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Genotoxicity and endocrine disruption potential of haloacetic acids in human placental and lung cells

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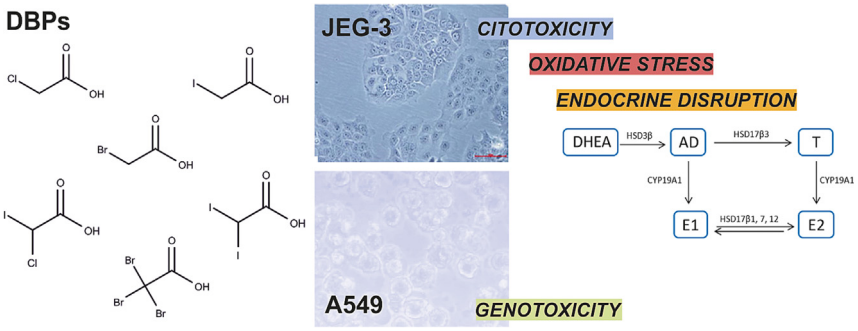
HIGHLIGHTS

- This work enlarges the toxicity database for selected haloacetic acids.
- Oxidative stress, endocrine disruption, and genotoxicity in human cells were studied.
- Toxicity potential of monohalogenated acids decreased in the order IAA > BAA> > CAA.
- Haloacetic acids are potential endocrine disruptors.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorination of water results in the formation of haloacetic acids (HAAs) as major disinfection byproducts (DBPs). Previous studies have reported some HAAs species to act as cytotoxic, genotoxic, and carcinogenic. This work aimed at further exploring the toxicity potential of the most investigated HAAs (chloroacetic (CAA), bromoacetic (BAA), iodoacetic (IAA) acid) and HAAs species with high content of bromine (tribromoacetic acid (TBAA)), and iodine in their structures (chloroiodoacetic (CIAA) and diiodoacetic acid (DIAA)) to human cells. Novel knowledge was generated regarding cytotoxicity, oxidative stress, endocrine disrupting potential, and genotoxicity of these HAAs by using human placental and lung cells as *in vitro* models, not previously used for DBP assessment. IAA showed the highest cytotoxicity (EC_{50}: 7.5 μM) and ability to generate ROS (up to 3-fold) in placental cells, followed by BAA (EC_{50}: 20–25 μM and 2.1-fold). TBAA, CAA, DIAA, and CIAA showed no significant cytotoxicity (EC_{50} > 250 μM). All tested HAAs decreased the expression of the steroidogenic gene *hsd17b1* up to 40 % in placental cells, and IAA and BAA (0.01–1 μM) slightly inhibited the aromatase activity. HAAs also induced the formation of micronuclei in A549 lung cells after 48 h of exposure. IAA and BAA showed a non-significant increase in micronuclei formation at low concentrations (1 μM), while BAA, CAA, CIAA and TBAA were genotoxic at exposure concentrations above 10 μM (100 μM in the case of DIAA). These results point to genotoxic and endocrine disruption effects associated with HAA exposure at low concentrations (0.01–1 μM), and the usefulness of the selected bioassays to provide fast and sensitive responses to HAA exposure, particularly in terms of genotoxicity and endocrine disruption effects. Further studies are needed to define thresholds that better protect public health.

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1. Introduction

Disinfection by-products (DBPs) are formed by the reaction of disinfectants (*e.g.* chlorine, chloramines, chlorine dioxide, ozone, UV) with natural organic matter and other water constituents like anthropogenic organic contaminants (*e.g.* pesticides, pharmaceuticals, and detergents) and inorganic ions (*e.g.* bromide and iodide) (Richardson and Kimura, 2020). The nature and quantity of the DBPs formed depends directly on the disinfectant used, the characteristics of the water to be treated in terms of its organic and inorganic matter content, and the conditions (*e.g.*, disinfectant dose, contact time, pH, temperature) under which the disinfection is done (Hua and Reckhow, 2007; Liang and Singer, 2003). DBPs are always present in drinking water distribution networks that rely on disinfection to deliver safe water, and therefore research efforts in this field are directed to comprehensively characterize the DBP mixtures (Li et al., 2021; Postigo et al., 2021; Powers et al., 2020; Sanchís et al., 2022) and determine the risk that they pose to human health (Lau et al., 2020; Postigo et al., 2018; Stalter et al., 2020). The latter is of relevance because DBPs are suspected to be carcinogenic for the liver, bladder, kidney, and intestine, and to induce leukemia and reproductive system diseases and alter embryonic development (Cantor et al., 2010; Hebert et al., 2010; Manasfi et al., 2017; Mashau et al., 2018; Richardson et al., 2007).

Among DBPs, haloacetic acids (HAAs) are characterized by their high polarity and acidic properties. They present very low acidity constant values (pKa: 0.7 to 3.1), low octanol-water partition coefficients (log K_{ow}: 0.22 to 2.66), and high aqueous solubility (water solubility: 1102 to 1,750,000 mg/L) (Postigo et al., 2020) (Table S1 in Supplementary Material (SM)). HAAs constitute the most common and abundant DBP class in chlorinated water together with trihalomethanes (THMs). Levels of HAA5, *i.e.*, the sum of five HAA species (chloroacetic acid (CAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromoacetic acid (BAA), and dibromoacetic acid (DBAA)) in drinking water from cities located in the Mediterranean basin (Barcelona, Athens, Heraklion, Nicosia, and Limassol) ranged from 0.2 to 28.3 μg/L (Kargaki et al., 2020). The sum of DCAA and TCAA was between 2 and 43 μg/L in the drinking water from a large water distribution system located in the city of Huzhou (China), where re-chlorination is required in areas located at a long distance from the water treatment plant to avoid microorganism regrowth (Dong et al., 2022). The concentration of ten HAA species (all brominated and chlorinated species (HAA9) and iodoacetic acid (IAA)) in chlorinated swimming pool water ranged between 28 and 80 μg/L in pools filled with drinking water (with DCAA and TCAA as the major species) and from 16 to 115 μg/L in pools filled with thermal water (with DBAA and dibromochloroacetic acid (DBCAA) as the major species) (Usman et al., 2022). Comparatively higher concentrations of HAA9 in swimming pool water (up to 653 μg/L) were reported elsewhere (Kargaki et al., 2020).

The pervasive occurrence of HAAs in chlorinated drinking water has motivated the study of their potential risk to human health. HAAs have been shown as mutagenic, cytotoxic, genotoxic, teratogenic, and carcinogenic substances in a variety of bioassays (Cortés and Marcos, 2018; Richardson et al., 2007). Their genotoxic and cytotoxic potency to mammalian cells (Chinese hamster ovary, CHO) is influenced by the number and type of halogens present in their molecular structure. In this sense, monohalogenated acids are more toxic than dihalo- and trihalogenated species and iodo-acids are more toxic than bromine- and chlorine-containing DBPs (I > Br>> Cl) (Wagner and Plewa, 2017). HAAs are also genotoxic to human small intestine epithelial cells (FHs 74-Int) (Attene-Ramos et al., 2010), human hepatoma cells (HepG2) (Zhang et al., 2012), spermatocytes, lymphocytes (Ali et al., 2014), and mouse embryonic fibroblast cells (NIH/3 T3) (Wei et al., 2013). Various studies pointed out that DNA damage is likely induced by the generation of reactive oxygen species (ROS) rather than direct interaction with the genomic material. The upregulation of the expression of oxidative stress-responsive genes in HepG2 reporter cells after exposure to monohaloacetic acids (EC_{50}: 1.17 μM IAA, 3.16 μM BAA and 16.2 μM CAA) supports this hypothesis (Pals et al., 2013).

A decade ago, Narotsky et al. (2011) reported reprotoxic effects such as pregnancy loss and eye malformations in the offspring of F344 rats exposed to HAA5, but at doses 17,000-fold higher than the maximum allowable concentration established for HAA5 in drinking water in the US (USEPA, 2010) and Europe (EU, 2020) (<60 μg/L), assuming 70 kg body weight and 3 L water consumption. More recently, HAAs have been recognized as endocrine disruptors, although this effect has been scarcely investigated. Jeong et al. (2016) demonstrated that 72 h of exposure to HAAs (EC_{50}: 10 μM IAA, 10 μM BAA, and 0.25 mM CAA) permanently inhibited both, E2 production (> 50 %) and the growth of mouse ovarian follicles. As for genotoxicity, the authors proposed the generation of ROS leading to cellular oxidative stress as a possible mechanism. In a different study, IAA (EC_{50}: 22 μM) was revealed as estrogenic in recombinant yeast expressing the human estrogen receptor (hERa) (Kim et al., 2020).

The placenta plays an important role in mammal reproduction. Most of the enzymes (*i.e.* P450 aromatase) involved in the synthesis of estrogens (estrone, estradiol) are highly expressed in this tissue. The human placental JEG-3 cell line has been used for the assessment of potential endocrine disruptors, *viz.* compounds that can modulate the activity of the enzyme P450 aromatase, encoded by *cyp19a1* (Marqueño et al., 2019; Pérez-Albaladejo et al., 2019; Xu et al., 2019), or the expression of other genes involved in the synthesis of estradiol, such as 3β-hydroxysteroid dehydrogenase (HSD) (*hsd3b1*) and 17β-HSD (*hsd17b1*). All these genes are highly expressed in placental JEG-3 cells (Samson et al., 2009).

In this context, this study aimed to further explore the toxic potential of selected HAAs, *viz.*, monohalogenated HAAs, tribromoacetic acid (TBAA), chloroiodoacetic acid (CIAA), and diiodoacetic acid (DIAA) in human cells. To this end, we used a battery of *in vitro* assays that allowed the detection of oxidative stress and endocrine disruption in placental JEG-3. Moreover, the potential genotoxicity to alveolar A549 cells was investigated, since these cells have previously shown to respond to the presence of genotoxic chemicals (Palacio et al., 2016). The selection of HAAs aimed at enlarging the toxicity database for the most investigated HAAs (CAA, BAA, and IAA), and exploring DBPs that are expected to be potentially toxic due to the high content of bromine (TBAA) and the presence of iodine in their structures (CIAA and DIAA).

2. Material and methods

2.1. Chemicals and reagents

Eagle's Minimum Essential Medium, Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, nonessential amino acids, sodium pyruvate, fetal bovine serum (FBS), penicillin, and streptomycin were from Gibco BRL Life Technologies (Paisley, Scotland, UK). Alamar Blue (AB), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) were from Invitrogen (Eugene, OR, USA). 1β-^{3}H-androst-4-ene-3,17-dione (^{3}H-AD) was from PerkinElmer, Ink (Boston, MA, USA). 3-Morpholinosydnonimine hydrochloride (SIN-1), 4-androstenedione (AD), mitomycin C, and bisbenzimide Hoechst 33342 were from Sigma-Aldrich (Steinheim, Germany). As for the selected HAAs (Table S1 in SM), CAA, BAA, IAA, and TBAA with a purity above 98 % were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), while CIAA and DIAA with a purity above 90 % were obtained from CanSyn Chem. Corp. (Toronto, ON, Canada). Stock solutions of HAAs for toxicity analysis were prepared in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) and stored at −20 °C in the dark.

2.2. Cell culture

JEG-3 placental cells (HTB-36™, ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium supplemented with 5 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L sodium bicarbonate, 50 U/mL penicillin, and 50 μg/mL streptomycin. The human alveolar epithelium lung cell line A549 (Merck KGaA) was maintained in DMEM-high glucose (Sigma Aldrich, Merck KGaA) with

10 % FBS, 4 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Both, JEG-3 and A549 cells were kept at 37 °C (5 % CO_2) in a humidified incubator. When 90 % confluence was reached, cells were trypsinized (0.25 % *w/v*) for subculturing and conducting experiments.

2.3. Analysis of placental cell viability and generation of reactive oxygen species (ROS)

Placental cell viability was evaluated using two fluorescent dyes, viz., AB and CFDA-AM (Schirmer et al., 1997). JEG-3 cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After attachment, cells were exposed for 24 h at 37 °C to different individual HAA concentrations (0.5 to 500 μM) or 0.1 % DMSO (solvent control) in a serum-free medium. Cell viability was measured using 5 % AB and 4 μM CFDA-AM as described in Pérez-Albaladejo et al. (2021) in a Varioskan microplate reader (Thermo Electron Corporation) (Ex/Em 530/590 nm and 485/530 nm for AB and CFDA-AM, respectively). Cell viability was expressed as the percentage of fluorescence referred to control cells (cells exposed to DMSO), as mean ± standard deviation (SD) of three independent experiments with six replicates each.

The generation of ROS was assessed by measuring the fluorescence emitted by the oxidized H_2DCF (LeBel et al., 1992). JEG-3 cells (5×10^4 cells per well) were allowed to attach overnight in 96-well plates and incubated for 30 min at 37 °C with 20 μM H_2DCF-DA diluted in a 1:10 Dulbecco's Phosphate Buffered Saline (DPBS) solution supplemented with 10 mM glucose (DPBS-glucose). After washing, cells were exposed to individual HAAs, 0.1 % DMSO (carrier), or 5 μM SIN-1 (positive control) in DPBS-glucose. Fluorescence of the oxidized H2DCF was measured after 15, 30, 60, and 120 min of exposure to individual HAA concentrations (0.5 to 100 μM) in the microplate reader at the Ex/Em wavelength pair of 485/528 nm. The generation of ROS in exposed cells was expressed as a fold change of the basal fluorescence in solvent control cells (mean ± SD of three independent experiments with six replicates each).

A schematic overview of the assays conducted to evaluate cell viability and ROS generation is provided in Fig. S1 as SM.

2.4. P450 aromatase activity

P450 aromatase activity in placental JEG-3 cells after 24 h of exposure to HAAs was determined by using the method of Lephart and Simpson (1991), with some modifications (Fig. S1 in SM). Cells were seeded in 48-well plates (7.5×10^4 cells per well), allowed to attach overnight, and exposed for 24 h at 37 °C to individual HAA concentrations (0.001 to 500 μM) or 0.1 % DMSO (carrier). After exposure, cells were incubated in a solution of DPBS-glucose containing 20 nM ^{3}H-AD and 20 nM AD for 30 min at 37 °C. The reaction was stopped by placing the plate on ice and immediately aspirating the culture medium that was extracted with dichloromethane (x3). The amount of tritiated water (^3H_2O) was determined by liquid scintillation counting (Tri-Carb 2100TR, Packard) in an aliquot of the aqueous phase. Aromatase activity in exposed cells was expressed in fmol/min/mg protein or as the percentage of the activity measured in solvent control cells.

2.5. Gene expression with real-time PCR

The relative expression of genes involved in steroidogenesis was assessed in placental JEG-3 cells after 24 h exposure to HAAs (Fig. S1 in SM). Cells were seeded in 6-well plates (10^6 cells per well), incubated overnight, and exposed to individual HAAs (5 μM) or 0.1 % DMSO (carrier) for 24 h at 37 °C ($n = 6$). This concentration was below the EC_{10} for all the HAAs, except for IAA. The isolation of total RNA was performed by using TRIzol (Invitrogen Life Technologies, Carlberg, Denmark). The amount of total RNA was measured in a NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Inc.). The obtained RNA (5 μg) was treated with DNase I (Ambion, Austin, TX, USA). Subsequently, the treated RNA (1 μg) was reverse-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche, Basel, Switzerland) according to the manufacturer's protocol. Taqman Gene Expression Assays (Applied Biosystems, Thermo Fisher Scientific, Inc) were used to quantify the expression levels of the following selected genes: *cyp19a1* (cytochrome P450 family 19 subfamily A member 1), which encodes for the enzyme responsible for the aromatization of androgens into estrogens; *hsd3b1* (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1); *hsd17b1* (hydroxysteroid 17-beta dehydrogenase 1); *hsd17b7* (hydroxysteroid 17-beta dehydrogenase 7); *hsd17b12* (hydroxysteroid 17-beta dehydrogenase 12), and *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), the latter used as the reference gene. Quantitative real-time PCR was carried out in a LightCycler 480 II (F. Hoffmann-Roche) using a cDNA amount equivalent to 50 ng of total RNA. Thermal cycling conditions were: 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 3 s (denaturation) and 60 °C for 30 s (annealing and primer extension). All reactions were run as technical duplicates. Table S2 (SM) shows the assay ID and the efficiency obtained for the target and reference genes. The quantification cycles (Cp) for each gene were determined by the second derivative maximum of their respective amplification curve. Relative mRNA abundance for each gene was calculated using the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001).

2.6. Genotoxicity: Micronuclei formation

The ability of HAAs to induce genotoxicity was estimated as the frequency of micronuclei in exposed human lung A549 cells as described in Schnell et al. (2013), with some modifications (Fig. S1 in SM). Briefly, 1.5×10^5 cells were plated in a 6-well plate containing 3 glass coverslips (Ø 12 mm; Marienfeld, Germany) per well. After 24 h attachment, cells were exposed for 48 h to 1 μM, 10 μM and 100 μM of individual HAAs, 0.25 μM mitomycin C (positive control), or 0.1 % DMSO (solvent control) (at 37 °C). Non-toxic exposure levels were selected in all cases. After a rinse step, cells were fixed with 4 % formaldehyde, stained with 5 μM bisbenzimide Hoechst 33342, and the coverslips mounted with Vectashield H-1000 (Vector Laboratories, CA, USA). A scan of 100 tiff-data images containing >2000 cells from each coverslip was obtained with an EVOS M7000 Cell Imaging System (Thermo Fisher Scientific) using x40 objective. The automated mode was configured to obtain 100 individually autofocused images and a tiled image grouping them in only one picture, which was exported to Celleste 5.0 Image Analysis software for the automatic counting of the nuclei. The software was configured to detect objects with bright, rounded shape and to filter the nuclei with an area ranging between 50 and 600 μm^2. Although the area of A549 cell nuclei was around 100 μm^2, the upper margin was set to allow the detection of overlapped nuclei. The split tool was set to allow the scoring of these nuclei separately. To validate the automated counting, five randomly chosen pictures were counted manually and compared to the automated process. To score micronuclei, the criteria described by Fenech (2000) were adopted. Thus, only micronuclei with round or oval shapes, with the same staining intensity as the main nucleus, no linked or connected to it, no refractive, and readily distinguished from artefacts, such as staining particles were considered and manually counted on individual data images.

2.7. Statistical analysis

The concentrations causing a 10 % and 50 % decline in cell viability (EC_{10} and EC_{50}, respectively) and dose-response curves were obtained with Sigmaplot 13.0. One-way ANOVA with Dunnett's *post hoc* tests were performed to detect significant differences from control cells in ROS generation and gene expression using SPSS Statistics 27.0, and micronuclei formation using GraphPad Prism 9. The level of significance was set at $p < 0.05$.

3. Results

3.1. Placental cell viability and generation of ROS

The highest cytotoxicity was observed for iodine and bromine monosubstituted compounds. Thus, IAA (EC_{50}s: 7.1 ± 0.2 and 7.7 ± 0.3 μM

for AB and CFDA-AM assays, respectively) and BAA (EC_{50}s: 20 ± 0.9 and 25 ± 3.1 μM) were the most cytotoxic compounds, far followed by TBAA (EC_{50}s: 458 ± 31 and 258 ± 44 μM) (Fig. 1). The other HAAs, *viz.* CAA, CIAA, and DIAA did not show significant cytotoxicity at the highest concentration tested (500 μM) (Table S3 in SM). EC_{10} values were 3.5 ± 0.1 and 3.2 ± 0.3 μM for IAA in the AB and CFDA-AM assays, respectively; 9.3 ± 1.0 and 15.7 ± 2.8 μM for BAA; and 242 ± 44 μM and 87 ± 17 μM for TBAA. The solvent control (0.1 % DMSO) did not induce cytotoxicity when compared to the negative control (0 % DMSO).

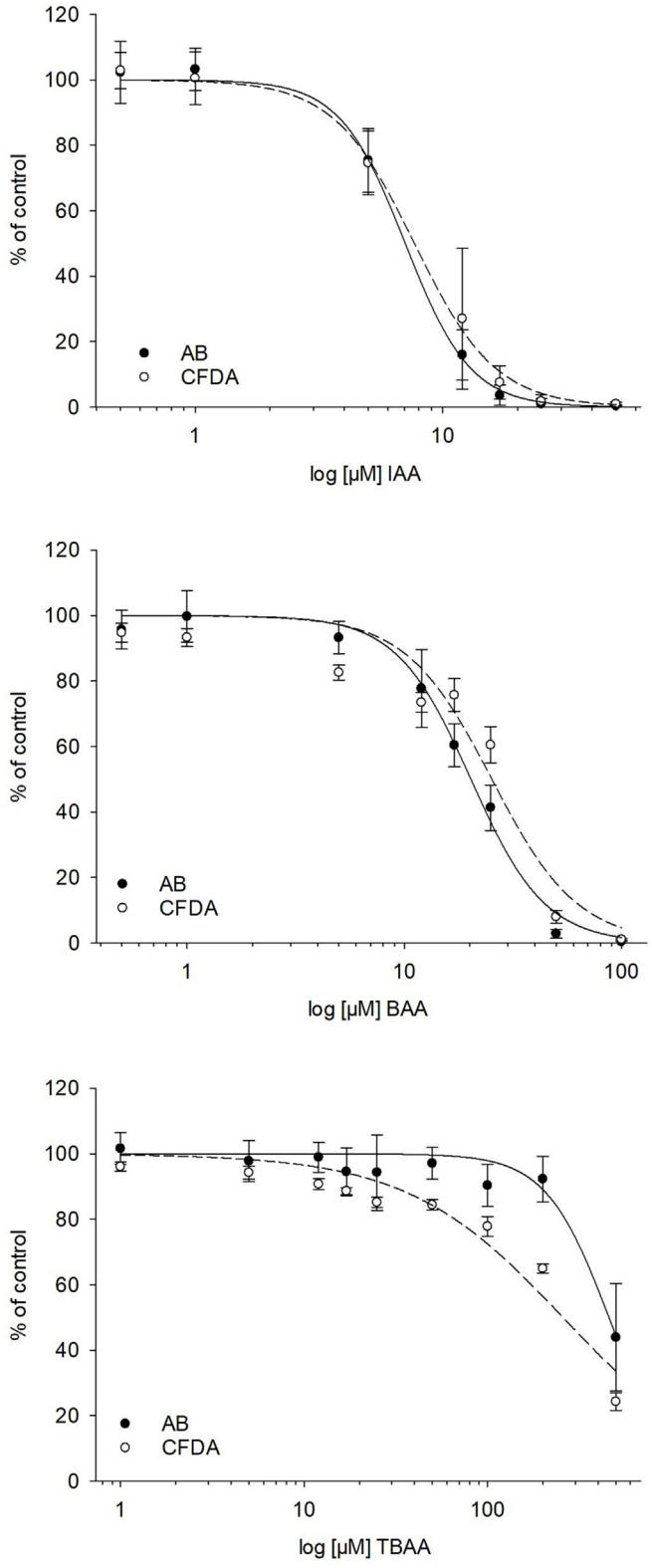

Fig. 1. Dose-response curves of cell viability in placental cells after 24 h of exposure to IAA, BAA and TBAA assessed by Alamar Blue (AB, solid line) and CFDA-AM (dotted line) assays. Results are expressed as percentage of cell viability of control cells (mean ± SD of at least 3 different assays).

Regarding oxidative stress, IAA showed the highest ability to induce the generation of ROS (3.0-fold increase after 120 min exposure) (Fig. 2), followed by BAA (2.1-fold). TBAA, CAA, CIAA, and DIAA induced ROS up to 1.9-fold, but ROS generation decreased over time (Fig. 2). 5 μM SIN-1 was used as the positive control and led to 2.3 ± 0.10, 4.1 ± 0.24, 8.3 ± 0.38, and 11 ± 0.53 fold induction of ROS, after 15, 30, 60, and 120 min of exposure, respectively.

3.2. Gene expression with real-time PCR

The expression of *hsd17β1, hsd17β12, hsd17β7, hsd3β1,* and *cyp19a1* was measured in JEG-3 cells exposed for 24 h to 5 μM HAAs. CAA, TBAA, CIAA, and DIAA down-regulated the expression of *hsd17β1* (up to 40 %) (Fig. 3). A small, not significant down-regulation was also observed for IAA and BAA. The expression of *hsd17β12, hsd17β7, hsd3β1,* and *cyp19a1* was not modulated by the exposure to HAAs (Fig. 3).

3.3. P450 aromatase activity

The basal activity of P450 aromatase in JEG-3 cells under our assay conditions was 235 ± 43.2 fmol/min/mg of protein. Exposure to IAA and BAA, at concentrations higher than 12–17 μM, led to an inhibition of P450 aromatase activity, which was attributed to cytotoxicity (Figs. 1 and 4). A small inhibition (20 %) of aromatase activity was detected at low concentrations of IAA (0.01 and 0.1 μM), BAA (0.5 and 1 μM), and TBAA (5 and 50 μM) (Fig. 4), while exposure to CAA, CIAA, and DIAA (0.01 to 500 μM) did not alter aromatase activity in exposed JEG-3 cells. The inhibition observed for TBAA at concentrations >50 μM could be attributed to cytotoxicity.

3.4. Genotoxicity: micronuclei formation

The frequency of micronuclei formation in alveolar A549 cells after exposure to the positive control mitomycin C (0.25 μM), solvent control (0.1 % DMSO) and negative control (DMEM) were 31, 2.4, and 1.9 ‰, respectively. IAA and BAA induced a non-significant increase in micronuclei formation at a concentration of 1 μM (Table 1). CAA, CIAA, and TBAA significantly induced the formation of micronuclei when tested at 10 μM, whereas DIAA only showed genotoxicity at the highest concentration tested (100 μM) (Table 1).

4. Discussion

This work investigates the effects of selected HAAs in human cell models (JEG-3 and A549) by assessing their toxicity, ability to generate ROS and genotoxicity. Special emphasis has been placed on evaluating their endocrine disruption potential, an effect that has been scarcely investigated. The mono-substituted HAAs IAA and BAA (EC_{10}: 3.5 to 15.7 μM; EC_{50}: 7.1 to 25 μM) were the most cytotoxic compounds for JEG-3 cells. However, the observed EC_{10}s (equivalent to 644 μg/L in the case of IAA and 2176 μg/L in the case of BAA for CFDA-AM assays) are about 1000 times higher than the usual concentrations reported for these compounds in drinking water, usually below 1 μg/L in the case of IAA and below few μg/L in the case of BAA (Postigo et al., 2018; Weinberg et al., 2002). The other investigated HAAs, *viz.*, CAA, TBAA, CIAA, and DIAA were not cytotoxic at doses below 80 μM, which are also much larger than the few μg/L usually measured for these substances in chlorinated water (Manasfi et al., 2017; Postigo et al., 2018; Weinberg et al., 2002; Yeh et al., 2014). In agreement with previous studies, iodinated and brominated HAAs revealed more cytotoxic than chlorinated ones (Attene-Ramos et al., 2010; Hall et al., 2020; Plewa et al., 2010; Procházka et al., 2015). EC_{50} values

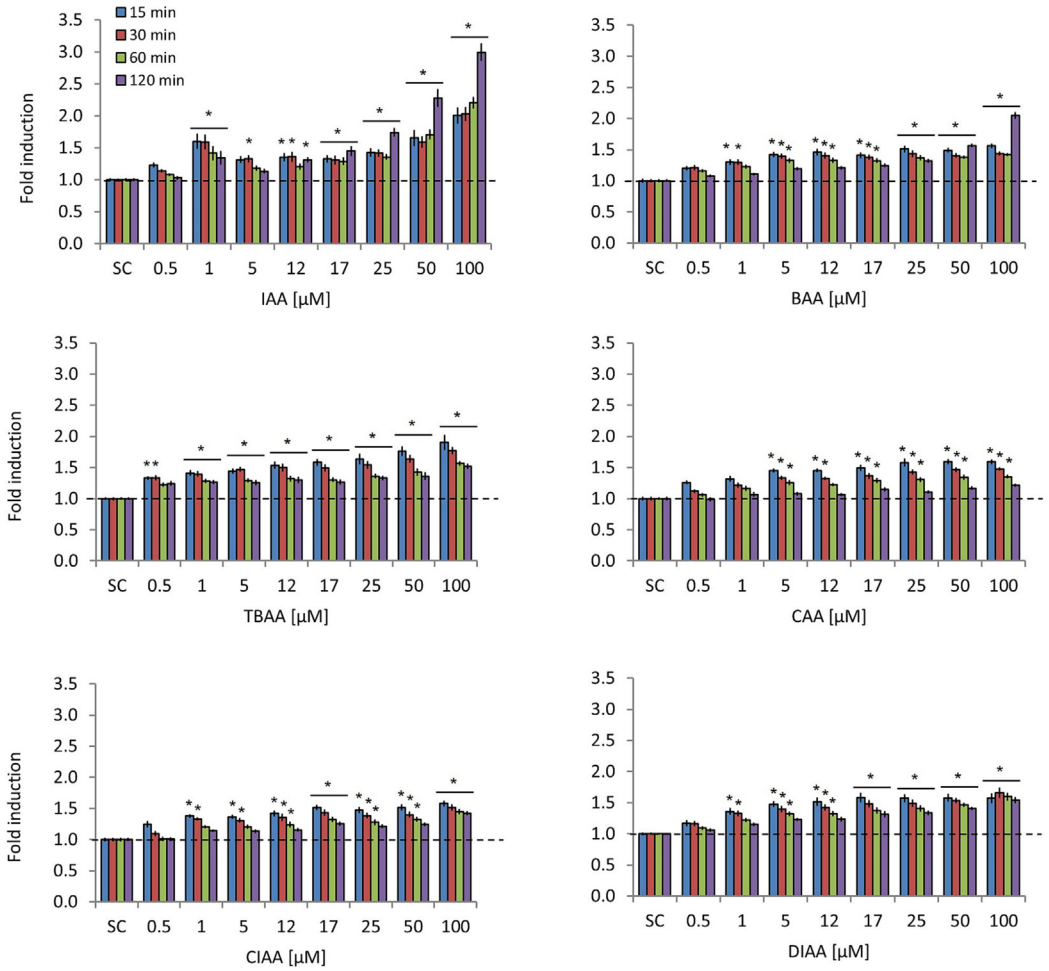

Fig. 2. ROS generation in JEG-3 cells after 15, 30, 60 and 120 min of exposure to HAAs. Results are expressed as fold induction over control cells (mean ± SEM). Dotted line: control cells. *Statistically significant differences from control ($p < 0.05$). SC: solvent control.

in CHO cells exposed for 72 h were 3, 10, 85, 330, and 810 μM for IAA, BAA, TBAA, DIAA, and CAA, respectively. An equivalent effect on cell viability (IC_{50}) was obtained at slightly higher HAA concentrations in human colorectal adenocarcinoma cells (Caco-2) exposed for 4 h (34 μM for IAA, 42 μM for BAA and 1200 μM for CAA) (Procházka et al., 2015). Similarly, human breast adenocarcinoma cells (MCF7) exposed for 16 h showed EC_{50} below 50 μM for IAA and BAA and higher than 100 μM for CAA (Hall et al., 2020). Although less sensitive to monohalogenated acetic acids, human intestinal epithelial cells exposed for 24 h also showed the same cytotoxicity pattern, *i.e.*, IAA > BAA>> CAA (EC_{50} > 50 μM for IAA, >150 μM for BAA and ~ 7000 μM for CAA) (Attene-Ramos et al., 2010). Plewa et al. (2004) postulated that this toxic profile could be attributed to the fact that HAAs are alkylating agents and react according to a nucleophilic substitution (S_N2) mechanism. Consequently, there is a relationship between the cytotoxic potential of mono-halogenated HAAs and their electrophilic reactivity, carbon – halogen length, and bond dissociation energy (Plewa et al., 2004). In addition, Dad et al. (2013) hypothesized that the alkylating power of IAA, BAA, and CAA leads to the inhibition of glyceraldehyde-3-phosphate dehydrogenase and the depletion of glycolytic ATP, increase of mitochondrial stress, and disturbance of normal oxidative

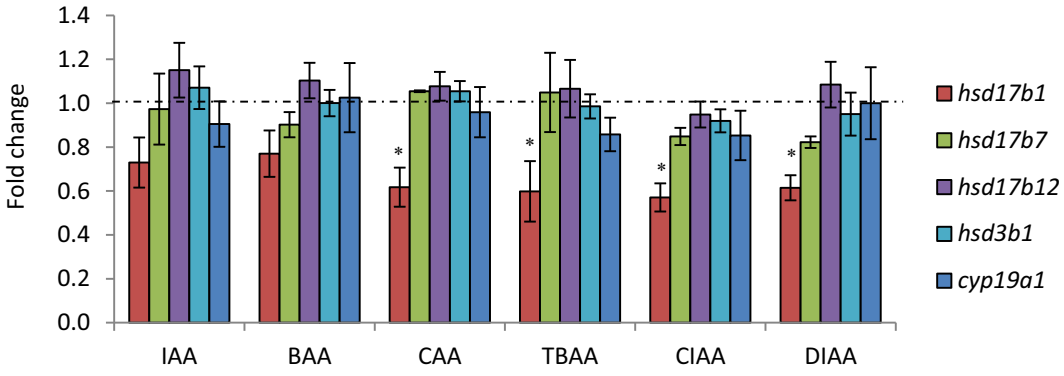

Fig. 3. Gene expression in JEG-3 cells after exposure to 5 μM HAAs. Results are expressed as fold change (mean ± SEM; $n = 6$). *Statistically different from control cells.

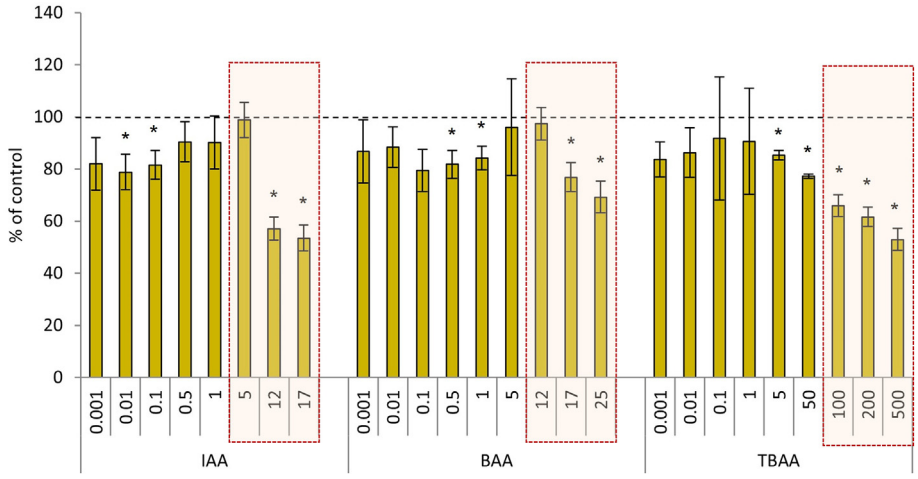

Fig. 4. P450 aromatase activity in JEG-3 cells exposed to different concentrations (μM) of HAAs for 24 h. Results are expressed as percentage of aromatase activity respect control cells (mean ± SD) of six replicate assays. Dotted line represents control value (100 %), and shadowed areas represent exposure concentrations at which cell viability was lower than 80 % (Fig. 1). *Statistically significant differences from control cells. Aromatase activity in control cells was 235 ± 43 fmol/min/mg of protein.

phosphorylation, which eventually generates ROS and depletes mitochondrial ATP. The strong depletion of cellular ATP levels after exposure of CHO cells to mono-HAAs confirmed this hypothesis (Dad et al., 2013). Based on this mode of action, the present study reveals the Alamar Blue assay, which measures cell mitochondrial metabolism (the non-fluorescent dye resazurin is reduced to the fluorescent dye resorufin by mitochondrial reductases), as a suitable method to detect the toxicity of mono-HAAs and highlights that placental cells are sensitive to exposure to these compounds.

There is also evidence that oxidative damage is involved in the cytotoxic effect of HAAs (Pals et al., 2013; Procházka et al., 2015). All the tested HAAs, but particularly the most cytotoxic ones (IAA and BAA), induced significant levels of ROS (up to 3-fold) in placental cells. Recently, Wang et al. (2020) demonstrated the linkage of up to 8 molecules of CAA to one molecule of catalase by van der Waals forces and hydrogen bonds, causing a bell-shape response of catalase activity in mouse primary hepatocytes after 24 h of exposure to 1 mM CAA. When antioxidant enzymes are not functional, levels of ROS will increase and generate oxidative stress, which will induce the partial loss of functionality of cell macromolecules, *viz.,* DNA, lipids, and proteins. Accordingly, in a previous study, we observed up to 30–40 % inhibition of aromatase activity in JEG-3 cells exposed to 4-heptylphenol, a compound that highly induced the generation of ROS (Pérez-Albaladejo et al., 2019).

In addition, genotoxicity has been often linked to oxidative stress (Dad et al., 2013; McCord, 2000). The excess of ROS can affect the functionality of key cellular components with a role in the correct distribution of chromosomes during mitosis, leading to micronuclei formation. Oxidative stress is the mechanism involved in the genotoxicity of mono-HAAs in CHO cells (Cemeli et al., 2006) and human peripheral blood lymphocytes (Ali et al., 2014). In both models, DNA damage was significantly reduced when the antioxidants butylated hydroxyanisole (BHA) and catalase were added to the system. The genotoxic potential of HAAs assessed by the comet assay was also evidenced in human small intestine FHs 74 cells. The alteration of genes responsible for encoding key enzymes for the cell cycle regulation and repair of double-strand DNA breaks was reported as a possible mechanism (Attene-Ramos et al., 2010; Plewa et al., 2004). Such effects were observed for monohalogenated HAAs at concentrations of 22, 57, and 3420 μM, for IAA, BAA, and CAA, respectively (Attene-Ramos et al., 2010), which are in all cases above drinking water concentrations.

In the present study, four of the six HAAs investigated (viz., BAA, CAA, CIAA, TBAA) induced significant genotoxicity in A549 alveolar cells, estimated as increased micronuclei frequency, at concentrations above 10 μM. IAA, together with BAA, induced a non-significant increase in micronuclei formation at a concentration of 1 μM. Similarly, 1.5 to 3 μM IAA did not induce the formation of micronuclei in NIH-3 T3 mouse embryonic fibroblasts (Wei et al., 2013). Furthermore, Liviac et al. (2010) reported a non-significant increase in the frequency of micronuclei in TK6 human lymphoblastoid cells exposed to concentrations up to 5 μM IAA, 10 μM BAA, and 500 μM CAA for 48 h. They suggested that this lack of response could be partially explained by the efficient repair of the initial primary DNA damage of TK6 cells, which may prevent its translation to fixed genetic damage. In a recent study, Font-Ribera et al. (2019) reported a non-significant increase in the frequencies of micronuclei in lymphocytes and reticulocytes (immature erythrocytes) in swimmers with an accumulated concentration of HAAs in serum up to 10 μg/L (equivalent to <0.1 μM HAAs, which is at least 10 times lower than the concentration at which we observed a non-significant increase of micronuclei formation for IAA and BAA in this study) after swimming for 1 h in an indoor swimming pool. An increased incidence of micronuclei means unrepaired damage of the DNA and consequently, it manifests genetic damage and risk for subsequent cell generations. A high micronuclei frequency has been related to several syndromes, including infertility, diabetes, cardiovascular and neurodegenerative diseases, and represents a predictive biomarker of cancer risk in humans (Bonassi et al., 2011; Fenech, 2000).

Endocrine disruptors often affect the activity of steroidogenic enzymes leading to an imbalance of sex hormones. In the steroidogenesis pathway for JEG-3 cells proposed by Samson et al. (2009), DHEA is converted to AD *via* 3β-hydroxysteroid dehydrogenase (HSD3β), and AD is irreversibly aromatized to produce estrone (E1) by P450 aromatase. E1 can be transformed into estradiol (E2) through the activity of HSD17β1, 7, and 12 (Fig. S2 in SM). When JEG-3 cells were exposed to HAAs (5 μM) for 24 h, a significant decrease in the expression of *hsd17b1* was observed in all cases, except for IAA and BAA, the only two compounds that inhibited aromatase activity at low concentrations (0.1 to 1.0 μM). The expression of

Table 1
Frequency of micronuclei formation in lung A549 cells after 48 h exposure to different concentrations of HAAs, expressed as the number of micronuclei per 1000 cells (mean ± SEM). Micronuclei frequencies in cells exposed to positive and solvent controls were 31 ± 1.4 and 2.4 ± 1.2 ‰, respectively. *Statistically different from solvent control. IAA and BAA were not tested at high concentrations due to cytotoxicity.

Compound	1 μM	10 μM	100 μM
IAA	3.8 ± 1.1	–	–
BAA	4.5 ± 0.4	6.3 ± 0.2*	–
CAA	1.4 ± 0.9	4.7 ± 0.3*	6.4 ± 0.9*
CIAA	0.7 ± 0.2	8.0 ± 0.1*	9.7 ± 2.3*
DIAA	2.3 ± 0.6	3.3 ± 0.1	5.8 ± 0.8*
TBAA	1.3 ± 0.7	7.0 ± 0.6*	9.3 ± 2.9*

hsd17b1 is specific to placental and ovarian granulosa cells, with low expression in the endometrium, adipose tissue, and prostate. It encodes for a reductive enzyme located in the cytoplasm, which converts E1 to E2, and to a minor extent, transforms AD into testosterone (He et al., 2016; Lukacik et al., 2006). It has been postulated that the expression of *hsd17b1* positively correlates with E1 reduction and E2 levels (Aka et al., 2010; He et al., 2016). Like in our study, the expression of *hsd17b1, hsd3b1,* and *cyp19a1* was not significantly altered in ovary cells after dosing female mice through drinking water to a wide range of IAA concentrations (0.5–500 mg/L) (Gonsioroski et al., 2021). A rather small decrease of *cyp19a1* expression in ovary cells was observed at the highest IAA concentration (500 mg/L), associated with a decrease of E2 levels in mice serum (Gonsioroski et al., 2021). In line with this finding, our results show a small inhibition (20 %) of the aromatase activity at low concentrations of IAA (0.01 and 0.1 μM IAA) with no modulation of *cyp19a1* expression, but a down-regulation of the expression of *hsd17β1* (up to 40 % at 5 μM of CAA, TBAA, CIAA, DIAA). Note that drinking water concentrations may not be fully bioavailable to cells and that high concentrations in drinking water may turn into small exposure cell concentrations. Also, a non-toxic exposure concentration (below EC_{10}) was selected for all investigated HAAs except IAA, which was slightly toxic at the tested concentrations (5 μM). Lower exposure concentrations of IAA should be further investigated so that relevant effects on the expression of these genes are not missed. Overall, both gene expression and aromatase measurements point to an overall decrease of E2 levels after HAA exposure as reported in other studies (Gonsioroski et al., 2021), but contrast with our previous study, where we measured E2 levels in the culture medium of JEG-3 cells after exposure to HAAs and found a 2.5-fold increase of E2 levels in cells exposed to 0.5 μM IAA (Mestres et al., 2022). This estrogenic effect of IAA in placental JEG-3 cells occurred at a single dose, and no dose-response curve was detected. It is common when studying endocrine disrupters to see lower dose effects that are not observed at higher dosages and *vice versa* or narrow dose effects (Vandenberg, 2014). Furthermore, the mechanisms through which HAAs can act as endocrine disrupters may be multiple. Apart from interaction with aromatase activity, IAA has been reported to induce toxicity in the female pituitary, to decrease the expression of key mRNA related to reproduction, and to act as a hypothalamic-pituitary-gonadal axis toxicant (González et al., 2021). There is also evidence in the literature of the estrogenic effect of HAAs (Kim et al., 2020). IAA activated the human estrogen receptor (hERα) in recombinant yeast cells (EC_{50} = 21.5 μM). CAA also weakly induced estrogenicity (EC_{10} = 43.4 μM), while BAA did not show a significant effect.

5. Conclusions

Overall, while general cytotoxicity in placental JEG-3 cells was observed mainly for IAA and BAA at exposure concentrations much higher (1000- and 10,000-fold) than levels usually found in drinking waters (a few μg/L); ROS generation was observed for all the investigated HAAs (except for CAA) at a concentration as low as 1 μM. IAA and BAA induced a non-significant increase in micronuclei frequency in alveolar A549 cells at this concentration (1 μM), while the other HAAs were genotoxic at higher concentrations (above 10 μM in the case of CAA, CIAA, and TBAA; 100 μM in the case of DIAA). Generally, cytotoxicity and to some extent genotoxicity, of monohalogenated acids followed the pattern observed in previous studies, with potencies in the order IAA > BAA>> CAA. Gene expression (down-regulation of *hsd17β1*) and inhibition of aromatase activity in JEG-3 cells pointed to an overall decrease of estrogen synthesis after HAA exposure, and consequently their potential as endocrine disruptors. All in all, these cell-based bioassays are reliable methods with enormous applicability to identify highly toxic DBPs, so that appropriate measures can be adopted to minimize their formation and protect public health. Further studies should address the toxicity of complex mixtures of DBPs and when possible, include the determination of these compounds in the culture medium of the assays, since many DBPs are unstable. This would help to increase the sensitivity of the *in vitro* tests and to reduce the discrepancy with *in vivo* studies.

CRediT authorship contribution statement

Elisabet Pérez-Albaladejo: Investigation, Visualization, Writing – original draft. **Raquel Pinteño:** Investigation. **María del Carmen Aznar-Luque:** Investigation. **Marta Casado:** Investigation. **Cristina Postigo:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Cinta Porte:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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