the organic carbon could be removed by materials such as granulated activated carbon prior to disinfection. Behavior modification by swimmers may be critical in reducing the genotoxicity of pool water. Actions such as requiring showering before entering the water and informing patrons about the potential harm from urinating in a pool could reduce the precursors of toxic DBPs.

Acknowledgments

This research was supported by grants from the National Science Foundation (CBET-0651732 and CBET-0651333). We appreciate the support by the Center of Advanced Materials for the Purification of Water with Systems, National Science Foundation Science and Technology Center, under Award CTS-0120978. D.L. was supported in M.J.P.'s laboratory from a postgraduate fellowship from the Universitat Autònoma de Barcelona.

Supporting Information Available

Information about single cell gel electrophoresis assay and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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ES903593W

ANEXO 5

Genotoxic effects in swimmers exposed to disinfection by-products in indoor swimming pools

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Environmental and Health Perspectives (enviado)

Genotoxic Effects in Swimmers Exposed to Disinfection By-products in Indoor Swimming Pools

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Running title: Genotoxicity in swimmers of a chlorinated pool

Key Words: swimming pools, water, chlorination, disinfection by-products, cancer, genotoxicity, mutagenicity, genetics

Acknowledgments: We thank Lourdes Arjona for assistance with the study. Research supported by Plan Nacional Grant SAF2005-07643-C03-01/02/03, Spain and FIS CP06/00341, Spain. CM Villanueva supported by the ISCIII (CP06/00341), Spain, L Font-Ribera by a predoctoral fellowship (FI06/00651), Spain, and D Liviac by a postgraduate fellowship UAB (PIF409-009), Barcelona. This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Competing Interests: None of the authors has any competing interests with regard to this work.

List of Abbreviations and Definitions

$CHBr_3$	Bromoform
$CHCl_2Br$	Bromodichloromethane
$CHCl_3$	Chloroform
$CHClBr_2$	Chlorodibromomethane
DBPs	Disinfection by-products
GSTT1	Glutathion-transferase theta-1
MN	Micronucleus
OTM	Olive tail moment
PBL	Peripheral blood lymphocytes
SCGE	Single Cell Gel Electrophoresis (Comet Assay)
SD	Standard deviation
THMs	Trihalomethanes

Outline

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Abstract

BACKGROUND: Exposure to disinfection by-products (DBPs) in drinking water has been associated with cancer risk. A recent study found an increased bladder cancer risk among subjects attending swimming pools.

OBJECTIVES: To evaluate whether swimming in pools is associated with biomarkers of genotoxicity.

METHODS: We collected blood, urine, and exhaled air samples from 49 non-smoking adult volunteers before and after they swam for 40 min in an indoor chlorinated pool. We compared the concentrations of four trihalomethanes in exhaled breath to the following biomarkers: micronuclei and DNA damage (comet assay) in peripheral blood lymphocytes before and 1 h after swimming, urine mutagenicity (Ames assay) before and 2 h after swimming, and micronuclei in exfoliated urothelial cells before and 2 weeks after swimming. We determined genetic polymorphisms in genes related to DNA repair or DBP metabolism.

RESULTS: After swimming, the total concentration of the four trihalomethanes in exhaled breath increased sevenfold. The frequency of micronucleated lymphocytes increased relative to the exhaled concentrations of the brominated trihalomethanes (p = 0.03 for CHCl2Br, p = 0.05 for CHClBr2, p = 0.01 for CHBr3) but not chloroform. Swimming caused no DNA damage detectable by the comet assay. Urine mutagenicity increased significantly after swimming relative to the concentration of exhaled CHBr3 (p = 0.004). No significant increase was seen for micronucleated urothelial cells.

CONCLUSIONS: Our findings support potential genotoxic effects of exposure to DBPs from swimming pools. The positive health effects gained by swimming could be increased by reducing the potential health risks of pool water.

Introduction

Swimming in pools is an important recreational activity followed by hundreds of millions of people worldwide that has been associated with significant positive health benefits (Zwiener et al. 2007). Hygiene and water quality, especially infections caused by feces-associated microbes and protozoa, have been a priority for regulators and researchers (WHO 2006). However, concerns have been raised regarding potential adverse health effects resulting from exposure to chemically disinfected swimming pool water (Zwiener et al. 2007).

As with drinking water, chlorination is the most common method of disinfection for swimming pools. The addition of chlorine to water results in the formation of hundreds of chlorination by-products due to the presence of organic matter (Richardson et al. 2007). Levels of disinfection by-products (DBPs) in swimming pool water are not necessarily higher than those in drinking water (Richardson SD et al., submitted). Swimming in an indoor pool, however, leads to a high uptake of compounds such as trihalomethanes (THMs), which are inhaled and absorbed by the skin (Font-Ribera L et al., submitted; Whitaker et al. 2003; Xu and Weisel 2004, 2005). High levels of haloacetic acids also have been reported in swimming pools; however, these DBPs are likely not taken up at significant levels because they are non-volatile, and uptake occurs mainly through ingestion. Another chemical class identified recently in chlorinated pools is nitrosamines (Walse and Mitch 2008), but their uptake via swimming has not been studied.

Epidemiological studies have shown that long-term consumption of chlorinated water and exposure to THMs at levels found currently in drinking water in many industrialized countries are associated with an increased risk of bladder cancer (Villanueva et al. 2004). A large study on bladder cancer in Spain was the first to examine exposure to THMs through ingestion of water and through inhalation and dermal absorption during showering, bathing, and swimming in pools (Villanueva et al. 2007). A twofold bladder cancer risk was found for household THM levels >49 µg/L versus <8 µg/L. The risks associated with tasks resulting in high exposure via inhalation and dermal absorption were higher than those for ingestion. In the same study, an increased risk was found for subjects attending swimming pools (odds ratio of 1.6, 95% CI 1.2 to 2.1).

Several DBPs, including some THMs, are genotoxic and as reviewed by Richardson et al. (2007), all four regulated THMs (chloroform, bromoform, bromodichloromethane, and chlorodibromomethane) are carcinogenic in rodents. Chloroform is not mutagenic; however, the brominated THMs are, and they are activated to mutagens by GSTT1-1 (DeMarini et al. 1997b; Pegram et al. 1997). Bromodichloromethane induced mutagenic urine in humans (Leavens et al. 2007). All but chlorodibromomethane induce DNA damage detected by the comet assay, and bromodichloromethaen and bromoform induce micronuclei (MN) (Richardson et al. 2007). Extensive quantitative testing of the mutagenic and genotoxic potency of DBPs has shown that iodinated compounds are generally more toxic than brominated DBPs, and DBPs that are both iodinated and brominated are more genotoxic than chlorinated DBPs (Richardson et al. 2007).

Metabolism of DBPs is mediated by enzymes from the GST and CYP families. An evaluation of polymorphisms in *GSTT1-1*, a gene involved in the metabolism of brominated THMs, indicated that subjects with functioning *GSTT1-1* (+/+ or +/- genotypes) were at significantly higher risk than subjects with deletions in both alleles (-/-) (Cantor et al. 2006). This was consistent with early studies showing that GSTT1-1 activated the brominated THMs, but not chloroform, to mutagens in a transgenic strain of *Salmonella* (DeMarini et al. 1997b; Pegram et al. 1997). *GSTZ1* catalyzes the oxygenation of dichloroacetic acid (DCA) to glyoxylic acid and plays a critical role in the tyrosine degradation pathway and in alpha-haloacid metabolism (Board et al. 2005). *CYP2E1*, *CYP1A2*, *CYP3A4,* and *CYP2A6* have been involved in the metabolism of chloroform and bromodichloromethane (Allis et al. 2002; Gemma et al. 2003; Leavens et al. 2007; Zhao et al. 2002), and *CYP2D6* has been found to modify THM blood levels after showering (Backer et al. 2007). The activation of bromodichloromethane by *GSTT1-1* produces 8-oxoguanidine DNA adducts, a mark of DNA oxidative stress (Ross et al. 2004), that are repaired by base-excision repair (BER) through the action of genes such as *OGG1*, *APEX1,* and *XRCC1*. There exists some evidence that genetic variants of *XRCC1,* which is part of nucleotide-excision repair (NER), influences MN formation, as might the *ERCC2* gene (Iarmarcovai et al. 2007). On the other hand, chloroform exposure results in increased expression of *APEX1* in rat liver (Zidek et al. 2007). Finally, these DNA repair genes have been associated with risk for bladder cancer, which is the main cancer produced by DPB exposure.

Genotoxicity evaluations are used extensively in studies of health effects of environmental exposures. These assays can be carried out using easily obtainable human cells, such as peripheral blood lymphocytes and exfoliated urothelial cells from urine, and have been used to evaluate the genotoxicity of DBPs (Ranmuthugala et al. 2003; Richardson et al. 2007; Liviac et al. 2009). Biomarkers detecting primary DNA damage include the comet assay, which detects single- and double-strand breaks, alkali-labile sites, and transient DNA repair breaks (Dusinska and Collins 2008). Primary DNA damage can be repaired easily; thus, it is necessary to use also biomarkers of fixed damage, which is probably more relevant for human risk assessment. In this context, the MN assay is a well-validated methodology that provides a measure of both chromosome breakage and chromosome loss, and it has been shown to be a relevant biomarker for cancer risk (Bonassi et al. 2007).

Urinary mutagenicity has been examined in occupational and environmental settings for more than three decades as a general cost-efficient assessment of systemic genotoxicity (Cerná and Pastorková 2002). Although several experimental studies have evaluated the mutagenicity of DBPs, only one study has evaluated the effect of a DBP on urine mutagenicity in humans in a controlled dermal and oral exposure (Leavens et al. 2007). The authors found that urine mutagenicity levels increased after exposure to bromodichloromethane, particularly among subjects exposed percutaneously compared to orally.

To evaluate the genotoxicity of swimming pool water in swimmers, we examined the above-mentioned biomarkers of genotoxicity in an experimental study in which adults swam for 40 min in a

chlorinated, indoor swimming pool. We compared the biomarker results to an internal measure of exposure, i.e., the concentrations of four THMs in exhaled breath as determined in a companion study (Font-Ribera L et al., submitted). We also evaluated the impact of genotype on the biomarker responses relative to THM exposure.

Methods

Main design. Fifty non-smoking volunteers aged 18 to 50 years were recruited through open advertisements on the Internet and at local universities, avoiding any direct personal contact, e.g., E-mailing because this is prohibited in research centers in Spain. A screening questionnaire was used to verify eligibility. Compensation was provided to subjects, who signed an informed consent form that acknowledged this compensation. A single, indoor, 25-m long chlorinated swimming pool in Barcelona, Spain, was selected for the study. One to four volunteers participated daily during May, June, September, and October 2007. The main design of the study is shown in Figure 1.

Subjects were requested not to swim for one week prior to the swimming experiment. Subjects were asked to swim for 40 min in the pool, and the timing of swimming and distance swam were measured for each individual. We selected 40 min based on our estimate of the usual time that non-competitive swimmers swim. Biological samples (exhaled breath, blood, and urine) were collected both before and after swimming at specified time periods (Font-Ribera L et al., submitted), and questionnaires were completed. THMs in exhaled breath were measured within a few minutes after swimming and before taking a shower. Blood samples were collected on average 1 h after swimming, urine samples were collected on average 2 h after swimming, and a second urine sample was collected 2 weeks after swimming for the micronuclei analysis in exfoliated urothelial cells. One subject failed to complete adequately the procedures for the measurement of THMs in exhaled breath and was excluded, leaving 49 subjects for the final analysis. All laboratory analyses were blind concerning pre- or post-swimming. The study protocol was approved by the ethics committee of the research centre.

Questionnaire. Subjects completed a self-administered questionnaire that included information on socio-demographics (e.g., age, education, occupation, basic residential and commuting information), detailed water-related habits (e.g., fluids ingestion, showers, baths, swimming pools), physical activity, medical history and drugs, lifestyle (e.g., past smoking, second-hand smoke, use of hair dyes), a food-frequency questionnaire validated in the Spanish population, and a short 24-h activity and recent-disease questionnaire. Measurement of physical activity during swimming was done indirectly by recording each individual's swimming pattern (number of laps).

Exposure assessment. A detailed description of measurements of THMs in air and exhaled breath before and after swimming is reported in a companion paper (Font-Ribera L et al., submitted), along with determinations of DBPs in the water and water mutagenicity (Richardson SD et al., submitted). THMs in air and water were determined at each swimming session following a specific protocol. Exhaled breath has distinct advantages for the assessment of THM intake. It is non-invasive and provides a

representative estimate of the concentration of contaminants in blood due to the gas exchange in the blood/breath interface in the lungs. Air and exhaled breath involved solid-phase adsorption on Tenax TA. Exhaled breath samples were collected using a portable system that consisted of a Haldane-Priestley tube modified to concentrate aliquots of exhaled breath from one or more exhalations. Water samples were analyzed with a purge-and-trap concentrator equipped with a Tenax® silica gel-charcoal trap.

Comet analysis in peripheral blood lymphocytes. The comet assay was performed as described previously (Singh et al. 1988) with minor modifications. Blood samples were collected in vacutainers with EDTA. Samples were kept chilled, and the length of time between blood collection and sample processing was a few hours (Anderson et al 1997). A drop (7 µL) of whole blood was resuspended in 75 µL of 0.5% low-melting-point agarose, layered onto microscope slides pre-coated with 150 µL of 0.5% normal-melting-point agarose (dried at 65ºC), covered with a coverslip, and kept at 4ºC until solidified. Then the coverslips were removed, and the cells were lysed overnight at 4ºC in a dark chamber containing fresh, cold lysing solution (2.5-M NaCl, 100-mM Na_2EDTA, 10-mM Tris, 10% DMSO, 1% Triton X-100, and 1% laurosylsarcosinate, pH 10). To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 20 min in a horizontal gel electrophoresis tank filled with fresh, cold electrophoresis solution (1-mM Na_2EDTA and 300-mM NaOH, pH 13.5). Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. Unwinding and electrophoresis steps were done in an ice bath.

After electrophoresis, slides were neutralized with 2, 5-min washes with 0.4-mM Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, slides were stained with 60 µL of ethidium bromide (0.4 µg/mL). The images were examined at 400X magnification with a Komet 5.5 Image Analysis (Kinetic Imaging Ltd, Liverpool, UK) fitted with a Olympus BX50 fluorescent microscope equipped with a 480-550-nm wide band excitation filter and a 590-nm barrier filter. One hundred cells selected randomly (50 cells from each of the two replicate slides) were analyzed per sample. Olive tail moment (OTM) and percentage of DNA in the tail were used as measures of DNA damage and computed using Komet Version 5.5 software.

MN analysis in peripheral blood lymphocytes. Blood was obtained from each subject by venipuncture using heparinized vacutainers and sent immediately to the laboratory for the lymphocyte cultures. Lymphocyte cultures were set up by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2-mM L-glutamine (all provided by PAA Laboratories GmbH, Pasching, Austria). Lymphocytes were stimulated by addition of 1% phytohaemagglutinin (Gibco, Life Technologies, Paisley, UK) and incubated for 72 h at 37°C. Two replicate cultures were prepared for each blood sample. Cytochalasin B (Cyt-B) (Sigma, St Louis, MO) at a final concentration of 6 µg/mL (Surrallés et al. 1994) was added to the cultures after a 44-h incubation to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 800 rpm for 8 min. Next, in order to eliminate red blood cells and to preserve cytoplasm, the cell pellet was treated with a hypotonic solution (7 min in 0.075-M KCl at 4°C).

Cells were then centrifuged, and a methanol/acetic acid (3:1 v/v) solution was added gently. This fixation step was repeated twice, and the resulting cells were re-suspended in a small volume of fixative solution and dropped onto clean slides. Finally, they were stained with 10% Giemsa (Merck, Darmstadt, Germany) in phosphate buffer, pH 6.8, for 7 min.

To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of micronuclei, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. In addition, 500 lymphocytes were scored to evaluate the percentage of cells with one to four nuclei, and the cytokinesis block proliferation index (CBPI) was calculated (Surrallés et al. 1995). Microscopic scoring was performed on coded slides.

MN analysis in urothelial cells. Urine samples were collected before swimming and again 2 weeks later in plastic vials (~50 mL) and sent to the laboratory where they were processed the same day. Two weeks was selected because this amount of time is required for exfoliation of cells from the urothelium (Espinoza et al. 2008). Cell samples were concentrated by centrifugation (10 min at 1500 rpm), the supernatant was discarded, and cells were washed in a NaCl solution (0.9%). Cells were centrifuged again and re-suspended in a new NaCl solution (0.09%). After another centrifugation, cells were fixed in 5 mL of a fresh fixative solution (methanol/acetic acid, 3:1), added drop by drop. After 1-2 washings with the fixative solution, the pellet was re-suspended in 1 mL of fixative solution and dropped onto pre-cleaned microscope slides. Cell density was confirmed by using a phase-contrast microscope (400 x) and adjusted by adding fixative solution. Slides were air dried overnight and stored at 4ºC in the dark until staining. Cells were stained with the DNA-specific stain 4',6-diamino-2-phenylindole dihydrochloride (DAPI) at 1 µg/mL, which avoids possible scoring artifacts. One scorer using an Olympus BX50 fluorescence microscope (1000 x) scored a total of 2000 cells/donor, wherever possible, on coded slides.

The criteria for MN evaluation were those suggested by Stick et al. (1983) as updated by subsequent guidelines by Fenech et al (2003). The frequency of cells with MN and the total number of MN were determined for each analyzed subject. Only those cells with a typical morphology corresponding to the urothelial cells were scored. This criterion avoids any kind of bias, especially in women where many squamous cells not of urothelial origin are observed. Although bacteria were present in a few urine samples, they did not interfere with the scoring.

Urine mutagenicity. Urine samples (30 mL) were collected prior to and 90 to 120 min after exposure and were evaluated for mutagenicity in the *Salmonella* (Ames) mutagenicity plate-incorporation assay (Maron and Ames 1983) in strain YG1024 with S9 mix. YG1024 is a frameshift strain derived from TA98 (*hisD3052, ΔuvrB, rfa,* pKM101) that contains acetyltransferase activity (Watanabe et al. 1990); it has been used extensively for urinary mutagenicity studies (Cerná and Pastorková 2002). Only one strain was used because of the limited availability of sample.

To extract urinary organics, 25 ml of urine was passed through C18 resin, and the organics were eluted by methanol and then solvent exchanged into dimethyl sulfoxide (DMSO) at 150x for bioassay as described (DeMarini et al. 1997a). Extracts were evaluated once in single plates per dose at 0.3, 0.6, 1.2,

3, 6, and 12 mL-equivalents per plate. Controls consisted of DMSO (100 µl per plate), C18 resin blanks prepared by passing 40 mL of distilled deionized water instead of urine through the columns (15 mL equivalent per plate), and 2-acetylaminiofluorene at 1 µg per plate (positive control). Control values (revertants per plate) were 9–14 (DMSO), 5–10 (resin blanks), and 377–595 (2-acetylaminofluorene). The mutagenic potencies of samples, expressed as revertants (rev) per mL-equivalent, were calculated from the slope of the regression over the linear portion of the dose-response curves. Slopes were calculated for only 43 subjects who met our criteria of having at least 4 points of observation (blank and at least 3 doses) both before and after swimming.

Gene selection and genotyping. Blood was collected in EDTA tubes and stored in the same tube at -80ºC for DNA extraction. DNA was extracted using the Chemagic Magnetic Separator Technology (Chemagen) at the Spanish National Genotyping Centre (CEGEN) and quantified using PicoGreen dsDNA fluorescent detection system (PicoGreen, Molecular Probes). We examined genetic variants, including single-nucleotide polymorphisms (SNPs), and copy-number variants (CNVs) in three genes involved in the metabolism of DBPs (*GSTT1-1, CYP2E1,* and *GSTZ1),* four additional genes that may play a minor role in the metabolism of DBPs (*GSTT2B, GSTM1, CYP1A2* and *CYP2D6*), and four DNA repair genes that could be relevant when examining results, particularly for the comet assay (*APEX1, ERCC2, OGG1, XRCC1*). We selected tagSNPs combined with functional variants most likely to influence gene expression or function. In total, three CNVs and 20 SNPs were genotyped. For a complete list see Supplementary Tables 1-4.

SNP genotyping was performed at the Santiago de Compostela node of the "Centro Nacional de Genotipado" (CEGEN) in Spain (http://www.cegen.org) using the Sequenom® platform. Individuals with low genotyping frequency (<75%) or non-Caucasians were excluded from the genetic analyses (n = 4). Genotyping failed for the following SNPs: rs11101815 and rs915908 in *CYP2E1*, rs1799793 in *ERCC2,* and rs28903081 in the *XRCC3* gene. The remaining 16 SNPs had a call frequency >90%. Genotyping quality was controlled by using positive controls consisting of one HapMap reference trio. CNVs for *GSTT1-1*, *GSTT2B,* and *GSTM1* were genotyped as described previously with minor modifications (Buchard et al. 2007; Zhao et al. 2009). Briefly, the genotyping consisted of multiplex PCR amplifications, where deleted and non-deleted alleles gave different fragment sizes that were resolved in agarose gels. The CNV call frequency was >90%. Genotyping was repeated for selected samples and give consistent genotypes.

Statistical analysis. Paired t-tests were used to examine changes in the level of biomarkers before and after swimming. These analyses are unadjusted for exposure and potential confounders. Associations between exposure to THMs measured in exhaled breath after swimming and changes in markers of genotoxicity before and after swimming were evaluated using linear regression. Beta-coefficients estimate the change in the biomarker measured in relation to a 1 µg/m^3 increase in total or specific THM concentration in exhaled breath. All analyses were adjusted for age and sex. Several other variables were included in the models to evaluate potential confounding effects, including water

consumption, source of water, antioxidant intake from diet, number of laps swum during the experiment (an indication of physical activity), and leisure physical activity (see list in Supplemental Figure 1). Because results were modified only marginally by the inclusion of these variables in the models, results shown are adjusted for only age and sex. In order to estimate the amount of variance in an endpoint due to the concentration of THMs in exhaled breath, we used unadjusted models to calculate the r^2 values.

We also tested for genotype deviations from Hardy-Weinberg equilibrium (HWE) (Wigginton et al. 2005). Analysis of single-marker effect was performed assuming both a dominant and an additive (not shown) genetic model, considering the most frequent allele as a reference category, and using logistic regression implemented in the SNPassoc package (version 1.5-1) from R statistical software (Version 2.6.1) (R Development Core Team 2007).

Results

Among the 50 subjects, 66% were women, 96% were Caucasian, most were highly educated, and by selection criteria, all were non-smokers, with approximately one-third being ex-smokers (Table 1). A high percentage was exposed regularly to second-hand smoke. About half did regular sports (once/week), and 11 (22%) swam at least once/month. As reported by Font-Ribera L (submitted), the average free chlorine level in the pool water was 1.17 mg/L (standard deviation, SD = 0.4), and the average total THM levels were 45.4 µg/L (SD = 7.3). In pool air the average total THM levels were 74.1 µg/ m^3 (SD = 23.7). In exhaled breath, THM levels increased, on average, about 7 times during swimming. The average total THM levels after swimming were 7.9 µg/m^3, and the average levels for the individual THMs were 4.5 µg/m^3 for chloroform, 1.78 µg/m^3 for bromodichloromethane, 1.2 µg/m^3 for chlorodibromomethane, and 0.5 µg/m^3 for bromoform (tribromomethane).

The average number of binucleated cells with MN in peripheral blood lymphocytes per 1000 binucleated cells increased non-significantly from 3.4 before swimming to 4.0 after swimming (Table 2). Likewise, the average frequency of MN in urothelial cells and the level of urinary mutagenicity also increased non-significantly after swimming relative to before swimming. In contrast, we observed a small but statistically significant decrease in the average amount of DNA damage in blood lymphocytes after swimming relative to before swimming (Table 2).

In the multivariate analysis, we associated the change in the frequency of MN in peripheral blood lymphocytes before and after swimming with the combined concentration of all four THMs measured in exhaled breath. Using this exposure metric, we found that there were 0.296 more MN per each 1 µg/m^3 increase in total THMs in exhaled breath; however, this increase was not significant (p = 0.09) (Table 3). When we analyzed the MN data with respect to the concentrations of the individual THMs in exhaled breath, the largest increases were observed for the brominated THMs (Table 3), and statistically significant increases in MN frequency were observed for bromodichloromethane (beta = 1.9 MN per 1000 cells per 1 µg/m^3, p = 0.03) and bromoform (beta = 5.04 MN per 1000 cells per 1 µg/m^3, p = 0.01). Based on the r^2, the fraction of the variance in changes in MN frequency that was explained by exposure to

bromodichloromethane was 10%; for bromoform this was 13%. Adjustment for several potential confounding factors resulted in minor changes in the results as compared to those when adjusting only for age and sex. Supplementary Figure 1 shows results for the main model (adjusted for age and sex) for total THMs and also for models with consecutive adjustment of 12 other variables. Adjustment for the number of laps swum during the experiment as a measure of physical activity resulted in a similar beta.

No associations were observed between total or individual THM concentrations in exhaled breath and the level of DNA damage in peripheral blood lymphocytes as assessed by the comet assay regardless of whether the results were expressed by OTM (Table 3) or the percent of DNA in the tail (data not shown).

In urine samples collected before swimming and 2 weeks after the experiment, the average MN frequency in exfoliated urothelial cells increased only in relation to exposure to $CHClBr_2$ (beta = 2.4) and $CHBr_3$ (beta = 4.3 MN per 1000 cells per 1 $\mu g/m^3$), but the results were not statistically significant (Table 3).

An increase in urine mutagenicity, measured as an increase in the slope of the dose-response curve (mutagenic potency) before and approximately 1.5 h after swimming, was observed for the combined concentration of the four THMs in exhaled breath as well as for the concentration of each individual THM (Table 3). However, these increases were statistically significant only for bromoform (beta = 5.27, p = 0.004). Based on the r^2, the fraction of the variance in change in urine mutagenicity that was explained by exposure to bromoform was 16%.

A potential effect modification of THMs by genetic variation included specific genes involved in the metabolism of DBPs or in DNA repair. Results of an interaction between exposure to bromoform (the THM showing the most significant associations with the effect biomarkers) and genetic polymorphisms in the three main genes associated with metabolism of DBPs are shown in Table 4. Results for all genes examined are shown in Supplementary Tables 1, 2, 3 and 4.

For *GSTT1-1*, which activates brominated THMs to mutagens, subjects with the null *GSTT1-1* genotype (-/-, a deletion in both copies of the gene) did not have significantly different frequencies of MN in blood (beta=7.8) or urothelial cells (beta = -5.2) or mutagenic potency of urine (beta = 1.9) compared to those with one (+/-) or none (+/+) of the copies being deleted (beta=3.4, beta = 1.7 and beta = 7.6, respectively) (Table 4). Statistically significant interactions were found for *GSTZ1* (rs3177427) for MN in blood and for *CYP2E1* (rs915906) for MN in urine (Table 4). Among the other genes examined, statistically significant interactions were found for *GSTT2B*, and *APEX1* for MN in blood (Supplementary Table 1) and for *GSTM1* for MN in urine (Supplementary Table 2). No statistically significant interactions were found for urine mutagenicity (Supplementary Table 3) or for the DNA repair genes for the comet assay (Table 4 and Supplementary Table 4).

Discussion

This is the first study of the genotoxicity of exposure to DBPs among swimmers in a chlorinated pool. Biomarkers of genotoxic effects have been used extensively to evaluate potential health effects of environmental exposures, and the MN assay has been shown to be a predictive biomarker of cancer risk within a population of healthy subjects (Bonassi et al. 2007). We identified increased responses in biomarkers of genotoxicity (MN in blood and urinary mutagenicity) after swimming relative to the concentration of brominated THMs in exhaled breath, and we found no associations between any of the biomarkers and the concentration of chloroform in exhaled breath, consistent with the fact that chloroform is not genotoxic (Richardson et al. 2007). There was no association between exposure to DBPs in the pool and DNA damage in the blood as measured by the comet assay. Results were not dependent on confounding factors. There was some indication that response to these compounds may be affected by genetic variation in genes shown to metabolize these compounds, but the study had low power to evaluate gene-environment interactions.

The four THMs we evaluated are the most common DBPs in swimming pool water (Richardson SD et al., submitted). Although the THMs are not considered to be the most toxic of the DBPs, all four are carcinogenic in rodents (Richardson et al. 2007). The brominated THMs have been shown to be mutagenic after activation by *GSTT1-1,* and some of them induce chromosomal aberrations, sister chromatid exchanges, and/or MN (IARC 1999; Richardson et al. 2007). In contrast, chloroform is not genotoxic (Richardson et al. 2007), and unlike the brominated THMs, it is not activated by *GSTT1-1* (Pegram et al. 1997). Thus, our finding of an association between exposure to brominated THMs and an increased response among various genotoxicity biomarkers, but the absence of such an association with chloroform exposure, is consistent with the toxicology of these THMs.

Our results are also consistent with extensive quantitative genotoxicity data on DBPs showing that brominated DBPs are generally more genotoxic and carcinogenic than chlorinated DBPs (Richardson et al. 2007; Plewa et al. 2008). In our study, we evaluated only THMs that are known to be the most common DBPs in swimming pool water and that exhibit high uptake by swimmers (Zwiener et al. 2007). Levels of haloacetic acids can be high in swimming pools; however, the uptake of these DBPs may be low because the haloacetic acids are not volatile and are not adsorbed efficiently by the skin (Xu et al. 2002). Among the chemical classes of DBPs, the rank order of the combined cytotoxicity and genotoxicity in CHO cells was halonitromethanes > haloacetamides > haloacetonitriles > haloacetic acids > halomethanes (Richardson et al. 2007). Future studies of swimmers should evaluate more completely the uptake and potential effects of a range of DBPs and other compounds present in pool water.

Evaluation of the modification of the environmental exposure by genetic polymorphisms was of low statistical power given the relatively small sample size, and interpretation of these findings should be done with caution. The main hypotheses focused on a potential modification of the effect by a few genes (*GSTT1-1*, *GSTZ1*, *CYP2E1*) that have been shown to code for enzymes that are important in the metabolism of DBPs (Richardson et al. 2007). Similar to what has been shown for mutagenesis in

bacteria and DNA adducts in rodents (DeMarini et al. 1997b; Pegram et al. 1997; Ross and Pegram 2003), individuals with the *GSTT1-1* null genotype had lower frequencies of MN in urothelial cells and lower urinary mutagenicity than those with one positive allele; however, in our case, the differences were not statistically significant. These differences were not observed in MN in peripheral blood lymphocytes probably because *GSTT1-1* is not expressed in lymphocytes (Wang et al. 2000). By contrast, individuals bearing *GSTT2B* +/+ had higher numbers of MN in blood. The CNV encompassing *GSTT2B*, which modifies *GSTT2* gene expression, is in linkage disequilibrium with the *GSTT1-1* CNV (Zhao et al. 2009). All three genes are located in the same cluster, and combined effects cannot be excluded; however, the role of *GSTT2* and *GSTT2B* genes on DBP detoxification is unknown. Limited experimental data are available for *GSTZ1* and *CYP2E1* in relation to DBP exposure. In this study, we identified differences between subjects of different genotypes, with those associated with *CYP2E1* being statistically significant. These findings should be verified in further studies.

The effect of confounding is unimportant in our study, which involved a comparison of individuals to themselves before and after an exposure over a limited time period. Essentially any change in biomarker levels could be attributed to only three factors: (i) effect of exposure to a mixture of chemicals in the pool; (ii) a potential effect of physical activity; and (iii) chance. Control of other lifestyle factors and environmental exposures, as expected, did not modify results. Physical activity has been shown in some studies (Schiffl et al. 1997) to be associated with genotoxicity through an effect on oxidative stress, but the results are not consistent (Stephanie et al. 2008; Battershill et al. 2008). In addition, adjustment in the analysis for the intensity of physical activity during swimming did not modify the results obtained relative to exposure to DBPs.

Confounding could be more of a problem for the analysis of MN in exfoliated urothelial cells, which were collected two weeks after swimming. Although we did control for several lifestyle factors in the analysis, it is still possible that results in urine could be affected more by uncontrolled confounding. Chance could be an explanation for some of the results and is particularly a problem for the evaluation of gene-environment interactions. In the main analyses of exposure and effect biomarkers; however, statistical tests indicated that the issue of chance findings due to multiple comparisons was minor. In addition, the identification of stronger genotoxic effects, particularly for the potentially more toxic brominated compounds, argues against an effect of chance.

The timing of the collection of biological samples is crucial when evaluating biomarkers of effect. Due to constraints in the study protocol, the first collection of samples had to be done during a 2-h period after swimming and 2 weeks after swimming for exfoliated urothelial cells. Due to the lack of previous studies of this type with swimmers, we had no precedence to follow, and the timing of blood collection that we used might not have been the most appropriate for some of the assays. Specifically, it is possible that collecting blood approximately 1 h after swimming for chemicals that are metabolized rapidly and that are of relatively low toxicity might not necessarily be appropriate for the comet assay because DNA damage induced by DBPs may be repaired fast (Liviac et al., 2009, Komaki et al 2009). However, studies in

rodents frequently assess DNA damage in lymphocytes 3-4 h after exposure. We collected urine 2 h after swimming for mutagenicity analysis and also two weeks after swimming for MN analysis. This last time period was selected to allow time for the exfoliation of cells from the urothelium exposed at the time of the experiment (Espinoza et al. 2008).

Swimming has significant positive health efffects related to the benefits of exercise and has some advantages over land-based activities for people of all ages and physical abilities (Zwiener et al. 2007). To retain the positive aspects of aquatic activities, regulators and researchers have turned their attention to the hygienic aspects of the quality of pool water, as well as of its chemical composition. It will be important to maintain microbial disinfection while minimizing potentially harmful DBPs. The goal would be to maintain the positive health effects of swimming through exercise while reducing other potential adverse health risks.

In conclusion, we found that exposure to brominated THMs through swimming in pools is associated with responses to genotoxicity biomarkers. Our findings were consistent when we examined different genotoxicity and mutagenicity assays, and we found that only brominated THMs were associated with higher genotoxicity; chloroform was not. The results are also consistent with the presence of mutagenic and genotoxic DBPs in pool water and the mutagenicity of the pool water. As noted by SD Richardson et al. (submitted), the general levels of DBPs and concentration of free chlorine that we found in pool water, as well as the mutagenicity of the water (~1200 rev/L-eq in strain TA100 of *Salmonella*), were similar to those found in drinking water. However, the concentrations of nitrogen-containing DBPs were higher in pool water than in drinking water. Although our study had low power to evaluate unambiguously the genetic variation in the response to chemical exposure during swimming, it appears plausible that such variation exists. Our findings, which should be verified in larger studies, indicate that the positive health effects gained by swimming could be increased by reducing the potential heath risks of pool water.

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Table 1. Characteristics of the study population

Characteristic	Number	%
Sex		
Men	17	34
Women	33	66
Age (mean ± SD)	30.1 ± 6.1	
Ethnicity		
Caucasian	48	96
Other	2	4
Education[a]		
Secondary	4	8
University	45	92
Tobacco		
Never smokers	36	72
Ex-smokers	14	28
Second-hand smoke		
Yes	34	68
No	16	32
Regular swimming (≥ once/month)		
Yes	11	22
No	39	78
Regular sport (≥ once/week)		
Yes	27	54
No	23	46

[a]Missing value for one subject.

Table 2. Change in mean values of biomarkers before and after swimming, peripheral blood lymphocytes (PBL) and urine.

	No. of subjects		Mean value ± SD		
Biomarker[a]	Before	After	Before	After	*p*-value[b]
MN-PBL	49	49	3.4 ± 2.4	4.0 ± 2.8	0.235
OTM-Comet-PBL	49	49	1.5 ± 0.7	1.3 ± 0.6	0.008
MN-urothelial cells	33	33	9.1 ± 9.1	10.3 ± 7.4	0.350
Urine-mutagenicity	43	43	0.6 ± 2.3	1.2 ± 2.2	0.257

[a] MN-PBL: Micronucleated cells per 1000 binucleated cells; OTM-Comet-PBL: Olive tail moment per 100 cells; MN-urothelial cells: micronucleated cells per 2000 cells; Urinary mutagenicity: Rev/ml-eq.

[b] Paired t-test.

Table 3. Changes in micronuclei (MN) frequency and olive tail moment (OTM) in peripheral blood lymphocytes (PBL), MN frequency in exfoliated urothelial cells and urine mutagenicity, relative to an increase of 1 μg/m^3 of total or individual trihalomethanes (THMs) in exhaled breath

Biomarker[a]	Exposure	Beta-coefficient[b]	95 % confidence interval	*p*-value
MN-PBL	Total THMs	0.30	-0.05 - 0.64	0.09
	$CHCl_3$	0.29	-0.27 - 0.85	0.31
	$CHCl_2Br$	1.92	0.21 - 3.63	0.03
	$CHClBr_2$	1.71	-0.02 - 3.44	0.05
	$CHBr_3$	5.04	1.23 - 8.84	0.01
OTM-Comet-PBL	Total THMs	-0.02	-0.07 - 0.046	0.53
	$CHCl_3$	-0.02	-0.10 - 0.06	0.64
	$CHCl_2Br$	-0.04	-0.30 - 0.23	0.79
	$CHClBr_2$	-0.14	-0.40 - 0.13	0.30
	$CHBr_3$	-0.23	-0.83 - 0.37	0.45
MN-urothelial cells	Total THMs	-0.10	-1.19 - 1.01	0.86
	$CHCl_3$	-0.47	-2.11 - 1.18	0.57
	$CHCl_2Br$	-0.46	-6.50 - 5.58	0.88
	$CHClBr_2$	2.44	-3.32 - 8.20	0.40
	$CHBr_3$	4.29	-6.87 - 15.45	0.44
Urine mutagenicity	Total THMs	0.24	-0.11 - 0.58	0.17
	$CHCl_3$	0.33	-0.22 - 0.89	0.23
	$CHCl_2Br$	0.61	-1.12 - 2.35	0.48
	$CHClBr_2$	0.92	-0.75 - 2.59	0.27
	$CHBr_3$	5.27	1.80 - 8.75	0.004

[a] MN-PBL: Micronucleated cells per 1000 binucleated cells; OTM-Comet-PBL: Olive tail moment per 100 cells; MN-urothelial cells: micronucleated cells per 2000 cells; Urinary mutagenicity: Rev/ml-eq.

[b]Beta-coeffficients represent a change in the biomarker level for an increase of 1 μg/m^3 of THMs in exhaled air.

Table 4. Interaction between the effect of exposure to bromoform on micronuclei and the comet assay in peripheral blood lymphocytes, micronuclei in exfoliated urothelial cells, and urine mutagenicity in relation to polymorphisms in the 3 main genes associated with metabolism of DBPs

		Micronuclei in lymphocytes			Micronuclei in exfoliated urothelial cells			Urine mutagenicity			Comet assay		
Gene	Geno-types	n	Beta-coefficient	*p*-value interaction	n	Beta-coefficient	*p*-value interaction	n	Beta-coefficient	*p*-value interaction	n	Beta-coefficient	*p*-value interaction
GSTT1-1	-/-	16	7.77	0.37	10	-5.19	0.70	10	-5.19	0.70	16	-0.04	0.47
	-/+, +/+	30	3.39		23	1.73		23	1.73		30	-0.43	
CYP2E1													
rs2070673	TT	27	4.35	0.45	21	10.55	0.07	21	10.55	0.07	27	-0-31	0.35
	AT_AA	16	-0.03		10	-11.49		10	-11.49		16	0.05	
rs915906	TT	28	6.65	0.14	22	11.62	0.02	22	11.62	0.02	28	0.22	0.33
	CT/CC	14	-5.91		9	-23.16		9	-23.16		14	0.91	
rs915907	CC	30	3.68	0.66	22	12.11	0.37	22	12.11	0.37	30	0.39	0.19
	CA_AA	14	6.55		9	9.76		9	9.76		14	0.85	
rs2515641	CC	35	5.76	0.91	25	7.58	0.84	25	7.58	0.84	35	-0.30	0.71
	CT	9	-0.36		6	-10.96		6	-10.96		9	0.78	
rs2249695	CC	25	4.22	0.38	21	10.55	0.07	21	10.55	0.07	25	-0.31	0.61
	CT_TT	19	7.45		10	-11.49		10	-11.49		19	0.12	
GSTZ1													
rs3177427	GG	22	10.16	0.04	19	-0.18	0.52	19	-0.18	0.52	22	-0.23	0.64
	AG_AA	22	1.38		12	8.66		12	8.66		22	-0.50	
rs1046428	CC	29	3.26	0.73	20	5.79	0.14	20	5.79	0.14	29	0.36	0.42
	CT_TT	14	-5.37		11	21.13		11	21.13		14	0.57	

Figure legend

Figure 1. Main design of the swimming pool study. Samples were collected before and after swimming in a period of minutes (exhaled breath), 1 h (blood for micronuclei and comet), 2 h (urine for mutagenicity testing), and 2 weeks (urine for micronuclei in exfoliated cells).

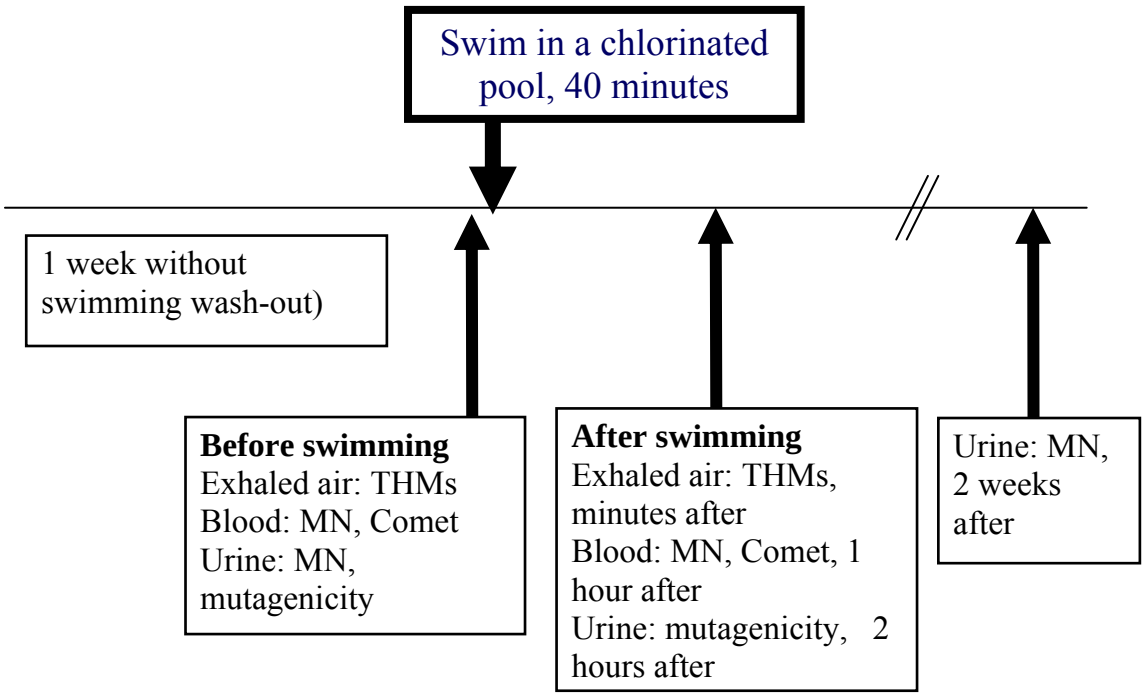


Figure 1

Supplementary Table 1. Interaction between the effect of exposure to bromoform on micronuclei in peripheral blood lymphocytes in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number Subjects	Beta-coefficient	95%CI		*p*-value for each genotype [a]	*p*-value interaction [a]
GSTT1-1	-/-	16	7.773	1.90	13.65	0.014	0.367
	-/+, +/+	30	3.389	-1.55	8.33	0.170	
GSTT2B	-/-, -/+	35	1.595	-2.32	5.51	0.412	0.016
	+/+	11	9.608	-0.11	19.33	0.052	
GSTM1	-/-	22	5.401	-0.67	11.47	0.078	0.892
	-/+, +/+	24	5.075	-0.94	11.09	0.094	
CYP2E1	TT	27	4.349	-0.41	0.91	0.071	0.447
rs2070673	AT_AA	16	-0.027	-7.52	7.46	0.994	
CYP2E1	TT	28	6.652	2.24	11.06	0.005	0.143
rs915906	CT/CC	14	-5.910	-14.11	2.29	0.139	
CYP2E1	CC	30	3.676	-1.78	9.13	0.178	0.655
rs915907	CA_AA	14	6.554	-1.98	15.09	0.118	
CYP2E1	CC	35	5.758	1.66	9.85	0.007	0.907
rs2515641	CT	9	-0.363	-14.07	13.34	0.948	
CYP2E1	CC	25	4.215	-0.81	9.24	0.096	0.381
rs2249695	CT_TT	19	7.448	1.09	13.80	0.025	
GSTZ1	GG	22	10.155	2.98	17.32	0.008	0.039
rs3177427	AG_AA	22	1.384	-3.73	6.50	0.577	
GSTZ1	CC	29	3.256	-0.96	7.48	0.125	0.733
rs1046428	CT_TT	14	-5.371	-18.49	7.75	0.383	
CYP1A2	AA	20	6.288	2.61	9.97	0.002	0.886
rs762551	AC_CC	21	2.899	-5.61	11.41	0.482	
CYP2D6	GG	32	4.958	0.74	9.17	0.023	0.558
rs3892097	AG	11	7.111	-7.59	21.81	0.290	
APEX1	TT	17	9.456	3.30	15.61	0.006	0.016
rs11304009	GT_GG	27	-2.457	-9.39	4.47	0.471	
ERCC2	TT	17	9.999	2.02	17.97	0.018	0.092
rs13181	GT_GG	27	2.660	-1.38	6.70	0.186	
OGG1	CC	28	5.760	1.14	10.37	0.017	0.727
rs1052133	CG/GG	16	2.608	-7.36	12.57	0.579	
XRCC1	GG	16	4.531	-1.77	10.83	0.143	0.674
rs25487	AG_AA	28	6.181	1.04	11.32	0.020	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplementary Table 2. Interaction between the effect of exposure to bromoform on micronuclei in exfoliated cells from urine in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype [a]	*p*-value interaction [a]
GSTT1-1	-/-	10	-5.189	-48.63	38.25	0.780	0.699
	-/+_+/+	23	1.732	-11.14	14.61	0.781	
GSTT2B	-/-_-/+	26	-0.054	-12.45	12.34	0.993	0.387
	+/+	7	-1.419	-59.90	57.06	0.943	
GSTM1	-/-	12	-23.145	-50.45	4.16	0.086	0.004
	-/+_+/+	21	8.108	-2.07	18.29	0.111	
CYP2E1	TT	21	10.546	0.90	20.19	0.034	0.068
rs2070673	AT_AA	10	-11.492	-41.07	18.09	0.378	
CYP2E1	TT	22	11.618	2.45	20.79	0.016	0.021
rs915906	CT/CC	9	-23.161	-52.44	6.11	0.098	
CYP2E1	CC	22	12.107	-1.32	25.53	0.074	0.366
rs915907	CA_AA	9	9.762	-12.32	31.85	0.321	
CYP2E1	CC	25	7.584	-3.21	18.38	0.159	0.842
rs2515641	CT	6	-10.963	-23.57	1.64	0.065	
CYP2E1	CC	21	10.546	0.90	20.19	0.034	0.068
rs2249695	CT_TT	10	-11.492	-41.07	18.09	0.378	
GSTZ1	GG	19	-0.176	-19.36	19.01	0.985	0.521
rs3177427	AG_AA	12	8.657	-5.62	22.93	0.200	
GSTZ1	CC	20	5.788	-5.65	17.22	0.299	0.138
rs1046428	CT_TT	11	21.132	-7.55	49.82	0.125	
CYP1A2	AA	15	6.238	-8.45	20.93	0.373	0.452
rs762551	AC_CC	16	13.721	-5.57	33.01	0.147	
CYP2D6	GG	23	11.135	0.44	21.83	0.042	0.394
rs3892097	AG	7	-5.176	-48.69	38.34	0.730	
APEX1	TT	10	6.778	-4.64	18.19	0.197	0.317
rs11304009	GT_GG	21	1.555	-19.82	22.93	0.880	
ERCC2	TT	13	1.693	-26.50	29.89	0.895	0.146
rs13181	GT_GG	18	12.883	3.48	22.29	0.011	
OGG1	CC	20	4.112	-7.17	15.40	0.451	0.095
rs1052133	CG/GG	11	29.044	-3.13	61.21	0.070	
XRCC1	GG	14	14.641	4.66	24.62	0.008	0.157
rs25487	AG_AA	17	-5.626	-25.28	14.03	0.547	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplementary Table 3. Interaction between the effect of exposure to bromoform on urine mutagenicity in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype[a]	*p*-value interaction[a]
GSTT1-1	-/-	14	1.902	-4.15	7.96	0.500	0.112
	-/+_+/+	26	7.590	2.97	12.21	0.003	
GSTT2B	-/-_-/+	30	6.296	1.34	11.25	0.015	0.410
	+/+	10	2.806	-3.19	8.81	0.296	
GSTM1	-/-	18	6.066	0.92	11.21	0.024	0.734
	-/+_+/+	22	4.679	-1.58	10.94	0.134	
CYP2E1	TT	22	6.893	0.74	13.04	0.030	0.341
rs2070673	AT_AA	15	3.010	-2.93	8.95	0.288	
CYP2E1	TT	22	5.844	1.83	9.86	0.007	0.381
rs915906	CT/CC	14	3.142	-3.95	10.23	0.347	
CYP2E1	CC	27	4.865	-0.85	10.58	0.091	0.986
rs915907	CA_AA	11	5.375	-5.07	15.82	0.263	
CYP2E1	CC	29	5.529	1.10	9.96	0.017	0.609
rs2515641	CT	9	2.881	-9.74	15.51	0.583	
CYP2E1	CC	21	7.332	2.78	11.88	0.003	0.083
rs2249695	CT_TT	17	2.457	-2.71	7.62	0.323	
GSTZ1	GG	21	3.478	-1.78	8.74	0.181	0.213
rs3177427	AG_AA	17	8.697	2.02	15.37	0.015	
GSTZ1	CC	24	6.523	1.86	11.18	0.009	0.708
rs1046428	CT_TT	13	-2.092	-18.87	14.68	0.784	
CYP1A2	AA	17	5.183	-2.48	12.84	0.168	0.274
rs762551	AC_CC	19	10.293	3.04	17.54	0.009	
CYP2D6	GG	26	4.800	1.40	8.20	0.008	0.810
rs3892097	AG	11	3.342	-9.75	16.44	0.565	
APEX1	TT	15	4.709	-3.14	12.55	0.213	0.543
rs11304009	GT_GG	23	0.447	-4.44	5.34	0.850	
ERCC2	TT	16	2.253	-4.05	8.55	0.451	0.121
rs13181	GT_GG	22	8.454	3.04	13.87	0.004	
OGG1	CC	24	5.563	0.68	10.44	0.028	0.630
rs1052133	CG/GG	14	0.473	-4.72	5.67	0.843	
XRCC1	GG	15	8.835	3.62	14.05	0.003	0.073
rs25487	AG_AA	23	2.431	-2.89	7.76	0.351	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

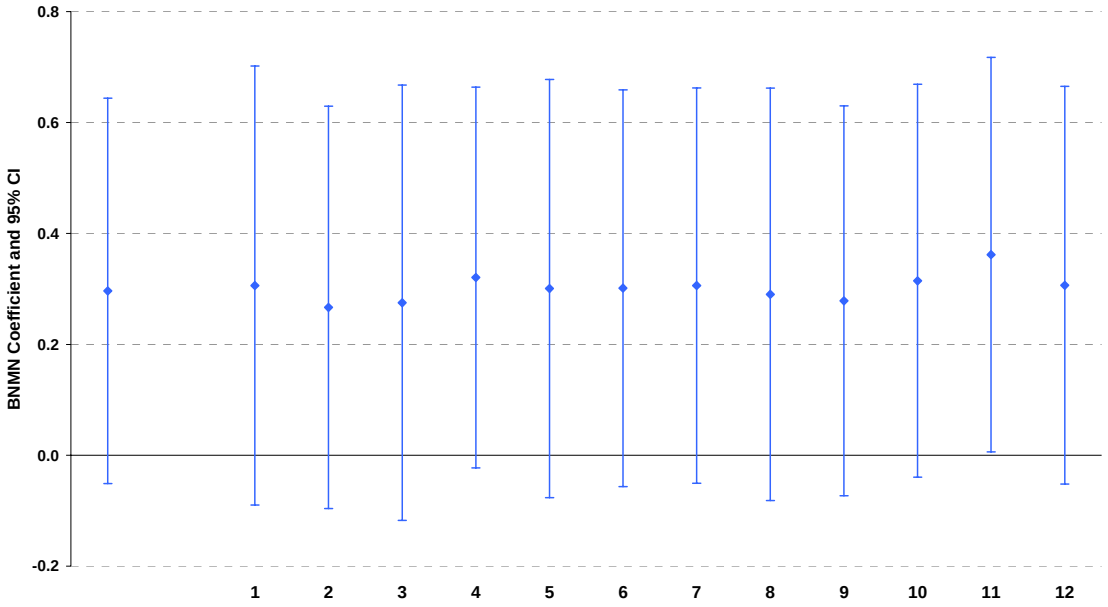
Supplementary Table 4. Interaction between the effect of exposure to bromoform on the comet assay in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype[a]	*p*-value interaction[a]
GSTT1-1	-/-	16	-0.043	-0.81	0.72	0.905	0.468
	-/+_+/+	30	-0.433	-1.25	0.38	0.285	
GSTT2B	-/-_-/+	35	-0.402	-1.18	0.37	0.299	0.437
	+/+	11	0.117	-0.71	0.94	0.748	
GSTM1	-/-	22	0.312	-0.64	1.27	0.500	0.065
	-/+_+/+	24	-0.629	-1.35	0.96	0.085	
CYP2E1	TT	27	-0.308	-1.10	0.49	0.430	0.347
rs2070673	AT_AA	16	0.048	-1.34	1.44	0.941	
CYP2E1	TT	28	-0.394	-1.03	0.24	0.215	0.330
rs915906	CT/CC	14	-0.108	-2.24	2.02	0.912	
CYP2E1	CC	30	0.391	-0.50	1.28	0.374	0.191
rs915907	CA_AA	14	-0.846	-1.65	-0.04	0.042	
CYP2E1	CC	35	-0.303	-0.94	0.34	0.343	0.707
rs2515641	CT	9	0.781	-1.96	3.52	0.497	
CYP2E1	CC	25	-0.305	-1.10	0.49	0.436	0.613
rs2249695	CT_TT	19	-0.124	-1.08	0.83	0.786	
GSTZ1	GG	22	-0.227	-1.16	0.71	0.617	0.637
rs3177427	AG_AA	22	-0.496	-1.48	0.49	0.303	
GSTZ1	CC	29	-0.348	-1.11	0.41	0.357	0.420
rs1046428	CT_TT	14	0.668	-1.85	3.19	0.567	
CYP1A2	AA	20	-0.513	-1.43	0.41	0.256	0.139
rs762551	AC_CC	21	0.753	-0.62	2.13	0.264	
CYP2D6	GG	32	-0.515	-1.12	0.09	0.094	0.062
rs3892097	AG	11	1.251	0.07	2.43	0.041	
APEX1	TT	17	-0.348	-1.32	0.62	0.452	0.210
rs11304009	GT_GG	27	0.353	-0.77	1.48	0.522	
ERCC2	TT	17	0.241	-0.73	1.21	0.601	0.378
rs13181	GT_GG	27	-0.295	-1.02	0.43	0.408	
OGG1	CC	28	-0.159	-0.79	0.47	0.605	0.394
rs1052133	CG/GG	16	-1.058	-3.04	0.92	0.267	
XRCC1	GG	16	-0.573	-1.51	0.37	0.209	0.171
rs25487	AG_AA	28	0.094	-0.65	0.84	0.797	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Legend to Figure in Supplementary Material

Supplementary Figure 1. Association between total THMs in exhaled breath and changes in the formation of micronuclei when adjusting for age and sex (main model) or additionally adjusting for 12 other variables (*n* = 49 subjects)[a].



[a]All models include age and sex. Models 1 to 12 include also the following variables: Model 1 water consumption, 2 source of water, 3 antioxidant intake from diet, 4 swimming at least once per month, 5 number of laps during experiment (an indication of physical activity), 6 leisure physical activity at least once per week, 7 self-reported work exposure to chemical, physical of biological agents, 8 exposure to second-hand smoke, 9 use of permanent hair dyes, 10 shower before the experiment, 11 leisure time physical activity last 24 h, 12 vitamins last 24 h.

Supplementary Figure 1

THE ORIGIN OF THE THESES