In Vitro Effects of Environmental Chemicals on Pancreatic β-cells

Possible relevance for type 1 and type 2 diabetes

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ABSTRACT

Plastic products are widely used in our modern life, and unbound chemicals such as bisphenol A (BPA) and phthalates can leak out into the surrounding environment. Special attention has been directed toward these chemicals due to high production volume, widespread use, and endocrine disrupting effects. Both BPA and phthalates are detected in urine and serum samples from the majority of the investigated populations. Increased exposure to environmental chemicals has been linked to the increasing incidence of type 2 diabetes (T2D), while possible associations between such chemicals and type 1 diabetes (T1D) has received less attention. However, experimental studies have indicated that these chemicals may affect the immune system and promote autoimmunity and autoimmune diseases like T1D.

The main objective of this study was to investigate whether BPA, and three phthalate metabolites (Mono-iso-butyl phthalate (MiBP), Mono-n-butyl phthalate (MnBP) and Mono(2-ethylhexyl) phthalate (MEHP)) alone or in combination could affect the β-cell viability, susceptibility to cytokine-induced apoptosis, or insulin secretion. These endpoints were chosen since they may be linked to an accelerated development of T1D and T2D. In addition, additive, synergistic or inhibitory effects of combined exposures were examined. The pancreatic rat β-cell line INS-1E cells was used as a model system, and concentrations of BPA, MiBP, MnBP and MEHP relevant for environmental exposures were included (1, 10, 50, 100, 500 nM), as well as higher concentrations (5, 50, 500 μM).

For the chemical concentrations relevant for environmental exposures (1-500 nM), no reduced β-cell viability, increased sensitivity to cytokine induced apoptosis or increased insulin secretion was detected. However, decreased viability was observed at 50 and/or 500 μM BPA, MEHP and the combinatory chemical exposures. BPA seemed to be the most potent of the chemicals. Moreover, the combination of the phthalates and the combination of phthalates and BPA appeared to result in additive effects.

The INS-1E cells are commonly used in mechanistic studies of cytokine-induced apoptosis as well as insulin secretion in response to glucose. However, in the present study, the cell line appears to be insensitive to the environmental chemicals tested. This suggests that INS-1E cells may not be a suitable model system for evaluation of environmental chemicals, since BPA has previously been reported to affect both viability and insulin secretion in primary islets and cell lines.

SAMMENDRAG

Plast produkter er mye brukt i vår moderne verden, og ubundne kjemikalier som bisfenol A (BPA) og ftalater kan lekke ut i omgivelsene. Spesiell oppmerksomhet har blitt rettet mot disse kjemikaliene på grunn av høyt produksjonsvolum, utbredt bruk, og hormonforstyrrende effekter. Både BPA og ftalater er funnet i urin og blodprøver fra hovedandelen av undersøkte populasjoner. Økt eksponering for miljøkjemikalier er nært knyttet til den økende forekomsten av type 2-diabetes (T2D), mens mulige sammenhenger mellom slike miljøkjemikalier og type 1 diabetes (T1D) har fått mindre oppmerksomhet. Imidlertid har eksperimentelle studier vist at disse kjemikaliene kan påvirke immunforsvaret og fremme autoimmunitet og autoimmune sykdommer som T1D.

Hovedmålet med denne studien var å undersøke om BPA, og de tre ftalat metabolittene Mono-iso-butyl ftalat (MiBP), Mono-n-butyl ftalat (MnBP) og Mono (2-ethylhexyl) ftalat (MEHP) alene eller i kombinasjon kunne påvirke β -celleviabilitet, sensitiviteten for cytokin-indusert apoptose, eller insulinutskillelse. Disse endepunktene ble valgt fordi de kan være knyttet til en fremskyndet utvikling av T1D og T2D. I tillegg ble additive, synergistiske eller antagonistiske effekter av kombinasjonseksponeringene undersøkt. Bukspyttkjertel β-cellelinjen INS-1E fra rotte ble brukt som modell system og konsentrasjoner for BPA, MiBP, MnBP og MEHP relevante for miljøeksponering ble inkludert (1, 10, 50, 100, 500 nM), i tillegg til høyere konsentrasjoner (5,50,500 µM).

Kjemikaliekonsentrasjoner som var relevante for miljøeksponering (1-500 nM) førte ikke til redusert β-celle viabilitet, økt følsomhet for cytokinindusert apoptose eller økt insulinsekresjon. Redusert viabilitet ble imidlertid observert ved 50 og/eller 500 µM av BPA, MEHP og kombinasjonseksponeringene, men BPA var det mest potente av kjemikaliene i vårt modellsystem. Videre, så det ut til at kombinasjonen av de tre ftalatene og kombinasjonen av ftalater og BPA resulterte i additive effekter.

INS-1E celler har ofte blitt brukt i mekanistiske studier av cytokin indusert apoptose, og insulin sekresjon etter glukosestimulering. I denne studien viste cellelinjen seg imidlertid å være ufølsom for de undersøkte miljøkjemikaliene. Dette kan tyde på at INS-1E cellene ikke er et passende modell system for å studere effekter av miljøkjemikalier, siden tidligere studier har vist at BPA kan påvirke både viabilitet og insulinsekresjon i primære øyer og cellelinjer.

1 INTRODUCTION

The use of plastics products is widespread in our modern world, and involves simultaneous exposure to many chemicals that may leak from the plastics (Alonso-Magdalena et al. 2006; Howard & Lee 2012). The daily exposure of various types of chemicals from plastics involves ingestion via contaminated food and beverage, absorption through skin contact, or inhalation (Kamrin 2009; Rubin 2011). Two major classes of chemicals leaching from plastics are phthalates, used as plasticizers in polyvinylchloride products, and bisphenol A (BPA), used in polycarbonate plastic. These chemicals are produced in several million tons per year (Grün & Blumberg 2007; Koch & Calafat 2009), and have also been detected in blood and urine samples from the majority of the population (Koch & Calafat 2009). In recent years, there has been a growing concern about these environmental chemicals and their adverse health effects, based on knowledge gained from animal studies (Alonso-Magdalena et al. 2012; Heudorf et al. 2007; Kang et al. 2006; Meeker et al. 2009; Rubin 2011). Therefore, regulations have been introduced in Norway and the EU on product content and production of phthalates and BPA, but they still occur in many consumer products that we are in daily contact with (Environment.no 2012a; Environment.no 2012b; Wormuth et al. 2006).

Interestingly, exposure to BPA and phthalates has been linked to several metabolic effects in both epidemiological and experimental studies, including diabetes (Alonso-Magdalena et al. 2011; Alonso-Magdalena et al. 2012; Bodin et al. 2013; Lang et al. 2008; Lin et al. 2011; Nadal et al. 2009; Shankar & Teppala 2011; Svensson et al. 2011). Diabetes is a common metabolic disorder worldwide, but the reason for its increasing incidence the last 30 years is still unknown (Vehik & Dabelea 2011). The increase has been suggested in part to be a result of unhealthy lifestyle changes associated with industrialization and rapid economic development, but it also coincides with a dramatic increase in exposure to synthetic chemicals (Makaji et al. 2011) including endocrine disruptors (Bodin et al. 2013).

The substantial economic and social costs associated with the increasing incidence of diabetes represents a major public health issue (The World Health Organization 2012b). Even though several environmental chemicals have been associated with type 2 diabetes (T2D), their potential role in the development of the autoimmune disease type 1 diabetes (T1D), has not received much attention. However, these environmental chemicals are thought to act as endocrine disruptors and may affect the immune system which further can promote autoimmunity (Howard & Lee 2012).

The aim of this study was to investigate whether two ubiquitous environmental contaminants; BPA and phthalates, may affect pancreatic β-cells *in vitro*, a cell type that has a central role in the development of diabetes (Song et al. 2012).

1.1 Diabetes prevalence and health consequences

The World Health Organization estimated that more than 346 million people worldwide were diagnosed with diabetes in 2012 (The World Health Organization 2012a). Additionally, many people are undiagnosed, and unaware that they are living with diabetes. There are several variations of diabetes, and this thesis focuses on the two main forms, type 1 and type 2 diabetes. For T1D, that usually occurs in the early childhood or in the young adult years, the prevalence has increased the recent years (The International Diabetes Federation 2011; Vehik & Dabelea 2011). The reason for this rapid increase in the very young is still unknown, but a contribution from both genetic background and environmental risk factors has been suggested (Vehik & Dabelea 2011). If this trend continues the number of new T1D cases may be doubled between 2005 and 2020 (Patterson et al. 2009). The Nordic countries have the world's highest average incidence rate of this disease, including Norway with an incidence rate of 27.9 per 100.000 in 20013 (Iacobucci 2013). Thus, although the increase in T2D incidence has received most attention, the rise of T1D in the very young should not be overseen (Patterson et al. 2009).

In contrast to T1D, T2D usually develops in the adult age and among the elderly, and is considered to account for more than 90% of all cases of diabetes (The World Health Organization 2012b). The accurate incidence of T2D in Norway is not known, as many patients do not use medications or may be undiagnosed. However, in 2011 103.000 Norwegians used medications for T2D, which is an increase from 71.000 in 2006 (Midthjell 2011).

Diabetes has severe impact on life quality and requires a careful daily diet control. If not controlled, it may result in acute and/or severe complications later in life. The delayed effects include macrovascular and microvascular changes that may lead to kidney failure, blindness, and amputations and enhanced risk of heart disease and stroke (Triplitt 2012). The World health organization (WHO) estimated that 3.4 million people died from complications due to

T2D in 2004 on world basis (The World Health Organization 2012b). Thus, the economic and social costs associated with T2D represent a major public health issue. Globally, the direct cost of diabetes ranges from 2.5 to 15% of the annual health care budgets, depending on local incidence and the treatment available (The World Health Organization 2012b).

The rising incidence in children is also a concern because these young individuals may experience several complications later in life, as well as early mortality (Triplitt 2012). It is therefore of importance to improve the understanding of the determinants in the disease development, such knowledge may help provide both individual and societal benefits through a more efficient prevention.

1.2 Mechanisms and Pathogenesis of Diabetes

Insulin and blood glucose regulation

Diabetes is characterized by a relative or absolute deficiency in the secretion or function of insulin. Insulin is a hormone contributing to the transport of glucose into the cells, thereby reducing blood glucose levels (Lin & Sun 2010). The insulin biosynthesis is controlled by glucose, but is also influenced by other signalling molecules including hormones (Nadal et al. 2009). Insulin is produced and secreted by β-cells, which is the dominating cell type found within the pancreatic islets of Langerhans (Triplitt 2012). Factors resulting in β-cell dysfunction may therefore disrupt glucose homeostasis and in this way contribute to the development of diabetes.

1.2.1 **Diabetes type 1**

T1D is an autoimmune disease characterized by an extensive loss of β-cells, causing total insulin deficiency. Thus, the patients depend on artificial insulin supply (Wyller 2005). The disease is only partly due to a genetic predisposition, as illustrated by studies on identical twins, where the risk of developing T1D is only approximately 50% even if one twin has already developed the disease (Howard & Lee 2012; Hyttinen et al. 2003). This implies that environmental factors like viral infections, diet, stress and toxins might accelerate this autoimmune disease when they occur together with genetically predisposed factors (Bresson

& von Herrath 2004). The etiology of T1D still remains incompletely understood, but is considered to result from an autoimmune process where the immune system attacks and destroys the insulin producing β-cells (Bresson & von Herrath 2004). The inflammatory process, insulitis, which is suggested to contribute to T1D can be divided into three phases (Figure1); (i) induction, (ii) amplification, and (iii) maintenance or resolution (Eizirik et al. 2009).

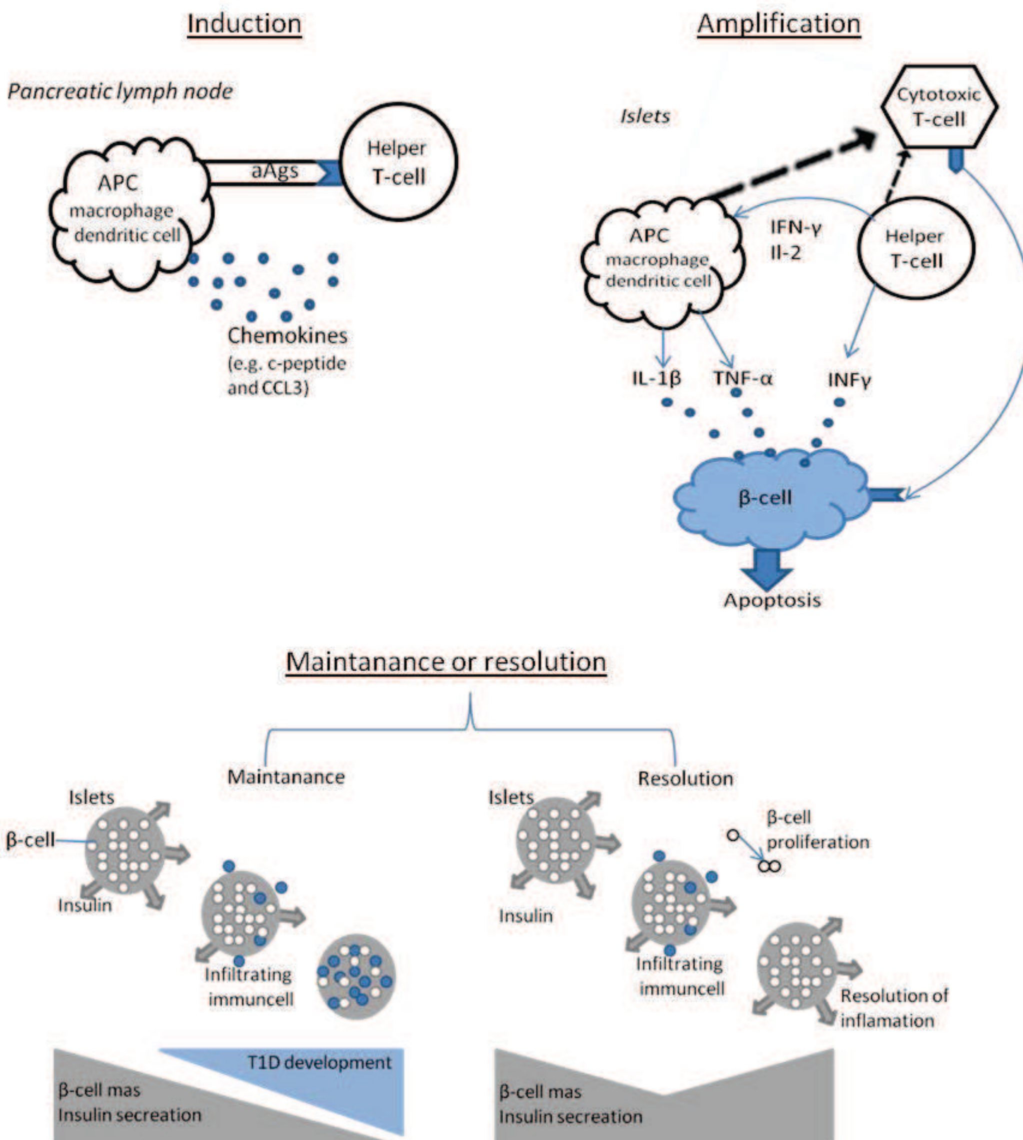


Figure 1: The autoimmune attack on the β-cells and the three stages of development of T1D.
Induction; The antigen presenting cells (APC) recruit and activate helper T-cells via presentation of β-cells auto antigens (aAgs) and secretion of chemokines. ***Amplification;*** Helper T-cells secrete cytokines which further stimulates the APC to secrete other cytokines. The stippled lines show that APC and helper T-cells contribute to recruitment and activation of cytotoxic T-cells indirectly via cytokine secretion. The cytokines and cytotoxic T-cells binds to the β-cells surface receptors and induce apoptosis via intracellular signalling. ***Maintenance or resolution*;** The maintenance is characterized by persistent infiltration of immune cells causing β-cell death and finally overt diabetes. On the other hand, a proliferation of β-cells and resolution of the inflammation may lead to normal islet function. The figure is based on figures from; Bresson and von Herrath (2004); Eizirik et al. (2009); Pirot et al. (2008).

Induction

The earliest sign of autoimmunity against β-cells is the circulation of autoantibodies (aAbs); proteins that recognize some of the β-cells own proteins (autoantigens, aAgs). Such aAbs might accelerate the disease by presenting aAgs to macrophages and T-cells (immune cells) (Pirot et al. 2008). However, many antibody positive individuals never develop insulitis or diabetes, which indicates that additional environmental triggers like viral infections, vaccination or toxins, may be required for the activation of autoreactive T-cells. T-cells might be activated through a molecular mimicry between viral proteins and β-cell antigens (Pirot et al. 2008). Several hypotheses have been proposed for the initial phase of T1D, but the exact mechanisms are not known. The attraction of immune cells to the pancreas by a local production of chemokines by antigen presenting cells, is however considered to play an important role in this process (Pirot et al. 2008).

Amplification

The progression of insulitis in the pancreatic islets is a characteristic feature of the amplification phase. This inflammatory state is characterized by infiltration of the pancreatic islet by immune cells, where macrophages are among the first immune cells to immigrate the pancreatic islets. An interaction between β-cells and immune cells is considered to occur during the course of insulitis, where activated macrophages and T-cells secrete cytokines such as tumor necrosis factor α (TNF-α), interferon-γ (INF-γ) and interleukin -1β (IL-1β) (Eizirik et al. 2009). The combinatory effect of these cytokines is suggested to promote the migration of cytotoxic T-cells into the islets and stimulate the β-cell release of cytokines and chemokines which further promotes the insulitis (Pirot et al. 2008).

Another consequence of increased cytokine levels is β-cell death, where apoptosis, the cells "autocrine suicide", has been suggested to be the main form of β-cell death (Bresson & von Herrath 2004; Pirot et al. 2008). Activated cytotoxic T-cells and pathogenic cytokine production can result in specific destruction of the pancreatic β-cell (Atkinson et al. 2011). The cytokines bind to receptors on the β-cell surface and cause activation of different complex intracellular pathways that leads to β-cell apoptosis (Pirot et al. 2008). Exposure to the cytokines in combination is suggested to trigger endoplasmic reticulum (ER) stress. The ER is the organelle responsible for the synthesis and folding of proteins in the cell, a process required for insulin secretion. Alterations of the normal ER function may contribute to impaired β-cell function and increased β-cell death (Makaji et al. 2011; Pirot et al. 2008).

Maintenance or resolution

The last phase can develop in two different directions. Most commonly, the inflammation is maintained with further suppression of β-cell function resulting in β-cell death, and eventually overt T1D. This sustained inflammatory process has also been hypothesised to cause insulin resistance (Pirot et al. 2008). Alternatively, an active resolution of the inflammation may occur in parallel with a stimulation of β-cell proliferation, but the mechanism behind this resolution process is still unknown (Pirot et al. 2008).

1.2.2 ***Diabetes type 2***

Unlike T1D, patients with T2D almost never have a total lack of insulin, but their insulin stimulation is not sufficient to regulate the blood glucose levels adequately (Ashcroft & Rorsman 2012). Patients with this disease therefore have abnormally high blood and plasma glucose levels (Lin & Sun 2010), and fasting plasma concentration of glucose above 7.0 mmol / l indicates T2D (Wyller 2005). Development of T2D is influenced by different lifestyle factors like diet, pregnancy, obesity and lack of activity, but genetic components are also thought to have a strong impact. The disease is irreversible at an early stage, but can often be controlled by an active lifestyle and diet regulations (Ashcroft & Rorsman 2012). The pathophysiology of T2D is very complex and varies within different tissues and organs (Triplitt 2012). However, the two central defects include (i) insulin resistance in peripheral tissues (i.e. diminished tissue response to insulin, predominantly in muscle, fat and liver) and (ii) islet β-cell dysfunction causing reduced insulin secretion (Aston-Mourney et al. 2008).

Insulin resistance

There are a large number of different insulin signalling pathways. The phenotype of insulin resistance will depend on the exact components affected and the exact tissues in which they are affected (Biddinger & Kahn 2006). Insulin resistance in the liver may cause increased hepatic glucose production, resulting in hyperglycemia, which itself is damaging to the β-cell function. High levels of circulating free fatty acids (FFA), due to decreased uptake of FFA in adipose tissue, may exacerbate insulin resistance through accumulation of lipid inside the muscle, liver and the β-cells (Hilsted et al. 2011).

β-cell dysfunction

The exact mechanisms involved in β-cell dysfunction remain somewhat unclear, but the proposed mechanisms are summarized in Figure 2. High blood glucose levels and FFA concentrations are thought to induce hypersecretion of cytokines from the β-cell, causing an inflammation, with β-cell dysfunction and increased apoptosis as possible consequences (Westermark et al. 2011). When insulin resistance has developed, the β-cells hypersecrete insulin to maintain normal blood glucose levels thus causing hyperinsulinemia (Nadal et al. 2009). Hyperinsulinemia is hypothesised to exacerbate the state of insulin resistance (Ueno et al. 2005), but also to deteriorate β-cell function due to a prolonged insulin demand. This may cause β-cell exhaustion, or promote stress responses that could cause apoptotic cell death (Figure 2) (Aston-Mourney et al. 2008). Paradoxically, hyperinsulinemia may therefore be an important mechanism contributing to T2D, a disease characterized by insufficient insulin levels. Whether insulin resistance precedes hyperinsulinemia or hyperinsulinemia precedes insulin resistance in the T2D development remains controversial, but they seem to walk hand in hand (Ropero et al. 2008).

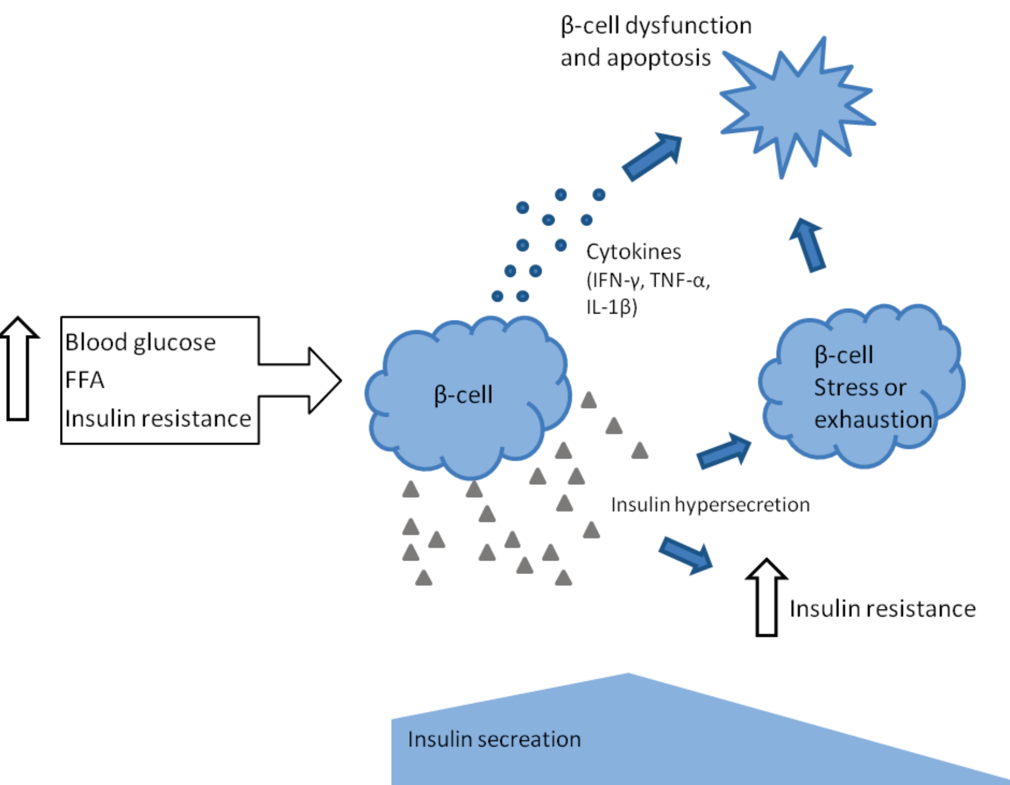


Figure 2: β-cell dysfunction in T2D.
High levels of blood glucose, circulating free fatty acids (FFA) and insulin resistance in different tissues, stimulate hypersecretion of cytokines and insulin from the β-cells. Cytokines may lead to an inflammation and further β-cell dysfunction and apoptosis. Hyperinsulinemia may lead to β-cell stress or exhaustion that can cause apoptosis. This eventually leads to reduced β-cell mass and insufficient insulin release.

1.3 Environmental chemicals

1.3.1 ***Bisphenol A***

BPA is a monomer used in the production of polycarbonate plastics, and is present in many consumer products that we are in daily contact with, like food containers, drinking bottles, receipts and resins lining the inside of metal cans (Rubin 2011). The chemical structural formula is shown in Table 1 together with chemical name and the acronym.

Table 1: BPA. Chemical structural formula, chemical name and the acronym of BPA, the formula is applied from Kang et al. (2006).

Chemical structural formula	Chemical name	Acronym
	Bisphenol A	BPA

BPA is one of the chemicals produced in the highest volume worldwide, with more than 8 billion pounds annually (Rubin 2011). It is not produced in Norway, but is imported for the manufacture of products (mostly plastic), or in finished products. The consumption of BPA in Norway in 2009 is estimated to be approximately 29 tons, but these estimations do not include imported products. Therefore, the actual amount of BPA in products sold in Norway is assumed to be significantly larger (Environment.no 2012). In Europe, consumption of BPA increased from 0.7 million tons in 1996/1999 to 1.2 million tons in 2005/2006. Recent information suggests that the consumption in Europe will continue to grow in the following years. This may also lead to increased consumption in Norway as a result of import of manufactured products from Europe (Environment.no 2012). The extent of BPA exposure is emphasized by Calafat et al. (2008), who estimated that BPA was present in urine samples of 92,6% of the U.S population. Interestingly, results from the Norwegian Mother and Child Cohort Study (MoBa) showed that BPA levels in urine samples from pregnant women in Norway was higher compared to urine samples from the Generation R-study in the Netherlands as well as the National Health and Nutrition Examination Survey (NHANES) in the United States (Ye et al. 2009).

BPA is not chemically bound to the plastic, and can leach out into for instance food or beverages. Ingestion is considered to be the major route of BPA exposure, although exposure through inhalation and absorption through the skin also occurs (Rubin 2011). Ingested BPA is rapidly conjugated with glucuronic acid in the liver, a process that is very important for the removal and detoxification of BPA (Kang et al. 2006), thus the biological half-life of BPA is short, around four hours (Chapin et al. 2008). BPA has been detected in human body fluids like blood and urine. The concentrations of free BPA in serum after ingestion are usually very low, in the 0.4 – 3 nM range (Völkel et al. 2002). However a bio-monitoring study has detected serum levels of BPA above 100 nM, in some highly exposed subjects (Olsén et al. 2012). BPA glucuronide is the major metabolite present, which implies that only a small part of the actual body burden of BPA is measured (Völkel et al. 2002). Additionally, a fraction of absorbed unmetabolized BPA is hypothesised to allocate into adipose tissue, resulting in a slow leaking of BPA into the bloodstream (Koch & Calafat 2009).

The daily intake of BPA for humans has been estimated to be less than 1 μg per kg body weight per day. Based on epidemiological data it has been suggested that BPA may have adverse endocrine disruptive effects at concentrations as low as 0.025 – 0.2 μg per kg body weight per day (Kang et al. 2006). Moreover, the highest levels were found in children, which may show increased vulnerability to BPA exposure during development (Rubin 2011). Accordingly, BPA has been associated with several health outcomes, such as cardiovascular disease, asthma, allergy, diabetes and increased numbers of premature deliveries and miscarriages (Donohue et al. 2013; Lang et al. 2008; Rubin 2011). Furthermore, diabetic patients have higher levels of BPA in urine compared to the non-diabetic population (Shankar & Teppala 2011). The growing concern about adverse health effects from BPA has contributed to regulations of BPA in Norway, and the use of BPA in baby bottles was banned in 2011 (Environment.no 2012).

A possible mechanism for the suggested BPA effects may be via binding to estrogen receptors in the plasma membrane (Alonso-Magdalena et al. 2006). Interestingly, overstimulation of estrogen reseptor α by an environmental such as BPA, has been reported to induce hyperinsulinemia, insulin resistance, and β-cell exhaustion in mice (Nadal et al. 2009). This pathway is thought to be related to the development of T2D (Makaji et al. 2011).

1.3.2 ***Phthalates***

Phthalates constitute a large group of industrial compounds that share basic chemical similarities. They are used as plasticizers in the production of soft polyvinyl chloride and other plastics in numerous consumer items, such as plastic gloves, paint, toys, and several personal care products (Kamrin 2009; Wormuth et al. 2006). Similar to BPA, phthalates are not chemically bound to the plastic, and may leak into the environment. Different phthalates have been found in a wide range of food items such as milk, meat, fish, seafood and vegetables (Kappenstein et al. 2012), but there is little documentation concerning phthalate levels in food sold in Norway. EU legislation on phthalates in materials with food entered into force in 2008 (Petersen & Jensen 2010). Thus, phthalate occurrence is probably mostly associated with imported products, contamination in production or cooking at home (NOU 2010). In addition, consumer products such as shampoos, cosmetics and skin creams contain various phthalates (Koch & Calafat 2009; Wormuth et al. 2006).

Ingestion is suggested to be the major exposure route, but inhalation and skin contact with clothing, toys or other products containing phthalates can also result in absorption through the skin (Kamrin 2009; Wormuth et al. 2006). In recent years, infants and toddlers mouthing of plastic objects have received particular attention. The infants are constantly in contact with plastic products directly through the mouth or through slipping on fingers after contact with the products (Wormuth et al. 2006). Children are particularly vulnerable to exposure because they are in their development phase (The Norwegian Institute of Public Health 2008; Wittassek & Angerer 2008). The use of phthalates in toys for children under 3 years, was therefore prohibited in the EU in 1999 (Wormuth et al. 2006). Structural formula of three of the major phthalate metabolites due to oral exposure are represented in Table 2 (Koch & Calafat 2009; Wormuth et al. 2006).

Table 2: Phthalat metabolites. Chemical structural formulas, chemical name and the acronym of the three phthalate metabolites included in this study. The formulas are applied from Koch and Calafat (2009).

Chemical structural formula	Chemical name	Acronym
	Mono(2-ethylhexyl) phthalate	MEHP
	Mono-iso-butyl phthalate	MiBP
	Mono-n-butyl phthalate	MnBP

Metabolism and elimination of phthalates is complex. In general, phthalates are diesters which are cleaved into their respective hydrolytic monoesters which can be further modified by different oxidation reactions. Secondly, both the hydrolytic monoesters and the oxidized metabolites conjugate with glucuronic acid, and are finally secreted through the urine. The biological half-life and the metabolite concentrations in urine differ between the various phthalates and their metabolites due to differences in phthalate structure and chain length. For instance, while approximately 70% of di-n-butyl phthalate (DnBP) is excreted as the primary metabolite mono-n-butyl phthalate (MnBP) (Koch & Calafat 2009), only 7% of di (2-ethylhexyl) phthalate (DEHP) is excreted as the primary metabolite MEHP (Wittassek & Angerer 2008). Individual phthalates are used in different products, thus exposure route and burden differs between the various phthalates, and between age and gender groups (Koch & Calafat 2009). The highest phthalate exposure through ingestion is seen for DnBP, diisobutyl phthalate (DiBP), benzyl butyl phthalate (BBzP), and DEHP (Wormuth et al. 2006). The concentrations of phthalate metabolites in serum vary between the different metabolites and between individuals (Frederiksen et al. 2010; Hogberg et al. 2008; Lind et al. 2012a; Olsén et al. 2012), reflecting differences in exposure within the population. Some phthalate metabolites, such as Mono-iso-butyl phthalate (MiBP), and Mono(2-ethylhexyl) phthalate (MEHP), may be detected in almost all subjects (Lind et al. 2012a; Olsén et al. 2012). Phthalates have also been detected in urine (Frederiksen et al. 2010) and breast milk (Meeker et al. 2009).

In rodents, exposure to phthalates has been associated with adverse effects in liver and kidney as well as the reproductive system (Heudorf et al. 2007), but the induced effects differ between the phthalates (Kamrin 2009). In epidemiological studies, several conditions like obesity, diabetes, and asthma have been associated with increased levels of phthalate metabolites in urine (Bornehag & Nanberg 2010; Grün & Blumberg 2007; Svensson et al. 2011). Phthalate metabolites may activate the peroxisome proliferator-activated receptor family of nuclear receptors (PPAR`s) (Koch & Calafat 2009), which play a major role in the regulation of insulin sensitivity, lipid storage and inflammation (Yessoufou & Wahli 2010), thus PPAR's are often suggested as a mechanism for phthalate-induced effects.

*1.3.3 **Serum levels of environmental chemicals***

BPA and phthalate metabolites have been detected in human serum samples in a number of studies, providing mean, median and minimum and maximum values, as summarised in Table 3 (Cobellis et al. 2009; Dirtu et al. 2008; Frederiksen et al. 2010; He et al. 2009; Hogberg et al. 2008; Kandaraki et al. 2011; Lind et al. 2012a; Olsén et al. 2012). The reported values are expressed in both mg/ml and nM, which illustrates that application of concentrations in nM ranges in *in vitro* studies are relevant for human environmental exposure. For a more detailed table see Appendix 1.

Note that the study reporting the highest mean phthalate levels did not report the maximum values (Lind et al. 2012a). Further, the standard deviations are large, reflecting large variations in serum levels of BPA and phthalate metabolites. The BPA values are from European studies (reviewed in Olsén et al. (2012)), but a number of studies from Asia report greater values, up to 9 ng/ml which corresponds to approximately 40 nM (Olsén et al. 2012). This also supports that there are large variations in exposure between individuals and that there are differences between the different bio-monitoring studies.

Table 3: Phthalate and BPA concentrations in serum samples, based on values from; (Cobellis et al. 2009; Dirtu et al. 2008; Frederiksen et al. 2010; He et al. 2009; Hogberg et al. 2008; Kandaraki et al. 2011; Lind et al. 2012a; Olsén et al. 2012), as specified in Appendix 1. Serum concentrations in ng/ml are represented as the range of mean, median and maximum values reported in the studies. Corresponding values in nM are listed in the second part of the table to show the relevance to the concentrations used in this study.

Serum concentrations of phthalates and BPA in ng/ml			
Chemical	Range Mean	Range Median	Range Maximum
MEHP Mono-2-ethylhexyl phthalate	0.77-20.3	0.49-7.88	0.47-514
MiBP Mono-isobutyl phthalate	0.72-44.6	0.50-13.5	0.50-1820
MnBP Mono-*n*-butyl phthalate	0.43-1.8	<LOD-0.54	0.54-20
BPA Bisphenol A	0.16-4.94	3.6-3.89	0.79-27.3

Serum concentrations of phthalates and BPA in nM			
Chemical	Range Mean	Range Median	Range Maximum
MEHP Mono-2-ethylhexyl phthalate	2.6-68.1	1.6-26.4	1.6-1724
MiBP Mono-isobutyl phthalate	3.2-200.7	2.2-60.7	2.2-8189.3
MnBP Mono-*n*-butyl phthalate	1.9-8.1	<LOD-2.4	2.4-90
BPA Bisphenol A	0.7-21.6	15.8-17	3.5-119.6

*LOD = Limit of detection

1.4 Chemical toxicity

Toxicity is usually defined as any harmful effect induced in a cell and/or organ (Yassi et al. 2001). Some toxicants may exert their effects on specific cells/organs and may there alter the DNA or other cellular molecules by interfering with their normal functions (Yassi et al. 2001). Whether a cell survives or dies in the presence of chemical exposure often depends on its capacity to respond to the stress, to maintain enzyme activity and to induce proteins that may promote or inhibit cell death. Exposure of chemicals does not always induce overt tissue injury, but may nevertheless affect cell functions and increase the sensitivity to other stressors. Therefore, some compounds may not have observable acute effects, but they might show long term consequences (Orrenius et al. 2011). Various toxicants may induce different

types of cell death, and low doses often results in apoptosis, whereas higher doses might induce necrosis (Orrenius et al. 2011).

As part of the normal development of an organism, apoptosis results from induction of an active processes within the cell and is then often described as a programmed cell death or "cellular suicide" (Orrenius et al. 2011). Typical, morphological changes include condensation and margination of cellular volume, cytoplasmic shrinkage, nuclear fragmentation and plasma membrane blebbing (Kroemer et al. 2009; Orrenius et al. 2011). In contrast, necrotic cell death is often considered as an accidental uncontrolled type of cell death, characterized by increased cell volume, swelling of organelles and rupture of the plasma membrane with subsequently loss of intracellular content (Kroemer et al. 2009). However, recent research suggest that some type of necrosis may also be regulated by signal pathways and mechanisms (Festjens et al. 2006; Golstein & Kroemer 2007).

1.5 Prior knowledge

The precise mechanisms underlying T1D and T2D, as well as the environmental factors contributing to their development, are not fully characterised. Urinary BPA levels have been associated with T2D in NHANES participants (Lang et al. 2008; Silver et al. 2011), and other epidemiological studies also report associations with alterations in hormonal and physiological processes such as energy balance, thyroid levels, metabolism as well as obesity which may play a role in the development of T2D (Carwile & Michels 2011; Hatch et al. 2010; Meeker & Ferguson 2011; Melzer et al. 2010). Several experimental studies have examined whether BPA exposure affects the pancreatic β-cells, and suggested associations between BPA exposure and T2D (Makaji et al. 2011; Nadal et al. 2009; Ropero et al. 2008). Recently, BPA exposure was also found to increase plasma insulin and simultaneously decrease plasma glucose in mice. Furthermore, BPA increased β-cell insulin content, hyperinsulinemia and resulted in insulin resistance (Ropero et al. 2008). Similarly, a study on islets from BPA exposed mice reported an increased glucose-induced insulin secretion, and a further increased insulin resistance (Alonso-Magdalena et al. 2006). A recent *in vitro* study (Song et al. 2012) on isolated pancreatic islets, reported increased insulin secretion at low BPA concentrations, but declining secretion with increasing concentrations. Interestingly, the BPA induced increase in insulin secretion was greater in a high glucose environment (16.7 mM). The increased insulin secretion at concentrations as low as 0.4 nM, implies that BPA

concentrations relevant for environmental exposures might affect insulin secretion. Recently, BPA was also reported to impair cell viability at relatively low concentrations, both *in vivo* and *in vitro* (Lin et al. 2013; Song et al. 2012).

While there is emerging evidence suggesting that BPA may influence the development of T2D, a possible impact on T1D has so far not received much attention. Some, associations between certain environmental chemicals and T1D have been suggested (Howard & Lee 2012). Furthermore, endocrine-disrupting compounds such as BPA have been associated with adverse effects on human immune function (Clayton et al. 2011), which is interesting as T1D is an autoimmune disease. In the non-obese diabetic (NOD) mouse model, BPA exposure resulted in increased infiltration of immune cells and increased apoptosis in the pancreatic islets accompanied by a tendency towards accelerated T1D development (Bodin et al. 2013).

Phthalates have received less attention both in relation to T1D and T2D, but have been linked to several other adverse health effects (Heudorf et al. 2007). However, epidemiological studies have reported associations between urinary levels of phthalate metabolites and diabetes, but also associations with poor insulin secretion (Svensson et al. 2011) (Lind et al. 2012b) (James-Todd et al. 2012). In addition, some phthalates have been suggested to increase the risk of obesity, which may be linked to T2D development (Grün & Blumberg 2007). Urinary concentrations of MEHP have also been associated with increased waist circumference, while MnBP was linked to increased insulin resistance (Stahlhut et al. 2007). Moreover, *in vitro* studies indicate associations between the phthalate metabolite MEHP and adipocyte differentiation, which might provide an additional link to obesity (Campioli et al. 2011; Feige et al. 2007). Recently developmental exposure of DEHP has been shown to impair β-cell function and glucose homeostasis in rats (Lin et al. 2011), but limited acute effects of DEHP on insulin secretion were detected in β-cells in vitro (Hectors et al. 2013). Thus, further investigations are necessary on the relationship between exposure to phthalates and β-cell function.

Mixture effects due to combined exposure to different toxicants, also known as “cocktail effects”, are recognized as an important aspect of toxicology (Backhaus & Faust 2012; Feron & Groten 2002). Experimental evidence suggests that chemicals in combination can produce additive or synergistic effects at concentrations that are not associated with an individual dose response (Hass et al. 2007; Howdeshell et al. 2008). The knowledge of biological effects due

to combined exposure to BPA and phthalates is however sparse. One study reported that a mixture of phthalates and BPA in combination with other chemicals induced adverse health effects on male sexual development (Christiansen et al. 2012). However, to our knowledge, no previous studies have examined the effects of combined exposure to BPA and phthalates on β-cell function.

1.6 Aim of the study

The main objective of this study was to investigate whether BPA and a selection of phthalate metabolites alone or in combination could influence cellular functions of pancreatic β-cells that may be linked to an accelerated development of diabetes type 1- and type 2.

Our main hypotheses were:

1. *Long-term exposure to BPA and phthalate metabolites may induce decreased β-cell viability.*
2. *Exposure to BPA and phthalate metabolites may increase the β-cell susceptibility to cytokine-induced apoptosis.*
3. *BPA and phthalate metabolite exposure entails increased glucose-induced insulin secretion.*
4. *The combinatory exposure of the chemicals may induce additive, synergistic or inhibitory effects.*

This approach allows for assessment of both the direct effect of each individual chemical on the β-cells, as well as a possible combinatory effect. The findings of this study may help to gain better knowledge and understanding of the associations between environmental chemicals and the increasing incidence of both T1D and T2D, which have been suggested based on epidemiological studies (Lang et al. 2008; Svensson et al. 2011)

2 MATERIALS AND METHODS

In this *in vitro* study, three model-systems were chosen to study possible effects of bisphenol A (BPA) and phthalate metabolites on the viability of the β-cells, as well as cytokine-induced apoptosis and glucose-induced insulin secretion.

2.1 Cell culturing conditions

Reliable β-cell models are essential for diabetes research. It is preferable to use primary cells, but this requires large quantities of isolated pancreatic islets, which are work intensive, expensive and represent a mixed population of cells. However, rodent β-cell lines have shown to be useful in mechanistic research (Mergelen et al., 2004). We chose the INS-1E β-cell line because it has proven its usefulness both in studies of insulin secretion in response to glucose (Mergelen et al., 2004) and with respect to cytokine-induced apoptosis (Hanzelka et al., 2012). INS-1E cells is a rodent β-cell line which was provided by Prof. C.B. Wollheim of the University of Geneva, Switzerland. INS-1E cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 in complete medium composed of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, as described in Merglen et al. (2004).

For environmental chemical exposure, the cells were seeded in Falcon 24 well plates, with a density of 120.000 to 140.000 cells/well in 1 ml medium. Seeding of cells was performed on Friday, with medium shift at Monday, and then exposure for environmental chemicals Monday or Tuesday with harvesting of cells at Thursday or Friday, respectively. The density were based on our pilot tests to determine appropriate cell number for 72 h chemical exposure and to ensure that there was a linear growth phase without limitations due to high cell density (Appendix 2). Pilot studies showed that the cells growth appeared to level off at 200.000 cell/well while 50.000 cells/well appeared to be too sparse (Figure A1, Appendix 2). Based on these pilots, cell densities between 100.000 and 150.000 cells/well were chosen.

2.2 Exposure to environmental chemicals

The cells were exposed to either BPA (TCI Europe nv, Zwijndrecht, Belgium) or the phthalate metabolites Mono-iso-butyl phthalate (MiBP), Mono-n-butyl phthalate (MnBP) and Mono(2-ethylhexyl) phthalate (MEHP) (Orchid Cellmark, New Westminister, BC Canada), either alone or in combination. MnBP, MiBP and MEHP are the primary metabolites of Di-n-butyl phthalate (DnBP), Di-isobutyl phthalate (DiBP) and di (2-ethylhexyl) phthalate (DEHP), respectively (Koch & Calafat 2009). MnBP is also a metabolite of Benzyl butyl phthalate (BBzP), but the main metabolite of the latter is Monobenzyl phthalate (MBzP), which was not included in this study. The three phthalate metabolites were selected since they have been shown to be the major metabolites due to oral exposure (ingestion of food and beverages), which is the major environmental exposure route (Koch & Calafat 2009; Wormuth et al. 2006).

The applied concentrations were chosen based on concentrations detected in human blood and serum samples (Frederiksen et al. 2010; Hogberg et al. 2008; Olsén et al. 2012), as described in section 1.3.3 where Table 3 shows reported serum concentrations in the nM range. However, we also used concentrations that where 100 to 200 times higher than the lowest relevant concentrations for some of the applied chemicals. For each chemical, five different concentrations were included in the low dose experiments; 1 nM, 10 nM, 50 nM 100 nM and 500 nM. Cellular exposure was performed by means of stock solutions of environmental chemicals in Dimethyl sulfidoxide (DMSO; Sigma-aldrich, Spruce Street, St.Luis, USA). Cells exposed to DMSO only were used as controls.

To investigate possible mixture effects of these chemicals, two scenarios for combinatory exposure were applied; (i) the combination of all the chemicals in the five different concentrations, and (ii) the combination of the three phthalate metabolites in the five different concentration. For investigating the mixture effects we combined the lowest individual concentrations of the chemicals, the second lowest individual concentrations, and so on. These mixed exposures can be considered to have relevance for individuals with different levels of chemical exposure ranging from low to highly exposed individuals. Since low dose exposure did not affect any endpoints, we also chose to examine higher concentrations of the environmental chemicals in high dose experiments, using concentrations between 5 - 500 µM, to test the sensitivity of the INS-1E cells as well.

2.3 Model systems

Three different exposure scenarios were used to study the effects of the chemicals alone (non-diabetic model), or their effects on the cytokine-induced apoptosis (T1D-model) and glucose-induced insulin release (T2D-model), and are referred to as model-systems throughout the text. In all three model-systems, INS-1E cells were exposed to environmental chemicals for 72 h at the concentrations and combinations described above. A schematic figure of these three model systems and the measured endpoints are included in Figure 3a-c.

I. Non-diabetic model-system

This model system reflects a normal non-diabetic situation, and is used to study if the chemicals can affect the β-cells viability, independently of other factors characterizing T1D and T2D. After 72 s exposure to environmental chemicals the β-cell viability was measured by the Methyl Thiazylol Tetraolium (MTT) assay. Then, if the treatments showed alterations in viability, this was further examined to determine if this was due to altered proliferation or apoptosis/necrosis (Figure 3a).

II. T1D model-system

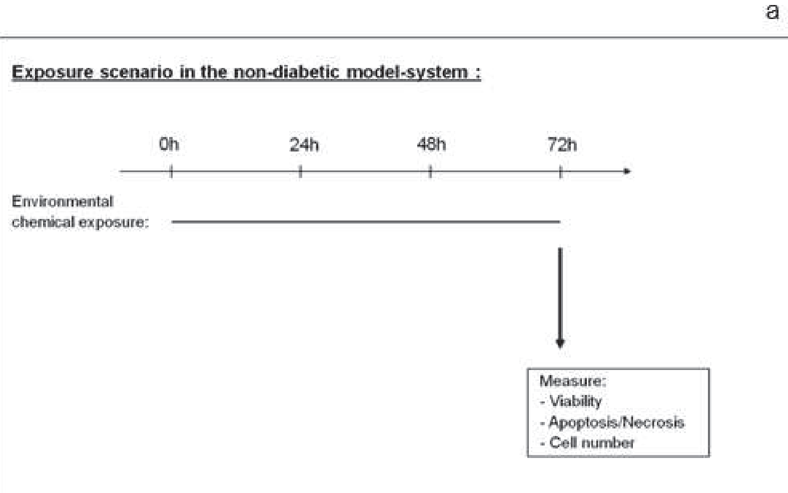
This exposure scenario reflects the *amplification phase* in T1D development, as described in section 1.2.1. Cytokine-induced apoptosis is one of the early events in the development of T1D (Pirot et al. 2008), and a mixture of cytokines (IL-1β, IFNγ and TNFα) has previously been shown to induce β-cell apoptosis (Pirot et al. 2008). Based on pilot studies (Figure A2-A4, Appendix 2) , the cells were exposed to a mixture of cytokines (5 ng/ml IL-1β, 25 ng/ml IFNγ and 25 ng/ml TNFα) in combination with environmental chemical exposure the last 24 or 48 h of the exposure. The cytokines were purchased from PromoKine, Heidelberg, Germany. At the end of exposure, the viability was determined by MTT. As for the non-diabetic model, significant changes in viability were further examined with respect to proliferation and apoptosis/necrosis (Figure 3b).

III. T2D model-system

It is hypothesised that enhanced glucose-induced secretion of insulin, may contribute to the development of T2D through β-cell exhaustion (Aston-Mourney et al. 2008) or exacerbated

insulin resistance (Biddinger & Kahn 2006) as described in section 1.2.2. Therefore, the effect of the environmental chemicals on glucose-induced insulin release was examined. Pilots were performed to determine cell numbers appropriate for measurement of insulin secretion, and based on these results, seeding of 120.000 cell/ml were used (Figure A4, Appendix 2) After nearly 72 h environmental chemical exposure, the cells were incubated for 1 h in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH), as described by Merglen et al. (2004). Furthermore, the cells were stimulated for 30 min with two different glucose concentrations (6.7 and 16.7 mM) in KRBH buffer, before determination of insulin release (Figure 3c). Two different glucose concentrations were included to study whether the glucose environment affected the cellular response. In mouse models, random blood glucose levels at 13,9 mM are used, independent of fasting levels, as diagnosis of diabetes (Rajagopalan et al. 2003). In *in vitro* studies concentrations between 5-7 mM and 15-17 mM are commonly used to measure glucose induced insulin secretion (Alonso-Magdalena et al. 2006; Merglen et al. 2004). In this study, the high glucose concentration (16.7 mM) will reflect the abnormal high plasma glucose levels in T2D patients, and the lowest concentration (6.7 mM) is a more normal plasma glucose concentration, as described in section 1.2.2.

a



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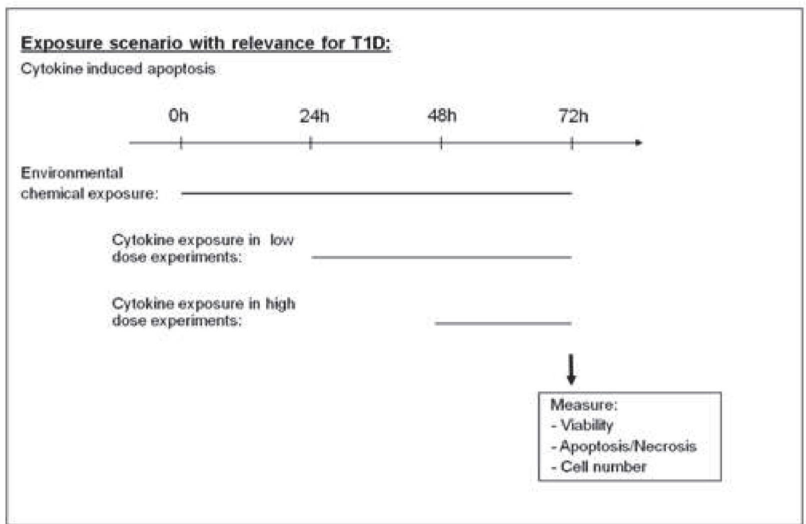

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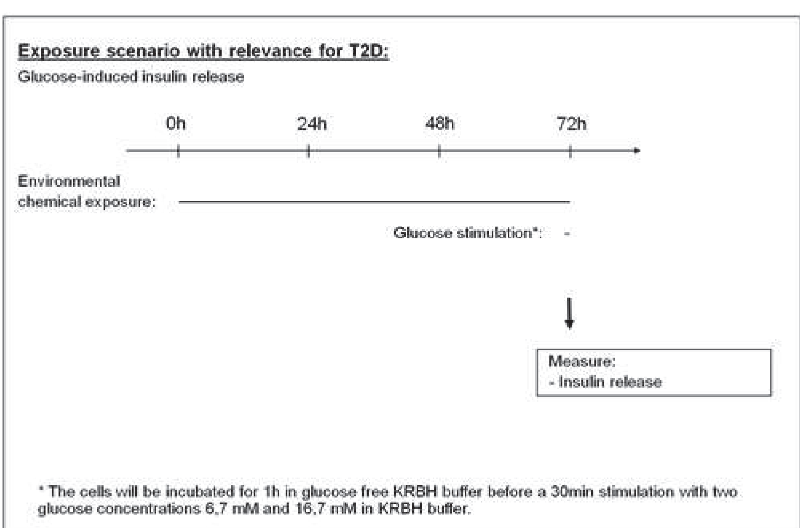


Figure 3a-c: Model-systems. The figure represents the difference between the three model systems in a schematic drawing, where exposure intervals and measure methods are illustrated. In non diabetic model system, cells are exposed to different concentrations of environmental chemicals only, while in the T1D model system cells are exposed to either 24 cytokines or 48 h cytokines in combination with either low or high concentrations of environmental chemicals, respectively. In the T2D model system the cells will be incubated with glucose after the environmental chemical exposure.

2.4 Description of analytical methods

After exposure, the medium was collected and centrifuged for 10 min to remove cells (300 x g). These cells combined with cells detached by trypsination were further analysed for changes in cell number and necrosis/apoptosis. In the non-diabetic and T1D model-systems, β-cell viability, cell number and necrosis/apoptosis were measured, as indicated in Figure 3a-c whereas glucose-induced insulin secretion was measured in the T2D model-system. The applied methods are described below.

Cell toxicity

MTT assay. The cellular "viability" was first measured using the MTT assay, which predicted the relative amount of "living" cells compared to controls. MTT produces a yellowish solution that is converted to dark blue, formazan crystals by living cells with active mitochondria. At the end of exposure, MTT was added to each well to a final concentration of 0.5 mg/ml. The cells were then incubated at 37° C for 30 min before the cell culture medium was removed and 300 µl DMSO was added to each well to solubilise the cells and the formazan crystals. Finally, absorbance was measured at 570 nm, using a Fluostar Optima plate reader (BMG-labtech, Offenburg, Germany)/Galaxy instrument (Nerliens Meszansky AS, Oslo, Norway). The MTT data are presented as relative cell viability, which was obtained by dividing the absorbance levels by the mean value of the controls in the same experiment, before multiplication with 100. For the low dose experiments all treatments were performed in duplicates for the MTT tests, while only singlets were used for the high dose experiments.

Trypan blue test. To further investigate whether the environmental chemicals affect the total cell number and/or the fraction of viable versus necrotic cells, the cells were stained 1:1 with 40% trypan blue Bio Rad and counted in an automated cell counter. The toxicity is presented as the number of trypan blue positive cells relative to the total cell number, while the number of viable cells is presented as the number of trypan blue negative cells per ml. Changes in these parameters will reflect increased cytotoxicity and/or modified proliferation rate.

Propidium iodide/Hoechst staining. To determine the relative amount of living cells versus necrotic and apoptotic cells, the beta-cells were stained with propidium iodide (PI; 10 µl/ml) and Hoechst 33342 (5 µl/ml), both commonly used nuclear fluorescent dyes which bind to DNA. Hoechst 33342 is a cell-permeant nuclear marker which provides information about

nuclear morphology of all cells, while propidium iodide (PI) is a cell-impermeant nuclear dye which marks cells with disrupted plasma membranes (Hubbard et al. 2012). These nuclear dyes colours the cell nucleus in different colours and the condition of each individual cell can be visualized; uniform blue fluorescence indicates living cells, red indicates necrotic cells, and cells with a condensed and/or fragmented luminous nucleus indicates apoptotic cells. After staining with PI (10 µg/ml) and Hoechst 33342 (5 µg/ml) the cells were smeared on glass slides and dried quickly with a hairdryer. The fractions of cells in the different groups were determined using fluorescence microscopy, counting a minimum of 300 cells per slide. The percentage of apoptotic and necrotic cells are presented as a fraction of the total number of counted cells.

Glucose-induced insulin secretion

After 30 min glucose stimulation, cells were kept on ice while harvesting, and the cell medium in each well was transferred to 96 well plates and centrifuged at 750 rpm for 10 min to remove cells. The supernatant was then transferred to new 96 well plates, diluted 20:1 with KRBH and stored at -70° C. The insulin release was measured with a commercially available enzyme linked immunosorbent assay (ELISA) according to the manufacturer`s manual. The increase in colour intensity was measured and quantified using a plate reader (TECAN Sunrise, Phoenix Research Products, CA, USA) with software (Magellan V 1.10). As for the MTT data, the insulin levels were normalised by dividing obtained data on the mean value of the insulin levels in the controls in the same experiment, before multiplication with 100.

2.5 Data processing and statistical analysis

To examine if combined exposure to the chemicals resulted in additive, synergistic or antagonistic effects on the cell viability, the effect induced by the combined exposure to the three phthalates (MnBP, MiBP and MEHP) and the combination of all four chemicals (MnBP, MiBP, MEHP and BPA) were compared to the calculated sum of the effects induced by exposure to the individual chemicals. This was achieved by calculating the relative reduction in viability for each chemical exposure (1- relative viability), then adding the relative viability reduction induced by each chemical exposure alone, before calculating the corresponding relative viability reduction (1- sum of the relative reduction of the individual

chemicals). These calculations allowed for statistical comparison of the calculated cell viability due to exposure of the individual chemicals and the response induced by the combined environmental chemical exposures. This was meant as an initial investigation of combinatory effects, rather than a characterizing of the chemicals concentration-effect curve to investigate whether they act through the same mechanisms (Christen et al. 2012).

The statistical analysis was performed in GraphPad Prism (GraphPad Software, CA, USA). Two-way analysis of variance (ANOVA) was used to analyse the data sets, with Bonferroni post-tests to compare different treatment groups. ANOVA allows for a statistical comparison of different groups or treatments, and the study design of our *in vitro* experiments are suitable for a two factor design with the different environmental chemicals and the varying concentrations as the two factors (Quinn 2002). ANOVA was used both to look at effects of treatments compared to control, and to compare the different treatments. Data were obtained from 3-4 independent experiments. Bars in columns are presented as mean ± SEM and and p values < 0.05 are generally considered as significant. ANOVA also assumes that any differences between SDs are due to random sampling. We therefore tested that the mean and the SDs were randomly distributed prior to two-way ANOVA.

2.6 Ethical considerations

Since the study only used a rat cell-line, there were no ethical precautions, and an application to Regional ethics committee (REC) was not necessary. Practical training required for the laboratory experiments and handling of toxic chemicals was completed at the Norwegian Institute of Public Health prior to the study.

3 RESULTS

3.1 Low dose exposure

3.1.1 ***Non-diabetic model***

To investigate a possible effect of the environmental chemicals on β-cell viability, the cell viability was initially measured using the MTT assay, comparing exposed wells with DMSO treated controls. After 72 h exposure to low concentrations of chemicals (1 - 500 nM), the INS-1E cells did not exhibit any significant change in cell viability in the non-diabetic model system (Figure 4).

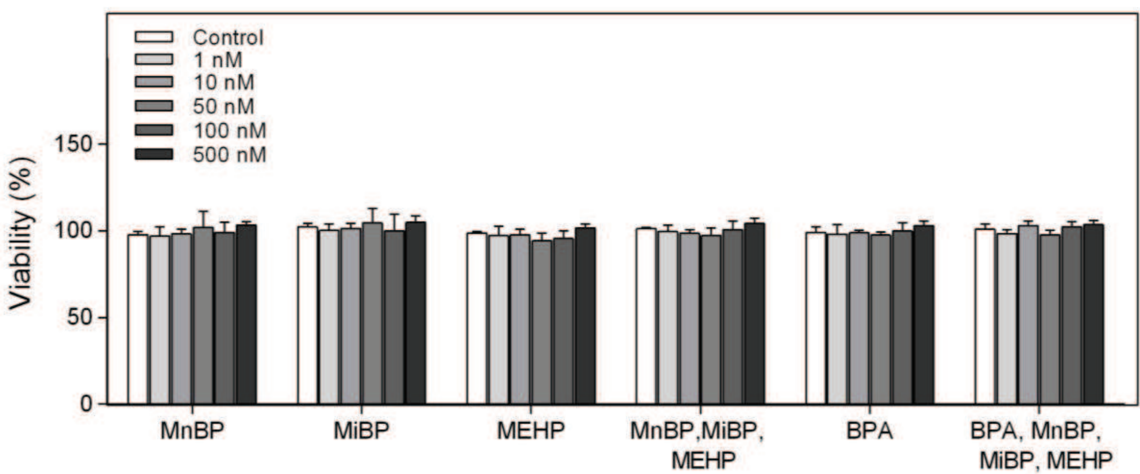


Figure 4: Non-diabetic model system: cell viability determined by MTT. INS-1E cells were seeded at 120 000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals, as indicated. In the combinatory exposures the given concentrations were added of each chemical and do not indicate the total dose of all chemicals. The relative cell viability was measured by the MTT assay as described in materials and methods (2.5). Cells exposed to DMSO only were used as controls. Data were obtained from duplicates in 4 independent experiments, and were normalized for each experiment, *i.e.* the mean of the duplicates were divided by the mean of all the controls in that experiment. The bars represent mean ± SEM. Two-way ANOVA with Bonferroni post-test showed no significant differences.

3.1.2 ***T1D model***

To examine if environmental chemicals increased the β-cell susceptibility to cytokine-induced apoptosis, cell viability was measured by MTT after exposure to INF-γ, TNF-α and IL-1β the last 48 h of the chemical exposure. The β-cell viability did not differ between cells treated with any of the chemicals at 1-500 nM compared to cytokine exposure alone in response to

the 48 h cytokine exposure (Figure 5). Thus, the chemicals did not appear to increase the β-cell susceptibility to cytokine-induced apoptosis.

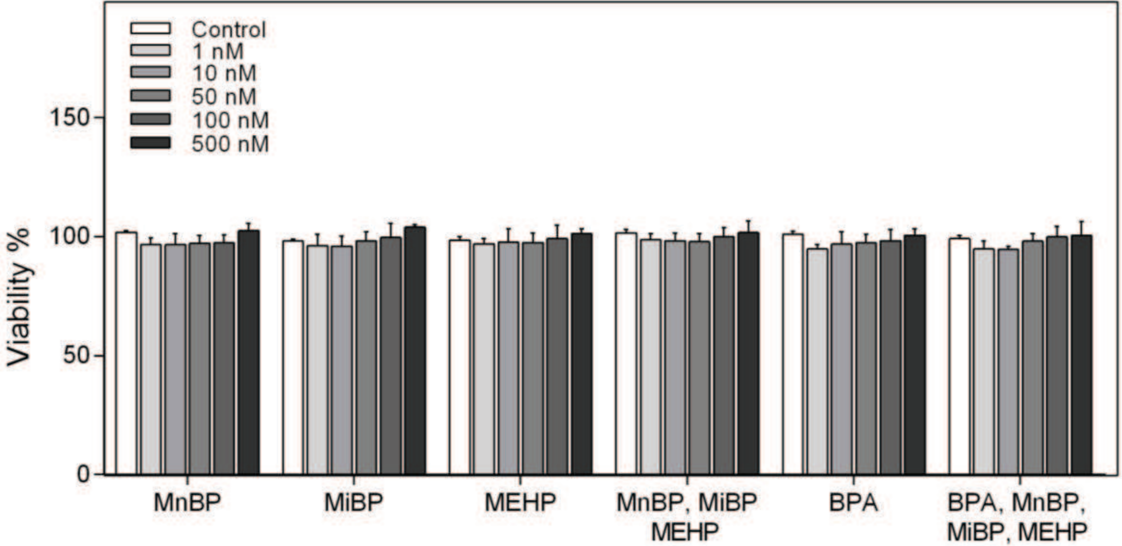


Figure 5: T1D model system: cell viability determined by MTT. INS-1E cells were seeded at 140.000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals, as indicated. The last 48 h of this exposure, the cells were co- exposed to the pro-inflammatory cytokines IL-1β, TNFα and INF γ at 5 + 25 + 25 ng/ml, respectively. Cells only exposed to the cytokines and DMSO were used as controls. Relative cell viability was then measured by MTT assay, as described in materials and methods (2.5). The data represent duplicates from 4 independent experiments, and were normalized for each experiment, *i.e.* the mean of the duplicates were divided by the mean of all the controls in that experiment, and expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test showed no significant differences.

The results from non-diabetic model (Figure 4) and T1D model (Figure 5) are presented relative to controls in each model system, and do not show the difference between the model systems. The effect of cytokine exposure in the T1D model system is illustrated in Figure 6a, showing that the 48 h cytokine exposure in INS-1E cells decreased the relative cell viability with approximately 65% compared to the no-cytokine group (Figure 6a). The relative viability decreased with increasing exposure time for the cytokines (24 h and 48 h).

In addition to MTT, we also used other techniques to characterize cell death (Figure 6b-c), but since there was no altered viability due to the chemical exposures, only the highest concentration of the combined exposure of all four chemicals (500 nM of BPA, MnBP, MiBP and MEHP) was analysed using staining with Trypan blue and Hoechst/PI. Cells stained with Hoechst/PI showed no significant increase in the number of apoptotic and necrotic cells after chemical exposure relative to DMSO treated controls (Figure 6b). Note that the bars in Figure 6b reflect the sum of necrotic and apoptotic cells, since very few apoptotic cells were detected

as illustrated in Figure A6 (Appendix 3) where only necrotic cell death are depicted due to the low proportion of apoptotic cell death. The results from Hoechst/PI coincide with the

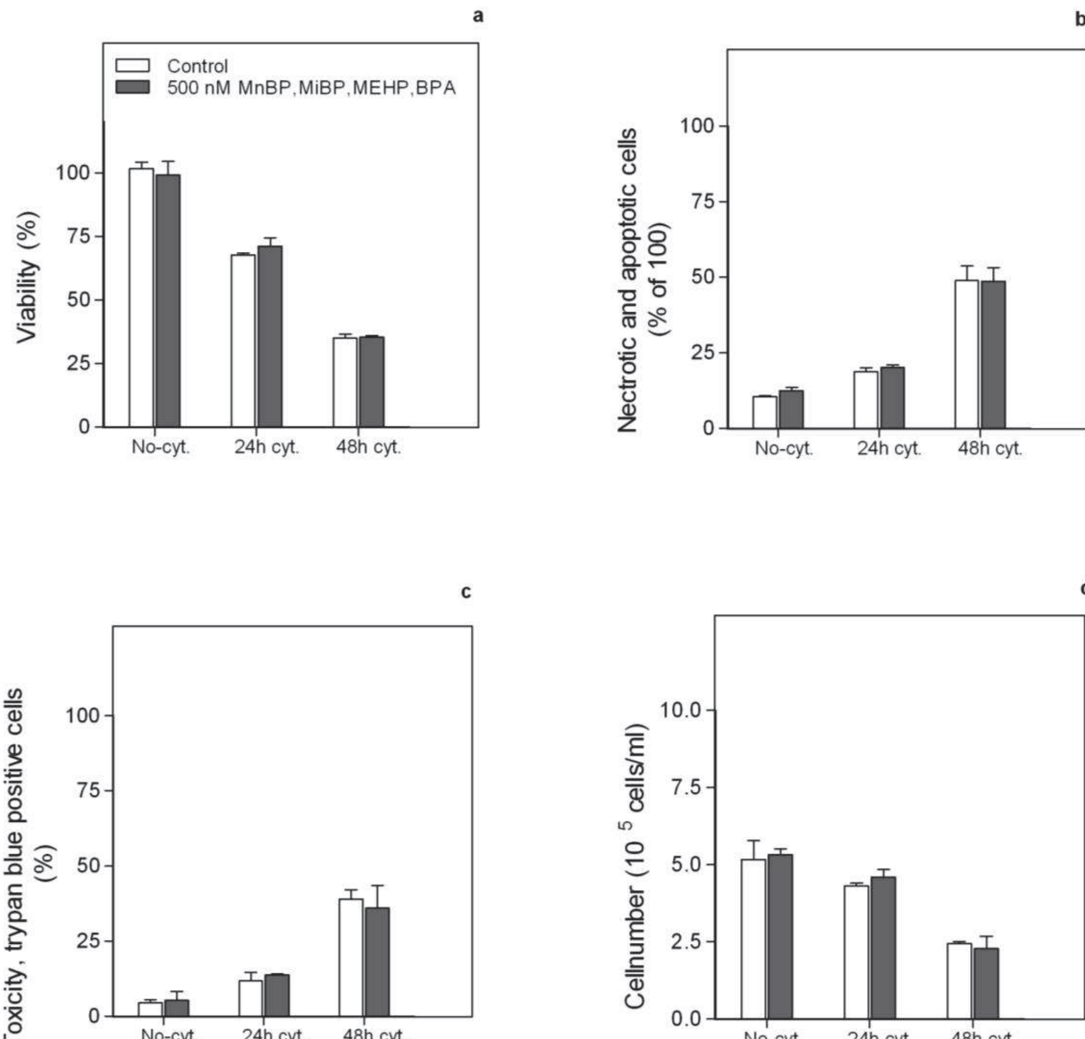


Figure 6a-d: Comparison of non-diabetic and T1D model systems for different viability measures. To illustrate the effect of cytokine exposure in the T1D model system, experiments were performed to compare the model systems on the same plate. INS-1E cells were seeded at 140.000 cell/ml, with a medium shift onday 3. 'No cytokine' corresponds to the non-diabetic model system, while '24h cyt.' and '48h cyt.' correspond to T1D model systems. As in Figure 5 the cytokine exposure (5 ng/ml IL-1β, 25 ng/ml IFNγ and 25 ng/ml TNFα) was done in combination with environmental chemical exposure the last 24 or 48 h of the exposure. Bars represent mean ± SEM for 3 independent experiments. (a) Relative cell viability was measured by the MTT assay. Data were normalized for each experiment, *i.e.* divided by the mean of **the non-diabetic controls** in that experiment. (b) The fractions of necrotic and apoptotic cells were determined by Hoechst/PI staining. Bars represent the relative numbers of the sum of necrotic and apoptotic cells, but very few apoptotic cells were detected. (c) Toxicity was expressed as the fraction of trypan blue positive cells relative to total cell count. (d) Number of viable cells, corresponding to fraction of trypan blue negative cells. Two-way ANOVA with Bonferroni post-test showed no significant differences.

cytokine-induced effects on viability detected by MTT, in that the number of dead cells (necrotic and apoptotic) increases with time after cytokine addition, but the environmental chemical exposure caused no further increase in cytotoxicity. The Trypan blue test (Figure

6c), also showed similar results as Hoechst/PI and MTT, with no significant increased toxicity induced by the chemical treatment, compared to DMSO treated controls. The cell number, determined by counting trypan blue negative cells, was not affected by chemical exposure, but was decreased by the cytokine exposures (Figure 6d), reflecting the decreased viability in Figure 6a-c. Overall, the results presented in Figure 6a-d confirmed the toxic effect of the cytokines, which was also reflected in the cell morphology observed in the light microscope (Figure A7, Appendix 3).

3.1.3 ***T2D model***

To investigate if the environmental chemicals could increase glucose-induced insulin secretion, the cells were stimulated for 30 min with two different glucose concentrations (6.7 and 16.7 mM) after 72 h chemical treatment, before determination of insulin release by ELISA. Generally, the high glucose concentration (16.7 mM) showed increased insulin secretion compared to the low glucose concentration (6.7 mM) (Figure A9, Appendix 4). However, the insulin secretion at 16.7 mM glucose showed a very high degree of variation in initial experiments but appeared to be unaffected by the chemicals. Thus, this high glucose concentration was not included in further experiments. The insulin secretion in response to 6.7 mM glucose was not affected by any of the individual exposures to phthalate metabolites or the mixed chemical exposures (Figure 7). Exposure to BPA showed a tendency to a u-shaped effect on insulin secretion with a decrease at 1 -100 nM compared to controls, but these decreases were not significant. Overall, there were no significant effects of the environmental chemicals in the concentration range 1-500 nM on insulin secretion for either of the two glucose concentrations.

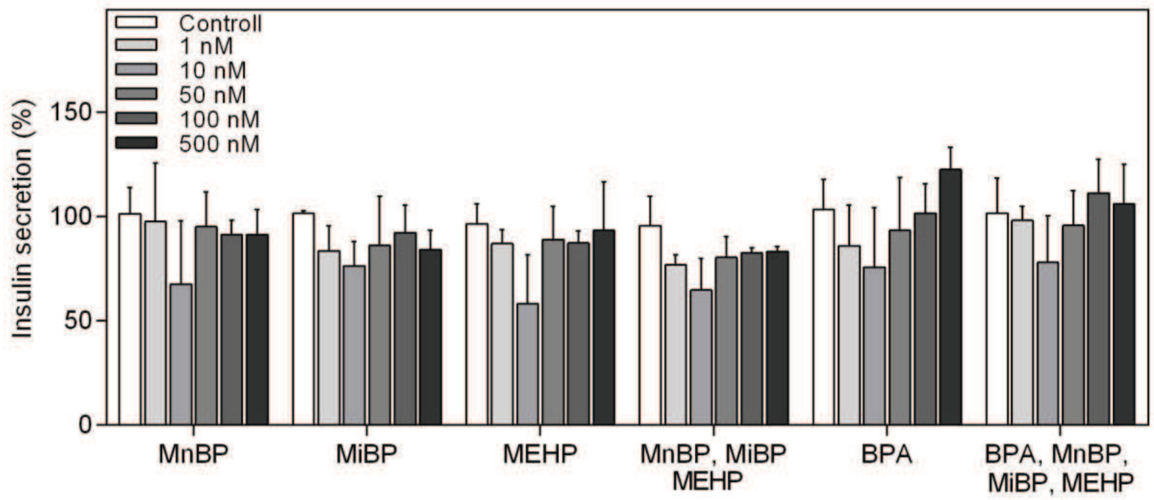
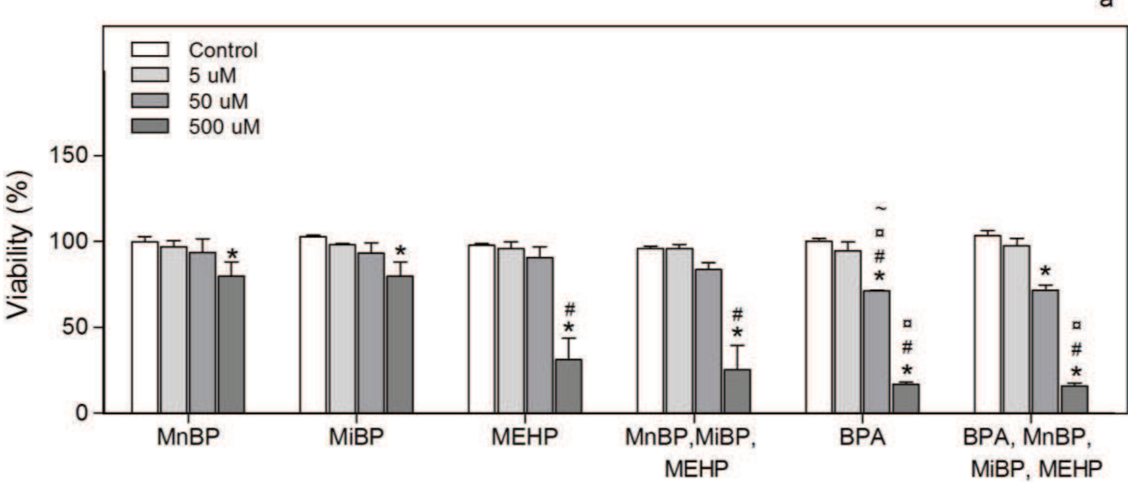


Figure 7: T2D model system: insulin secretion after 6.7 mM glucose stimulation. INS-1E cells were seeded at 120.000 cells/ml, with medium shift on say 3, before 72 h environmental chemical exposure, as indicated. Cells exposed to DMSO alone were used as controls. After 72 h exposure, all wells were incubated in glucose free KRBH buffer for 1 h, before 30 min incubation with 6.7 mM glucose in KRBH buffer. Insulin secretion in cell culture supernatants was determined by ELISA. Data were obtained from duplicates in 3 independent experiments, and were normalized for each experiment, *i.e.* the mean of duplicates were divided by the mean of all the controls in that experiment, and expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test showed no significant differences.

3.2 High dose exposure

*3.2.1 **Non-diabetic model***

Due to the lack of significant effects at the low concentrations of environmental chemicals, we chose to also include three higher concentrations; 5, 50 and 500 µM. The effects of 50 µM environmental chemical exposure on cell morphology are illustrated in Figure A8 (Appendix 3). The cell viability measured by the MTT assay was significantly decreased for the highest concentration of all the applied chemical exposures (Figure 8a).



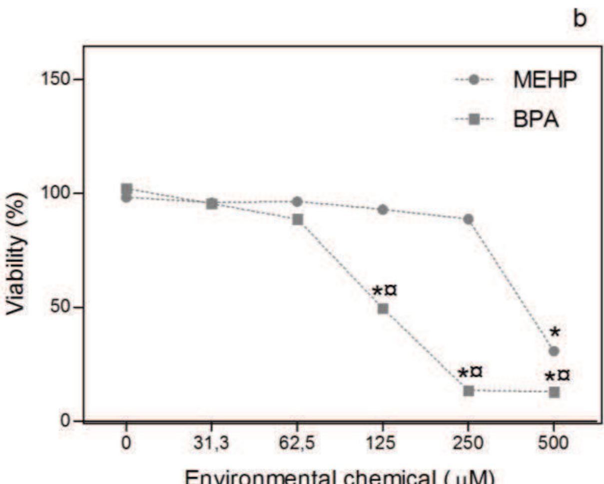


Figure 8a-b: Cell viability by MTT in non-diabetic. INS-1E cells were seeded at 120 000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals, as indicated. In the combinatory exposures the given concentrations were added of each chemical and do not indicate the total dose of all chemicals. The relative cell viability was measured by the MTT assay as described in materials and methods (2.5). Cells exposed to DMSO only were used as controls. Figure b shows the difference in viability reduction in concentrations from 50- 500 µM between BPA and MEHP. Data were obtained from 3 independent experiments, and were normalized for each experiment, *i.e.* the mean of the duplicates were divided by the mean of all the controls in that experiment. The bars and the points represent mean ± SEM. * Indicates significant decrease compared to controls, # indicates a significant decrease compared to MnBP and MiBP (same concentration), ¤ indicates significant decrease compared to MEHP (same concentration), ~ indicates significant decrease compared to combinatory exposure of MnBP, MiBP and MEHP (same concentration), when analysed by two-way ANOVA with Bonferroni post-test.

The most potent exposures were MEHP, the three phthalates, BPA and the combination of the four chemicals, where the 500 µM exposures caused reductions in viability of approximately 65, 70, 85 and 90%, respectively. In contrast, only a 20-25% reduction in viability was detected for MnBP and MiBP. BPA and the combination of the four chemicals also showed a significant decrease in cell viability at 50 µM of approximately 30%. Furthermore, the cell viability was also significantly attenuated for BPA compared to MEHP and the combination of the three phthalates for 50 µM (¤ or ~, Figure 8a). Thus, BPA appeared to be the most

potent of the four environmental chemicals. Between the three phthalates, MEHP clearly showed the strongest effect on cell viability as it caused significantly lower viability than both MnBP and MiBP at 500 µM (#, Figure 8a). To further examine the difference in effect between the individual exposure to BPA and MEHP, four additional concentrations (32.3, 62.5, 125 and 250 µM) were included for these two chemicals (Figure 8b). BPA showed a reduced viability already at 125 µM, while the concentration-effect curve for MEHP did not decline until 500 µM, which confirms that BPA is considerably more potent than MEHP in this model system.

To explore possible additive, synergistic or antagonistic effects of the combinatory exposure we compared the combined exposures and the calculated sum of the individual exposures (Figure 9), as described in Materials and methods (section 2.5). The 500 µM exposures were not included in these analyses, since the reduction in viability for the individual exposures was so high that the calculated data would result in negative values, while the 5 µM exposures were excluded since they did not cause any significant reductions in viability. The cell viability after 50 µM combined exposure to the three phthalates showed a similar viability as the calculated sum of the individual exposure of 50 µM MnBP, MiBP and MEHP (6% difference), suggesting an additive effect of the chemicals. For combined exposure to all the four chemicals the viability was 23% higher than the calculated sum of the individual exposures. This difference was not statistically significant, but implies an antagonistic tendency during combined exposures to the chemicals.

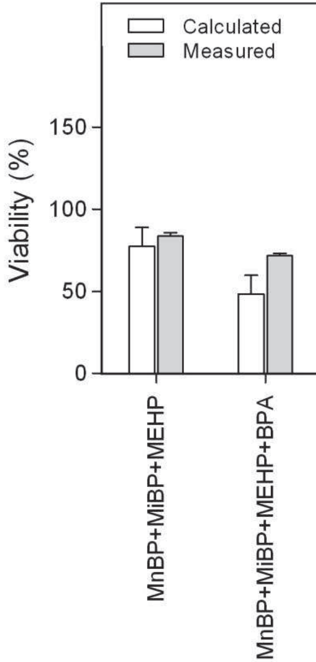


Figure 9: Comparison of effects due to combined and calculated exposures. Data from Figure 8a were used to evaluate if combined exposures resulted in additive, synergistic or antagonistic effects in the non-diabetic model system. The effect of the combined exposure of 50 µM of the three phthalates (MnBP, MiBP and MEHP) and the combined exposure of the four environmental chemicals (MnBP, MiBP, MEHP and BPA) was compared to the calculated sum of the effects of the individual chemicals as described in materials and methods (section 2.6). Two-way ANOVA with Bonferroni post-test showed no significant differences.

3.2.2 ***T1D model***

Exposure to environmental chemicals significantly decreased the cell viability in presence of 24 h cytokine exposure (INF-γ, TNF-α and IL-1β) compared to the cytokine exposure alone (Figure 10). The response pattern was similar as in the non-diabetic model, with highest reductions at 500 µM exposure of MEHP, the three phthalates, BPA and the combination of the four chemicals, whereas the three phthalates, BPA and the combination of the four chemicals also showed a significantly decreased cell viability at 50 µM. Thus, there were more significant differences between the effects of chemical treatments in the T1D model system than the non-diabetic model (Figure 8a).

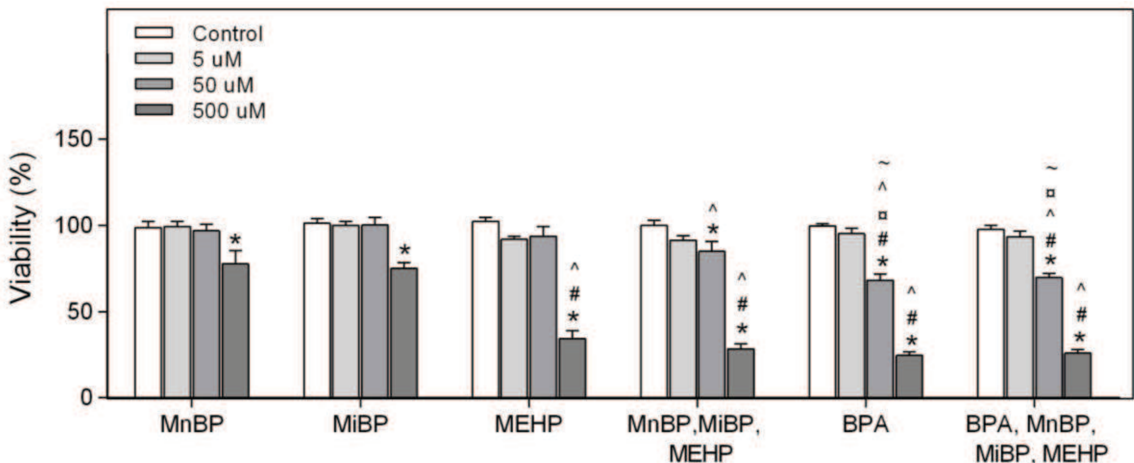


Figure 10: T1D model system: cell viability determined by MTT. INS-1E cells were seeded at 140.000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals, as indicated. The last 48 h of this exposure, the cells were also exposed to the pro-inflammatory cytokines IL-1β, TNFα and INFγ in 5 + 25 + 25 ng/ml, respectively. Cells exposed to the cytokines and DMSO were used as controls. Relative cell viability was then measured by MTT assay, as described in materials and methods (2.5). The data represent duplicates from 3 independent experiments, and were normalized for each experiment, *i.e.* the mean of the duplicates were divided by the mean of all the controls in that experiment, and expressed as mean ± SEM. * Indicates significant decrease compared to controls, # indicates significant decrease compared to MnBP (same concentration), ^ indicates significant decrease compared to MiBP (same concentration), ¤ indicates significant decrease compared to MEHP (same concentration), ~ indicates significant decrease compared to the combinatory exposure of MnBP, MiBP and MEHP (same concentration).

The responses in the non-diabetic and the T1D model are compared in Figure 11, showing the effects of the chemicals relative to the respective controls. There were however no significant differences in the effects induced by the various chemicals in the two model systems, suggesting that the high concentrations of environmental chemicals did not affect the cellular sensitivity to cytokine-induced cell death.

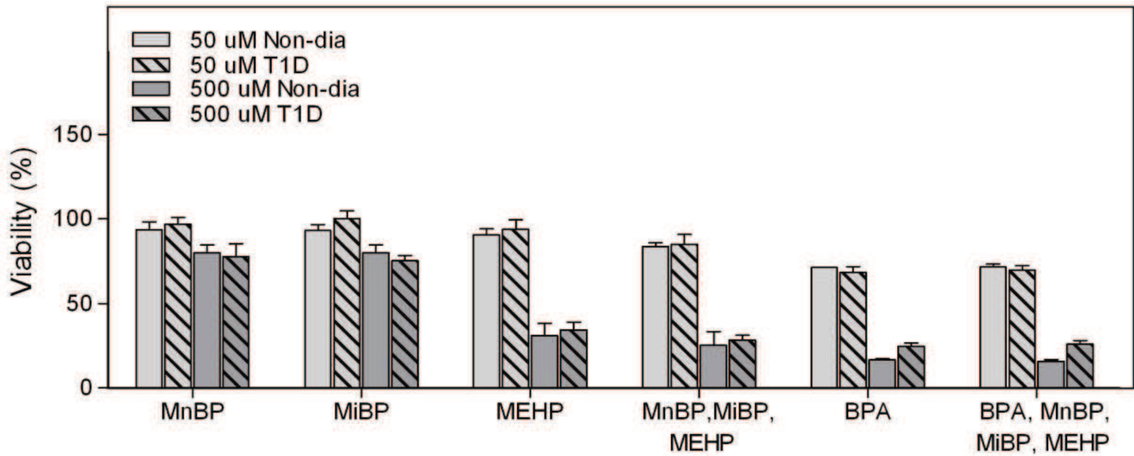


Figure 11: Comparison of sensitivity to environmental chemicals in the non-diabetic and T1D models. Data from non-diabetic (Figure 8a) and T1D (Figure 10) for the two highest chemical concentrations (50 µM and 500 µM). As in Figure 8a and 10 the data are normalised with the controls in the respective model systems, which allows for a comparison of the environmental chemical sensitivity in the two model systems. No significant differences were detected between environmental chemical exposures in the non -diabetic and T1D model systems when analysed by two-way ANOVA with Bonferroni post-test

As for the non-diabetic model, additive, synergistic or antagonistic effects were explored for 50 µM exposures only, by comparisons between the measured effects due to combined exposure and the calculated sum of the individual exposures. There were no significant differences between the measured combined exposures and the calculated sum of the individual exposures, suggesting an additive effect (Figure 12).

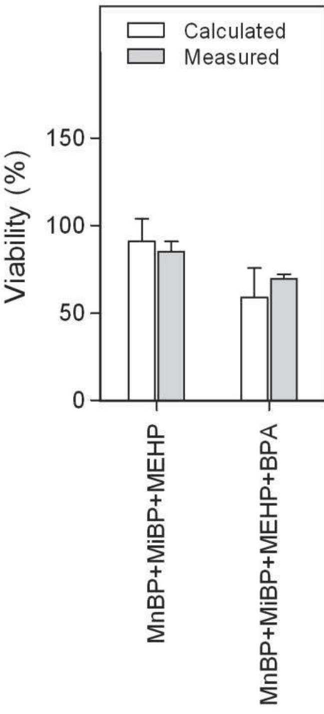


Figure 12: Comparison of effects due to combined and calculated exposures. Data from Figure 10 were used to evaluate if combined exposures resulted in additive, synergistic or antagonistic effects in the non-diabetic model system. The effect of the combined exposure of 50 µM of the three phthalates (MnBP, MiBP and MEHP) and the combined exposure of the four environmental chemicals (MnBP, MiBP, MEHP and BPA) was compared to the calculated sum of the effects of the individual chemicals as described in materials and methods (section 2.6). Two-way ANOVA with Bonferroni post-test showed no significant differences.

The MTT test was compared with other toxicity measures for both the non-diabetic and the T1D model systems, for exposure to 50 µM of the three phthalates, BPA and all four chemicals (Figure 13a-d). In the same experiments, the effects of 24 and 48 h cytokine exposures were compared. Figure 13a displays the increasing reduction in cell viability at the two different cytokine incubation times (24 h and 48 h). Similar to low dose experiments (Figure 6a), the 24 h and 48 h cytokine exposure (INF-γ, TNF-α and IL-1β) decreased the viability of INS-1E cells with about 30 and 65% compared to untreated controls, respectively. Furthermore, BPA and the combinatory exposure of the four chemicals induced significant reductions in viability in the no-cytokine and the 24 h cytokine exposure group compared to

controls, while the viability was not affected by chemical exposure after 48 h cytokine exposure. Thus, 48 h cytokine exposure appeared to attenuate the effects of the environmental chemicals.

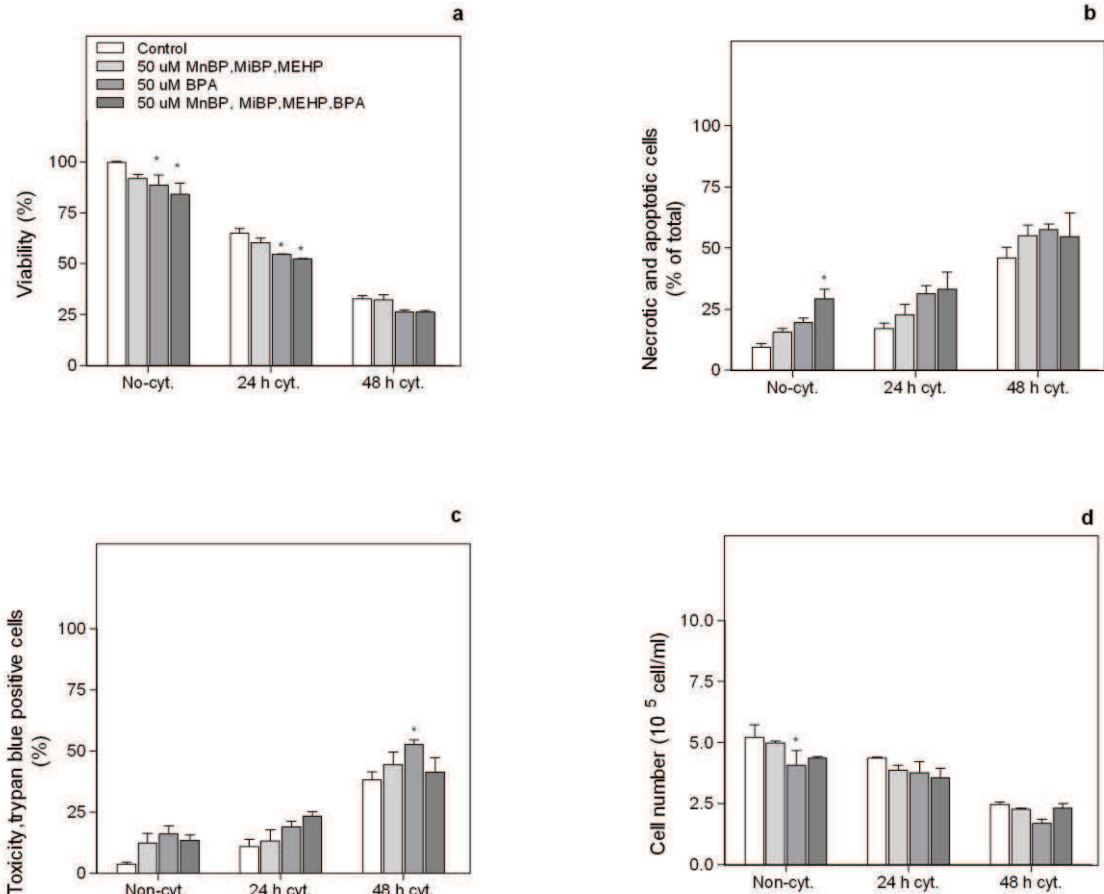


Figure 13a-d: Comparison of non-diabetic and T1D model systems for different viability measures – high dose. To illustrate the effect of cytokine exposure in the T1D model system, experiments were performed to compare the model systems on the same plate. INS-1E cells were seeded at 140.000 cell/ml, with medium shift on day 3. 'No cytokine' corresponds to the non-diabetic model system, while '24h cyt.' and '48h cyt.' correspond to T1D model systems. As in Figure 5 the cytokine exposure (5 ng/ml IL-1β, 25 ng/ml IFNγ and 25 ng/ml TNFα) was done in combination with environmental chemical exposure the last 24 or 48 h of the exposure. Bars represent mean ± SEM for 3 independent experiments. (a) Relative cell viability was measured by the MTT assay. Data were normalized for each experiment, *i.e.* divided by the mean of **the non-diabetic controls** in that experiment. (b) The fractions of necrotic and apoptotic cells were determined by Hoechst/PI staining. Bars represent the relative numbers of the sum of necrotic and apoptotic cells, but very few apoptotic cells were detected. (c) Toxicity expressed as the fraction of trypan blue positive cells relative to total cell count. (d) Number of viable cells, corresponding to fraction of trypan blue negative cells. * Indicates significant decrease compared to controls.

The percentage of apoptotic and necrotic cells presented in Figure 13b were in accordance with the reduced viability observed by the MTT, in that the number of necrotic and apoptotic cells increased with all the chemical exposures in the non- cytokine and the 24 h cytokine exposure group. The increase was only significant for the combined exposure to all four chemicals in absence of cytokines, but these experiments were only performed three times. Similarly, the toxicity determined by percent of trypan blue positive cells increased after

exposure to chemicals and cytokines, but the increase was only significant for 50 µM BPA exposure together with the 48 h cytokine treatment (Figure 13c). Thus, overall the chemical-induced toxicity detected by the MTT assay was reflected by the other toxicity assays. The cell numbers, measured by trypan blue exclusion, were not significantly affected by chemical exposures, although a slight tendency to decreased cell numbers after chemical exposure was observed.

3.2.3 ***T2D model***

After 72 h treatment, MnBP and MiBP did not affect insulin secretion, while MEHP, BPA and the two combinatory exposures caused a significant reduction in insulin secretion at 500 µM compared to controls (Figure 14). The reduction in insulin secretion was more than 50% compared to control, and appeared to reflect the chemical-induced toxicity. BPA did however show a slight non-significant tendency to increase the insulin secretion at 5 and 50 µM.

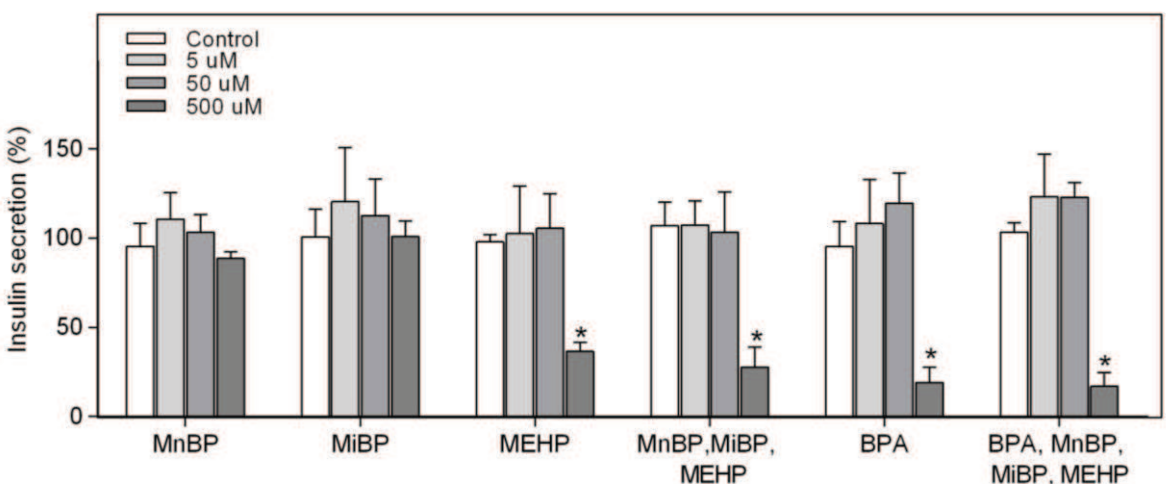


Figure 14: T2D model system: insulin secretion after 6.7 mM glucose stimulation. INS-1E cells were seeded at 120.000 cells/ml, with medium shift on day 3, before 72 h environmental chemical exposure, as indicated. Cells exposed to DMSO alone were used as controls. After 72 h exposure, all wells were incubated in glucose free KRBH buffer for 1 h, before 30 min incubation with 6.7 mM glucose in KRBH buffer. Insulin secretion in cell culture supernatants was determined by ELISA. Data were obtained from duplicates in 4 independent experiments, and were normalized for each experiment, *i.e.* the mean of duplicates were divided by the mean of all the controls in that experiment, and expressed as mean ± SEM. * Indicates significant decrease compared to controls.

4 DISCUSSION

The use of BPA and phthalates in a large variety of consumer products has resulted in a widespread human exposure, and epidemiological studies have suggested a link between exposure to these environmental chemicals and diabetes (Lang et al. 2008; Lind et al. 2012b; Shankar & Teppala 2011; Svensson et al. 2011). However, whether this association is causal remains highly controversial. BPA and phthalates, are possible candidates to exacerbate and accelerate the development of T2D. Several *in vivo* studies reports a variety of effects on β-cell function after BPA exposure (Alonso-Magdalena et al. 2006; Makaji et al. 2011; Nadal et al. 2009; Ropero et al. 2008), whereas phthalates have received less attention in experimental studies. An association between endocrine disruptors and the autoimmune disease T1D has also been suggested (Howard & Lee 2012), and recently BPA was reported to show a tendency to accelerate development of T1D in NOD mice (Bodin et al. 2013).

Here, we hypothesised that exposure to BPA and three phthalate metabolites (MiBP, MnBP and MEHP) would directly decrease viability, increase the sensitivity to cytokine induced apoptosis and increase insulin secretion from the rat pancreatic INS-1E β-cells. In our model system, treatment with 1-500 nM BPA and phthalate metabolites did not have any effect on β-cell viability, sensibility to cytokines or insulin secretion, but higher concentrations (50 and 500 μM) reduced the viability of the INS-1E cells. BPA was however the most potent chemical and MEHP was the most potent between the three phthalate metabolites. Moreover, the combinatory exposures of the three phthalates and all four chemicals appeared to induce additive effects on β-cell viability at 50 μM.

4.1 Non-diabetic

Three different model systems were used to examine effects of BPA and the phthalate metabolites; MiBP, MnBP and MEHP after 72 h exposure, where the non-diabetic model system reflects a normal non-diabetic individual. The viability, as determined by MTT, was not reduced at concentrations of 1-500 nM of any of the environmental chemical treatments. Our negative results of BPA exposure were surprising since a recent study in a similar cell line (INS-1) showed decreased cell viability after BPA exposure as low as 200 nM for 12 h, with an accelerated rate of apoptosis in a dose dependent manner (Lin et al. 2013). In addition, mitochondrial morphology and mass was affected, suggesting that the BPA induced

apoptosis was associated with mitochondrial defects in the β-cells (Lin et al. 2013). Moreover, 110 nM BPA exposure has also caused mitochondrial swelling in isolated rat islets (Song et al. 2012). In the TC-6 cell-line 400 nM BPA did not seem to affect mitochondrial activity (Makaji et al. 2011), but caused an ER stress response that may eventually lead to β-cell dysfunction and apoptosis (Aston-Mourney et al. 2008). These discrepancies could be explained by a number of experimental factors such as slight genetic differences between cell lines, or experimental setup (Spinnler et al. 2013). In addition, it should be noted that cell lines often are less sensitive than primary cells or islets (Hohmeier et al. 2000).

Since previous studies, in contrast to the present study, have shown effects of BPA exposure in the nM range, we also wanted to examine potential effects of higher concentrations in our model system, to test whether the INS-1E cells could respond to environmental chemicals. Three concentrations were chosen for these high-dose experiments; 5, 50 and 500 µM. These concentrations are higher than the serum concentrations reported in most biomonitoring studies, but for some of the phthalate metabolites there might be an overlap with the ranges reported for some populations, since the maximum concentrations for MiBP have been reported to be more than 8 µM (Appendix 1, table 3). The highest concentration reduced cell viability for all the environmental chemical treatments, but BPA appeared to be the most potent resulting in a significantly higher reduced viability also at 50 µM. A more detailed concentration-effect curve of BPA and MEHP also confirmed that BPA was the most potent chemical. Since BPA was more potent than MEHP in our model system, and MEHP was more potent than the other phthalate metabolites, it is tempting to suggest that phthalates might induce less effect on β-cells than BPA. However, further studies are necessary to clarify this assumption.

Changes in absorbance in the MTT assay provide a relatively rough measure of viability, which may reflect both toxicity, altered proliferation or reduced mitochondrial activity (Werner et al. 2013). Overall, the Hoechst/PI and the trypan blue test confirmed the results from the MTT assay for both low and high concentrations. However, fewer significant effects were detected with the Hoechts/PI and trypan blue assays compared to the MTT test, which may be due to inclusion of only 3 experiments in the statistical analyses.

Exposure to 50 µM BPA mainly increased the number of necrotic cells and with little changes with regard to apoptotic cells. This is in contrast to a recent study on BPA which found an

accelerated rate of apoptosis (Lin et al. 2013). The previous study (Lin et al. 2013) used a shorter exposure time of 48 h, as opposed to the 72 h exposure used in our study. A possible explanation for our result is that apoptotic cells might have been converted to necrotic cells during the last 24 h of exposure.

4.2 T1D

T1D is thought to be mediated by an insulitis, including infiltration of immune cells to the pancreas, and cellular secretion of cytokines. Due to the reported tendency of accelerated T1D development in non-obese (NOD) mice (Bodin et al. 2013) by BPA, we hypothesised that the environmental chemicals could increase the sensitivity to cytokine-induced cell death.

In the T1D model, the cells were exposed to cytokines the last 24 or 48 h of the environmental chemical exposure, but there were no further changes in cell viability neither for low nor high concentrations of the environmental chemicals. These data suggest that the environmental chemicals did not increased the β-cells sensitivity to cytokine induced cell death. Overall, the toxicity data from the trypan blue positive cells and necrotic/apoptotic cells (Hoechst/PI) confirmed these conclusions based on the MTT data.

Interestingly, the 48 h cytokine exposure seemed to level out the environmental chemical induced cell death in the MTT data, when compared to 24 h or no cytokine treatment. Even though this trend was not so clear in the Hoechst/PI and trypan blue test, it may suggests that 24 h cytokine exposure would have been a better experimental design in the low dose experiments also, as applied in the high dose follow up experiments. However, the low dose chemical exposure did not appear to increase the sensitivity to the cytokine-induced cell death during the 24 h cytokine treatment either, when assessed for the combined exposure to all four chemicals for highest concentration (500 nM, Figure 6a-d)).

The present reduction in viability after cytokine exposure, with approximately 30 and 65% after 24 and 48 h, respectively, suggested a strong cytotoxic effect of the cytokines. Hoechst/PI analyses showed that this reduced viability was primarily dominated by necrosis with below 1% apoptosis, similar to the effects induced by the high concentration of the environmental chemicals. This is in contrast to our hypothesis of cytokine-induced apoptosis

and the relevance for T1D. However, whether apoptosis is a major cell death pathway during diabetes development is highly controversial. On the one hand, there is a general agreement that cytokines appear to have a direct role in inducing pancreatic β-cell death leading to a decrease in β-cell viability. However, whether the cell death induced by pro-inflammatory cytokines occurs by necrosis, apoptosis, or both is still debated (Delaney et al. 1997; Fehsel et al. 2003; Saldeen 2000). Our data arguing for a cytokine induced β-cell death by necrosis differ from some prior studies that claim an important contribution from apoptosis (Jensen et al. 2005; Kutlu et al. 2003). On the other hand, some studies on INS-1 cells and isolated islets support our results that necrosis is the predominant type of cell death by cytokine-induced killing of β-cells (Collier et al. 2006; Collier et al. 2011; Saldeen 2000). A factor that might contribute to these conflicting results with respect to type of cell death could be differences in the applied cytokine concentrations, and it has been postulated that the dose of cytokines might determine which cell death pathway is preferentially activated (Grunnet et al. 2009). It is possible that the long-term exposure of cytokines (24-48 h) used in our study, may preferentially induce non-apoptotic β-cell killing, while β-cell apoptosis might be induced at lower exposure time. Since shorter exposure times were not investigated presently, we cannot exclude the possibility that this could explain the observed low rate of apoptosis.

To our knowledge the present study is the first to examine if environmental chemicals could increase the β-cell sensitivity to cytokine induced cell death *in vitro,* with relevance for T1D. The cytokines reduced the cell viability and increased the toxicity, however, neither BPA nor phthalate exposure increased the sensitivity to this cytokine induced cell death. This may suggests that the accelerated T1D development by BPA exposure recently reported, may be due to an direct effect of the chemical, rather than increased sensitivity to cytokine induced cell death. However, the lack of effects may also be explained by insensitivity in our model system. Moreover, these results cannot rule out contributions from other possible mechanisms in accelerated T1D development, thus further studies should be performed, also in other cell types.

4.3 T2D

In the present study, levels of MiBP, MnBP, MEHP and BPA relevant for human environmental exposures (1-500 nM) did not affect insulin secretion in INS-1E cells, neither

for exposures alone or in combination. Even high concentrations in the µM range did not increase the insulin secretion, and the only significant effect was decreased insulin secretion after exposure to 500 µM of MEHP, BPA and the combinatory exposures. This reduction was most likely due to the chemical- induced toxicity observed at these concentrations. The lack of effect of BPA on insulin secretion was surprising, given that approximately 0.4 - 4 nM BPA has been shown to increase insulin secretion in other β-cell lines as well as primary cells (Adachi et al. 2005; Lin et al. 2013; Makaji et al. 2011; Song et al. 2012; Soriano et al. 2012). Suggested mechanisms for the insulin hypersecretion in response to BPA include ER stress, and dysregulation in normal function of the ER and mitochondria have shown associations with impaired β-cell function and increased β-cell death (Drews et al. 2010; Jitrapakdee et al. 2010; Laybutt et al. 2007; Thomas et al. 2010).

BPA has previously been shown to induce increased insulin secretion at concentrations as low as 0,4 nM in primary cells (Adachi et al. 2005; Song et al. 2012). While higher concentrations of 400 nM and 4 µM BPA were necessary to increase insulin secretion in the TC-6 cell line. This might imply that there are differences in sensitivity between cell lines and primary cells, which is also in accordance with previous reports in that INS-1E cells cannot substitute primary β-cells (Hohmeier et al. 2000; Merglen et al. 2004; Spinnler et al. 2013). However, BPA recently increased insulin secretion in INS-1 cells at concentrations as low as 20 nM (Lin et al. 2013). Even though INS-1 cells are rather similar to the INS-1E cells used presently, the INS-1E cells are further improved and more stable with respect to insulin secretion (Merglen et al. 2004). A recent study investigating the acute (2 h) effects of 1, 10 and 100 nM BPA and DEHP on insulin secretion in an other INS-1 subclone cell line (INS-1 832/13), also found limited effects of these chemicals, and concludes that the cell line appears to lack certain characteristics needed to respond appropriately to environmental chemicals (Hectors et al. 2013). This clone of the INS-1 cell line is also known for its stable insulin secretion, and a possible explanation is that in gaining stability with respect to insulin secretion, the sensitivity to environmental chemicals might be lost.

The lack of effect in the present study may also be explained by application of different exposure conditions, since no previous studies have examined 72 h chemical exposure in β-cell lines. Lin et al. (2011) reported effects after 48 h in the INS-1 cells, while Adachi et al. (2005) found effect in the primary cells after 24 h but no acute effects after 1 h BPA exposure. Interestingly the TC-6 cells showed increased insulin secretion after only 1 h exposure, but in

that study the cells were exposed to glucose and BPA simultaneously (Makaji et al. 2011). It should also be noted that it would have been feasible to include an appropriate reference compound, i.e. positive and negative controls in our experiments, to verify whether general exposure conditions affects the function of our cell line (Hectors et al. 2013). Possible candidates for positive and negative controls for insulin secretion include diazoxide and 1-methyl-3-isobuthylxanthine (Hectors et al. 2013).

There have previously been some concerns that the plastic in the equipment used in cell culturing, exposure and harvesting may contain both BPA and phthalates, which may increase the exposure dose beyond the studied concentrations and might lead to variations in the data. However, the plastic in the equipment used presently was polysteren (Falcon cell culture flasks and plates) or polypropylene (pipette tips), both are plastic types that are free of BPA and phthalates. Another source of contamination could be the foetal calf serum used in the cell culture medium, but we did currently not have the possibility to investigate this. Phenol red, which is a standard ingredient in most cell culture medium has previously been reported to have a weak estrogenic activity (Glover et al. 1988) and effects on cell proliferation and growth in different cell cultures (Glover et al. 1988; Walsh-Reitz & Toback 1992; Wesierska-Gadek et al. 2007). Since the increased insulin secretion reported by BPA exposure is suggested to be mediated through estrogen receptors (Adachi et al. 2005; Alonso-Magdalena et al. 2006; Alonso-Magdalena et al. 2012), one might suggest that this could have an impact on our results. However, whether phenol red has a particular impact in cell line models or not, is highly controversial (Hubert et al. 1986; Rajendran et al. 1987; Welshons et al. 1988). Results from a previous study using nine estrogen reseptor-positive cell lines have concluded that phenol red in culture medium is insufficient to cause estrogenic effects (Moreno-Cuevas & Sirbasku 2000). Additionally, the previous studies who found effect on insulin secretion from BPA (Lin et al. 2013; Makaji et al. 2011) also seemed to use phenol red containing medium, as application of phenol red-free medium was not indicated. Thus, even though we cannot exclude that the phenol red in the medium presently may have contributed to the large variability in our insulin secretion levels, it is not likely to explain the lack of effects on increased insulin secretion.

Presently, the variations in the levels of insulin secretion at 6.7 mM glucose stimulation showed relatively large variability. The insulin release data was normalized for each experiment, in an attempt to remove some of the variations, but the variability in our model

system appeared to be larger than in previous studies. However in previous studies insulin secretion per protein or insulin content has most commonly been used previously rather than inulin levels in ng/ml or normalised to control levels (Lin et al. 2013; Makaji et al. 2011; Merglen et al. 2004). In Merglen et al. (2004) there were large variations in insulin content, while the insulin secretion expressed as relative to insulin content, showed less variation. This implies that the insulin content reflects the insulin secretion, and that this presentation form may remove some of the variability in the insulin measurements. Therefore, expressing insulin secretion relative to protein or insulin content rather than relative to control might be a more feasible method. Nevertheless, it might not be correct relating to insulin content or to cellular protein content when studying toxic components, since high toxicity may give a false increase in insulin secretion, as illustrated for data from one of our experiments in Figure A10, Appendix 5.

To the best of our knowledge, no other *in vivo* or *in vitro* studies have investigated whether phthalate metabolites affect β-cell function. One recent study has however investigated the acute effect of DEHP, but only in order to get a first indication of the relevance of their model system (Hectors et al. 2013). They reported limited effect of acute exposure, but only three concentrations were included (Hectors et al. 2013). However, in rats exposed to DEHP in utero, the glucose homeostasis was disrupted, suggesting that this phthalate may induce a β-cell dysfunction (Lin et al. 2011). Interestingly, DEHP reduced the pancreatic insulin content, and the authors suggested that defects in insulin action early in life could be compensated by higher insulin sensitivity in offspring. Then, β-cell failure may occur with age, as shown by both decreased pancreatic insulin content and reduced β-cell mass, because the production of new β-cells in adulthood is low (Lin et al. 2011). DEHP has also been reported to alter glucose tolerance in rats, due to abnormal glucose content in liver and skeletal muscle (Lin et al. 2011). These results suggest that MEHP, which is a metabolite of DEHP might be involved in altered glucose metabolism, and may impact on T2D development through other pathways than a direct effect on the insulin secretion from pancreatic β-cells. An epidemiological study has however reported associations between MiBP and other phthalate metabolites, such as Mono-methyl phthalate (MMP) and Mono-ethyl phthalate (MEP), and diabetes (Lind et al. 2012b). Even though our results showed no significant effects of the phthalate metabolites MiBP, MnBP and MEHP on insulin secretion, further studies should be performed in other cell lines or primary cells where BPA already has shown effects.

4.4 *Combinatory exposure*

Humans are exposed to multiple phthalates and BPA and risk assessment of individual compounds may lead to an underestimation of the risk if the compounds have common action. It is therefore important to examine the activity of environmental chemicals as a mixture (Backhaus & Faust 2012). We have defined additive effects as an outcome of no interaction, where the observed toxicity of the mixture is the sum of each chemicals toxicity observed individually (Laetz et al. 2009). If the mixture effects causes a larger effect than the extrapolated effect from each chemical it is referred to as a synergistic effect, while a smaller effect is referred to as antagonistic (Laetz et al. 2009).

In the INS-1E cells, a combinatory exposure to 50 μM of the different chemicals did not cause significant differences in response from the sum of the effects of the individual chemical, thereby implying additive effects. A possible explanation for the additive effects from the combined exposure to phthalates and BPA in our study is that these chemicals may interact with the same genes/proteins, and they have also been shown to induce similar effects in other tissues and are associated with similar adverse health outcomes (Singh & Li 2012). However, the concentrations we applied to study additive effects were relatively high and at sufficiently high concentrations most compounds can induce non-specific toxicity (Yassi et al. 2001). On the other hand, not all the applied substances induced effects, suggesting that the concentrations were within a non-toxic range.

Interestingly, phthalates have previously shown to induce additive effects on fetal steroidogenesis in rats (Howdeshell et al. 2008), while mixed exposures to phthalates and BPA has shown coinciding additive effects in animals, with a tendency to additive effects at low doses, and synergistic effects at higher doses (Christen et al. 2012). A possible consequence of additive or synergistic effects of the phthalates is that risk assessments should consider total phthalate exposure rather than exposure to individual phthalates (Christen et al. 2012). The methods used to calculate the mixture effects in these previous studies differ from the method used presently. Christen et al. (2012) characterized the concentration-effect curve to each individual chemical, and subsequently estimated the mixture exposure by regression based on the equally potent levels of the chemicals, which is a more formalized method for examination of mixture effects, particularly to investigate whether the chemicals act through the same mechanisms. However, since we aimed to study whether low concentrations of the

combinatory exposure may induce greater effects than the individual chemical exposures, we did not characterize the concentration-effect curve for each individual chemical.

The present study appears to be the first investigation of combinatory effects of phthalate metabolites and BPA on β-cell function. Since our model system seems to have low sensitivity to environmental chemicals, further studies in other model systems are necessary to investigate combinatory effects of these chemicals in other cell types and model systems where BPA has been shown to affect β-cell function.

4.5 Methodological considerations

Limitations of model systems

Even though *in vitro* studies with cell lines are commonly used and readily performed, it involves several limitations. For instance, *in vitro* model systems only provide information about signalling molecules and viability which cannot predict the more complex biological responses, in humans. Furthermore, the altered characteristics and limited communication with other cells compared to cells in tissues is important to take into account. Thus, observed effects in *in vitro* studies need to be further investigated in *in vivo* model systems. Furthermore, one should also be aware that *in vivo* studies in rodents cannot be directly extrapolated to humans, as rodents and humans might differ from each other in both with regard to physiology and toxicokinetics (Kamrin 2009). Furthermore, the percentage of β-cells is lower in human pancreatic islets than in rodent islets, while humans percentage of α-cells is higher (Cabrera et al. 2006). This might influence on the interactions between the cell types and might be of more importance in humans than in rodents.

Measures of insulin secretion

Presently, the insulin secretion was expressed as relative to mean control in each experiments, rather than normalized to cellular protein or insulin content as done previously (Lin et al. 2013; Makaji et al. 2011; Soriano et al. 2012). To compare these two methods for presenting insulin secretion, we measured the protein content in one high dose experiment. Then the insulin release (ng/ml) was normalized to the protein content (mg/ml) and expressed as

insulin per mg protein as in the previous studies (data shown in Appendix 5, Figure A10). The measured protein content (mg/ml) was in accordance with results from the MTT assay, in that it showed reduced protein content for the highest concentration of the environmental chemicals. Interestingly, when insulin secretion was normalized to cellular protein content, we also observed an increased insulin secretion, but only at the chemical levels inducing high toxicity simultaneously. This might suggests that although the viability was reduced, the insulin secretion per living cell was increased.

However, Alonso-Magdalena et al. (2006) reported increased insulin content and secretion in *in vivo* expressed as plasma insulin normalized to control values, and not only per protein content. Their results still imply that BPA can increase both plasma insulin and β-cell insulin content, emphasising our suggestion that use of INS-1E cells are not suitable to examine insulin release mediated by chemical exposures.

4.6 Further studies

Due to lack of effects at concentrations relevant for environmental exposures in the present study, experiments with high concentrations were included. A more ideal approach would have been to examine effects of the low dose concentrations in a different cell line or in primary cells, but this was not possible due to time limitations. However, further experiments are currently conducted in the INS-1 cell line at NIPH, since BPA exposure was recently reported to accelerate apoptosis and induce increased insulin secretion in these cells (Lin et al. 2013). In these additional experiments, the low environmentally relevant chemical concentrations will be tested in all the three model systems applied presently. Thus both cell viability and insulin secretion will be investigated in relevance to T1D and T2D development. Moreover, a positive control for insulin secretion will be included as well as shorter exposure times (1-48 h). , This will allow for investigation of the influence of incubation time on insulin release and fraction of necrotic vs apoptotic cells. Finally, use of primary cells as a follow-up study will be considered for selected chemicals and model systems.

If a suitable model is established another interesting aspect would be the possible effects of other phthalate metabolites including more oxidised metabolites on pancreatic β-cell function. Even though the present study found no effect of the chemicals on sensitivity to cytokine

induced apoptosis, other mechanisms relevant for T1D should be further examined, including chemical-induced effects on macrophage function or the functionality of other immune cells. Further studies in animal models is also important to characterize possible alterations in more complex biological responses with relevance for both T1D and T2D after exposure to both BPA and phthalates.

5 CONCLUSION

Our main hypothesis, that exposure to BPA and the phthalate metabolites MiBP, MnBP and MEHP, either individually or in combination would affect β-cell function in environmentally relevant concentrations, was not supported by our results. Only high concentrations, not relevant for environmental exposure levels, reduced the cell viability, possibly due to direct toxic effect of the chemicals. However, the environmental chemicals did not affect the sensitivity to cytokine-induced cell death. Since our results are in conflict with other studies, they suggests that although INS-1E cells are commonly used in mechanistic studies of cytokine-induced apoptosis and insulin secretion, this cell line appears to be insensitive to environmental chemicals.

In conclusion, the INS-1E cell line does not appear to be suitable for studies of metabolic effects of environmental chemicals, and the data obtained cannot be used to further characterize the effect of BPA and phthalates on β-cell function. However, experiments with INS-1 cells are now in progress, and obtained data will be submitted for peer-reviewed publication together with the data from the present study.

6 PUBLIC HEALTH PERSPECTIVES

Presently BPA and phthalates showed no effects on pancreatic INS-1E β-cell function at concentrations relevant for human exposure, possibly due to a low chemical-sensitivity of the INS-1E cells. Thus, our results do not exclude the more general aspect of our hypothesis; that environmental chemicals used in plastic products may affect the pancreatic β-cell functions, and thereby contribute to development of diabetes type 1 or type 2.

Accumulating evidence from *in vitro* and *in vivo* studies has shown that BPA exposure accelerated β-cell death, altered expression of key proteins in the cellular and ER stress response and disturbed insulin secretion (Adachi et al. 2005; Lin et al. 2013; Makaji et al. 2011; Nadal et al. 2009; Ropero et al. 2008; Song et al. 2012). Unfortunately, there are few human studies on the association between BPA and diabetes, and available studies have reported conflicting results (Lang et al. 2008; Melzer et al. 2010). These previous human studies are often based on self-reported diagnoses of diabetes (Lang et al. 2008; Melzer et al. 2010; Shankar & Teppala 2011), which may lead to a miss classification in that the patients who actually have diabetes are classified as non-diabetic which could lead to an underestimation of a possible association (Shankar & Teppala 2011).

Shankar and Teppala (2011) and Lang et al. (2008) both found positive associations between increased urinary BPA levels and T2D, based on different data from NHANES, but it is not clear whether this is due to cause or consequence. That is, whether the disease itself leads to alterations in normal functions which may impact on the chemical levels in urine and serum, or whether highly exposed individuals actually have higher risk of developing the disease. For instance, T2D may lead to changes in energy metabolism (Lin & Sun 2010) and the majority of diabetes patients are overweight or obese (Ameican Diabetes Assosiation 2008). It is therefore conceivable that they might also have different metabolism and excretion of environmental chemicals compared to normal-weight subjects. The associations between T2D and urinary BPA levels may therefore be a consequence of already having developed the disease rather than reflecting that the higher BPA levels cause higher risk of developing T2D. To address this question in future studies it would be necessary to do longitudinal rather than cross sectional studies.

BPA exposure is suggested to play a role in weight gain and obesity development through several mechanisms (Ben-Jonathan et al. 2009; Masuno et al. 2005; Phrakonkham et al.

2008). An excessive insulin signalling produced by an overstimulation of β-cells by BPA exposure may provoke insulin resistance in liver and skeletal muscle (Nadal et al. 2009). Furthermore, it may induce alteration in liver and adipose tissues, resulting in dyslipidemia, obesity and glucose intolerance (Biddinger & Kahn 2006). As a consequence, blood glucose concentrations increase, further promoting hyperinsulinemia (Alonso-Magdalena et al. 2006) and thereby β-cell exhaustion, contributing to the development of T2D (Nadal et al. 2009). Thus, BPA could also contribute indirectly to the development of T2D through other mechanisms and tissues contributing to obesity, rather than exerting direct effects on β-cell function.

BPA has also been reported to show a tendency to accelerate T1D development in mice (Bodin et al. 2013). The mechanisms involved are not fully determined, however, the acceleration is suggested to be due to an increased insulitis and β-cell death and reduced numbers of tissue resident macrophages in pancreatic islets (Bodin et al. 2013). Additionally, BPA may alter immune responses, and induce changes as altered T-cell subsets, β-cell functions, and dendritic cell and macrophage biology, through multiple actions like estrogen receptor and PPARs signalling (Rogers et al. 2013). Since T1D is suggested to be mediated through autoimmunity and alterations in the immune system, further examinations of a possible association between BPA and T1D would be feasible in both experimental and epidemiological studies.

Although phthalate metabolite exposure did not affect β-cell viability or insulin secretion, and all the phthalate metabolites showed less potency compared to BPA, there are number of ways phthalates potentially could impact diabetes development. It is well known that some phthalate metabolites may bind to PPARs, which is involved in regulation of lipid and glucose metabolism and may also alter immune cell functionality (Ferre 2004; Luquet et al. 2005). By binding to PPARγ, it has been asserted that phthalates may influence adipogenic genes leading to increased obesity, which in turn can lead to insulin resistance, metabolic dysregulation and increased risk of T2D (Grün & Blumberg 2007). Another potential pathway is binding to PPARα which impact on lipid handling and regulate circulating glucose levels (Casals-Casas et al. 2008; Desvergne et al. 2009; Feige et al. 2007) and thereby may affect diabetes risk by altering β-cell insulin secretion. It is also well known that both obese individuals and individuals with higher levels of circulating glucose levels has higher risk of developing T2D (Lin & Sun 2010; Triplitt 2012). Therefore, one might suggest that

phthalates could contribute to diabetes by other mechanisms than directly through β-cell dysfunction. One possible mechanism for phthalate-induced effects could be altered inflammatory responses, as reported for MEHP (Bolling et al. 2012; Jepsen et al. 2004; Vetrano et al. 2010), which could indirectly contribute to the development of autoimmune diseases like T1D.

Presently, additive effects were shown from the exposure of both the combination of the three phthalate metabolites and the combination of the phthalate metabolites and BPA. But these additive effects were only shown at one of the highest concentrations, not relevant for human exposure. Nevertheless, humans are exposed to multiple phthalates in combination, simultaneously with other environmental chemicals that may produce adverse effects together (Stahlhut et al. 2007). Although individual phthalate exposures in humans are generally asserted to be below the no observed effect level (NOAL), the additive effects of various phthalates and in combination with BPA, as shown in previous studies (Christen et al. 2012; Howdeshell et al. 2008), should be implemented in risk characterisations.

There is an ongoing debate concerning the determination of safe levels of BPA exposure based on the LOAEL (lowest-observed-adverse-effect-level) used up to now (50 mg/kg/day) and a new risk assessment by The European Committee of Food Safety (EFSA) is now in its final phase. The determination of safe levels are most often based on the NOAEL (No-observed-effect-level), but since the NOAEL of BPA has not been detected, due to adverse responses even at the lowest dose administered, it is based on the LOAEL (Vandenberg et al. 2009). Despite several studies reporting links between BPA and adverse health effects, several controversies on BPAs impact on humans has sprung up and continued through several years (Vandenberg et al. 2009). However, previously reported BPA effects at low concentrations, suggests that this should be further investigated (Adachi et al. 2005; Lin et al. 2013; Song et al. 2012). Even though the present study did not show any increased risk of β-cell dysfunction by BPA and phthalates at levels relevant for human exposure, studies on mice have indicated associations between BPA and insulin resistance at much lower doses than the LOAEL (Alonso-Magdalena et al. 2006). It is well known that findings in mice cannot be extrapolated directly to humans, due to differences in mechanisms and cell interactions (Brissova et al. 2005; Cabrera et al. 2006; Kamrin 2009). However, the increased insulin secretion of BPA described in mice has also been shown in human islets, but in a stronger manner compared to that in mice (Soriano et al. 2012). This may indicate that at least

some of the adverse effect of BPA on glucose homeostasis described in mice may be relevant for humans, and that further studies should be performed in this field.

Results from the Norwegian mother and child Cohort Study (MoBa) (Ye et al. 2009) showed higher levels of both BPA and the phthalate metabolites MnBP and MiBP than the women participating in the NHANES in the United States, possibly due to consumption of canned food and use of personal care products. The estimated daily intakes in Norway were still below the estimated tolerable daily intake (TDI) (Ye et al. 2009). However, children often have higher concentrations of BPA and phthlates than adults, while women have higher concentrations than men (Calafat et al. 2008; Wittassek & Angerer 2008). This may be a matter of concern, since children may be more vulnerable to exposure during development and exposure during pregnancy might enhance mothers insulin resistance and disrupts foetuses glucose homeostasis later in life (Alonso-Magdalena et al. 2010).

This study failed to find any effect of BPA and phthalate metabolites with relevance for diabetes development, it is however highly likely that the applied cell line was chemical-insensitive and thus an inadequate model system to examine effects of environmental chemicals on β-cell function. Despite this, several population studies reports associations between these chemical exposures and T2D (James-Todd et al. 2012; Lang et al. 2008; Silver et al. 2011; Svensson et al. 2011) and there is accumulating evidences that BPA induces effects on β-cell function (Makaji et al. 2011; Nadal et al. 2009; Ropero et al. 2008), in addition both BPA and phthalate exposure may induce other effects with relevance for diabetes development (Ben-Jonathan et al. 2009; Clayton et al. 2011; Feige et al. 2007; Lin et al. 2011). Furthermore, due to the economic and social costs associated with diabetes type 1 and type 2, and not to mention the severe implications for the affected individuals, the diseases represent a major public health issue. Thus, BPA and phthalate exposures deserve further investigations in both experimental and epidemiological study designs of type 1 and type 2 diabetes.

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APPENDIX 1

Table 3: Detailed overview of Phthalate and BPA concentrations in serum samples. Serum concentrations ng/ml are represented as mean, median and range with maximum values. The values are also shown converted to nM to show relevance to the concentrations used in this study. The grey fields represent the range of the reported values.

Serum concentrations of phthalates and BPA ng/ml (nM)			
Chemical	Mean	Median	Range/Maximum
MEPH Mono-2-ethylhexyl phthalate	0,77-20,3 (2,6-68,1)	0,49-7,88 (1,6-26,4)	0,47-514 (1.6-1724)
		4,69[a] (15,7)	0,47-514 (1.6-1724)
		4,33[aa](14,5)	0,51-415 (1,7-1391)
	6.74 [b] (22,6)	7.88 (26,4)	<LOD-15.93 (<LOD-53,4)
	0,77 [c] (2,6) 20,3 [d] (68,1) 18,9 [dd] (63,4)	0,49 (1,6) 4,3 (13,4) 4,7(15,8)	0,49-4,5 (1,6-15,1)
MiBP Mono-isobutyl phthalate	0,72-44,6 (3,2-200,7)	0,50-13,5 (2,2-60,7)	0,50-1820 (2,2-8189,3)
		13,4 [a] (60,3)	3,22-1390 (14,5-6254)
		13,5[aa] (60,7)	2,7-1820 (12,1-8189,3)
	0,72[b] (3,2)	<LOD	<LOD- 4,36 (<LOD-19,6)
	0,87 [c] (3,9) 53,6 [d] (241,1) 44,6 [dd] (200,7)	0,50 (2,2) 13,5 (60,7) 13,4(60,3)	0,50-11 (2,2-49,5)
MnBP Mono-*n*-butyl phthalate	0,43-1,8 (1,9-8,1)	<LOD-0,54 (<LOD-2,4)	0,54-20 (2,4-90)
	0,43 [b] (1,9)	<LOD	<LOD-1,51 (<LOD-6,8)
	1,8 [c] (8,1)	0,54 (2,4)	0,54-20 (2,4-90)
BPA Bisphenol A	0,16-4,94 (0,7-21,6)	3,6-3,89 (15,8-17)	0,79-27,3 (3,5-119,6)
		3,6[a] (15,8)	<LOD-27,3 (<LOD-119,6)
		3,89[aa] (17)	<LOD-24 (<LOD-105,1)
	4,94 [a2] (21,6)		<LOD-27,3(<LOD-119,6)
	0,20[e] (0,9)	<LOD	
	0,16[ee] (0,7) 0,98[f] (4,3)	>LOD	
	2,91[g] (12,8)		0,79-7,12

		(3,5-31,2)
	1,05 [h] (4,6)	

*LOD = Limit of detection
[a]Olsèn et al., (2012): 502 Swedish male seniors
[aa]Olsèn et al., (2012): 501 Swedish females seniors
[a2]Olsèn et al., (2012): Swedish males and Females together
[b]Fredriksen et al., (2010):60 young Danish men
[c]Högberg et al., (2008): 130 Swedish women
[d]Lind et al., (2012): 501Swedish males
[dd]Lind et al., (2012): 502 Swedish females
[e]He et al., (2009): 404 Chinese males
[ee]He et al., (2009): 482 Chinese females
[f]Dirtu et al., (2008):7 Belgium adults
[g]Cobellis et al., (2009):58 Italian women
[h]Kandaraki et al., (2011): 71 Greek women

APPENDIX 2

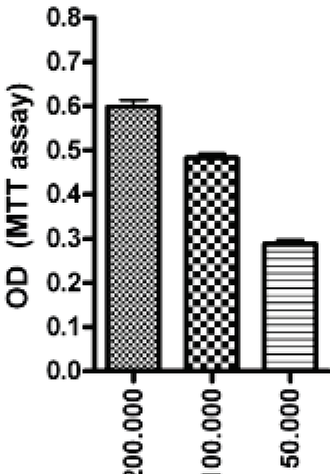


Figure A1: Test of different cell numbers. INS-1E cells were seeded with a density of 50.000, 100.000 and 200.000 cells/ml in parallel wells at Friday. Then the cells were harvested Thursday. Cell viability where measured using MTT. MTT values showed that seeding cells at 200.000/ml were no longer in a linear growth face. Visual inspection showed that 50.000 cells/ml was a bit sparse.

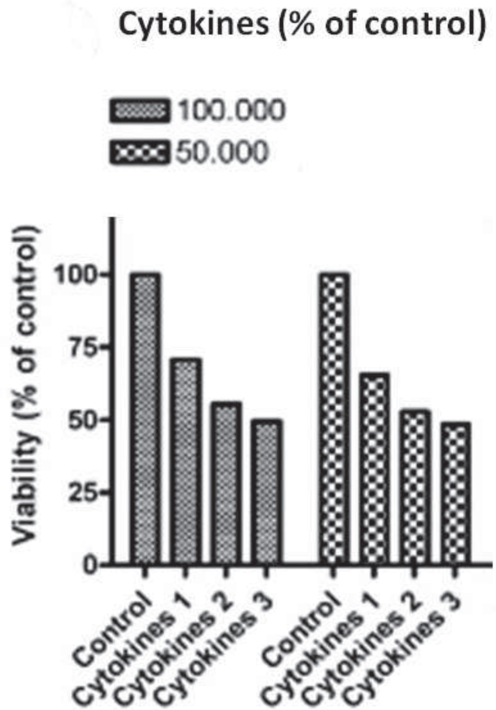


Figure A2: Test of different cytokine concentrations
INS-1E cells were seeded with a density of 50.000 and 100.000 cells/ml in parallel wells. Then the cells were exposed to either no cytokines in control wells or cytokines in one of the following concentrations; Cytokines 1= 1 + 5 + 5, Cytokines 2= 5 + 25 + 25, Cytokines 3= 10 + 50 + 50 ng/ml of IL-1β, TNFα and INFγ, respectively. Harvesting of cells was performed Friday. Furthermore, cell viability was measured by MTT. The MTT values for these cell densities showed that cytokine concentrations seem to have the same effect on cell viability.

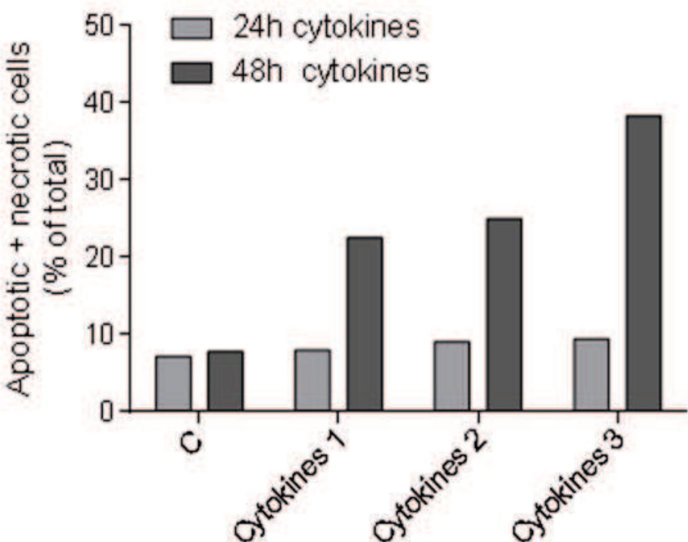


Figure A3: Test of exposure time of cytokines
INS-1E cells were seeded with a density of 150.000 cells/ml. The cells where then exposed to either no cytokines in control wells, or one of the following cytokine concentrations; Cytokines 1= 1 + 5 + 5, Cytokines 2= 5 + 25 + 25, Cytokines 3= 10 + 50 + 50 ng/ml of IL-1β, TNFα and INFγ, respectively. The 48 h exposure was performed at Tuesday and the 24 h exposure at Wednesday. At Thursday, cells were harvested and necrotic and apoptotic cells were measured in % of total cell number, using Hoechst/PI test. Results from Hoechst/PI show no differences in apoptotic/necrotic cells after 24 h, compared to controls. While, 48 h cytokine concentration shows an increased cell death compared to controls. Based on this pilot we chose cytokine concentration 2 (5 ng/ml IL-1β + 25 ng/ml TNFα + 25 ng/ml INFγ) in our T1D model system, because it showed considerable cell death, but not too high.

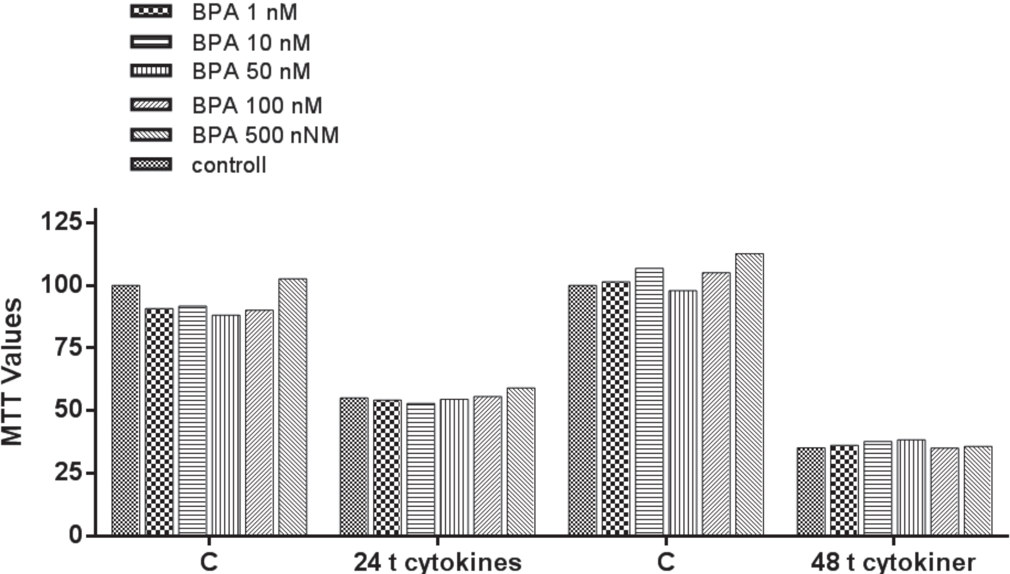


Figure A4: Test of cytokine incubation time together with chemicals (BPA).
The seeding of 120.000 cells/ml was performed at Friday. Exposure of cells with different BPA exposure was then performed Monday. Additionally, cells including controls were exposed to 24 and 48 h cytokines (20 mg/ml IL-1β+ 100 mg/ml TNFα+ 100 ng/ml INFγ) at Tuesday and Wednesday, respectively. Eventually, Thursday cells were harvested and cell viability was measured using MTT. The MTT values shows that cell death after 48 h cytokine concentrations do not prevent any further cell death from exposure to chemicals.

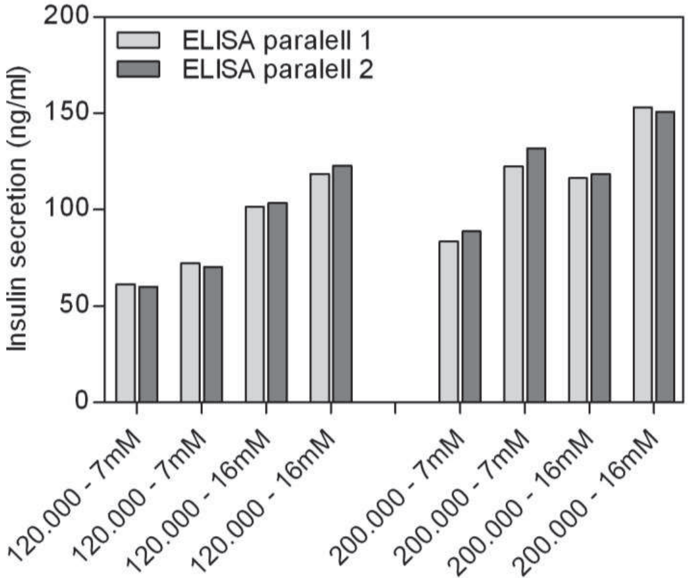


Figure A5: Test of appropriate cell number in relation to insulin secretion.
Seeding of INS-1E cells was performed at Friday, with a density of 120.000 and 200.000 cells/ml in parallel wells. Thursday, cells were incubated in KRB buffer for 1 h, then 30 min with the two different glucose concentration; 7 mM or 16 mM glucose. Harvesting was then performed by transfer of the supernatant into ELISA plates in parallel wells. The double columns next to each other represent the two parallel wells of the 24 wells falcon plate (seeding plate), and compares the variability within the two identical cell wells. While the two lateral columns with different colour combination reflects the parallel wells of the ELISA plate, which compare variability of the two identical ELISA wells. The ELISA variability was less than the cell well variability, and therefore we chose to run the experiments with parallel wells in the cell plate and only single wells in the ELISA plate. Moreover, the insulin secretion had less variance at 120. 000 cells, therefore this cell density was chosen.

APPENDIX 3

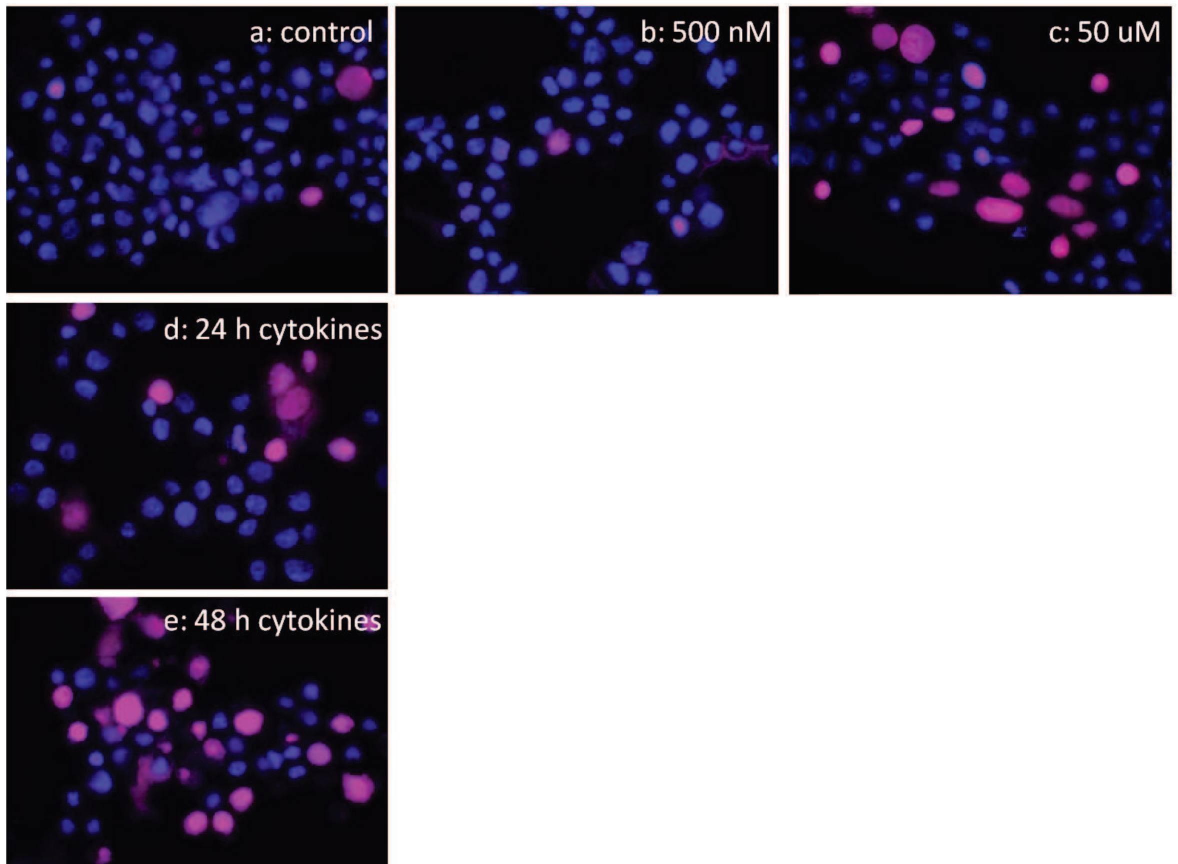


Figure A6: Amount of living and apoptotic/necrotic cells after low and high chemical concentrations and cytokines. INS-1E cells were seeded at 140.000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals or cytokines. Cells exposed to DMSO only were used as controls. Cells were then harvested and stained with Hoechst/PI. The pictures illustrates the differences in effects by low and high dose chemical exposure and cytokine exposure at different time point. (a); control, (b); 500 nM of each of the chemicals MiBP, MnBP, MEHP and BPA, (c); 50 µM of each of the chemicals MiBP, MnBP, MEHP and BPA, (d); 24 h cytokines, (e); 48 h cytokines.

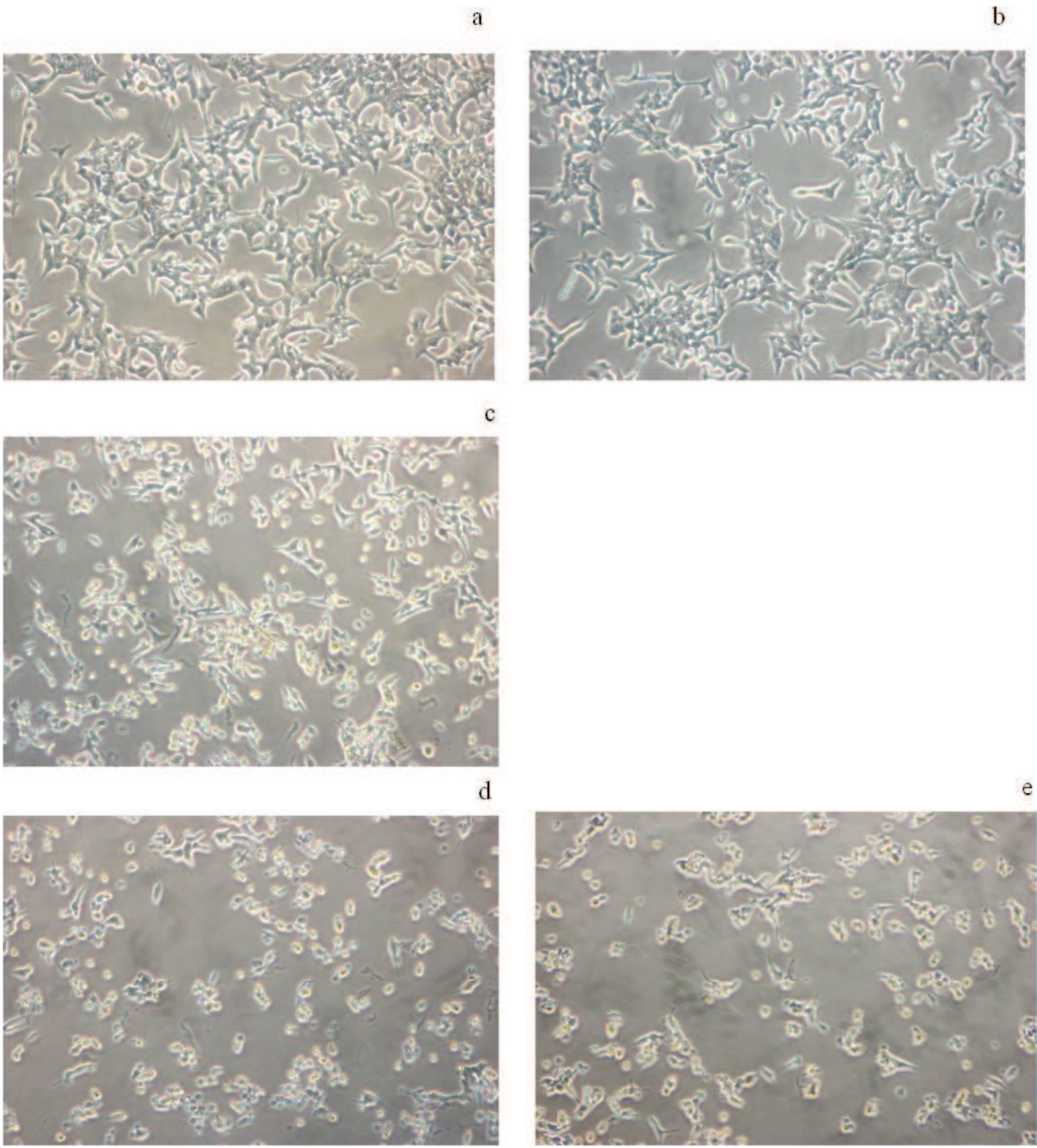


Figure A7: Effect of cytokine and low dose of environmental chemicals on cell morphology. To illustrate the effect of cytokine exposure in the T1D model system, experiments were performed to compare the non-diabetic and T1D model system, in both one low and one high chemical concentration. INS-1E cells were seeded at 140.000 cell/ml, with a medium shift on day 3, before 72 h exposure to environmental chemicals. The last 48 or 24 h of this exposure, the cells were co-exposed to the pro-inflammatory cytokines IL-1β, TNFα and INF γ at 5 + 25 + 25 ng/ml, respectively. The pictures are taken from microscope at the end of the exposures, before harvesting. Picture a,c and d at the first column illustrates the effects of the cytokines at different time-points. (a); control cells only exposed to DMSO, (b); 500 nM of each of MiBP, MnBP, MEHP, and BPA, (c); cells exposed to DMSO and 24 h cytokines, (d); cells exposed to DMSO and 48 h cytokines, (e); cells exposed to 48 h cytokines in combination with 500 nM of each of MiBP, MnBP, MEHP,and BPA.

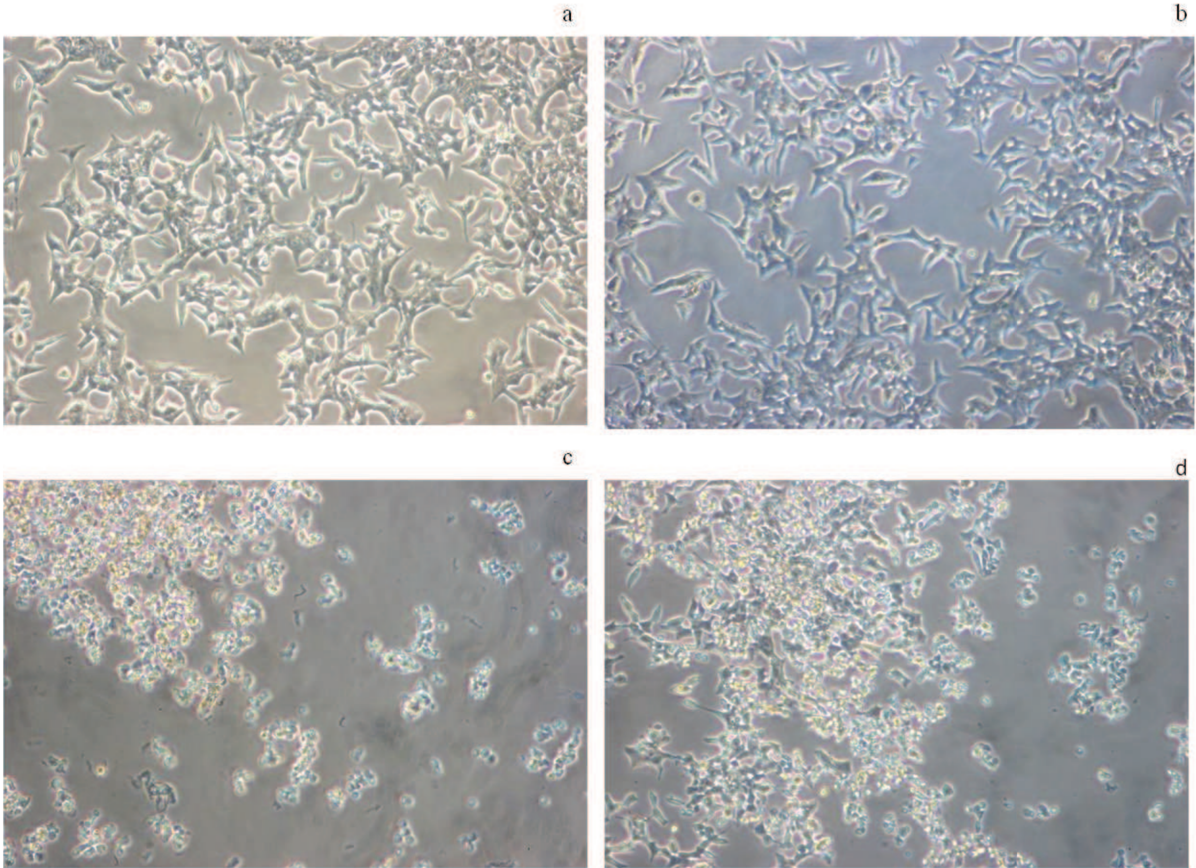
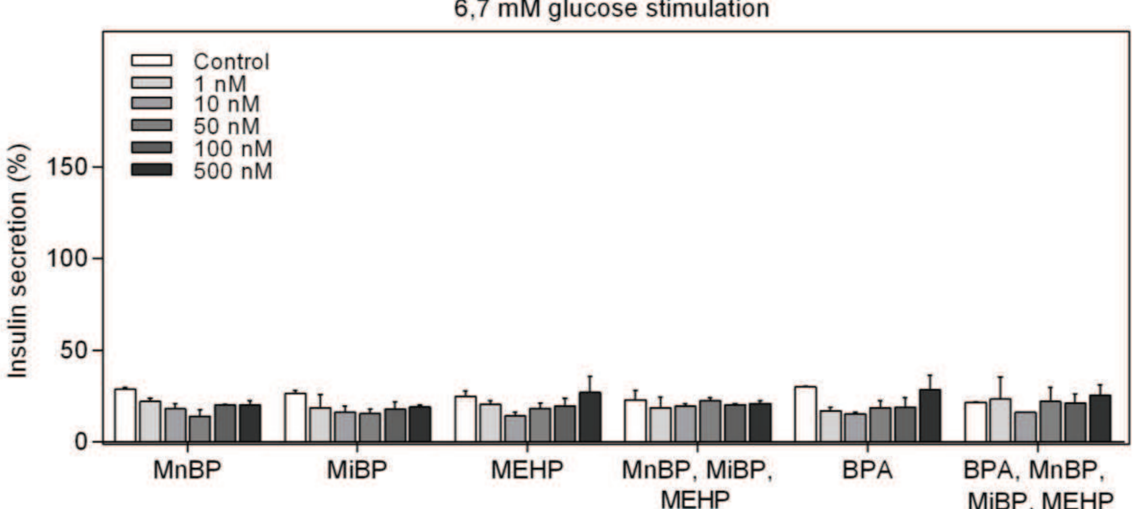


Figure A8: Effect of the high dose of environmental chemicals on cell morphology. INS-1E cells were seeded at 140.000 cells/ml, with medium shift on day 3, before 72 h exposure to environmental chemicals. Cells exposed to DMSO only were used as controls. The pictures are taken from microscope the last day of exposure, before harvesting. (a); control, (b); cells exposed to 50 µM of each of MiBP, MnBP and MEHP, (c); 50 µM BPA (d); 50 µM of each of MiBP, MnBP, MEHP and BPA.

APPENDIX 4



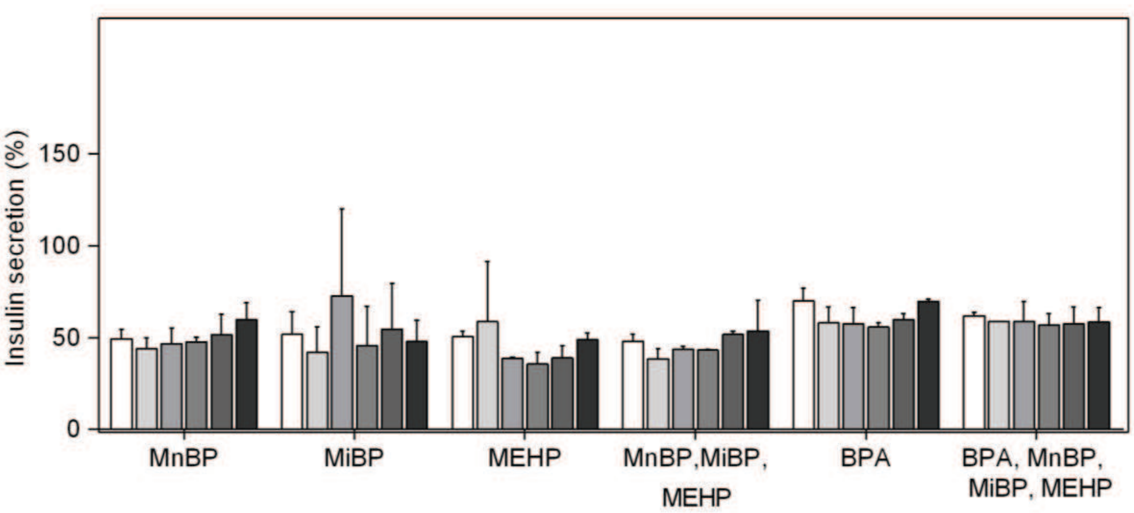
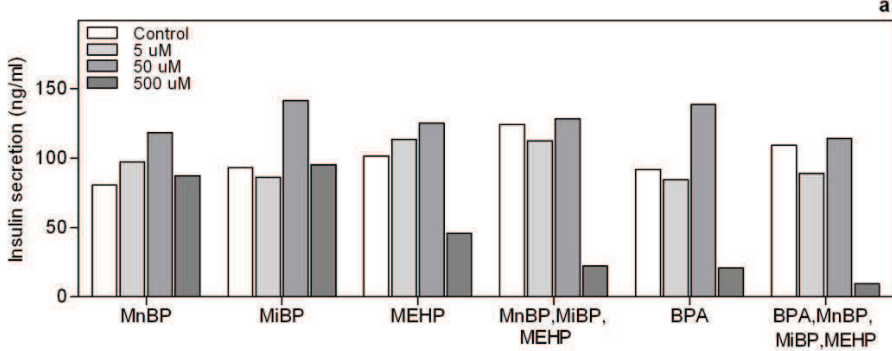
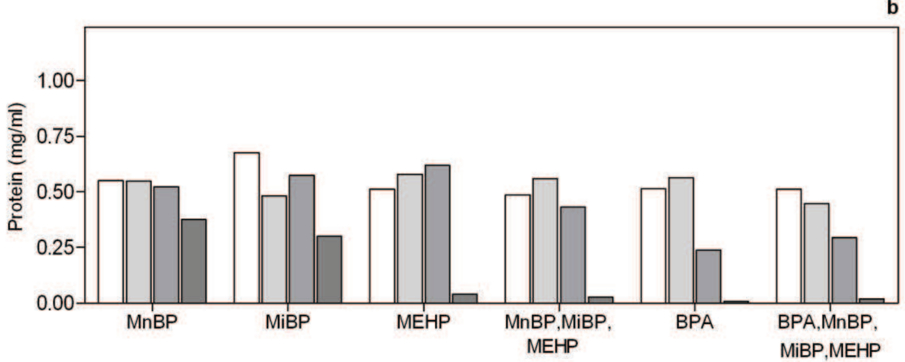


Figure A9: T2D model system: insulin secretion after 6.7 mM and 16.7 mM glucose stimulation. INS-1E cells were seeded at 120.000 cells/ml, with medium shift on day 3, before 72 h environmental chemical exposure, as indicated. Cells exposed to DMSO alone were used as controls. After 72 h exposure, all wells were incubated in glucose free KRBH buffer for 1 h, before 30 min incubation with 6.7 mM or 16.7 mM glucose in KRBH buffer. Insulin secretion in cell culture supernatants was determined by ELISA. The bars illustrates data obtained from duplicates in 1 experiment only. Data are normalized, *i.e.* the mean of duplicates were divided by the mean of all the controls in that experiment, and expressed as mean ± SEM. Two-way ANOVA with Bonferroni post-test showed no significant differences.

APPENDIX 5





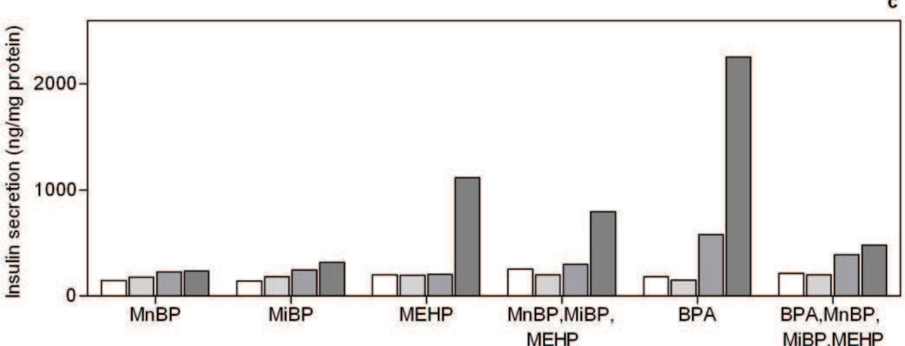


Figure A10: Insulin secretion related to protein level. The figure illustrates the difference between insulin secretion expressed as ng/ml, and ng insulin per mg protein. INS-1E cells were seeded at 120.000 cells/ml, with a medium shift on day 3, before 72 h environmental chemical exposure. Cells exposed to DMSO alone were used as controls. After 72 h exposure, all wells were incubated in glucose free KRBH buffer for 1 h, before 30 min incubation with 6.7 mM glucose in KRBH buffer. Data represents singlets from 1 experiment (a) Insulin secretion measured with ELISA. Data were normalized, *i.e.* the mean of duplicates were divided by the mean of all the controls in that experiment (b) Total protein content determined using a bio-rad dc protein assay according to the manufacturer`s instructions (Hercules, CA, USA). Data were normalized, *i.e.* divided by the mean of controls in that experiment. (c) Insulin release (ng/ml) was normalized to the protein content (mg/ml) and expressed as ng insulin per mg protein.