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The effects of Tetrabromobispehenol-A (TBBPA)on the cell viability, gap junction intercellular communication and DNA damage in the epithelial liver cell line IAR20 in vitro

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Abstract

Brominated flame retardants are released into the environment in many ways and have been shown to have negative effects on the health of living organisms. A disturbing increase in the use of the BFRs is considered a new environmental problem as many of the organic brominated compounds like tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), hexa-bromocyclododecane (HBCD) and polybrominated biphenyls (PBBs) are persistent and lipophilic, leading to a potential accumulation in the fat tissue of living organisms. Elevated levels of TBBPA have been found in human blood, maternal- and cord serum, adipose tissue and breast milk. The latest years it has been of increasing interest to investigate the possible relationship between exposures to brominated flame retardants and the risk of cancer development.

The main objective of this thesis was to investigate the negative effects of the toxicants Tetrabromobispehenol-A (TBBPA), using the epithelial liver cell line IAR20 *in vitro* with special emphasis on cell viability, gap junction intercellular communication (GJIC) and DNA damage.

Concentrations ranging from 0,06 µM to 25 µM were tested in the current study, based on a pilot dose range-finding viability assay for TBBPA. The objective was demonstrated through the Alamar blue viability assay, the scrape dye-loading assay and the single-cell gel electrophoresis assay (Comet assay). The comet assay was performed with a smaller range of concentrations of TBBPA than that used in the scrape dye load assay and the AB assay, 1,25 µM, 2,5 µM, 5 µM, 10 µM, 15 µM and 20 µM was used as exposure concentrations. TBBPA exercised an inhibiting effect on the intercellular communication via gap junctions in the highest dose range. The cells remained viable up to an exposure of 15 µM TBBPA and showed to have a GJIC significantly different from the negative control starting from 2,5 µM and up. This shows that the cells continued to be viable, but didn't communicate as well. The DNA damage in the cells was shown to be significant at a concentration of 2,5µM up to 20 µM.
In vitro results from this study cannot be directly compared to *in vivo* experiments, but it provides indications of exposure effects, as exposure to TBBPA for 24 hours can reduce cell viability, disrupt GJIC and cause DNA damage in IAR20 cells.

Sammendrag

Brominerte flammehemmere blir sluppet ut i miljøet på mange måter og har vist seg å ha negative effekter på dyr og menneskers helse. En urovekkende økning i bruken av brominerte flammehemmere er nå sett på som et nytt miljøproblem, da mange organiske brominerte forbindelser som tetrabromobisfenol A (TBBPA), polybrominerte difenyl etere (PBDEs), hexa-bromocyclododecane (HBCD) og polybrominerte bifenyler (PBBs) er persistente og lipofile, noe som kan lede til potensiell akkumulasjon i fettvevet til levende organismer. Forhøyede nivåer av TBBPA har blitt funnet i blod, fostervann, fettvev og brystmelk hos mennesket. De siste årene har det vært interesse for å undersøke forholdet mellom eksponeringer for brominerte flammehemmere og helserisiko.

Formålet med denne studien var å undersøke effektene av Tetrabrombisfenol A (TBBPA), ved å bruke leverepitelcellelinjen IAR20 *in vitro* med fokus på celleviabilitet, gap junction-intercellulær kommunikasjon(GJIC) og DNA-skade. Konsentrasjoner fra 0,06 µM til 25 µM ble testet i den aktuelle studien. Målet ble vist ved Alamar blue viabilitetsanalyse, scrape dye-loading-analyse og single cell gel elektroforese-analyse (Comet assay).

Comet analysen ble 1,25 µM, 2,5 µM, 5 µM, 10 µM, 15 µM and 20 µM brukt som eksponerings konsentrasjoner altså ble det utført med færre konsentrasjoner av TBBPA enn det brukt i scrape dye load-analysen og viabilitetstesten.

TBBPA hadde en hemmende effekt på den intercellulære kommunikasjonen via gap junctions innenfor det høyeste doseområdet. Cellene forble levende opp mot en konsentrasjon på 15 µM TBBPA og viste seg å ha en GJIC signifikant forskjellig fra den negative kontrollen begynner på 2,5 µM og oppover. Dette viser at cellene fortsetter å være levende, men ikke kommuniserer like godt. DNA skaden i cellene viste seg å være signifikant fra og med en konsentrasjon på 2,5µM opp mot 20 µM.

In vitro resultater fra denne studien kan ikke sammenliknes direkte med *in vivo* eksperimenter, men kan gi indikasjoner på eksponeringseffekter, da eksponering mot TBBPA i 24 timer kan redusere celleviabilitet, GJIC og forårsake DNA skade i IAR20 celler.

Abbreviations

8-OHdG =8-hydroxy-2'–deoxyguanosine

AB assay = Alamar blue Assay

BFR= Brominated flame retardant

BMP=2,2-bis(bromomethyl)-1,3-propanediol

CYP2B1= cytochrome P450, family 2, subfamily b, polypeptide 1

DEHP= di- (2-ethylhexyl) phthalate

DMEM=Dulbecco's Modified Eagle's Medium

DMSO=Dimethyl sulfoxide

FADH= Flavin adenine dinucleotide hydrogenase

FMNH= Flavin adenine dinucleotide hydrogenase

FRs= Flame retardants

GJ= Gap junctions

GJIC = gap junction intercellular communication

H2O2= Hydrogen Peroxide

HBCD =hexa- bromocyclododecane

IC= intercellular channels

LMP=Low melting point

LY= Lucifer yellow

M=medium control

MW=molecular weight

NADH= Nicotinamide adenine dinucleotide hydrogenase

NADPH= Nicotinamide adenine dinucleotide phosphate hydrogenase

NK=Human Natural killer

NRK= normal rat kidney epithelial cells

PBB=polybrominated biphenyls

PBDE=polybrominated diphenyl ethers

RNA=ribonucleic acid

SCGE =single-cell gel electrophoresis

T4= thyroxine

TBB=Tetrabromobenzoate

TBB=Tetrabromobenzoate

TBBPA= tetrabromobisphenol A

TBPH= bis(2-ethylhexyl) tetrabromophthalate

TBPH=2,3,4,5-tetrabromo-bis(2-ethylhexyl) phthalate

Triton X 100 = Polyethylene glycol p-(1.1.3.3-tetramethylbutyl)-phenyl ether

TTR = transthyretin

µM = Mikromolar

1. Introduction

1.1 Flame retardants

The last decades, modern technology has countered the challenge of fire by introducing flame retardant chemicals that can lower the risks of ignition and burning of materials used in the daily life. The use of flame retardant chemicals has saved many lives, millions of dollars in property damage every year, and the use of different types of FRs has increased the last years(Alaee & Wenning, 2002). Flame retardants (FRs) are substances added to a material in order to suppress, reduce or delay the flammability of the materials like plastics, textile, wood and paper (Alaee, Arias, Sjodin, & Bergman, 2003). FRs have a long history and were used already 2500 years ago when the Egyptians reduced the combustibility of wood with a mixture of aluminum and vinegar (Alaee et al., 2003). The initial chemical FRs were polychlorinated biphenyls (PCBs), but because of their toxicity they were forbidden in 1977 in the US (Legler & Brouwer, 2003). FRs are found in the environment mostly because of wastes and effluents from factories producing objects containing FRs.

Humans can get exposed to FRs through inhalation, skin contact and ingestion. Sources of exposure can be for example consumer products and manufacturing facilities. These routes are possible for industrial exposure as well, during producing, processing, transporting and recycling the FRs. Work-related exposure to the breakdown products may also occur during fire fighting (de Wit, 2002).
FRs are incorporated into many different polymers to fulfill regulatory requirements on flame retardancy. The halogenated organic flame retardants are used most because of their efficiency, low costs, and a low ability to damage the polymer's functionality (Jurgens et al., 2014).

1.1.1Brominated flame retardants

Brominated flame retardants (BFRs) are produced by a direct bromination of organic molecules or by bromine-addition to alkenes. The BFRs have a relative molecular mass ranging from 200 to that of large molecule polymers, by weight they normally contain 50 to 85% of bromine (-WHO, 1997). BFRs are organic compounds and produced in a big scale all over the world (Wang, 2013).

There are five main groups of BFRs with common uses, polybrominated diphenyl ethers (PBDEs) used in plastics, textiles, electronic castings and circuitry, Hexabromocyclododecanes (HBCDs) used in thermal insulation in the building industry, Tetrabromobisphenol A (TBBPA) and other phenols used in printed circuit boards and thermoplastics, Polybrominated biphenyls (PBBs) used in consumer appliances, textiles and plastic foams and other brominated flame retardants (authority, 2006).
The BFRs are proved to be the most efficient flame retardants in plastic and textiles. This group of environmental toxins has been in great focus the last years (de Wit. 2002). A disturbing increase in the use of the BFRs is considered a new environmental problem (Burkow 2001) as bioaccumulation within animals and man can take place.

1.1.2 Toxicity of brominated flame retardants

Many of the organic brominated compounds like tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), hexa- bromocyclododecane (HBCD) and polybrominated biphenyls (PBBs) are persistent and lipophilic, leading to a potential accumulation in the fat tissue of living organisms including humans (de Wit. 2002). Polybrominated diphenyl ethers (PBDEs) are abundant in the environment. For many years, these flame retardants have reduced the flammability of everyday objects like computers, furnishings and mattresses. Some PBDE compounds have been nominated for possible inclusion under the Stockholm Convention on Persistent Organic Pollutants, where Australia is a Party(Stockholm-covention, 2008).Studies under the Stockholm Convention has demonstrated the capacity of some PBDEs to persist and accumulate in the environment and to be carried long distances.

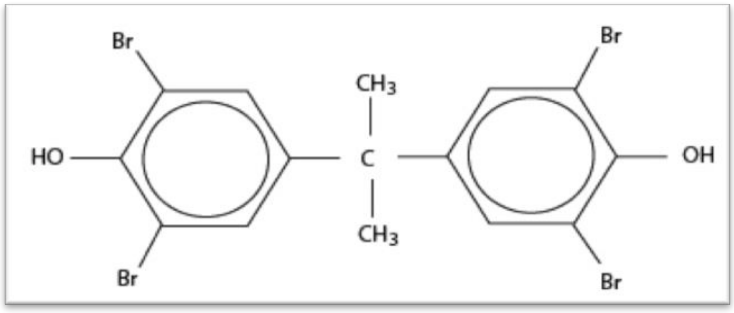
HBCD is a flame retardant that decreases the human natural killer cell´s lytic function and is likely to increase the cancer rate and viral infections (Hinkson & Whalen, 2010). In October 2012, a minor body of the Stockholm Convention on Persistent Organic Pollutants, recommended to include HBCD in the Convention's Annex A which is a list of the chemicals in production or use that need to be eliminated. The HBCD amendment will enter into force for Parties on 26 November 2014, except for those Parties that might opt out (Stockholm convention, 2014).
BFRs are being studied due to the concerns about health effects related to endocrine disruption, immunotoxicity, reproductive toxicity, and neurotoxicity (Gosavi, Knudsen, Birnbaum, & Pedersen,

2013). Some of the BFRs in use today have a neurotoxic effect, TBBPA and HBCD have been shown to inhibit neurotransmitter uptake into synaptic terminals from neurons (synaptosomes). Together with Pentabromodiphenylether (PBDE)-, TBBPA and HBCD also inhibit the uptake of dopamine into synaptic vesicles. This effect on dopamine uptake is compared to the drug ecstasy that selectively inhibits dopamine/serotonin transport (Mariussen & Fonnum, 2003). HBCD has been found in higher concentrations than TBBPA in the human body, this is explained by the differences in the usage and bioaccumulation potential of the two BFRs.TBBPA is bound chemically to the polymer structure; therefore the release of TBBPA into the environment is limited. It is also quickly metabolized by the mammalian liver and eliminated into bile (B. Johnson-Restrepo, Adams, & Kannan, 2008), (de Wit, 2002).

1.1.3 Tetrabrombisphenol A

Tetrabrombisphenol A (MW, 543.9 g·mol−1) is one of the 75 different BFRs and in terms of production volume globally, the largest and it is produced by a bromination of bisphenol-A(Jun HU, 2008) (see *figure 1*)

TBBPA is used to improve fire safety, mostly of laminates in electronic equipment (BSEF, 2012).



Figur 1 *Chemical structure of TBBPA (EBFRIP)*

TBBPA is highly lipophilic and has a small water solubility of 0.72 mg/. It is mainly found chemically bound to the material (about 90%) and is not meant to spread to the environment in larger amounts. Nevertheless, TBBPA is found in significant amounts in the nature and human samples (Reistad, Mariussen, Ring, & Fonnum, 2007). TBBPA was estimated to have a half-life of 2.2 days in occupationally exposed workers indicating a rapid turnover. TBBPA is normally bound chemically to

the polymer structure; therefore the release of TBBPA into the environment is limited, it is also quickly metabolized by the mammalian liver and eliminated into bile(B. Johnson-Restrepo et al., 2008) (de Wit, 2002).Despite a rapid metabolism it is assumed that continuous exposure to TBBPA may maintain constant serum levels (Janssen, 2005).

TBBPA has been found in air, soil, and sediment and in general not found in water samples(Boris Johnson-Restrepo, Adams, & Kannan, 2007). In regards to the human body, an experiment was done in 2008 where maternal- and cord serum, adipose tissue and breast milk were collected from 93 French volunteers during caesarean deliveries. TBBPA was present in 44% of the analyzed breast milk samples with concentrations ranging from 0.06 to 37.34 ng/g (up to 0,063µM) lipid weights and also in 30% of serum samples with the concentrations 0.97 to 3.34 $g \cdot L^{-1}$(up to 6mM) and 4.65 to 10.12 $g \cdot L^{-1}$ in cord and maternal serum. TBBPA was also found at concentrations ranging from 0.64 to 1.8 $ng \cdot g^{-1}$ (up to 0,003 µM) of lipid in the blood plasma samples from people who dismantle electronic components in Norway. Another finding in Sweden was in blood serum of four workers from an electronic dismantling plant and in blood ranging from 2.4 to 12.0 $\mu g \cdot kg^{-1}$(up to 0,203 µM) lipid weight of 54 volunteers (27 males and 27 females) from Japan (Cariou et al., 2008). All of these findings lead to concern about the possibility of TBBPA´s association with negative health consequences.

1.1.4 Toxicity of TBBPA

TBBPA was introduced as a substitute to selected usages of PDBEs, because of the persistency of the PDBEs in the environment and the interference with hormone signaling in the body.
TPPBA can induce transformation of SHE cell colonies, which is a sign of early cancer (Wang, thesis 2013)(Leo Morf et al., 2002).The acute oral toxicity of TBBPA for laboratory animals is low(IPCS, 1995).
TBBPA can act as an endocrine disruptor by interfering with both estrogens and androgens(Shaw et al., 2010). PBDEs and TBBPA resemble the thyroid hormone structurally; therefore the effects on the thyroid function have been much studied (Leo Morf et al., 2002).
The structure of thyroid hormone is shown below; as the structure of TBBPA is introduced in Figure 1, one can see that there is a similarity between the molecules.

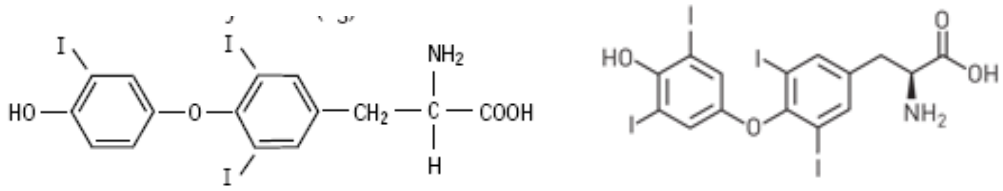


***Figure2**. Structure of the thyroid hormones; Triiodothyronine,T3 left and Thyroxin, T4 at right. T3 is the active form of the hormone.*

An analysis of different compound´s interaction with thyroxin (T4) through binding to the transthyretin (TTR), a thyroid hormone-binding transport protein in the plasma in vertebrates, showed that TBBPA had a competitive ability to bind to TTR.
The study showed that an increasing amount of bromine attached to the phenol increased the binding affinities to TTR (Meerts et al., 2000). TBBPA has been found in human blood, interference with the Human Natural killer (NK) cell function, may increase risk of tumor development and/or viral infection (Kibakaya, Stephen, & Whalen, 2009). *In vitro* studies of immune cells have shown that TBBPA can be a potent inhibitor of T-cell activation (Birnbaum & Staskal, 2004). *In vitro* and *in vivo* studies have proven the toxic effects of TBBPA on different cell types and tissues like the liver, neuronal tissue and neutrophils (Ogunbayo, Jensen, & Michelangeli, 2007).
Male ICR mice were exposed orally for 14 consecutive days to TBBPA, at the mid- and highest dose, a slight enlargement of hepatocytes, inflammatory cell infiltration and focal necrosis of hepatocytes took place (Environment & Canada, 2013).

TBBPA is a derivative of bisphenol-A (BPA) which is shown to be cytotoxic, mutagenic and employs various unfavorable effects on immune, endocrine, reproductive, developmental and nervous systems in animals and human and exhibits toxicity by all routes of exposure (Environment & Canada, 2013; Gowder, 2013).

DNA damage has also been reported because of TBBPA exposure. A recent study used the sperm of starlet (*Acispenser ruthenus*) to investigate the effect of the TBBPA on sperm quality variables, where

one variable was DNA damage. Damage to the DNA was demonstrated in the sperm with exposures higher than ≥ 2.5 µg/L of TBBPA (Linhartova, Gazo, Shaliutina-Kolesova, Hulak, & Kaspar, 2014).
A recent report described oxidative DNA damage in rat testis and kidney, by induction of 8-hydroxy-2'–deoxyguanosine (8-OHdG), which is one of the major products of DNA oxidation. The results suggest that active oxygen produced by CYP2B1-induced oxidative stress may contribute to oxidative DNA damage(Linhartova et al., 2014),(Valavanidis, Vlachogianni, & Fiotakis, 2009).

Most of the different *in vitro* and *in vivo* models that demonstrate negative effects of TBBPA are limited to only give an indication on how it would affect human health in real life.

1.2 Cell viability

The measurement of cell viability plays a fundamental role in all forms of cell culture and is important when measuring the cytoxicity of a compound. Cells exposed to a cytotoxic compound can have different response reactions. If the compound is toxic, the cells may undergo necrosis, which means they will lose membrane integrity and die quickly. The cells can also undergo apoptosis which is a programmed cell death (Life.tecnologies, 2014).

There are many different methods to detect cell viability, most of these tests rely on a breakdown in membrane integrity measured by the uptake ranging from the most routine Trypan blue dye exclusion assay to the complex analysis of individual cells, utilizing RAMAN microscopy (Stoddart, 2011).

One frequently used viability assay is the MTT-assay, which measure the activity of mitochondrial dehydrogenases colorimetrically. Nevertheless, the MTT-procedure leads to cell death, an alternative dye that is not toxic to the cells Alamar Blue dye(Gloeckner, Jonuleit, & Lemke, 2001). A way to assess cell health after exposure to TBBPA, an Alamar Blue Assay (AB Assay) was used to measure the viability (Al-Nasiry, Geusens, Hanssens, Luyten, & Pijnenborg, 2007). When added to cell cultures, the oxidized form of Alamar Blue enters the cell cytosol and is converted by mitochondrial activity to the reduced form by accepting electrons from NADPH, FADH, FMNH, NADH and the cytochromes. The

redox reaction that take place can be distinguished by a change in color of the culture medium from blue to pink/red, which can be measured and read by a spectrophotometer (Nakayama, Caton, Nova, & Parandoosh, 1997)

1.3 Gap junctions intercellular communication (GJIC)

Gap junctions are narrow water-filled channels that directly connect to the cytoplasms of neighboring epithelial cells (Alberts, 2008; Leithe, Kjenseth, Bruun, Sirnes, & Rivedal, 2010).

The gap junction channels are found in most animal tissue and are composed of two connexons; one from each communicating cell. Each connexon consists of six transmembrane proteins called connexins. Connexons float in the plasma membrane until a match is made to the nearest connexon in a neighbor cell (Holder, Elmore, & Barrett, 1993).

The GJIC allow inorganic ions and other small water-soluble molecules, up to the size of about 1,2 k Dalton, to pass directly from the cytoplasm of one cell to the cytoplasm of the other, thereby coupling the cells both electrically and metabolically (Alberts, 2008).

Gap junctions play an important role in embryonic development, cellular differentiation, and growth control (Wei, Xu, & Lo, 2004). The GJIC is important to maintain tissue homeostasis, its alternation associates with various abnormal cell activities, including cell transformation(Lee & Rhee, 2007). Many carcinogenic agents have been shown to inhibit and disrupt GJIC(Opsahl & Rivedal, 2000), and dysfunctional GJ relate with carcinogenesis as most tumor cells have reduced or absent GJIC. This is hypothesized to be an important step in carcinogenesis (Kjenseth, Fykerud, Rivedal, & Leithe, 2010). The importance of GJ makes it necessary to have good methods for qualitative and quantitative determination.

Today there are several methods used successfully to measure GJIC both *in vitro* and *in vivo*. Studies of IC are carried out either by measuring dye transfer, using techniques like microinjection, scrape loading, electroporation, gap-FRAP (fluorescence recovery after photo bleaching), preloading assays, local activation of molecular fluorescent probe (LAMP), or by measuring electrical conductance and metabolic cooperation(Abbaci, Barberi-Heyob, Blondel, Guillemin, & Didelon, 2008).

In this study scrape loading of the fluorescent dye, introduced in 1978, Lucifer yellow (LY) has been used (Hanani, 2012). In 1987 el Fouly et *al.* published a method for assessing GJIC *in vitro.* He suggested that counting cells containing LY as a good possible technique to quantify GJIC. With the use of image analysis software, quantitative scrape loading is a rapid method that gives a reliable quantum of GJIC.

1.4 Genotoxicity

A genotoxin is an agent that can cause DNA or chromosomal damage. A growing number of genotoxic pollutants enter the environment all the time. The genomes of all living organisms are relatively stable, the cell`s DNA replicates during cell division and passes all the genetic information to their progeny which is important to certify the right function of their genetic information. This can result in a large variety of DNA lesions that could be cytotoxic or genotoxic to the cells, the actual biological effects of the damage depend on the chemical nature of the DNA damage (Aziz et al., 2012).

DNA damage is considered to be an important initial event in carcinogenesis (Moller, 2006). The types of damage detected in cells affected by a genotoxin are chromatid and chromosome gaps, chromosome breaks, chromatid deletions, fragmentation, translocation, complex rearrangements, and many more. The purpose of toxicological *in vitro* testing is to determine whether a substrate produces genetic damage. Test methods most commonly used for genotoxicity/mutagenicity testing are bacterial reverse mutation test (Ames test), mammalian chromosome aberration test, mammalian cell gene mutation test, *in vitro* mammalian cell micronucleus test (Kirkland, Aardema, Henderson, & Muller, 2005).

The single-cell gel electrophoresis (SCGE), also called Comet assay is one of the most common tests for genotoxicity (Magdolenova et al., 2014).The methods' main advantages include; only a small cell sample is required, possibility to measure damage in practically any mammalian, relatively fast and economical procedure, and various applications of the method, which allow measurement of a range of different DNA lesions as well as DNA repair (Azqueta, Lorenzo, & Collins, 2009). It is a widely used method to detect strand breaks as well as specific DNA lesions like oxidized purines or pyrimidines

and is considered e.g. useful for genotoxic testing *in vitro* and *in vivo* (MDU, 2013).

In the 70s, Peter Cook and Co developed a method to investigate nuclear structure based on lysis cells with nonionic detergent and high-molarity sodium chloride (Collins, 2004; Cook, Brazell, & Jost, 1976). That treatment removes membranes, cytoplasm, and nucleoplasm, and disrupts nucleosomes, almost all histones being solubilized by the sodium chloride. Left is the nucleoid, containing the nuclear matrix or scaffold composed of RNA (ribonucleic acid) and proteins, together with the DNA, which is negatively supercoiled because of the turns made by the double helix around the histones of the nucleosome.

Östling and Johanson were the first to demonstrate "comets". They described the tails as DNA with relaxed supercoiling, as a halo of relaxed loops pulled to the anode of the electrical field (Collins, 2004).

The lysis process removes membranes, cytoplasm, nucleoplasm, and disrupts nucleosomes, the high salt will solubilize almost all the histones. Electrophoresis at high pH results in "comets", these are scored and observed in a fluorescence microscope; the intensity or signal of the comet´s tail, comparative to the head, shows the degree of DNA damage (Collins, 2004; Lorenzo, Costa, Collins, & Azqueta, 2013). The damaged DNA from the nucleus has an increased migration to the anode under the electrical flow, this gives the structure and the look of a "comet tail" (Rojas, Lopez, & Valverde, 1999).

2. Aim of study

Because of the widespread industrial use of TBBPA and moderately slow breakdown in the nature, it is abundant in the environment and a likely threat to human- and animal health.
The compound has been associated with effects on the immune system, endocrine effects and neurotoxicity. Most recently, TBBPA has been shown to cause transformation in SHE cells, something that is an indication of early cancer.
The main objective of this study was to investigate the effects of the brominated flame retardant TBBPA, using the epithelial liver cell line IAR20 *in vitro* with special emphasis on cell viability, gap junction intercellular communication and DNA damage.

Specific aims were:

- Investigate cytotoxicity of TBBPA on the IAR20 cells using an Alamar Blue Assay.
- Investigate whether the exposure to TBBPA disrupts the cell communication through gap junctions in monolayer cultured IAR20 cells
- Investigate genotoxicity of TBBPA on the IAR20 cells and at which exposure-concentrations this would be the case.

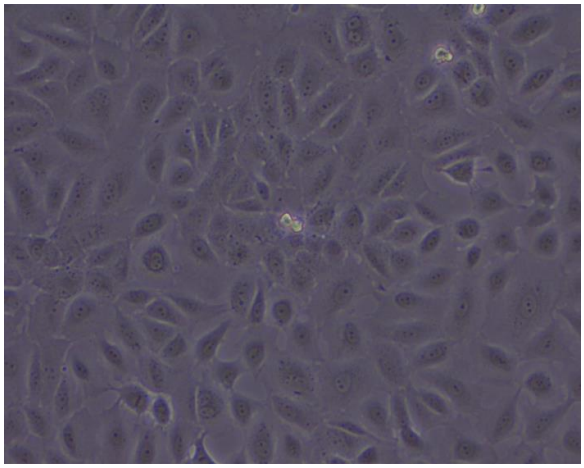
3. Materials and methods

3.1 Chemicals

H_2O_2 (Hydrogen peroxide) (#328385), Chlordane (#45378), Lucifer Yellow (#L0259-25MG) and TBBPA (3, 3'5, 5'-Tetrabromobisphenol A) (purity> 97.0 %) were all purchased in powder from Sigma-Aldrich® (St Louis, MO, USA).

3.2 The cell line IAR20

The cell line used for the experiments was IAR20, a non-tumorigenic epithelial cell line, was isolated from the liver of normal inbred BDVI rats. The cells were obtained from Dr Edward Leithe, the institute of Cancer Research, Oslo University Hospital HE-Norwegian Radium hospital. It was a generous gift to the research group. The culturing of the IAR20 cells has been performed in accordance to the protocol provided by Professor Edward Leithe. The morphology of the cell line cultured in a monolayer can be viewed in the figure below (Figure 2).



Figur 2 *Phase-contrast image of the morphology of IAR20 cells grown as monolayer. Taken with a Nikon TS1100 phase microscope at 10x objective. Scale bars 50µm*

3.2.1 Storage and retrieval of the IAR20 cells

Before all the experiments, the stock of cells was expanded and frozen. At 70-80% confluency the cells were trypsinized and centrifuged in a Heraeus 3SR+ multifuge (Thermo scientific, US) at 200g for

5minutes. The pellet of cells was re-suspended in 2ml growth medium, FBS and in the end DMSO added carefully to the mix reaching a concentration of 20%FBS and 8% DMSO. The cell suspension was aliquoted into cryotubes and frozen over night in -80^{o}C and then stored in a liquid nitrogen tank until retrieval. At retrieval the tube of cells were thawed in a 37^{o} C water bath (Lauda-Brinkmann, Delran, NJ, US) and seeded directly on a 100-mm culture dish with fresh growth.

3.2.2 Culturing of the IAR20 cells

The cells were grown as a monolayer in 10cm^{2} cell culture Falcon ^{TM} petri dishes (BD #353003) seeded 1 X 10^{6} cells per dish. Dulbecco's modified Eagle's medium (DMEM) (Lonza BE12-614F) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life technologies#26140079) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, US ref #25030081) at 37 °C in a humidified atmosphere of 5% CO_2 (HERA cell 150). When 70-80 % confluency was reached, the cells were trypsinized using 0,25% (w/v) trypsin (Sigma-Aldrich #25300-054, 0,05% EDTA) added to the monolayer and incubated at 37°C in a humidified, 5% CO_2 containing incubator for 5minutes to facilitate the enzymatic activity.
The cell detachment was viewed in an inverted light microscope (Nikon ECLIPSE, TS100). When the dishes were confluent, the cells were split 1:3-1:5 passage ratio. When necessary, the cells were thawed in a 37^{o} C water bath (Lauda-Brinkmann, Delran, US) and seeded directly onto a dish with new growth medium.

Cells passaged up to 11 times were used for the experiments, after that they were discarded. All additives and mediums were mixed and filtered through a 0.2µM filter (#514-0025) to get sterilized.

3.2.3 *In vitro* exposure of IAR20

The IAR20 cells were seeded onto the desired dish or plate 24 hours before the exposure to TBBPA and 48 hours before experiments. The growth medium with 10% FBS was replaced with DMEM supplemented with 1% FBS when being exposed. The cells were exposed to TBBPA with the following concentrations 0,0625µM, 0.625 µM, 1,25µM, 2,5 µM, 5 µM,10 µM,15 µM,20 µM, 25 µM, and DMSO as a solvent control, the concentration of DMSO was 1/1000 after dilution. For each method used in this study, three equivalent experiments were performed.

3.3 Scrape dye loading

Scrape dye loading is a technique to measure the degree of gap junction intercellular communication (GJIC) in a wide variety of mammalian cells. Scrape dye loading combined with image analysis has some limitations with regard to the cells that may be used. For optimum results it requires homogenous cells growing in a direction independent manner, and the cells need to be strongly attached to the dish to avoid cell detachment during the scrape loading procedure(Opsahl & Rivedal, 2000).

A quantitative determination of dye spreading takes place through image analysis. The fluorescent dye, Lucifer yellow CH (MW 457.2, Sigma Life science LO259-25MG,) is added to the cell monolayer and the degree of intercellular communication (IC) is calculated using the area percentage of the diffusion of Lucifer yellow (LY). The distance the LY wanders from the scalpel wounds indicate the communicative area for the cells. The IAR20 cells are communication- competent so the LY transmission takes place within minutes after loading with the dye (El- Fouly MH et al 1987).

3.3.1 Preparation for scrape dye loading

$1x10^6$ cells in growth medium were seeded onto 60 mm cell culture dishes (Falcon, Corning) and incubated at 37^oC for 24 hours, medium was replaced with medium with 1% growth medium and each dish exposed with the mixtures in similar dilution ranges as described above. After exposure, the cells were incubated at 37^oC for 24 hours. Three independent experiments were carried out.

3.3.2 GJIC *in vitro* assay on IAR20 cells

After 24 hours of exposure the IAR20 cells had reached a confluence of about 80%, the monolayer of cells was rinsed twice with DPBS (Ca2+/Mg2+, Life Technologies #14040-117). 3-4 ml of 0.05% (w/v) concentration of Lucifer Yellow (Sigma # LO259-25MG) dissolved in PBS (LONZA #BE17516F) was applied to cover the cells. A blade of a scalpel was surged gently down to make 5 scrapes in the cell monolayer to allow passage of the membrane impermeable dye into ruptured cells. The LY was left in the culture dish to stain the cells for 3,5 minutes. This was found to be the ideal time for the IAR20 cells (Opsahl & Rivedal, 2000).The culture dish was then washed four times with PBS to remove the excess dye. The monolayer was fixated with Formaldehyde 4% (m/v)(VWR #361387P) aqueous

solution covered with aluminum foil in a fume hood over night. The next day the dish was washed twice with DPBS, after that 55 µl of a high quality anti-fade medium; Mowiol^{R} (#101289165) was gently added to the dish and covered with a cover slip.

A positive control was provided through exposure to 30 µM chlordane (# 45378) for 1h. This was used to achieve a complete inhibition of GJIC in the IAR20 cells. Cell culture dishes were shielded with aluminum foil to protect the cells from light exposure and stored at 4°C before the wounds were photographed in the fluorescence microscope (Olympus IX2-UCB).

3.3.3 Image J

The images were quantified using Microsoft Excel, JMP and Image J. Image J is a public domain National Institute of Health image program that performed the analysis of the photos taken in the fluorescence microscope (Leithe, Cruciani, Sanner, Mikalsen, & Rivedal, 2003). Of the three similar experiments (parallels), 10 images with matrix size of 2080 x 1544 pixels where collected of each concentration or cell culture dish by using a fluorescence microscope (10xobjective). Image J converted the images to binary 8-bit contrast images. The migration of Lucifer Yellow is presented as a percentage of the solvent control value and the distance the LY migrated from the scalpel wound is revealing the degree of intercellular communication that has taken place. The LY dilithium, dissolved in PBS was stored and protected from light at 4^{o}C between each experiment. Mowiol 4-88 was dissolved according to manufacturer's procedure and stored at room temperature.

3.3.4 Challenges with fluorescence microscopy

Quantitative work on the fluorescence images acquired from the microscope began while performing the last parallel of the three doing the scrape dye load assay. The images came up with the right side of the image faded and much darker than the photos had used to be.

The source of the problem with the microscope was not detected, but solved by cutting away the dark part of the images (about 33% of the area) using Microsoft office. As this cutting was done for all of the images within one parallel it would not affect the result in comparison with the two other parallels, as the % GJIC was calculated as percentage of the solvent control (DMSO). An other

challenge microscoping was background noise in a few of the photos, this was corrected by adjusting the threshold.

3.4 Analysis of the viability of the cells

To check the viability of the TBBPA exposed cells, the Alamar Blue Assay (ABa) was applied; this is an assay to generate a quantitative measure of viability and cytotoxicity (Raz, Iten, Grether-Buhler, Kaminsky, & Brun, 1997). Healthy cells will continuously reduce resazurin, a blue non-fluorescent indicator dye to resorufin, a red and fluorescent dye.

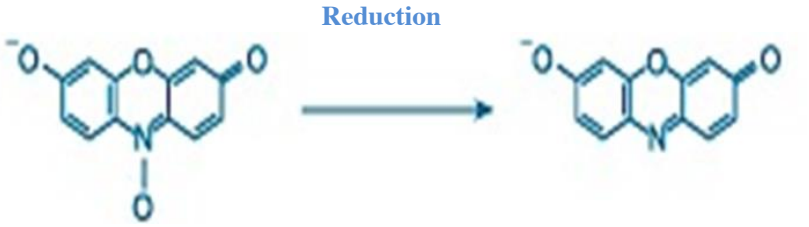


Figure 4 *Resazurin (left) is converted to bright red–fluorescent resorufin (right) via the reduction reactions of metabolically active cells. The amount of fluorescence produced shows the number of living cells ("alamarBlue®—Rapid & Accurate Cell Health Indicator,")*

If the color of the media in the wells change from blue to red, the cell´s mitochondric activity is still adequate, if the color of the wells remains blue, this indicates that the cells are unhealthy.
The amount of absorbance is proportional to the number of living cells and relates to the cells metabolic activity. Cells that are damaged and not viable have a lower innate metabolic activity and therefore generate a lower signal than healthy cells. 15000 cells/well were cultured in a 96 well plate (#734-2097). Three independent experiments were performed on three different plates with four equivalents in each plate. AB was added directly into the culture media at a final centration of 10%, the plate was then moved back to the incubator. The plates were exposed to an excitation wavelength of 570nm and an emission wavelength 600nm with a standard spectrophotometer at 3,5 –4 h after adding AB (Al-Nasiry et al., 2007).

3.5 The comet assay

The comet assay in its most general procedure consists of lysis with detergent and high salt after having embedded the cells in agarose, so that the DNA is immobilized for the electrophoresis that follows. Cells mixed with agarose are placed on a microscope slide and lysed with detergent and high salt to form nucleoids that contain supercoiled loops of DNA linked to the nuclear matrix. The comet assay used in this thesis is the alkaline version of comet assay, which detect single – or double stranded DNA breaks and the procedure is done according to Andrew Collins(Azqueta & Collins, 2013) as it will be described below.

First the lysis solution, the electrophoresis solution and the positive control (Hydrogen peroxide) solutions were prepared according to Hudocova et al. (Hudecova et al., 2012), (Hudecova et al., 2010). The microscope slides were pre-coated with 100 µl of 1 % normal melting point agarose in distilled water and the slides were placed to dry for 24 hours. The cells were re –suspended in 1 % low melting point agarose in PBS buffer (Ca^{2+} and Mg^{2+} free).
The lysis solution was made by weighing 146, 1 g NaCl, 37,22g EDTA(#E5134-500G), 1,21g Tri-hydroxymethyl aminomethane (#33621260), these chemicals were added to 900 ml of distilled water and placed on a magnet stirrer, while the pH was adjusted up to 10 by using a 10 M NaOH solution adding up to a volume of 1 L. The same day of the experiment 1% Triton X-100(#T-9284) was added to the lysis solution and it was stored at 4°C until use.
The electrophoresis solution was made by mixing 2l ddH2O with 24g NaOH, and 0,74g EDTA, pH(13).
Hydrogen peroxide (H_2O_2) was used as a positive control, it is an oxidative biocide that cause damage in the cells at a molecular level (Linley, Denyer, McDonnell, Simons, & Maillard, 2012).
H_2O_2 concentration: Solution A: 11 µl stock solution (30% w/v i.e 30 g / 100 ml; 9.82 M) in 1 ml of PBS = 100 mM. Solution B: 10 µl solution A in 1 ml of PBS = 1 mM. The final solution of H_2O_2 was a concentration of 30 µM.

The IAR20 cells were first cultured and exposed to TBBPA in 24 well plates as described in section 3.2, except the number of cells seeded was $3x10^5$ per well. The exposure-concentrations used in the comet assay were 2,5µM, 5µM, 10µM, 15µM, 20µM and 25µM. H_2O_2 was used as a positive control at 30 µM and DMSO as a negative control. After exposure and incubation the cells were trypsinized

with 200 µl per trypsin per well and transferred to 1,5ml eppitubes. The tubes were centrifuged at 300g for 10min. After the supernatant was taken out, the pellet was resuspended and 170 µl 1% low melting point agarose (#A9414-10G) in PBS (#BE17-516F) was added to the eppitubes. Then 140 µl of this suspension was placed on pre-coated slides. One cover slip was placed over each drop of cells and LMP agarose to form a flat small gel. The slides were then placed in the fridge to cool for 7min after this the cover slips were gently removed and the slides put into staining jars. The slides for positive control were divided into staining jars with hydrogen peroxide solution (#328385) and left for 5min,after this they were rinsed twice in PBS (#BE17-516F). All the slides were then put into staining jars filled with the lysis solution and left for 1hour in the fridge.

After lysis the slides were directly transferred to an electrophoresis tank containing electrophoresis solution for 20 min to unwind at 4°C. After this the electric source was turned on adjusted at 25 V, between 260-320mA and run for 30 min. The slides were then neutralized in PBS for 7 min and ddH_2O 7 min at 4°C and left to dry in room temperature over night. Before scoring the comets in the microscope (Leica DMI6000 B) the slides were stained either with 20µl of SYBR Gold (0,1 µl/ml in TE buffer (10 mM Tris –HCL, 1mM Na2EDTA, pH 7.5 – 8). The aliquots were stored at -20ºC until use. Prepared aliquots SYBR Gold was provided at UIO, Oslo university hospital.

The comet assay was performed with a smaller range of concentrations of TBBPA than that used in the scrape dye load assay and the AB assay. The reason for this was because the cells showed to have a very low viability already at an exposure of 10 µM and upwards. So there was no point in using the highest and the lowest concentration of exposure, as the cells were dead and very viable at those stages. The concentration 25 µM was not included, as necrosis would have taken place at that time, information was used from the Alamar blue assay on the cell viability to determine this before the comet assay started

The number of comets to be scored per gel was 50 (2 gels per concentration) and were scored by the image analysis program; Comet Assay IV 4.2, Perceptive Instruments Ltd. This program is designed to differentiate comet head from tail, and to measure a variety of parameters including tail length, and results were expressed as % of total DNA fluorescence in tail. Three experiments with identical cell samples were performed.

3.6 Statistical analysis

The statistical analysis was performed using JMP 9 software (SAS Institute Inc, Cary, NC, USA).
The distributions of dependent variables were tested for normality by the Shapiro –Wilk test. All studied endpoints gave a satisfactory fit to the normal distribution.
Hsu –Dunnett`s T –test was used to compare exposed groups to control. General linear models (GLM) were used. Percentage of DNA damaged cells, percentage of GJIC and percentage of living cells were dependent variables. Independent variables were experiment (n=3) and dose of TBBPA were entered as a discrete variable. Differences between exposure groups and controls were assessed with the Hsu-Dunnet`s test. Dose-response relationships were evaluated by fitting experiment as a discrete variable and dilution of the mixtures as a spline function. P-values < 0, 05 were considered statistically significant.

4. Results

4.1 The viability assay

The IAR20 cells were incubated with Alamar Blue as described in section 3.4. The results of the viability assay performed on the IAR20 cells are presented in a coupled diagram together with the results from the scrape dye load assay (Figure 6).

The results show that the viability of TBBPA exposed cells is stable until the concentration of 15 µM exposure where an immediate decline of the curve can be observed. There was no apparent effect of solvent control (DMSO) exposure alone and it did not show to decrease the cell viability significantly in comparison with the medium control.

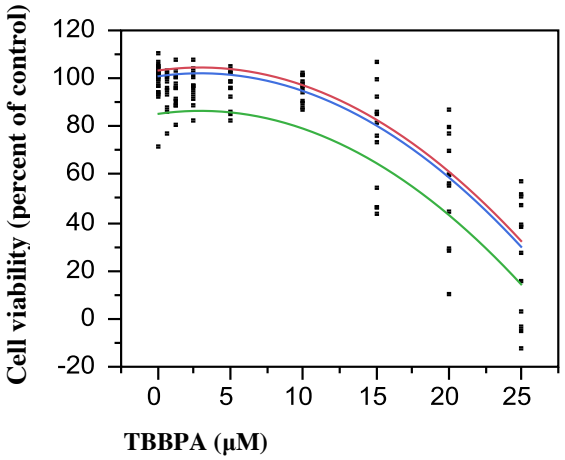


Figure 5 *Cell viability (%of solvent control) as a function of TBBPA, using the Alamar Blue assay, IAR20 cells in monolayer. The data were modeled with experiment t(shown separately) and TBBPA dose entered as spline function. Semi-logarithmic diagram: Log dose values were added as spline function (Y=x^2 +a) Log dose vs. percentage of viability (Percent viability %= Experiment+ TBBPA (continuous) + $TBBPA^2$ + error).*

To demonstrate the difference between the experiments the Figure 5 is attached above. The colored lines represent each individual experiment, as it shows one of the parallel experiments had an overall lower viability (green curve), the model explained, 0,83 (R^2) of total variation.

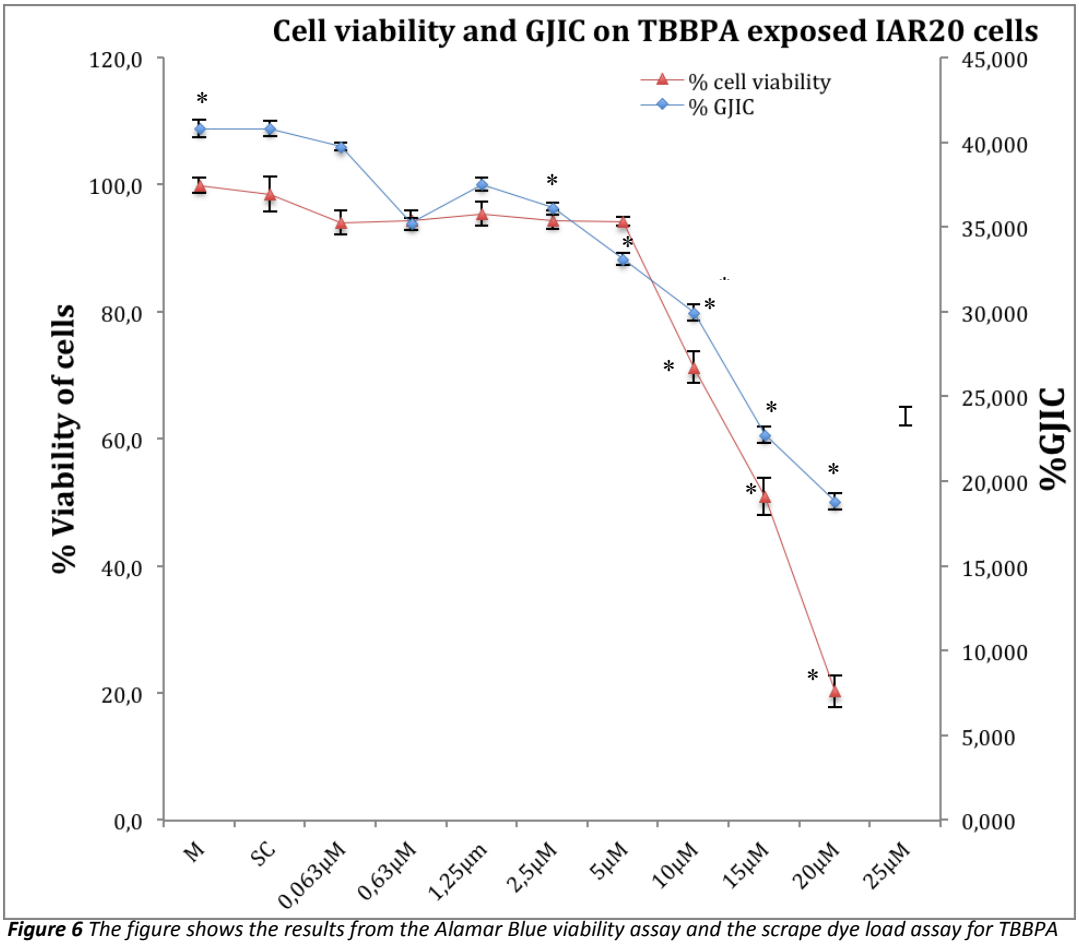
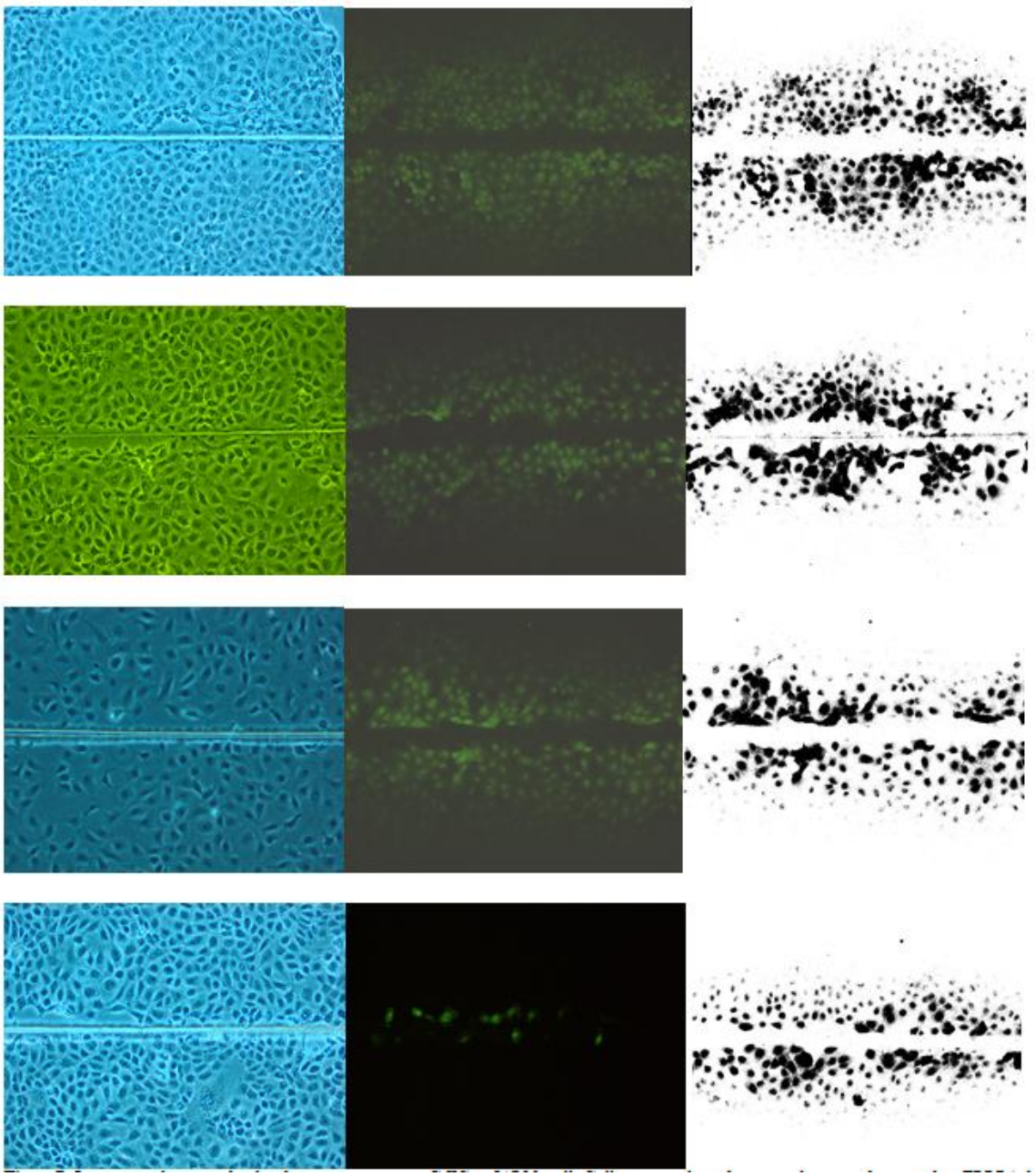


Figure 6 *The figure shows the results from the Alamar Blue viability assay and the scrape dye load assay for TBBPA exposed IAR20 cells. The viability (%of control) is shown on the left Y-axis and the GJIC on the right Y-axis. The curve for GJIC is shown in blue with triangles (the one starting above the other curve) and the lower curve in red represents cell viability. * Significantly different from solventcontrol (Hsu-Dunnets test; p < 0.5).*

4.2 The scrape dye loading

The quantification of GJIC in percent of DMSO is shown in Figure 6 above. The results demonstrate stable GJIC up to a concentration of 2,5µM TBBPA, whereafter there was a decline. The GJIC-curve declined along with the reduction in cell viability (fig. 6).



Figur 7 *Overview of scrape dye load assay to measure GJIC in IAR20 cell. Cells exposed to TBBPA for 24 hours. The images from left to right are; (1s) phase contrast microscopy, (2s) fluorescent microscopy and (3s) binary contrast 8-bit images. Rows from the top and down Row 1: Solvent control (DMSO) Row 2: 2,5 µM TBBPA. Row 3: 15µM TBBPA. Row 4:25 µM TBBPA. Scale bars 50 µm*

4.3 The comet assay

The photos below show how the comets appeared in both controls in comparison with the highest concentration of the range, 20 µM of TBBPA. DNA damage was calculated as the DNA tail area /whole DNA area (%) and the comet tail length, from the center of DNA head to the end of the DNA tail, in IAR20 cells. The bigger the DNA tail area (%) or the longer the DNA tail length, the more significant was the damage.

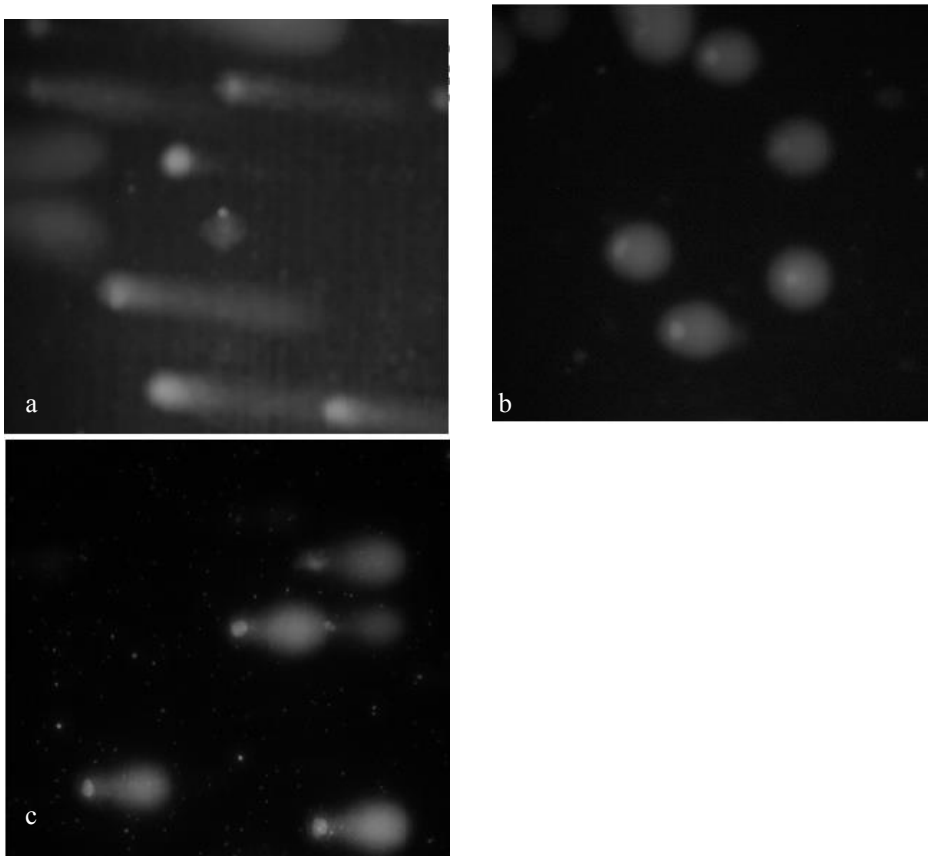
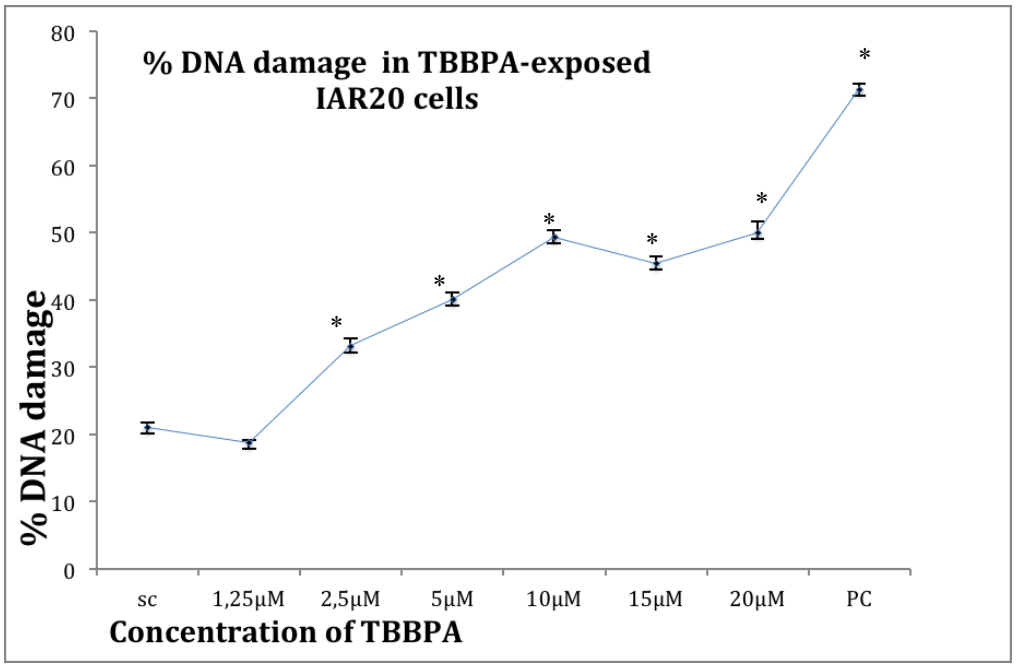


Figure 8 *Illustrative overview of comet assay to measure DNA damage in IAR20 cell. The images are a) positive control (H_2O_2), b) solvent control (DMSO) and c) 20 µM of TBBPA. Pictures taken with a 20X objective*



Figur 9 *Percentage DNA damage in the IAR20 cells after exposure to TBBPA for 24 hours. * Significantly different from solvent control (P<0,05;Hsu-Dunnett test)*

TBBPA exposure resulted in a significant increase in %DNA damage at concentrations> 2,5 µM TBBPA when compared with solvent control (P<0,05;Hsu-Dunnett test)

5. Discussion

The results showed that TBBPA can reduce cell viability, disrupt GJIC and cause DNA damage in IAR20 cells. In these experiments the cells were exposed to only one compound, the brominated flame retardant TBBPA. Under normal conditions outside the laboratory, humans are exposed to a mixture of different chemicals, which might influence the responses to single compounds like TBBPA alone.

The concentrations of TBBPA used in this study are similar to those found in lipids from Japanese workers at an electronic dismantling plant were 2.4 - 12.0 µg·kg^{-1} (up to 0,203 µM) was found. The highest concentrations used in the present study are higher than what has been found in living organisms.

It is difficult to predict outcomes in living organisms from *in vitro* experiments. In the present study cells were only exposed for 24 hours, while in living organisms exposure goes on for a longer time period and perhaps also during sensitive windows of development. However, although In vitro results

cannot be used to predict in vivo outcomes directly, it provides indications of exposure effects and useful information relevant in risk assessment.

5.1 Cell viability

Effect on cell viability by TBBPA exposure was tested with Alamar Blue against the solvent control (0.01% DMSO) after 48 hours of incubation. All of the nine exposure concentrations of TBBPA were included. Because TBBPA was diluted in DMSO, the effect of DMSO on cell viability was also examined by including a medium control (M).
The exposure concentrations 15, 20 and 25 µM were significantly different from the solvent control. The cells remained viable up to an exposure of 5µM TBBPA, while the intercellular communication showed inhibition already at 2,5 µM. This shows that even though the cells may still be viable at that concentration, the GJIC can have been inhibited long before they die.

Previously, the impact of TBBPA-exposure on viability was investigated on a human epithelial alveolar type II-like lung cell line, Cal-62 human thyroid anaplastic carcinoma cells and normal rat kidney epithelial cells (NRK) (Strack, Detzel, Wahl, Kuch, & Krug, 2007). The exposure of these cells to TBBPA showed a similar increase in cell viability as with the IAR20 cells, although at a higher exposure concentration dose than used in the present study. All of the cell lines had a significantly reduced viability in the concentration range 25 to 200 µM.

5.2 The gap junctional intercellular communication

In this study, one aim was to investigate how TBBPA affects the intercellular communication of monolayer cultured IAR20 cells. The GJIC was shown to be significantly reduced from a concentration of 5 µM and higher.
TBBPA is a compound is appropriate in studies of GJIC because it has been found to be homogenously distributed in the cell membrane of human adrenocortical carcinoma cell line (H295R) (Gutleb et al., 2012).

One way the TBBPA could work in the cells is by modifying the structural organization of the membrane lipids. As lipophilic compounds such as BPA can modify the structural organization of membrane lipids and dramatically affect functional properties of membrane proteins. The membrane proteins are embedded in a core of lipids that stay in a gel state. The gating of gap junction channels comes from conformational changes in channel proteins; therefore, the changes in the properties of the lipid bilayer can have effects on the functional stages of gap junction channels. In brief, TBBPA could maybe, as other lipophilic compounds like BPA, interact with plasma membrane components, and modulate GJIC (Lee & Rhee, 2007).

In one study Leydig TM3 cells derived from the testis of the immature BALB/c mouse were used to determine if estrogenic compounds inhibit gap junction function. The compounds used were: diethylstilbestrol (DES, a synthetic estrogen), 17β-estradiol (E2, a natural estrogen), and genistein (GEN, a phytoestrogen), they all caused GJIC inhibition in the TM3 cells after 24 hours (Iwase, Fukata, & Mori, 2006). TBBPA has shown to be estrogenic by means of estrogen receptor binding and growth stimulation in a rat pituitary tumor cell line, whose growth was stimulated by estrogens (Kitamura, Jinno, Ohta, Kuroki, & Fujimoto, 2002).

5.3 The genotoxicity, comet assay.

The results indicated that exogenous DNA damage was induced after exposure to TBBPA for 24 hours. The DNA damage was shown to be significant from a concentration of 2,5µM and up to 20µM of TBBPA.

A few challenges appeared during the comet assay as some of the slides had no comets at all, too many or that some had different signal and noise, this could be due to the use of a clouded SYBR gold reagent and cell debris following the samples that had been pipetted onto the pre-coated slides. On some of the slides the amount of cells were also very high something which caused difficulties finding individual comets.
The results above show a lower DNA damage between 10µM and 20µM of TBBPA, the DNA damage has a little drop in DNA damage from 10µM to 15µM. Cells have mechanisms that help them survive in the midst of extreme change in environment. The change of environment can activate the expression of different genes whose protein products shield the cell from the harmful effects of this change (Alberts B, 2002). Another explanation is that the slide had fewer comets and was randomly scored in an area of the slide with less damaged cells and therefore it appeared with an averagely lower % DNA damage. A solution to this could be to include more than just three equivalent experiments.

A previous report demonstrated that the brominated flame retardant 2,2-bis (bromomethyl)-1,3-propanediol (BMP) induced DNA strand breaks and oxidative base damages through generation of oxidative stress shortly after its exposure in UROtsap53 cells (an immortalized human urothelial cell line). Although the induced damage seemed rapidly repaired, the early genotoxic events may contribute to the BMP-induced carcinogenesis observed in rodents and help understand how other brominated flame retardants like TBBPA could induce DNA damage (Kong, Kuester, Gallegos, & Sipes, 2011).

A study was performed on fathead minnows, exposing them with the flame retardants Firemaster 550 and Firemaster BZ-54 that contained Tetrabromobenzoate (TBB), the nonbrominated flame retardant di-(2-ethylhexyl) phthalate (DEHP) and 2,3,4,5-tetrabromo-bis(2-ethylhexyl) phthalate

(TBPH). During the exposure period a significant increase in DNA strand breaks occurred in liver cells but not in blood cells. That study provided evidence for genotoxicity of these new flame retardant formulations in fish and can maybe add to understand how the liver cell line IAR20 can be subject to DNA damage when exposed with TBBPA (Bearr, Stapleton, & Mitchelmore, 2010).

BPA is reported to have possible genotoxic effects in cultured cells. Micronuclei are characterized in the cells that have some sort of DNA damage. Micronuclei are small, extranuclear bodies that are formed during mitosis from lagging chromosomes. They are often found in cancer cells, or cells that have been exposed to increased risk factors, as BPA has a similar chemical structure as TBBPA. There is support in knowing that BPA can act in a genotoxic manner in hamster cells and can confirm the DNA damage after TBBPA exposure in the present study (Strack et al., 2007),(Jaeg, 2004).

6. Conclusion

The results achieved through this thesis, demonstrate that TBBPA disrupts intercellular communication through gap junctions and can cause DNA damage in IAR20 cells, with significant effect.

The results showed that TBBPA disrupted intercellular communication through gap junctions and caused DNA damage in IAR20 cells at exposure concentrations below the level of cytotoxicity.
The effects were observed at concentrations relevant for what can be found in living organisms. This raises concerns with respect to risk assessment related to human health. Additional research on how exposure to TBBPA can affect living organisms is necessary to gain a better insight into how it works in real life.

7. Future perspectives

It would be interesting to involve further studies into the lower dose range of TBBPA, as a significant increase in DNA damage took place already at 2,5 µM.
C43 is a tumor suppressor gene both in cell culture and animal tests, and restores growth regulatory and differential properties of carcinoma cells (Kato & Kenne, 1996; Leithe et al., 2010). Since IAR20 also contains connexin 43 it would be interesting to involve western blotting in future experiments to investigate whether degradation of Cx43 expression would take place because of TBBPA exposure in the same dose range used in this study.

As TBBPA can act as an endocrine disruptor by imitating the shape of Thyroxin and estrogenic by means of estrogen receptor binding, it would also be of interest to use techniques and a different cell line to detect endocrine disruption in the same dose range of TBBPA as used in this study.

Further investigation should be carried out to elucidate whether similar results after TBBPA exposure would also occur *in vivo*, that would gain a better insight to how it works in real life.

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APPENDICES

PRODUCT	SUPPLIER	PRODUCT NUMBER
DMEM	Life tecnologies (LONZA)	BE12-614F
96-microwell plate	VWR (Thermo scientific)	734-2097
Mowiol 4 88	Sigma-Aldrich	81381
Alamar blue dye	Life Technologies	DAL1100
Lucifer yellow CH dilithium salt	Sigma-Aldrich	L0259
DPBS (Ca2+/Mg2+)	Life Technologies (Gibco)	14040-174
BD Tissue Dishes 60 mm	VWR (BD Biosciences)	353004
Formaldehyde 4%(m/v)	VWR	361387P
Chlordane 30μM	Sigma-Aldrich	45378
Sodium chloride (NaCl)	MERCK	106404
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich (Saint Louis, US)	E5134-500G
Tri- (hydroxymethyl) aminomethane	VWR	33621260
Sodium hydroxide	EKA (Elektrokjemiska aktebolaget)	MW40
Agarose, low gelling temperature	Sigma Aldrich (Saint Louis, US)	A9414-10G
Agarose for molecular biology	Sigma Aldrich (Saint Louis, US)	05066-50G
PBS	LONZA (Life Technologies	BE17-516F
Hydrogen peroxide(H_2O_2)	Norsk medisindepot	328385
Triton x 100	Sigma Ultra	T-9284
4',6-diamidino-2-phenylindole(DAPI)	In Vitrogen	D1306
SYBR® Gold Nucleic Acid Gel Stain	Life technologies™	unknown
L-Glutamine	Life tecnologies(Gibco)	25030081
Cryotube vials	Thermo Scientific	363401
15 ml tube	WVR(Falcon, Corning)	352096
50 ml tube	WVR(Falcon, Corning)	352070

APPENDICES

Sterile vacuum 0,2 µm filtration unit	Thermo scientific	514-0025

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