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JENNI REPO

ROLE OF PLACENTA IN FETAL TOXICITY OF CHEMICALS

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Role of placenta in fetal toxicity of chemicals

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ABSTRACT

Placental function is important for the growth and development of the fetus. Any disturbance to the normal function of the placenta will obviously harm the fetus. Exposure to certain chemicals, such as alcohol, tobacco and to some food toxins may be reduced by choice, but exposure to many other chemicals is unavoidable.

The function of placenta and fetal exposure cannot be studied by exposing pregnant women to harmful chemicals; other approaches have to be used. The structure and function of animal placentas are very different from their human counterpart, and thus animal placentas are not good models for predicting effects in human placenta. In Finland, human placenta is considered as a biological waste after the birth of the baby; it can be used for research purposes if the mother consents to donate her placenta.

The ultimate aim of this project was to evaluate fetal exposure to ethanol and nicotine and the molecular mechanisms related to these compounds by using human placental models. In addition, the effect of ethanol on the transfer of other toxic compounds through human placenta was studied. As models, a human placental trophoblastic cancer cell line BeWo, human placental perfusion and human placental villous explant cultures were used. In human placental perfusion, the newly born placenta remains functional with separate medium circulations under *ex vivo* conditions. In this work, human placental first trimester and full-term villous explants were cultured. Structures of human placental villi were isolated, cultured and exposed to the compounds being evaluated. In addition, to develop the method further, it was assessed whether glucose consumption and lactate dehydrogenase (LDH) release would be feasible ways to monitor the viability of the explants.

In human placental perfusion, nicotine (15μM) and ethanol (2‰) passed easily through placenta. Ethanol did not affect the transfer of nicotine (n=5) nor of the food carcinogens PhIP (2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, n=4) or NDMA (N-nitrosodimethyl-amine, n=5). The combination of ethanol and nicotine increased the levels of reactive oxygen species (ROS) in BeWo cells statistically significantly more than ethanol or nicotine alone ($p<0.01$, n=4). In BeWo cells, nicotine also increased the expression of the endoplasmic reticulum (ER) -stress associated protein GRP78/BiP ($p<0.05$, n=4). To confirm the effects of nicotine and ethanol on human placental primary tissue, a term human placental villous explant culture was set-up and developed further by analyzing the viability of explants. LDH release was clearly a better viability marker than glucose consumption. The studied compounds i.e. ethanol, nicotine and their combination increased by over 1.5 fold the expression of GRP78/BiP in both term and early human placental villous explants. However, the difference was statistically significant only in term explants and after ethanol treatment ($p<0.01$, n=5). Thus, oxidative and ER stress were detected in BeWo cells and placental explants. These results show for the first time that ethanol and/or nicotine cause both oxidative and ER stress in human placenta.

National Library of Medicine Classification: QV 84, QV 137, WP 465, WQ 210-212
Medical Subject Headings: Chorionic Villi; Endoplasmic Reticulum Stress; Ethanol/toxicity; Fetus/growth and development; Human; L-Lactate Dehydrogenase; Nicotine/toxicity; Oxidative Stress; Placenta/metabolism; Pregnancy

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TIIVISTELMÄ

Istukan normaali toiminta on tärkeää sikiön kasvun ja kehityksen kannalta. Häiriö istukan toiminnassa voi vaurioittaa sikiötä. Altistumista tietyille aineille, kuten alkoholille, tupakalle ja joillekin riskialttiille ruoka-aineille voidaan itse säädellä, mutta suurta osaa altistumisesta ei pystytä omilla valinnoilla säätelemään.

Istukan toimintaa ja sikiön altistumista ei voida tutkia altistamalla raskaana olevia naisia haitallisille aineille, vaan on käytettävä muita malleja. Eläinten istukat ovat rakenteeltaan ja toiminnaltaan hyvin erilaisia verrattuna ihmisen istukkaan, eikä eläinistukka ole hyvä malli kuvaamaan ihmisistukkaa. Suomessa ihmisistukat ovat jätettä synnytyksen jälkeen ja istukoita voidaan käyttää tutkimukseen, jos äidit haluavat luovuttaa istukan tutkimukseen.

Tämän tutkimuksen päätarkoituksena oli selvittää sikiön altistumista etanolille ja nikotiinille sekä selvittää näiden aineiden molekulaarisia mekanismeja käyttäen ihmisitukan malleja. Myös etanolin vaikutus muiden toksisten aineiden kulkeutumiseen ihmisistukan läpi tutkittiin. Tässä työssä käytettiin malleina ihmisistukan trofoblastisolulinjaa (BeWo), ihmisistukan perfuusiota sekä ihmisistukasta eristettyjen villusten viljelymenetelmää. Istukkaperfuusiossa vastasyntynyttä ihmisistukkaa pidetään elinkykyisenä laitteistossa erillisten nestekiertojen avulla. Tässä työssä viljeltiin sekä varhaisen ihmisistukan että täysiaikaisen ihmisistukan rakenteellisia osia, villuksia. Villukset eristettiin istukasta, viljeltiin ja altistettiin tutkittaville aineille. Lisäksi villusten elävyyttä arvioitiin glukoosin kulutuksella ja laktaattidehydrogenaasin (LDH) vapautumisella.

Nikotiini (15μM) ja etanoli (2‰) kulkeutuivat helposti ihmisistukan läpi istukkaperfuusiossa. Etanoli ei vaikuttanut nikotiinin (n=5) tai ruuan karsonigeenien PhIP:n (2-Amino-1-metyyli-6-fenyyli imidatsoli(4,5-b)pyridiini, n=4) ja NDMA:n (N-nitroso-dimetyyliamiini, n=5) kulkeutumiseen istukan läpi. Etanolin ja nikotiinin yhtäaikainen annostelu lisäsi reaktiivisten happiradikaalien (ROS) määrää istukan BeWo-soluissa tilastollisesti merkitsevästi enemmän kuin aineiden yksittäinen annostelu ($p<0.01$, n=4). BeWo-soluissa nikotiini lisäsi endoplasmakalvoston (ER) stressiin liittyvän GRP78/BiP -proteiinin määrää ($p<0.05$, n=4). Vahvistaaksemme tulokset myös normaalilla istukkakudoksella, pystytimme täysiaikaisen istukan villusten viljelymenetelmän ja kehitimme sitä edelleen analysoimalla villusten elävyyttä. LDH osoittautui selvästi paremmaksi elävyysmarkkeriksi kuin glukoosin kulutus. Kaikki tutkittavat aineet; etanoli, nikotiini ja niiden yhdistelmä lisäsivät GRP78/BiP proteiinin ilmentymistä yli 1.5 kertaisesti sekä aikaisen vaiheen istukan villuksilla että täysiaikaisen istukan villuksilla. Tilastollisesti merkitsevä ero havaittiin kuitenkin vain etanolilla käsitellyillä täysiaikaisen istukan villuksilla ($p<0.01$, n=5). Näin ollen oksidatiivinen ja ER stressi nähtiin BeWo soluissa ja istukan villuksilla. Tulokset osoittavat ensimmäistä kertaa, että etanoli ja/tai nikotiini aiheuttavat sekä oksidatiivista että ER stressiä ihmisistukassa.

Yleinen suomalainen asiasanasto: altistuminen; istukka; kemikaalit; myrkyllisyys; nikotiini; oksidatiivinen stressi; raskaus; sikiönkehitys;

‘Science is a way of thinking much more than it is a body of knowledge.’

- Carl Sagan

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List of the original publications

This dissertation is based on the following original publications:

I Veid J*, Karttunen V*, Myöhänen K, Myllynen P, Auriola S, Halonen T and Vähäkangas K. Acute effects of ethanol on the transfer of nicotine and two dietary carcinogens in human placental perfusion. *Toxicology Letters 205: 257-264, 2011.* * Equal contribution.

II Repo JK, Pesonen M, Mannelli C, Vähäkangas K and Loikkanen J. Exposure to ethanol and nicotine induces stress responses in human placental BeWo cells. *Toxicology Letters 224: 264-271, 2014.*

III Repo JK, Huovinen M, Ietta F, Paulesu LR and Vähäkangas KH. Human placental villous explants to study toxicity of ethanol and nicotine. *Manuscript submitted.*

Contents

Abbreviations

ABC-transporters	ATP-binding cassette -transporters
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ATF6	Activating transcription factor 6
BCRP	Breast cancer resistant protein (ABCG2)
CYP	Cytochrome P450 (enzyme)
DES	Diethylstilbestrol
ER	Endoplasmic reticulum
EtG	Ethyl glucuronide
FAEE	Fatty Acid Ethyl Ester
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FM-ratio	Feto-Maternal Ratio
GRP78	Glucose regulated protein 78
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen
IRE1α	Inositol requiring protein 1 α
JNK	Jun amino-terminal kinases
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinases
MDR-1	Multidrug resistance protein 1 (P-gp; ABCB1)
MRP	Multidrug resistance-associated protein (ABCC)
NDMA	Nitrosodimethylamine
nAChR	Nicotinic acetylcholine receptor
NRT	Nicotine replacement therapy
p38	Protein 38 (one of MAPKs)
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PERK	Pancreatic endoplasmic reticulum kinase
PGH	Placental growth hormone
PI	Propidium iodide
P-gp	P-glycoprotein
PhIP	2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine
PP13	Placental protein 13
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinases
UPR	Unfolded protein response

1 Introduction

The human placenta is a complex organ, which connects the mother and the fetus (for reviews, see Syme et al. 2004, Benirschke et al. 2006). The most important functions of placenta are to transfer nutrients and oxygen to fetus, assist in the removal of waste products and to produce pregnancy maintaining hormones. In addition, the placenta is an important organ in toxicokinetics, since it contains many transporter proteins and some metabolizing enzymes also for xenobiotics (for reviews, see Syme et al. 2004, Myllynen et al. 2007, Vähäkangas et al. 2011).

Some chemicals never reach the mother`s systemic blood circulation i.e. they may not be absorbed, or there may be effective xenobiotic metabolism in skin, lung and gastrointestinal tract. Many absorbed chemicals are rapidly metabolized to less harmful forms or excreted as such. However, metabolic intermediates may be more toxic than the parent chemical. Most chemicals in the maternal circulation do penetrate into placenta (for reviews, see Syme et al. 2004, Myllynen et al. 2007) and pass through placenta to the fetus, but there are also compounds that can accumulate in the placenta causing toxicity. Placental functions are essential for fetal development, wellbeing and growth and any kind of tissue damage or malfunction in placenta is obviously a risk also to fetus (Godschalk and Kleinjans 2008, Dimasuay et al. 2016).

Interestingly, some diseases are known to have their origin in the fetal period (for a review, see e.g. Vähäkangas 2011). The best known transplacental carginogen is diethylstilbestrol (DES), which was administered to pregnant women to prevent miscarriages before the 1970´s (Magee 1975). Subsequently, it was observed that maternal exposure to DES caused clear-cell adenocarcinoma in female offspring. Other examples of prenatal carcinogens include ionizing radiation, paternal smoking and exposure to some pesticides, which have been linked to childhood leukemia (Lafiura et al. 2007, Orsi et al. 2015, Spycher et al. 2015). In addition to cancer, some other diseases such as diabetes, cardiovascular diseases, hypertension and obesity have been suggested to have their origin, at least partially, in the prenatal period (for a review, see Avila et al. 2015). Maternal smoking during pregnancy has been linked to chronic fetal lung diseases, such as asthma, later in life (Chhabra et al. 2014).

Either accidental *in vivo* exposure or experimental models of human placenta have to be used to study placental toxicity of chemicals because mothers cannot be purposely exposed to harmful substances. When a pregnant woman is exposed during pregnancy and cord blood and maternal blood *in vivo* are available after birth, fetal exposure can also be evaluated. Cord blood samples taken from the umbilical cord of the placenta after birth represent fetal blood and thus fetal exposure. Fetal exposure to unavoidable chemicals, accidental exposures to harmful substances or medications during pregnancy, can be estimated also in other post-natal samples such as in fetal hair and meconium (Guo et al. 2013, Goecke et al. 2014, Sanvisens et al. 2016).

The development, structure and function of the human placenta differ from those of all other animal species; for this reason, placentas of other species do not truly resemble the human placenta (Orendi et al. 2011, Heinonen 2015, Grigsby 2016). The use of rodent models in research into fetal exposures is based on the fact that the placentas of rodents belong to the same category of hemochorial placentas found in primates (Grigsby 2016). In hemochorial placentas, fetally-derived trophoblast tissue is directly bathed in maternal blood. However, mice have three trophoblastic layers (for an extensive review, see Dilworth and Sibley 2013) and rats have two trophoblastic layers (for a review, see Furukawa et al. 2011) between the maternal and fetal circulations compared to the single layer of syncytiotrophoblasts in human placenta (for a review, see Benirschke et al. 2006). In addition, the villous structure is lacking in rat and mouse placentas (Orendi et al. 2011). There are many chemicals that behave

differently in animal placentas than in human placentas (for a review, see Heinonen 2015). In particular, there are often differences in the transport of chemicals through placenta in differrent species.

In Finland, placentas are considered as biological waste after the delivery (Halkoaho et al. 2010). Important toxicological issues may be studied using placental tissue after birth if mothers consent to the use of their placenta for research purposes (Halkoaho et al. 2011). Many efforts have been made to develop human placental models to study transfer and mechanisms of toxicity in placenta. Transfer studies require that there is the existence of a complete barrier e.g. as in placental perfusion experiments, whereas placental toxicity can be studied in any placental model. Primary trophoblastic cells isolated from placenta reflect the *in vivo* situation better than immortalized or cancer cell lines, but setting up such a model is challenging (Orendi et al. 2011). Another challenge in modelling placental toxicity is that the placenta undergoes extensive tissue development throughout the pregnancy (for a review, see Benirschke et al. 2006). Thus, for a complete picture, both early and term placentas should be experimentally modelled.

2 Review of the Literature

2.1 FETAL EXPOSURE TO CHEMICALS

2.1.1 Chemicals to which mothers are exposed

The time between conception and noticeable pregnancy, especially in the case of unplanned pregnancies, may lead to accidental exposure to many chemicals (Table 1, for a recent review, see Ross et al. 2015). Exposure to some life-style chemicals, for example food toxins and social and illegal drugs, can be decreased by choice (for reviews, see Myöhänen and Vähäkangas 2012, and Ross et al. 2015). Similarly, the exposure to food toxins can be reduced by avoiding risky foods, such as cured or smoked meat and shellfish or raw fish. However, it is impossible to avoid exposure to many chemicals, such as environmental and industrial chemicals, endocrine disrupters as well as toxins present in the natural environment and some food carcinogens, because they are ubiquitous (for an extensive review, see Mitro et al. 2015). The air may be polluted, drinking water has chemical residues and food can be contaminated by man-made chemicals (e.g. pesticides) as well as natural agents (e.g. mycotoxins) (Chen et al. 2006, Adebambo et al. 2015, Fang et al. 2015, Ferguson et al. 2015, Jedrychowski et al. 2015). Plastic dishes and containers storing food may release harmful chemicals (e.g. bisphenol A) especially when they are heated (Peretz et al. 2014). Cosmetics, such as toothpaste, mouthwash, creams, ointments, antiperspirants and make-up are another source of exposure; these are often complex mixtures of different chemicals (LaRocca et al. 2016).

The human body does not differentiate between chemicals from different sources; instead all compounds are handled similarly, according to their chemical structure. Various chemicals, such as pesticides (Sexton and Salinas 2014, Morello-Frosch et al. 2016), polycyclic aromatic hydrocarbons (PAHs) (Sexton and Salinas 2014, Jedrychowski et al. 2015), and parabens (Pycke et al. 2015) have been detected in umbilical cord blood, evidence that chemicals can cross the human placenta *in vivo*. In some cases, compounds may be retained in placental tissue (Myllynen et al. 2008b). Some chemicals even accumulate in the fetus; for instance, over two times higher levels of polychlorinated biphenyls (PCBs) have been found in umbilical cord than in maternal blood in families living near dump sites (Grumetto et al. 2015).

Placental permeability is not only a negative property. In some cases, the fetus can be treated by medicines, which need to pass through the placenta, e.g to combat infections during labour or to prevent premature birth (for a review, see Staud et al. 2012). In transplacental treatment, the drug is given to the mother. Examples of fetal medical treatment include glucocorticoids to promote fetal lung maturation in cases of threatening premature birth, antiretrovirals to prevent transmission of HIV from mother to fetus and cardiovascular drugs to treat life-threatening fetal cardiac arrhythmias. In addition to fetal illnesses, also maternal illness may require medication during pregnancy. The risk-benefit ratio of medical treatment needs to be considered carefully as it can be assumed that most drugs will gain access to the human placenta and those passing through it, can affect the wellbeing and development of the fetus (reviewed by Staud et al. 2012, Thomas and Yates 2012).

Exposure to chemicals may lead to congenital malformations or more subtle functional aberrations (for reviews, see Brent 2004, Obican and Scialli 2011). Anatomical congenital malformations are dependent on the chemical, its concentration and timing of the exposure. Organogenesis, which takes place during the first weeks of pregnancy is the most sensitive time for anatomical malformations. However, the central nervous system (CNS) not only develops throughout the pregnancy but also long after birth. In addition, exposure at any time to the so-called functional teratogens may disrupt the development of the pregnancy, e.g. by suppressing blood circulation.

Table 1. Examples of harmful/toxic chemicals to which mothers may be exposed during pregnancy.

Group	Examples of chemicals	Source	Reference
Social drugs	ethanol nicotine caffeine taurine	alcohol tobacco smoking coffee energy drinks	Ross et al. 2015
Illegal drugs	cocaine, buprenorphine, amphetamine	drug abuse	Ross et al. 2015
Medicines	epileptic drugs	pharmacotherapy	Myllynen et al. 2005
Industrial chemicals	dioxins polychlorinated biphenyls, metals, endocrine disrupters	contaminated food occupational and non-occupational exposure	Mitro et al. 2015 Wang et al. 2016 Frye et al. 2012
Agricultural chemicals	pesticides, insecticides	contaminated food	Mostafalou and Abdollahi 2016
Environmental chemicals	PAHs metals	combustion products polluted air, soil or water	Wang et al. 2016
Food carcinogens	Aflatoxin B1 PhiP, NDMA	contaminated food cooked, smoked or cured meat	Myöhänen and Vähäkangas 2012
Natural toxins	botulinum toxin saxitoxin	canned food shellfish	Myöhänen and Vähäkangas 2012, Leclair et al. 2013 Cusick and Sayler 2013

PAH=polycyclic aromatic hydrocarbon, PhIP=2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, NDMA=N-nitrosodimethylamine

In real life, people are exposed to a multitude of chemicals (for reviews, see Heinonen and Tähti 2013, Cedergreen 2014). The amount of different chemical combinations is massive and conclusions cannot be drawn simply by analyzing the structure of compounds. True synergistic reactions, where chemicals enhance the effects of other chemicals, seem to be rare and often occur at high concentrations (for a review, see Cedergreen 2014).

Nonetheless, some pesticides, metals and antifouling mixtures have given rise to concerns about synergistic health effects (for a review, see Cedergreen 2014). Thus, it seems that some specific chemical groups are more likely than others to exhibite synergism. If one considers the pesticides, cholinesterase inhibitors and azole fungicides have often been claimed to display synergism (for a review, see Cedergreen 2014). With respect to metals, true synergy occurs often at much higher concentrations (mg/l –level) than the levels (ng/l) found in metal polluted waters. There is only one published study where combined exposure to metals has been evaluated in placental cells. The combination of inorganic arsenic and cadmium increased synergistically mRNA expression of heme-oxygenase and metallothionein when compared to the treatment of inorganic arsenic or cadmium separately in Jeg-3 cells (Adebambo et al. 2015). Notably, the genomic responses were observed at significantly lower concentrations than those present in Chinese surface waters.

2.1.2 Food toxins and carcinogens

Many regulatory efforts have been made to enhance food safety in the western countries, but still many unfortunate exposures occur. Different countries have their own recommendations for pregnant women. For example, the Finnish Food Safety Authority has published a list of foodstuffs known to have potential risks for the fetus (Evira 2016). The list is extensive and avoidable foodstuffs include pike fish (high mercury levels), liver foods (high in vitamin A), false morel mushroom (gyromitrin toxin), energy drinks (high caffeine levels), herbal teas (safety not known), liqourice (glycyrrhizin), flaxseeds (heavy metals), ginger products (safe limits are not known), seaweed products (high iodine content) and herbal food supplements (safe limits are not known). In addition, many foodstuffs, such as raw or cured fish and meat, unpasteurised milk products, frozen vegetables and foreign frozen berries need to be heated up to 70–90 degrees Celcius for 2–5 minutes to eliminate the risk of bacterial and/or viral infection. (Evira 2016)

Toxins may end up in food from various sources. There are naturally occurring toxins, contaminants, bacterial toxins as well as toxins that are formed while cooking (for a review, see Myöhänen and Vähäkangas 2012). Typical naturally occurring toxins in food are gyromitrin in false morel mushroom, capsaicin in chili pepper, nicotine in the tobacco plant and marine biotoxins. Food may be contaminated by industrial products or industrial side-products, such as pesticides, metals, phthalates and dioxins. In addition, there are some very potent natural toxins e.g. aflatoxin B1 (hepatotoxic and carcinogenic) and ochratoxin A (toxic to kidney); these may contaminate nuts, grain and corn if the food is not stored in proper conditions. Bacterial toxins include botulinum toxin, which is produced by *Clostridium botulinum*. Some toxins, such as PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and acrylamide are formed when food is heated or cooked at high temperatures.

Some food toxins have carcinogenic properties or they become converted into carcinogenic metabolites in the human body (for a review, see Myöhänen and Vähäkangas 2012). Two significant carcinogenic compounds in food are PhIP (Group 2b, possibly carcinogenic to humans, IARC 1993) and N-nitrosodimethylamine (NDMA) (Group 2a, probably carcinogenic to humans, IARC 1978). PhIP is one of the most abundant heterocyclic amines present in cooked meat (Knize et al. 2002). PhIP requires metabolic activation by CYP1A2 to form the carcinogenic metabolite, 2-hydroxyamino-PhIP. In addition to CYP1A2, also CYP1A1 and CYP1B1 contribute to the metabolism of PhIP (Han et al. 2008). Daily exposure to PhIP varies between individuals from dozens of nanograms up to micrograms (Augustsson et al. 1997, Keating and Bogen 2004, Bogen et al. 2007). Estimates for PhIP intake in the European population are in the range 2.2–6.6 ng/kg/day while the level in the US population is thought to be about three times higher (Keating and Bogen 2004). PhIP has been clearly shown to cause breast (Ito et al. 1997), prostate (Tang et al. 2011) and colon (Imaida et al. 2001, Tang et al. 2011) cancer in rodents. In humans, it is known that high consumption of meat (especially grilled and cooked meat) is linked to prostate (Ferguson 2002), colon (Corpet 2011) and breast cancer (Sinha et al. 2000, Bennion et al. 2005). However, it is very difficult to elucidate exactly which heterocyclic amines and especially whether PhIP is the reason for the increase in some cancer-rates.

Nitrite is added to processed meats to improve their flavor and preservation. Nitroso-compounds, such as NDMA can also be formed in the stomach if alkylamine containing foods are consumed (Tricker 1997, Song et al. 2015). Heavy smokers, people who consume large amounts of cured meat and those who drink beer may expose themselves to over two times higher amounts of NDMA than the rest of population (Tricker et al. 1991, Tricker 1997). NDMA is activated by CYP2E1 (Lin and Hollenberg 2001) and highly reactive methylguanine metabolites with DNA-adducts may be formed (Chhabra et al. 1995, Ma et al. 2015). In animals, NDMA exposure has been linked to tumours in several organs (IARC 1978). Epidemiologic data in humans has shown that NDMA containing food is associated with the incidence of gastrointestinal cancer (Loh et al. 2011).

2.1.3 Social drugs

Unfortunately many pregnant mothers use 'social drugs', including drinks containing alcohol or caffeine as well smoking tobacco products (for a review, see Kuczkowski 2003). Often these compounds are used together (Niemelä et al. 2016); in surveys, mothers have a tendency to underestimate their substance abuse (Sanvisens et al. 2016). Alcohol drinking and tobacco smoking act synergistically, particularly in causing cancer (for a review, see Salaspuro 2003). Both tobacco smoking (IARC 2004) and ethanol (IARC 2012) have been classified as group 1 carcinogens (carcinogenic to humans) by IARC. Tobacco smoke contains dozens of carcinogenic compounds, and therefore it is not surprising that it evokes synergism with ethanol. However, the mechanisms behind synergism are not well understood, and chronic exposure in humans is very difficult to study, because of confounding factors.

Ethyl alcohol (ethanol) is toxic to the fetus, and unfortunately, many pregnant mothers drink ethanol despite warnings. It has been estimated that about 10% of American pregnant mothers use alcohol (Tan et al. 2015) and this percentage is probably at the same level or even higher in other western countries including Finland (Niemelä et al. 2016). In the Norwegian Mother and Child Cohort Study (MoBa) with 66 111 participants, 16% reported light alcohol use during the first trimester of pregnancy (Stene-Larsen et al. 2013). Light alcohol consumption was defined as 0.5–2 units of alcohol from one to four times per month. In the same cohort, 10% of mothers reported light alcohol use during the second trimester of pregnancy.

Ethanol passes through the human placenta with ease (Idanpaan-Heikkila et al. 1972). It is metabolized by alcohol dehydrogenase (ADH) to acetaldehyde, which is a highly toxic metabolite. Acetaldehyde is further metabolized by mitochondrial aldehyde dehydrogenase (ALDH) to acetate and eventually to CO_2 and water. Karl et al. (1988) utilized a human placental perfusion technique to demonstrate that the placenta metabolized ethanol to acetaldehyde and that the acetaldehyde from the maternal circulation could be transferred to the fetal circulation, reaching approximately 50% of the maternal concentration.

A small proportion (5–10%) of ethanol is metabolized by CYP2E1 (Pizon et al. 2007, Gemma et al. 2007). There is one study reporting that placental CYP2E1 protein expression increases due to heavy drinking (Rasheed et al. 1997). Rasheed et al. (1997) studied the placentas of mothers who reported that they drank on average more than two portions (24g) of absolute alcohol per day during their pregnancy. In six out of 12 placentas, CYP2E1 protein was expressed (Rasheed et al. 1997). However, discrepant data also exists (Collier et al. 2002, Czekaj et al. 2005). Collier and co-workers (2002) did not detect any activity of CYP2E1 in human first trimester placentas analyzed by chlorzoxazone in microsomal fractions and HPLC. Collier and co-workers (2002) did not detect any activity even in mothers who smoked and used alcohol. In addition, Czekaj and co-workers (2005) did not detect any CYP2E1 protein in normal term human placentas (*n=10*).

Nicotine is the addictive ingredient in tobacco smoke and it is the reason why smoking cessation is so difficult (for a recent review, see Wadgave and Nagesh 2016). Nicotine is especially found in the leaves of tobacco plants (*Nicotiana rustica, Nicotiana tabacum*) but in much smaller amounts also in many other plants e.g. in eggplant, tomato and potato. It functions as an antiherbivore agent in plants and, in fact, it has been used as an insecticide in the past. When smoking a cigarette, about 1–2 mg of nicotine are absorbed systemically in the body (for a review, see Benowitz et al. 2009). Nicotine replacement therapy (NRT), electronic cigarettes and chewed tobacco products (such as snuff) are other sources of nicotine (for reviews, see Khoudigian et al. 2016, Lipari 2013). In Finland, about 15% of pregnant mothers smoke tobacco (Finnish National institute for Health and Welfare, 2016) and an even larger proportion of pregnant women are exposed to passive smoking.

Nicotine binds to the nicotinic acetylcholine receptor (nAChR), which is formed as a pentamer from subunits (for a review, see Schuller 2009). Altogether sixteen subunits of nAChR have been identified in mammals: nine α-subunits, four β-subunits and one each of the δ, γ and ε subunits (Machaalani et al. 2014). The mRNAs of all subunits have been

detected from human placenta, although the mRNA expression of α2, α3, α4, α9, β2 and β4 subunits is higher than the expression of other subunits (Machaalani et al. 2014). At the protein level, the subunit α7 has been shown to be expressed in term human placenta (Kwon et al. 2007). Recently also the protein of subunit β1 was confirmed to be expressed in term human placenta (Aishah et al. 2017). Nicotine is very toxic to both animals and humans. The oral LD_{50} value of nicotine is 50mg/kg in rats and 3mg/kg in mice (CDC 2014). Smokers develop a tolerance to nicotine and the highest peak concentrations found in blood have been about 20μM (~3700μg/l) (Massadeh et al. 2009). The commonly found concentrations of nicotine in blood of regular smokers are about two orders of magnitude smaller (Russell et al. 1976, Russell et al. 1980).

Nicotine undergoes extensive metabolism with a wide interindividual variation (Vähäkangas and Pelkonen 1993) and is excreted into all body fluids, especially urine (for reviews, see Yildiz 2004, Benowitz et al. 2009, Abu-Bakar et al. 2013). Only 1% of nicotine is present unchanged in body fluids. Cotinine is a major metabolite of nicotine. In most individuals, about 70–80% of nicotine is metabolized by CYP2A6 to cotinine. Other important metabolites are nicotine-N-oxide (4–7%), nicotine glucuronide (3–5%), 3′-hydroxycotinine, 5′-hydroxycotinine and cotinine-N-oxide. Cotinine elimination from the body takes several days, i.e. the mean half-life for cotinine is about 16–19 hours (Jarvis et al. 1988), although it can be even longer (Vähäkangas and Pelkonen 1993). Cotinine has also been used as a biomarker for smoking (Pasanen et al. 1988b). A value of 3ng/ml has been used in U.S. as the cut-off point for plasma cotinine to distinguish smokers from non-smokers (see Benowitz et al. 2009). Nicotine has been shown to pass rapidly through human placenta (Pastrakuljic et al. 1998, Sastry et al. 1998).

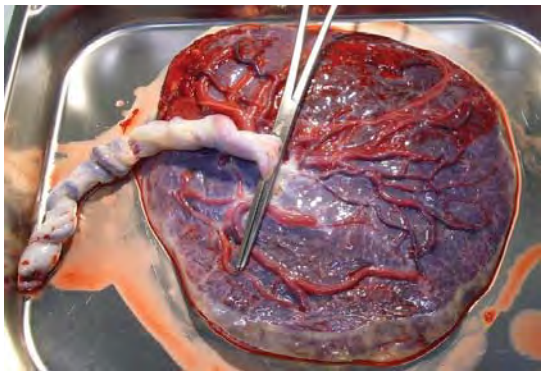
2.2 HUMAN PLACENTA

2.2.1 Anatomy of human placenta

Human placenta is a disk-like, flat and round organ that connects the mother and the fetus (Figure 1, for a review, see Benirschke et al. 2006). It is responsible for gas exchange, nutrient transport, secretion of hormones and excretion of waste products to the maternal circulation. On the fetal side of placenta, chorionic veins and arteries can be identified which converge towards the umbilical cord. The umbilical cord with two arteries and one vein connects the placenta and the fetus. The maternal side (*basal plate* or *decidua basalis*) of the placenta, on the other hand, is attached to the uterine wall and is divided into cotyledons (or lobes), which appear as slightly elevated areas of the tissue. Each maternal cotyledon is occupied by one or several chorionic villous trees. The space between fetal chorionic plate and basal plate is filled with maternal blood and called the intervillous space. Maternal blood surrounds the structures of villous trees that emerge from the chorionic part of the placenta.

An average full term placenta weighs about 500–600 g, has about 20 basal cotyledons and is 20–25 cm of diameter (Benirschke et al. 2006). The human placenta develops and changes throughout the pregnancy (Huppertz et al. 2014). For example, the diffusion distance between maternal and fetal blood is reduced from about 50μm in the first trimester to < 5μm at term. At the same time, the expression of proteins including transporter proteins and cytochrome P450 (CYP) enzymes changes.

A



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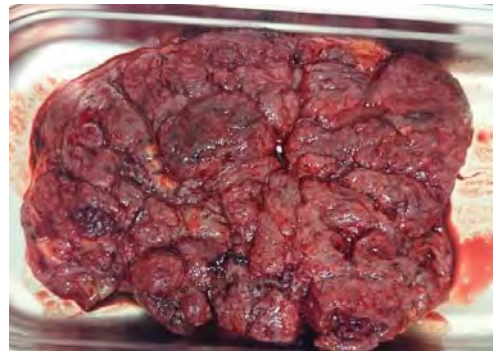


Figure 1. Full term human placenta. A) Fetal surface with umbilical cord, arteries and veins. B) Maternal surface with cotyledons. (Photos by Jenni Repo)

2.2.2 Structure and functions of placental villi

Human placental villous trees are finger- or tree-shaped structures of human placenta (Figure 2). The brush border apical membrane of the syncytiotrophoblast forms the surface of villous trees. Villous trees are functional units of the placenta, as most metabolic and endocrine activity takes places in syncytiotrophoblast (Benirschke et al. 2006). The syncytiotrophoblast is the site of hormone synthesis; progesterone, estrogen, human chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth hormone (PGH) and placental protein 13 (PP13) are all synthesized in the syncytiotrophoblast (for a review, see Costa 2016). At the end of pregnancy, the syncytiotrophoblast is one single layer with nuclei that cannot divide. Beneath the syncytiotrophoblast, some remaining mononucleated cytotrophoblasts cells exist (Huppertz et al. 2014). Cytotrophoblasts form a heterogenous and mononucleated stem cell population. A subset of cytotrophoblasts fuse to form the syncytiotrophoblast, another subset preserves proliferative activity throughout the pregnancy and the third subset differentiate into extravillous trophoblast cells and spread into maternal tissue (Benirschke et al. 2006).

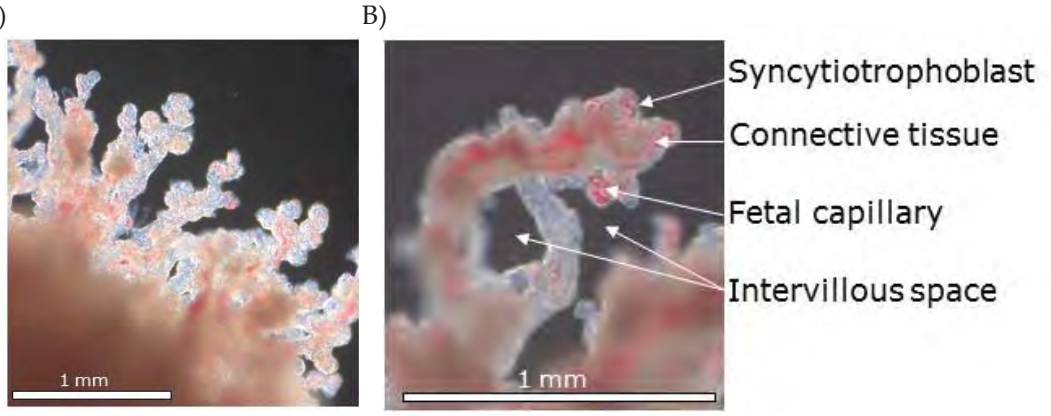


Figure 2. Structure of full term human placental villi. Photograph was taken under light microsope and the area shown is about 1-4 mm^2. (Photos by Jenni Repo)

Although the villi of the human placenta develop and change as the placenta grows, all villi exhibit the same basic structure i.e. consisting of syncytiotrophoblast, cytotrophoblasts, trophoblastic basement membrane, connective tissue (stroma) and fetal vessels (Benirschke et al. 2006). Five villous types have been described in the literature; they are mesenchymal, immature intermediate, mature intermediate, stem and terminal villus. In term human placental villous explant cultures, terminal explants are used, whereas in first trimester explant culture, either mesenchymal or immature intermediate villi may be used depending on the gestational weeks. From the 5th to the 7th week of gestation, mesenchymal villi are the only vascularized villous type (Benirschke et al. 2006). Mesenchymal villi are responsible for nearly all of the placenta`s endocrine activity during the first weeks of gestation. Some mesenchymal villi remain at term, although their volume from the total villous volume is extremely low (about 1%).

Immature intermediate villi and mature intermediate villi differentiate from mesenchymal villi (Figure 3). Immature intermediate villi are the principal sites of exchange only during the first two trimesters. Subsequently, other more specialized villous trees, such as mature intermediate villi and terminal villi, become differentiated and take care of most of the

fetomaternal exchange. Terminal villi originate from mature intermediate villi and due to the high vascularization and minimal maternofetal diffusion distance (< 5μm), this villous type is the most appropriate place for substance exchange at term (Benirschke et al. 2006).

The main function of the fifth villous type, stem villi, is to provide mechanical support for the structure of villous trees and thus no dirct fetomaternal exchange or endocrine activity takes place in these villous types (Benirschke et al. 2006).

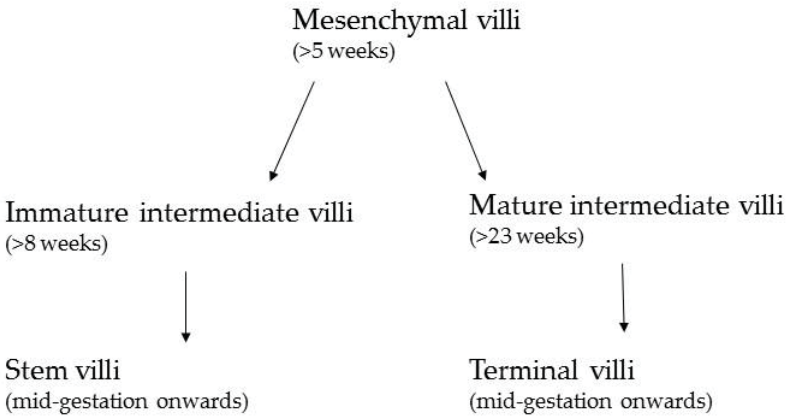


Figure 3. Formation and differentiation of placental villi (pregnancy weeks in brackets) (data from Castellucci et al. 2000).

2.2.3 Toxicokinetics in human placenta

Human placenta can metabolize xenobiotic compounds although at a much lower level than maternal liver (for a review, see Myllynen et al. 2007). In human placental perfusion studies, only a small number of compounds have been shown to been metabolized in placenta, and even then, to only minor degrees (Table 2).

Only the placental hormone metabolizing enzyme CYP19A1 (aromatase), is expressed in placenta at relatively high levels (Storvik et al. 2014). Expression of CYP19A1 in human term placenta is at least one degree of magnitude higher than the expression levels of CYP2 or CYP3 in liver, which indicates that human placenta is particularly vulnerable to endocrine disrupters. Other pharmacologically important CYP enzymes, such as CYP3 and CYP2 – families, are expressed in placenta at minor protein levels as compared to maternal liver (for reviews, see Hakkola et al. 1996, Myllynen et al. 2007).

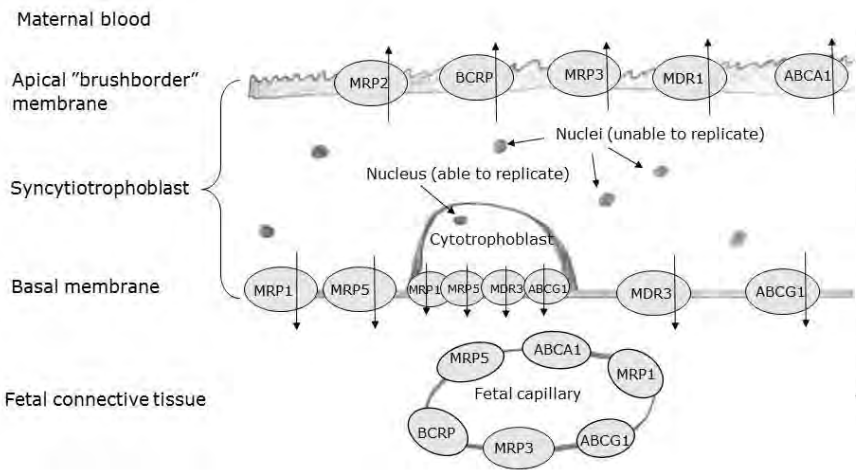
In most placentas, CYP1A1 is inactive, but the protein expression of placental CYP1A1 can be induced in some individuals by smoking (Pasanen et al. 1988a, Pasanen et al. 1990). CYP1A1 has been shown to metabolize benzo(a)pyrene only in the placentas of some smokers (Vahakangas et al. 1989) and to form benzo(a)pyrene-7.8-dihydrodiol-9,10-epoxide (BPDE) -adducts in human placenta (Manchester et al. 1988). In human placental perfusions, benzo(a)pyrene-7.8-dihydrodiol-9,10-epoxide (BPDE) -adducts were found in one of the two benzo(a)pyrene perfused placentas, but not in non-perfused control tissue of the same placentas, confirming that CYP1A1 protein had been able to activate benzo(a)pyrene in human placenta (Karttunen et al. 2010).

Table 2. Compounds that human placenta can metabolize according to perfusion studies.

Compound	Metabolite	Enzyme	Reference
Oxcarbazepine	10-Hydroxy-10,11-dihydro-carbamazepine	CYP450	Pienimäki et al. 1997
Ethanol	Acetaldehyde	Alcohol dehydrogenase (ADH)	Karl et al. 1988
Buprenorphine	Norbuprenorphine	CYP 19 (aromatase)	Nanovskaya et al. 2002, Deshmukh et al. 2003
Aflatoxin B	Aflatoxicol	NADPH-dependent reductase	Partanen et al. 2010
Benzo(a)pyrene	Benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)	CYP1A1	Karttunen et al. 2010

Placental transporters play an important part in placental function, because passive diffusion alone is not capable of fulfilling fetal requirements for substance transfer. Large (>500 Da) and hydrophilic compounds have to utilize transporters in order to cross the placental barrier (for a review, see Syme et al. 2004). Usually compounds that are actively transported through placenta are structurally similar to endogenous compounds. From a toxicokinetic point of view, transporters represent an important part of the so-called placental barrier (for a review, see Vähäkangas and Myllynen 2009). Transporter proteins are found both in the apical and basal membrane of the syncytiotrophoblast as well as in the fetal capillary endothelium (Figure 4). Transporters are localized in cell membrane and they may act as either efflux or uptake transporters; i.e. depending on their localization and function, they may either decrease or increase the transfer of substances from the maternal to the fetal circulation. The expression of the toxicologically most important ABC (ATP-Binding Casette) transporters changes with gestational age of the placenta (Bloise et al. 2016). Interestingly, also the localization of ABC transporters changes during gestation. The cholesterol transporter, ABCA1, has been found in large amounts from basal membrane of the syncytiotrophoblast in first trimester placentas but only from apical membrane and endothelial cells of fetal vessels in third trimester placentas (Bhattacharjee et al. 2010, Bloise et al. 2016).

Term placenta expresses less ABCB1/MDR1 (P-glycoprotein, P-gp) and ABCC/MRP transporter proteins than first trimester placenta (Mathias et al. 2005, Meyer zu Schwabedissen et al. 2006). However, there is still no consensus on the expression of ABCG2/BCRP (Mathias et al. 2005, Meyer zu Schwabedissen et al. 2006, Yeboah et al. 2006). First, Mathias et al. (2005) reported that there was no change in the protein expression of ABCG2/BCRP when first trimester and term placentas were compared. Later, Meyer zu Schwabedissen (2006) reported that the protein expression of ABCG2/BCRP declined towards term. However, in the same year, Yeboah et al. (2006) reported that the protein expression of ABCG2/BCRP was higher in term than in early placentas. Recently, Sieppi et al. (2016) published a study that is in line with the results of Meyer zu Schwabedissen (2006), showing that the protein expression of ABCG2/BCRP was much greater in first trimester placentas than in term placentas. They also revealed that bisphenol A and *p*-nonylphenol downregulate ABCG2/BCRP protein expression depending on gestational age (Sieppi et al. 2016). The protein expression of ABCG2/BCRP was decreased by *p*-nonylphenol and bisphenol A treatment in term placentas, but not in first trimester placentas (Sieppi et al. 2016).



Gene	Alternative name
ABCA1	-
ABCB1	MDR1 (p-gp)
ABCB4	MDR3
ABCB5	-
ABCC1	MRP1
ABCC2	MRP2
ABCC3	MRP3
ABCC5	MRP5
ABCG1	-
ABCG2	BCRP

Figure 4. ABC transporters in term human placenta. Arrows indicate the direction of transfer (data from Vähäkangas and Myllynen 2009, Bloise et al. 2016).

2.2.4 Transplacental transfer of compounds and tissue accumulation

Maternal blood from the intervillous space of the placenta is in direct contact with the syncytiotrophoblast layer of villi (Benirschke et al. 2006). All chemicals must go through the syncytiotrophoblast, connective tissue and fetal capillary endothelium to reach fetal circulation.

Most chemicals pass through human placenta by passive diffusion (for a review, see Syme et al. 2004). In passive diffusion, chemicals move from sites with a higher concentration to those with a lower concentration without the need of energy. Passive diffusion is dependent on membrane permeability, it is not saturable and it favours low-molecular weight (<500 Da) and lipid-soluble compounds. Only non-protein-bound chemicals can cross placenta by passive diffusion. Facilitated diffusion is a transfer mechanism which requires the presence of a carrier substance within the placenta and the system can become saturated. However, facilitated diffusion does not require any energy. Only a few chemicals (such as folic-acid and metformin) have been suggested to use facilitated diffusion as a transfer mechanism (Takahashi et al. 2001, Kovo et al. 2008).

In addition to passive and facilitated diffusion, transplacental transfer can be mediated by active transport or endocytosis (for reviews, see Syme et al. 2004, Akour et al. 2013). Active transport requires energy, usually obtained by the breakdown of adenosine triphosphate (ATP). All active transporters, such as organic anion and cation transporters, serotonin transporter and ABC-transporters may work against concentration gradient. Endocytosis may be further divided into pinocytosis, phagocytosis and receptor-mediated endocytosis (for a review, see Doherty and McMahon 2009). Some important substances from the developmental point of view, such as riboflavin and folic acid pass through human placenta via receptor-mediated endocytosis (Keating et al. 2006, Foraker et al. 2007).

Large molecules, which are not ligands of transporters or cannot utilize endocytosis pathways, do not cross the placental barrier. At present, from all the substances studied in human placental perfusion, only some rather large molecules e.g. pegylated gold-nanoparticles (Myllynen et al. 2008b), ochratoxin A (Woo et al. 2012) and pegylated liposomal doxorubicin (Soininen and Repo et al. 2015) have not crossed human placenta during

perfusion. Interestingly, ochratoxin A, which is a food contaminant and a relatively large mycotoxin (403.8 Da), did not pass through human placenta, even during a long (20h) perfusion (Woo et al. 2012). However, ochratoxin A has been found in human cord blood samples that represent fetal blood, and thus there can be *in vivo* exposure *(*Postupolski et al. 2006*)*. At present, ochratoxin A seems to be the only compound that has behaved differently in human placental perfusion than *in vivo*.

Some drugs may bind (Karttunen et al. 2010) or accumulate (Nanovskaya et al. 2002, Myllynen et al. 2008b) to placental tissue. Genotoxic compounds can bind to placental DNA, if the genotoxic metabolites are formed in placenta (Karttunen et al. 2010). High lipophilicity and binding to plasma or tissue proteins have been shown to increase the tendency towards placental accumulation (Ala-Kokko et al. 1995, Syme et al. 2004). If a chemical accumulates in placenta, it decreases the direct transplacental transfer from mother to fetus, but may cause other harmful effects in the placenta, for example by causing cell stress.

2.3 MECHANISMS OF PLACENTAL TOXICITY

2.3.1 Toxicity of ethanol in placenta

Toxic mechanisms of ethanol in human placenta have so far been studied in first trimester and term placental explants and two human placental trophoblastic cancer cell lines (Jeg-3 and BeWo) (Kay et al. 2000, Clave et al. 2014, Lui et al. 2014). Clave et al. (2014) noted that in Jeg-3 cells, ethanol increased the protein expression of P-H2AX, caspase-3 and PARP-1 in apoptotic pathways (Clave et al. 2014). A dose-dependent increase of the proteins was seen with the relevant concentrations of 25mM (~1.25 ‰) and 50mM (~2.5 ‰) of ethanol. Interestingly, number of viable cells and total protein concentation decreased with both concentrations (Clave et al. 2014).

Lui et al (2014) studied the effects of ethanol and acetaldehyde in BeWo cells and in villous explants of first trimester human placenta. They used 0.5 to 2 ‰ concentrations of both ethanol and acetaldehyde, and an exposure time of 48 hours in BeWo cells and 72 hours in placental explants. They observed that both acetaldehyde and ethanol decreased the proliferation of BeWo cells. In addition, ethanol, but not acetaldehyde, inhibited the transport of taurine across cell membrane both in BeWo cells and in human placental first trimester explants. In cats, the deficiency of taurine is known to cause congenital anomalies and blindness. It has been hypothesized that taurine is needed also for human retinal development (Militante and Lombardini 2002).

The first and so far only indication that ethanol could increase reactive nitric oxygen species in human placenta is based on the study of Kay and co-workers (2000). They used very high concentrations from 50 mM (~2.5 ‰) to 200mM (~10 ‰) and exposed term human placental explants to ethanol for 2 hours. A statistically significant increase in nitric oxide (eNOS) was seen only with the highest concentrations of 100mM and 200mM. The viability of the explants was not studied. Therefore, it remains unclear whether the high concentrations used evoked stress by decreasing the viability of explants.

All these findings indicate that ethanol has cytotoxic effects in human placenta, including early placenta.

2.3.2 Toxicity of nicotine in placenta

The toxicity and harmful effects of cigarette smoke to fetus and placenta have been recognized for a long time (for a review, see Brown 1996). Nicotine is an addictive compound in cigarette smoke, and recently many harmful and even carcinogenic effects have been linked to nicotine (for reviews, see Grando 2014, Mishra et al. 2015, Wadgave and Nagesh 2016). In animal studies, nicotine exposure during pregnancy has been shown to decrease the birthweight (Wang et al. 2009, Holloway et al. 2005), probably because of changes in energy and fatty acid metabolsim, as has been shown in adult rats (Phillips et al. 2015). In humans, there is one birth cohort -study showing that the use of more than one nicotine replacement therapy (NRT) product during pregnancy decreased the birthweight (Lassen et al. 2010). Moreover, decreased birthweight has been linked to many adverse effects later when the offspring reaches adulthood, such as high blood pressure and type 2 diabetes (Kaakinen et al. 2014, Hjort et al. 2015).

Machaalani et al. (2014) showed that both mRNA and protein expression of α9 subunit of nAChR are increased in smokers´ placentas when compared to placentas of non-smokers. The upregulation of nAChR subunits may have serious consequences, because some subunits of nAChR are known to stimulate cancer development (for an extensive review, see Schuller 2009). α7nAChR is the most powerful receptor to stimulate cancer cells, whereas the α4β2nAChR regulates predominantly inhibitory actions towards cancer. All these nAChR

subunits are present in healthy term human placenta (Machaalani et al. 2014) and the balance between different receptor subtypes may be disturbed by smoking.

Even though the effects of cigarette smoke on animal (Maccani et al. 2010) and human (Bruchova et al. 2010, Niu et al. 2015) placentas have been studied to some extent, very little is known about nicotine toxicity in placenta. The study of Wong et al. (2015) conducted with rat placenta, showed that prenatal nicotine exposure elevated ER stress, as indicated by the increased expression of GRP78/BiP. They also revealed that nicotine evoked ER stress in rat placental cancer cell line (Rcho-1), because the phosphorylation of the ER stress marker PERK was increased after nicotine treatment (Wong et al. 2016). In humans, it is almost impossible to exclude the effects of smoking from the effects of nicotine alone in *in vivo* -studies, because very often even the users of nicotine replacement therapies (NRT) have been smokers before or are still being exposed to passive or active cigarette smoking, even though they are using NRT. As far as we are aware, there are no published experimental studies investigating the toxic mechanisms of nicotine in human placenta.

2.3.3 Mechanisms and biomarkers of placental toxicity

Basically, any cell or protein can be a target of toxicity, also in placenta (for a recent review, see Vähäkangas et al. 2014). In addition to proteins, the corresponding gene expression at the mRNA level and epigenetic mechanisms, such as DNA methylation can be studied to estimate placental toxicity. Human placenta can metabolise xenobiotics to some extent, which means that toxic metabolites may be formed in human placenta (Vähäkangas et al. 1989, Pienimäki et al. 1997, Karl et al. 1988, Partanen et al. 2010, Karttunen et al. 2010). Toxic metabolites are usually unstable and have a tendency to react immediately in the tissue where they were formed.

In both the research setting and the clinic, biomarkers are a tool to estimate placental function and toxicity in placental tissue (Table 3, for a review, see Costa 2016). The syncytiotrophoblast secretes many hormones, not only into the fetal circulation, but also into the maternal circulation. By taking samples from maternal blood, important information of placental function can be gained. One of the most common types of placental insufficiency is preeclampsia (for reviews, see Hod et al. 2015, Phipps et al. 2016), which is a state of toxemia caused by circulating antiangiogenic proteins of 'soluble fms-like tyrosine kinase 1' (sFLT1) and 'soluble endoglin' (sEng). These two proteins are specific markers for preeclampsia and they become upregulated weeks before the appearance of other clinical signs of the disease (e.g hypertension and proteinuria). Many other biomarkers for preeclampsia, such as markers related to oxidative (Huang et al. 2015) and ER stress (Yung et al. 2014, Fu et al. 2015) or apoptosis (Sharp et al. 2014) have been identified, but their clinical relevance has been questioned.

Certain metabolites of ethanol such as ethyl glucuronide (EtG), ethyl sulfate (EtS) and fatty acid ethyl esters (FAEE) have been used to estimate fetal alcohol exposure in meconium (Bakdash et al. 2010, Cabarcos et al. 2014, Goecke et al. 2014). Earlier it was thought that FAEE are produced by the fetus from ethanol and that meconium is a more suitable matrix for assessing alcohol intake than placenta (Chan et al. 2004). Recently, also placental FAEE levels have been shown to correlate well with maternal alcohol intake (Gauthier et al. 2015). In addition to FAEE, also ethyl glucuronide (EtG) has been detected in placental tissue and proposed to be a good placental biomarker for alcohol exposure (Morini et al. 2011). A good correlation has been shown between specificity of both EtG and FAEE to detect maternal alcohol drinking (Cabarcos et al. 2014).

Table 3. Examples of potential biomarkers of human placental toxicity (for a review, see Vähäkangas et al. 2014).

Biomarker	Examples	Cause	Reference
DNA-adducts	PAH-DNA-adducts, organochlorine-related-DNA adducts	smoking, environmental PAHs	Laqueux et al. 1999, Annola et al. 2009, Karttunen et al. 2010, Obolenskaya et al. 2010
Hormones	hCG, hPL, estrogen, progesterone, placental growth hormone	cell stress	Polliotti et al. 1995, Sooranna et al. 1999, Vähäkangas et al. 2014
Expression of apoptotic proteins	p53, TNFR, MAPK	cell stress	Edinger and Thompson 2004, Halperin et al. 2000, Haider and Knofler 2009, Vähäkangas 2003
Expression of stress proteins	GRP78/BiP, MAPK	cell stress	Shu et al. 2014, Veerbeek et al. 2015
Pre-eclampsia markers	soluble fms-like tyrosine kinase 1, soluble endoglin	pre-eclampsia	Phipps et al. 2016, Hod et al. 2015
LDH	cell integrity marker	cell stress	Reti et al. 2007, Audette et al. 2010, Sengupta et al. 2011, Vaidya et al. 2011
Cotinine	metabolite of nicotine	tobacco products	Pasanen et al. 1988b, Vyhdial et al. 2013
Ethyl glucuronide (EtG) Fatty acid ethyl esters (FAEE)	metabolites of ethanol	alcohol	Morini et al. 2011 Gauthier et al. 2015
Acetylcholinesterase activity	carboxylesterase activity decreased in exposure	pesticide exposure	Vera et al. 2012

PAH=polycyclic aromatic hydrocarbon, hCG= human chorionic gonadotropin, hPL= human placental lactogen, p53= a 53 protein, TNFR = tumour necrosis factor receptor, LDH= lactate dehydrogenase, MAPK= mitogen activated protein kinases.

2.3.4 Cell stress

Cell stress covers a wide range of molecular changes that cells undergo in response to different stressors, such as toxins. Many endogenous and exogenous factors may cause cell stress. Inflammation and autoimmune diseases are endogenous inducers of cell stress (for a review, see Ortona et al. 2008), whereas various chemicals, radiation and bacterial infections are examples of exogenous inducers. Chemicals typically induce oxidative stress which is an important form of cell stress (for an extensive review, see Ray et al. 2004). Oxidative stress is characterized by the formation of reactive oxygen species (ROS). Another form of chemically induced cell stress is endoplasmic reticulum (ER) stress, which is also known as the 'unfolded protein response' (for a review, see Bhandary et al. 2012). Oxidative and ER stress are both recognized mechanisms of placental toxicity (Figure 5, for a review, see Burton and Jauniaux 2011). Genotoxic stress can occur due to chemically induced DNA adducts or DNA strand breaks (for reviews, see Rotman and Shiloh 1999, Ishikawa et al. 2006) and these have also been found in human placenta as shown by Tadesse et al. (2014).

Reactive oxygen species (ROS) are signaling molecules that are produced as a result of normal cell functions in all tissues, including placenta (for a review, see Bevilacqua et al. 2012). For example, ROS are generated in mitochondrial electron transport chain and in

membrane-bound NADPH oxidase. However, under abnormal conditions, such as inflammation and infection, the amount of ROS may increase drastically, leading to a condition defined as oxidative stress (for a review, see Poston and Raijmakers 2004). Oxidative stress arises when the production of reactive oxygen species (ROS) overwhelms the anti-oxidant defence systems. Anti-oxidant defences can be divided into enzymatic and non-enzymatic precesses (for a review, see Burton and Jauniaux 2011). Antioxidative enzymes such as superoxide dismutase and glutathione peroxidase have a transition metal at their core, which can exhibit different valences as electrons are transferred during the detoxification process. Thioredoxin, glutaredoxin and peroxiredoxins are known electron donors to glutathione peroxidase and thus ROS decomposing enzymes (Lehtonen et al. 2005). Non-enzymatic defences include vitamin C and α-tocopherol (vitamin E). Oxidative stress is known to play a central role in the pathophysiology of many different placental disorders, including preeclampsia and miscarriage (Adebambo et al. 2015, Charnock-Jones 2016). In addition, the expression levels of thioredoxin and glutaredoxin are decreased in preeclamptic placentas *in vivo* (Sahlin et al. 2000).

ER stress is expressed as an unfolded protein response (UPR). During pathological conditions, proteins become aggregated, causing accumulation of misfolded proteins in the ER lumen. Only the properly folded proteins are modified in the Golgi apparatus and translocated to their destinated sites. Misfolded proteins aggregate in the ER-lumen and ROS are generated as a part of this process. ER induced oxidative stress will further trigger the generation of mitochondrial ROS (for a review, see Bhandary et al. 2012). Many mechanisms have been described to induce mitochondrial ROS production under ER stress, including glutathione depletion, mitochondrial electron transfer chain system and calcium-related ROS generation.

In ER stress, expression of the ER chaperone protein GRP78/BiP is increased in an attempt to refold the misfolded proteins. GRP78/BiP is an upstream regulator of three molecular pathways; inositol-requiring protein -1α (IRE1α), pancreatic endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). These pathways may finally activate the transcription of the UPR target genes. Known ER stress related factors GRP78/BiP, IRE1α, ATF-6 and PERK are increased in human placenta in pre-eclampsia (Yung et al. 2014, Fu et al. 2015). However, while ER stress and oxidative stress have been studied in animal placentas and cell models, little is known about human placental ER stress and oxidative stress.

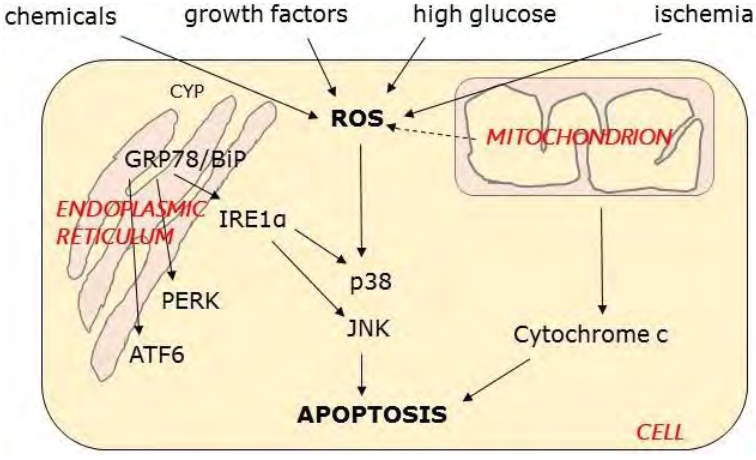


Figure 5. Crosslinking of ER stress and oxidative stress (Data from Malhotra and Kaufman 2007, Burton and Jauniaux 2011). ROS=reactive oxygen species, CYP=cytochrome P450 enzyme, IRE1α=inositol-requiring protein 1α, PERK=pancreatic endoplasmic reticulum kinase, ATF6=activating transcription factor 6. The protein 38 (p38) and Jun amino-terminal kinases (JNK) belong to mitogen activated protein kinases (MAPK).

2.4 HUMAN PLACENTAL RESEARCH MODELS

2.4.1 Existing models

Placental toxicity can be studied in many models; these can consist of placental cells or tissue (for a review, see Gohner et al. 2014). Cell culture and other i*n vitro* -methods, such as microsomes and isolated enzymes have been used for decades to study human placental metabolism and toxicity (Contractor and Krakauer 1976, Vähäkangas et al. 1989, DuBois et al. 2012). However, more advanced methods, such as isolated villous explants and human placental perfusion exist as well (Figure 6, for reviews, see Vähäkangas and Myllynen 2006, Myllynen and Vähäkangas 2013). These models can provide a wider perspective not only of placental toxicity, but also of many other aspects of fetal exposure. For example, villous explant culture can be used for comparing the responses of explants during the different stages of pregnancy and human placental perfusion can model the transfer across placenta. Both models, as well as cells of placental origin are being used for studies on placental endogenous and xenobiotic metabolism.

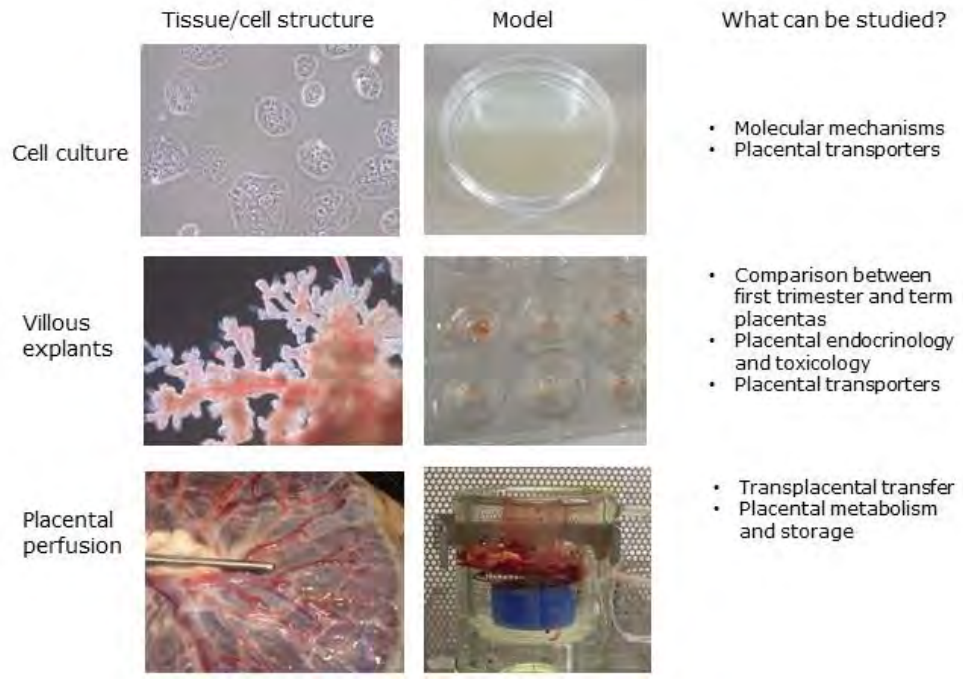


Figure 6. Experimental models to study placental toxicity.

2.4.2 Cell culture

Placental cell culture models may be roughly divided into four main groups: primary cells, cancer cell lines, immortalized cell models and cultures with tight junctions. Altough primary cells have the advantage of being normal cells, the establishment of primary trophoblastic cultures is laborious in comparison to immortalized or cancer cell lines (for a review, see Vähäkangas et al. 2011). Primary cells spontaneously syncytialize in culture, which means that the character of the cells is continuously changing during the culture (Orendi et al. 2011).

In addition, primary cells are difficult to isolate from term placentas and only short term cultures of a few days can be performed.

Three widely used trophoblastic secondary cell lines exist; and they are choriocarcinoma cells BeWo, Jeg-3 and JAr (Orendi et al. 2011). Jeg-3 and JAr cell lines are derivates of BeWo cells (for a review, see Prouillac and Lecoeur 2010). Of these cell lines, BeWo has been the most extensively used model for villous trophoblasts since it displays most of the characteristics of villous trophoblasts including secretion of hCG, hPL, progesterone and estradiol (Orendi et al. 2011). BeWo cells also express the BCRP transporter, similarly to normal human placenta, but many studies report no or only limited expression at the protein level of P-glycoprotein, although its mRNA can be found (Myllynen et al. 2008a, Evseenko et al. 2006). BeWo cells consist of cytotrophoblasts with few syncytialized cells and they do not syncytialize spontaneously (for a review, see Prouillac and Lecoeur 2010). BeWo cells express many similar biochemical marker enzymes and display the same morphological properties as normal placental trophoblasts (Orendi et al. 2011). The characteristics of Jeg-3 cells resemble the syncytiotrophoblast better than BeWo cells. Unlike syncytiotrophoblast in human placenta, Jeg-3 cells are mononucleated and proliferative. JAr cells, on the other hand, have characteristics more reminsiscent of undifferentiated cytotrophoblasts when compared to BeWo and Jeg-3 cell lines.

Over 20 immortalized trophoblastic cell lines were reported by King and co-workers (2000). Some of the most widely used are SV40 or human papilloma virus E6 and E7 infected lines, such as HTR-8SVneo, TCL-1, IST-1 and SGHPL group of cells. The problem with immortalized trophoblastic cell lines seems to be the different characteristics of each line. None of these cell lines fulfilled the minimum characteristic requirements for trophoblastic cells (cytokeratin 7 or $CK7^{+}$, HLA-class I^{-} and $CD9^{-}$) in the report of the International Federation of Placenta Associations –meeting (King et al. 2000). CD9 is known to be expressed in extravillous trophoblasts and the lack of HLA-class I expression is used as an evidence of villous trophoblastic origin (King et al. 2000)

The *in vitro* transfer studies can only be carried out with a cell line that forms a polarized monolayer with tight intercellular junctions. From cancer cell lines, the subclone of BeWo cells, BeWo b30, fulfills the criteria for being a continuous cell line (Heaton et al. 2008, Cartwright et al. 2012). In addition, Ikeda et al (2011) have described the use of Jeg-3 cells in transwell -studies after specialized culture conditions. Recently, it has also been reported that human primary trophoblasts isolated from term placenta can develop a confluent monolayer *in vitro* under optimized conditions (Huang et al. 2016).

2.4.3 Placental explant culture

The method to culture human placental villous explants was already established in the 1940's (Wang and Hellman 1943). Placental tissue has been cultured also without isolation of villi (Heikkilä et al. 2002). For years, human placental villous explant culture was only used for studies on physiology or pathology (for original articles, see e.g. Sooranna et al. 1999, Ietta et al. 2006, Ietta et al. 2007, Baczyk et al. 2009). Human placental villous explant cultures were rarely used in toxicology studies. Villous explants are primary tissue with a potentially similar expression profile of transporters and metabolizing enzymes as normal placenta *in vivo* (for a review, see Vähäkangas et al. 2011). For instance, the cancer cell line BeWo does not express P-glycoprotein, which is an important transporter in placenta (Evseenko et al. 2006).

Human placental villous explants cultures can be used for various types of studies. Firstly, explants from normal (uncomplicated) pregnancies can be exposed to chemicals and the changes at the molecular level can be compared with non-treated controls of the same placenta. Explants can also be used for investigation into how diseases affect placental function. The explants isolated from pre-eclamptic placentas can be compared with the

placental explants of normal pregnancies (Czikk et al. 2013). Villous explants of both first trimester and term placentas can be cultured (Miller et al. 2005). Second trimester explants can also be studied (Patni et al. 2015), but the only way to obtain second trimester placentas is after the delivery of a premature infant. The placentas of premature infants are often needed for pathology to confirm the health status of the baby.

There are two different techniques to culture human placental villous explants (for a review, see Miller et al. 2005). For placental development and invasion studies, the first trimester explants are cultured in a supporting matrix, usually Matrigel®. Third trimester explants are usually freely suspended in the well of a cell culture plate. For toxicological studies, floating cultures have been used with both first and third trimester explants.

According to the literature, the longest duration that term explant cultures have survived has been for up to 6 days (Haning et al. 1988, Audette et al. 2010) but first trimester explant cultures have been kept alive even longer (Polliotti et al. 1995). However, the viability of human placental explant cultures has not been thoroughly studied, and no established viability markers exist. Thus, especially in toxicological studies, it is very important to estimate the viability of explants to be sure that the molecular changes are due to chemical exposure and not overall decreased viability.

The two most widely used viability markers in villous explant cultures have been the release of lactate dehydrogenase (LDH) to the medium (Siman et al. 2001, Reti et al. 2007, Audette et al. 2010, Messerli et al. 2010, Sengupta et al. 2011, Vaidya et al. 2011) and glucose consumption (Chen and Lin 1998, Di Santo et al. 2003, Reti et al. 2007, Hulme et al. 2012). Of these two, LDH has been used more in placental explants and in isolated placental trophoblastic cells. In addition to these two, also hormone secretion (Polliotti et al. 1995, Sooranna et al. 1999), production of placental protein (PP13) in the culture medium (Huppertz et al. 2011) and expression of the proteins Bcl2 or PCNA (proliferating cell nuclear antigen) in the tissue (Menon et al. 2011) have been used. However, many of the viability markers have disadvantages. For example, the ability of term placental villous explants to produce hormones at measurable levels has been debated (Polliotti et al. 1995, Sooranna et al. 1999). In toxicological studies, normally all the tissue of explants (each explant ~100 mg) is needed for the analysis of the studied proteins and thus viability markers that can be measured from the culture medium would be preferable.

2.4.4 Placental perfusion

Schneider et al. (1972) established the method of perfusing one human placental cotyledon in early 70s, and the method was further developed by Brandes et al. (1983). In their work, Schneider and co-workers (1972) used the so-called 'once through' or 'open' -method, where perfusate was not recirculated, but collected after draining through the placenta. Later Brandes and co-workers (1983) established the dual recirculating method, which better reflected the *in vivo* -situation and enabled studies on metabolism in the placenta. Medical drugs (Ala-Kokko et al. 1995, Pienimäki et al. 1995, Pienimäki et al. 1997), food carcinogens (Annola et al. 2008, Annola et al. 2009, Partanen et al. 2010) and other xenobiotics, such as benzo(a)pyrene (Karttunen et al. 2010) have been studied in human placental perfusion.

The *ex vivo* -perfusion model of isolated human placental cotyledon is the only experimental model which is useful when examining the transfer of chemicals, which retains all the layers of placental barrier (Vähäkangas and Myllynen 2006, Myllynen and Vähäkangas 2013). In addition, human placental perfusion may be used to study acute toxicity and metabolism in placenta. Tissue accumulation can be estimated by analyzing the placental tissue after perfusion and comparing the results to the tissue of the same placenta that has not been perfused (Annola et al. 2008). Human placenta can retain its viability for hours after the delivery and the longest perfusions reported in the literature have lasted 48 hours (Polliotti et al. 1996). Normally perfusion is carried out for 2–6 hours, as almost all

compounds will be transferred from the maternal to the fetal side and equilibrated between circulations within this period of time (Karttunen et al. 2015).

Recently, the criteria for a successful perfusion have been quite generally accepted and an interlaboratory comparison as a part of prevalidation has been carried out (Myllynen et al. 2010, Mose et al. 2012, Karttunen et al. 2015). In short, placental perfusion predicts well placental transfer *in vivo*, when the most extensively used criteria (volume loss from fetal circulation <3ml/h) and the transfer of a reference compound antipyrine are used. The transfer of antipyrine is transferred through placenta with passive diffusion and used to estimate the overlap between maternal and fetal circulations (Schneider et al. 1972, Challier et al. 1983, Karttunen et al. 2015).

Placentas are usually disposed of in Western countries and mothers are very willing to donate them to research after delivery (Halkoaho et al. 2010). However, preparation of placentas has to be started within 10 minutes after the labour, and the time for collecting the tissue limits the use of this model. In addition, the success rate for set-up perfusions is only about 50%, which makes the method quite laborious (Karttunen et al. 2015).

2.4.5 Ethical aspects of placental studies

Pregnant women are regarded as a special group, because two individuals are involved and delivery can be a very emotional and sensitive situation. The process of asking for permission to participate in any study during such a special situation should be considered carefully before approaching mothers (Halkoaho et al. 2012). In Finland, human placenta is studied for any abnormalities after delivery by nurses and discarded if there is no medical need to send it for pathological examination. Therefore, it is ethically acceptable to use placenta for research if the mother consents to its use. The use of placenta in research does not affect the clinical treatment of the mother or baby in any way. Mothers in Finland are usually very willing to donate their placentas for research (Halkoaho et al. 2010). Halkoaho and co-workers (2010) found that mothers considered the studies on fetal exposure to chemicals very important. Two other existing studies on the ethics of placental studies, one from Brazil (Yoshizawa et al. 2015) and the other from Denmark (Lind et al. 2007), support these results. Mothers are willing to donate placentas, because among other things, they have a general trust in medical science and research (Lind et al. 2007, Yoshizawa et al. 2015).

The use of clinical placental samples poses some unique challenges to a study, not existing in cell culture studies. It is important that the collection, storage and handling of biological waste is conducted in the appropriate manner (Jeffers 2001). Furthermore, international regulations and national laws need to be taken into account. A recommendation of the study by the local ethics committee is legally required in Finland and ethical aspects such as respecting patients' autonomy by obtaining informed concent are included in the law. In addition, protection of personnel against infections mediated through human blood have to be taken into account when handling human tissues.

3 Aims of the Study

Most chemicals pass easily through human placenta, but they may also accumulate or cause toxicity in placental tissue. Any disturbance in placental function will harm fetal development or growth, depending on the gestational age and the chemical. Unfortunately, many pregnant women still use alcohol and smoke tobacco. Animal studies do not correlate well with human data in placental toxicity studies, because placenta varies more than any other organ between species. Thus, models to study placental toxicity with human primary tissue are urgently needed.

This thesis is a part of larger research program on fetal exposure to toxic compounds. The ultimate aim of the whole research program is to evaluate fetal exposure and the molecular mechanisms related to it. The harmful effects of the substances examined in this work, nicotine and alcohol, are widely known, but the mechanisms behind their placental toxicity are unclear.

The specific objectives of this study were as follows:

1. To develop further the method to culture term human placental villous explants
2. To determine the effect of ethanol on the transfer of other chemicals in human placenta
3. To reveal if ethanol and nicotine causes ER and oxidative stress in placenta

4 Materials and Methods

4.1 STUDY CHEMICALS AND OTHER MATERIALS (I, II, III)

Chemicals and reagents used in the present study are shown in the Table 4. All reagents were of analytical grade and all solvents used for HPLC and LC-MS/MS were of HPLC grade. Other materials used in this study are described in detail in the original papers (I, II and III).

Table 4.Chemicals and reagents used in the present study.

Chemical / reagent	CAS	Origin
Nicotine	54-11-5	Sigma-Aldrich, St. Louis, USA
d3-nicotine	69980-24-1	CDN isotopes, Quebec, Canada
Ethanol	64-17-5	Altia Corporation, Finland
PhIP (2-amino-1-methyl-6-phenyl-imidazo(4,5-b)pyridine)	105650-23-5	Toronto Research Chemicals, Ontario, Canada
14C-PhIP	210049-12-0	Toronto Research Chemicals, Ontario, Canada
Antipyrine	60-80-0	Sigma-Aldrich Chemie, Steinheim, Germany
NDMA	62-75-9	Sigma-Aldrich, St Louis, USA
14C-NDMA (n-nitrosodimethylamine)	62-75-9	Moravek Biochemicals, Brea, CA, USA
Sodium pyruvate	113-24-6	Lonza Biowhittaker, Belgium
Penicillin-streptomycin	3810-74-0	Lonza Biowhittaker, Belgium
Coomassie Brilliant Blue	6104-58-1	Bio-Rad, USA
L-Glutamine	56-85-9	Sigma, UK
EDTA	6381-92-6	Sigma, UK
Bovine serum albumin (BSA)	9048-46-8	Sigma, UK
Trypsin	9002-07-7	Gibco, UK
FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)	370-86-5	Sigma-Aldrich, St Louis, USA
H2O2 (hydrogen peroxide)	7722-84-1	Fisher Scientific, UK
Tunicamycin	11089-65-9	Sigma-Aldrich, St Louis, USA
MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide)	298-93-1	Sigma-Aldrich, St Louis, USA
H2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate)	4091-99-0	Sigma-Aldrich, St Louis, USA
Acetonitrile	75-05-8	J.T Baker, Deventer, Netherlands
Methanol	67-56-1	J.T Baker, Deventer, Netherlands

Table 4.Chemicals and reagents used in the present study.

Chemical / reagent	CAS	Origin
Complete protease inhibitor		Roche, Germany or Sigma-Aldrich, St. Louis, USA
Halt phosphatase inhibitor 100× cocktail		Thermoscientific, USA
Sodium orthovanadate		Sigma-Aldrich, St. Louis, USA
Antibody for p53		Novocastra laboratories Ltd, UK
Antibody for GRP78/BiP		Cell Signaling Technology, USA
Antibody for IRE1α		Cell Signaling Technology, USA
Antibody for p38		Cell Signaling Technology, USA
Antibody for ERK1/2		Cell Signaling Technology, USA
Antibody for JNK		Cell Signaling Technology, USA
Antibody for phopsho-p38		Cell Signaling Technology, USA
Antibody for phosphor-EKR1/2		Cell Signaling Technology, USA
Antibody for phosphor-JNK		Cell Signaling Technology, USA
Antibody for heme-oxygenase 1		Santa Cruz Biotechnology, Europe
Antibody for Nrf-2		Cell Signaling Technology, USA
Antibody for beta-actin		Sigma-Aldrich, St. Louis, USA or Cell Signaling, Massachusetts, USA
Anti-mouse antibody		Amersham, UK
Anti-rabbit antibody		Calbiochem, Germany
Non-essential amino acids		Lonza Biowhittaker, Belgium
Fetal bovine serum (FBS)		Lonza Biowhittaker, Belgium
Dulbecco's Phosphate Buffered Saline (DBPS)		Lonza Biowhittaker, Belgium
RPMI-1640, without phenol red		Lonza, Verviers, Belgium or Gibco, Paisley, Scotland, UK

4.2 PLACENTAL TISSUE AND ETHICAL CONSIDERATIONS (I, III)

For this study, both first trimester placentas (in Siena, Italy) and term placentas (in Kuopio, Finland) were used. First trimester placentas were collected after voluntary elective termination of pregnancies. In full-time pregnancies, placentas of mothers going through Cesarian sections were preferred to placentas of normal birth, as mothers had more time to think about the study before entering labour. The mothers of both first trimester and term placentas were informed about the study by a nurse. Both written and oral information was given to the mothers before written consent was obtained (Halkoaho et al. 2010, 2011, 2012). Placentas of healthy, non-smoking mothers who did not use alcohol during pregnancy were used. All placentas were anonymized (identifications were ripped off and placental samples were stored with a non-linkable code). Mothers were very willing to donate their placentas for this kind of study (Halkoaho et al. 2010). The Research Ethics Committee of the University Hospital District of Kuopio region approved the study 11.5.2005 (79/2005) and 30.5.2007 (54/2007).

4.3 HUMAN PLACENTAL VILLOUS EXPLANTS (III)

The isolation of both first trimester and term placental explants are explained in detail in publication III. The basic method is the same for first trimester and term placental explants. In this work, the methodology used for the culture of first trimester human placental villous explants was applied to human term placental villous explant culture (III).

Culture medium was prepared in advance by adding 5ml of penicillin-streptomycin to 500ml of RPMI-1640 without phenol red and the needed amount was warmed up to 37 ˚C in a water bath. For clarity, all the details of the placentas were collected in so-called explant form. The placenta was carried from the hospital to the primary cell laboratory within an hour after delivery. A maternal cotyledon from the central area was selected and cut to include the full depth of the placenta. The tissue was rinsed in a test tube with ice cold PBS at least three times to remove extra tissue and red blood cells from the villous trees. The tissue with villous structures was placed on a petri dish with about 10 ml of ice-cold PBS. Whole villous trees (volume approximately $10mm^3$) were cut under a stereomicroscope and placed on another petri dish with a drop of culture medium. Explants were transferred to the wells of 24-well plates with 300µl (first trimester explants) or 500µl (term explants) of culture medium in each well (one explant per well). A 24-hour incubation at 37 ˚C with no treatments was started to allow the explants recover from the isolation and the morphology of the explants was checked under a light microscope. A villous explant had to appear viable (almost transparent with visible blood vessels) to be accepted for the study. No changes in the color (to brown or gray) were accepted.

Each 24-well plate contained blanks (no explants), controls (only medium and explant) and treated explants. One experiment (one plate) included duplicate or triplicate explants (two or three explants in different wells from the same placenta with the same treatment or control). The compounds were dissolved in culture medium and a volume of 300 or 500µl was pipetted into each well. Explants were transferred randomly from the old 24-well plate to the new 24-well plate. At the end of each exposure period, the villous explants were snap-frozen in Eppendorf tubes and stored in a freezer (-80 ˚C) until analysis.

In the protein analysis, the villous explants (first trimester and term) were lysed with a sonicator. A volume of 30–40µl of RIPA (radioimmunoprecipitation assay buffer) solution with protease and phosphatase inhibitors was added immediately to each explant and the explants were kept on ice during the lysis. Each explant was sonicated three times for 10 seconds. Lysates and culture media were stored in a freezer (-80 ˚C) for further analysis.

4.4 HUMAN PLACENTAL PERFUSION (I)

Preparation of tissue, perfusion and tissue lysis

Human placental cotyledons were perfused using the dual recirculating method described earlier (Pienimäki et al. 1995, Annola et al. 2008, Karttunen et al. 2015). The details of the system are also explained in publication I.

In short, placentas were collected at 10 minutes after birth and a preperfusate with heparin was injected to the umbilical vein and arteries to prevent blood coagulation. An artery and a vein of the peripheral area of placenta were cannulated in the fetal circulation. The cotyledon was cut off and placed in the perfusion chamber. Two cannulas were inserted through basal plate to intervillous space for maternal circulation. The placenta was allowed to recover from hypoxia for 30 minutes, before the studied substances and the reference compound antipyrine were added. Liquid samples of the perfusate were collected from both circulations at designated time points. Perfused placental tissue and control samples from the unperfused areas of the same placenta were collected.

For further analysis of the samples, placental tissue sample (0.1g) was homogenized in 1ml of TRIS-HCL (0.1 M) with K-EDTA (1mM) with ULTRA-TURRAX®.

Criteria for successful perfusion

Strict criteria for usable perfusions were applied throughout the work. Recommendation for the criteria of an acceptable perfusion have been published (Mathiesen et al. 2010, Myllynen et al. 2010, Mose et al. 2012, Karttunen et al. 2015). A volume loss less than 3ml per hour from fetal to maternal circulation, transfer of the reference compound antipyrine with feto-

maternal ratio above 0.75 within 3 hours and consumption of glucose were used as criteria. In addition, blood gases (pO_2 and pCO_2) and pH were monitored throughout the study. pH was adjusted to the physiological level (7.35–7.42) with a drop of HCl when needed.

4.5 CELL CULTURE (I, II)

BeWo cells (ATCC® CCL-98™) were grown as described in original publication II and MCF-7 cells were grown as described in original publication I. Cells were tested to be free of mycoplasma contamination by measuring the presence of mycoplasma enzymes using the mycoplasma detection kit (MycoAlert, Lonza, USA). BeWo cells did not form a confluent monolayer on a petri dish even at 72h regardless of the number of plated cells, which is a typical feature of this cell line.

Preparation of total cell fractions is described in detail in original publication II. In short, cells were scraped from the plates in 1ml DPBS (Dulbecco's Phosphate Buffered Saline) and centrifuged. After this, the supernatant was removed and replaced with RIPA-like lysis buffer with protease and phosphatase inhibitors. The cell suspension was incubated on ice and centrifuged. Thereafter, supernatants were collected and the protein concentration was measured.

4.6 CYTOTOXICITY TESTS (II, III)

Propidium iodide method for cell viability (II)
Cells were treated with PI, which is excluded from viable cells, but binds to the DNA of non-viable cells. After PI treatment, digitonin is added. Digitonin damages the cell wall and enables PI binding to DNA in all cells (living and dead). This reflects the total cell amount and makes it possible to calculate cell viability. The fluorescence was measured at an excitation wavelength of 531nm and an emission wavelength of 615nm. Thereafter, cells were treated with digitonin to calculate the total number of cells (maximal fluorescence).

Lactate dehydrogenase (III)
LDH was analyzed with the in vitro Toxicology Assay Kit (Sigma-Aldrich, St. Louis). Shortly, 30µl of medium sample was pipetted in triplicates into the wells of a 96-well plate. A volume of 60µl of LDH assay mixture was added to the wells. After 30 minutes of incubation at room temperature, the absorbance was measured at the wavelength of 490nm and the background (at 690nm) was subtracted from the primary values.

MTT-Assay (III)
MTT-reduction assay was carried out as described by Pesonen et al (2012) with minor modifications. The explants were isolated and allowed to recover in 24-well plates (one explant per well) for 24 hours. Exposures were started by changing the explants to a new 24-well plate with exposure medium (500µl per well), control (cell culture medium) and blanks (no villous explants). After exposure for 24 hours, the medium was changed to MTT-containing (0.5mg/ml) medium (300µl per well) and the incubation continued at 37°C for 2h. Thereafter, the medium was removed and formazan crystals were solubilised with SDS-HCl-buffer (pH 4.7, 300µl per well) at room temperature for 40 minutes. Thereafter, an aliquot of the medium (200µl) was changed to a new 48-well plate and the absorbance was measured at 570nm.

Glucose measurement (III)
Glucose in the medium was analyzed with Bayer Contour glucose monitoring system (Bayer) according to the manufacturer's instructions. A volume of 100µl of culture medium was taken for glucose analysis from each well. The corresponding amount in the well was replaced with the same culture medium as used before to avoid diminishing the volume of medium in each well. In the Bayer Contour glucose monitoring system, a drop of supernatant is dispensed in the instrument and the value registered before freezing the samples.

4.7 REACTIVE OXYGEN SPECIES (II)

The analysis of ROS production was based on 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) accumulation in the cells and conversion into the highly fluorescent DCF when oxidized by intracellular ROS. Thereafter, the fluorescence was measured at an excitation wavelength of 485nm and an emission wavelength of 535nm. The details of the analysis are explained in publication II.

4.8 ANALYSIS OF SAMPLES (I, II, III)

Samples were analyzed by LC/MS-MS (nicotine), HPLC (antipyrine) or immunoblotting (proteins). Nicotine analysis was mainly done as described earlier (I and Raunio et al. 2008). HPLC was used for antipyrine analysis (I and Annola et al. 2008).

In the case of proteins, the total protein concentration was determined by the Quick Start Bradford Protein Assay (Bio Rad Microscience). Absorbance were read at the wavelength of 595nm using the UV Spectrophotometer (Pharmacia Biotech Utraspec 2000) for first trimester explants or (ELX800UV, Bio-Tek Instruments Inc.) for term explants.

In immunoblotting (Table 5), proteins (15, 20 or 30µg) in sample buffer were separated electrophoretically in 10–12% gel containing SDS (publication II) (Tampio et al. 2008, Bhattacharjee et al. 2010). The secondary antibody dilution was 1:2000 in all studies. Protein bands were visualized with ECL+ Plus or ECL Prime system according to the manufacturer′s instructions (Amersham, UK). Beta-actin was used as a loading control and densitometric analysis of the results was carried out by using QuantityOne program (Bio-Rad laboratories inc. USA).

Table 5. Proteins analyzed by immunoblotting.

Protein	Primary antibody dilution	Secondary antibody
GRP78/BiP	1:1000	anti-rabbit
IRE1α	1:1000	anti-rabbit
p-p38 and tot-p38	1:1000	anti-rabbit
p-ERK1/2,	1:1000	anti-rabbit
tot-ERK1/2	1:2000	anti-rabbit
p-JNK and tot-JNK	1:1000	anti-rabbit
beta-actin	1:2 mil.	anti-mouse
p53	1:2000	anti-mouse

GRP78/BiP=glucose regulating protein 78, IRE1α= inositol requiring enzyme 1α, p-p38=phosphorylated protein p38 (MAPK), tot-p38=total protein p38 (MAPK), p-ERK1/2= phosphorylated ERK1/2 (MAPK), tot-ERK1/2 = total protein ERK1/2 (MAPK), p-JNK=phosphorylated JNK protein (MAPK), tot-JNK= total JNK protein (MAPK), p53= the protein 53 (MAPK).

4.9 STATISTICAL ANALYSIS

Two-way ANOVA followed by a Bonferroni post hoc test was the main statistical test used in the perfusion studies. For cell culture and other *in vitro* –studies, one way ANOVA followed by a Dunnett`s test was used. $p < 0.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 or 7.0.

5 Results

5.1 OPTIMUM CONDITIONS FOR TERM HUMAN PLACENTAL VILLOUS EXPLANTS CULTURES AND COMPARISON OF VIABILITY TEST RESULTS WITH FIRST TRIMESTER EXPLANTS (III)

The method for culturing term human placental villous explants was further developed (Figure 7) to determine optimum conditions so that the explant would remain viable and to identify the most representative viability measurement, because no consensus exists about how to assess explant viability. Two term placentas were used to assess the optimal culture time as well as reliable viability markers and positive controls. Firstly, the viability of explants was assessed by microscopy at 24h, 48h and at 72h (Figure 7; 1st step). Normal explants appeared almost transparent with visible blood vessels (Figure 2). At 24 hours, no changes in the color or morphology of term placental villous explants were seen indicating that there were normal biophysiological conditions. However, at 48 hours, the color of some explants and the morphology had changed, as analyzed by microscopy. More visible changes in color (turned to grey or brownish) and morphology were seen in many explants at 72h. Because of the changes in morphology at 48 and 72 hours, the timepoint of 24h was selected for further experiments.

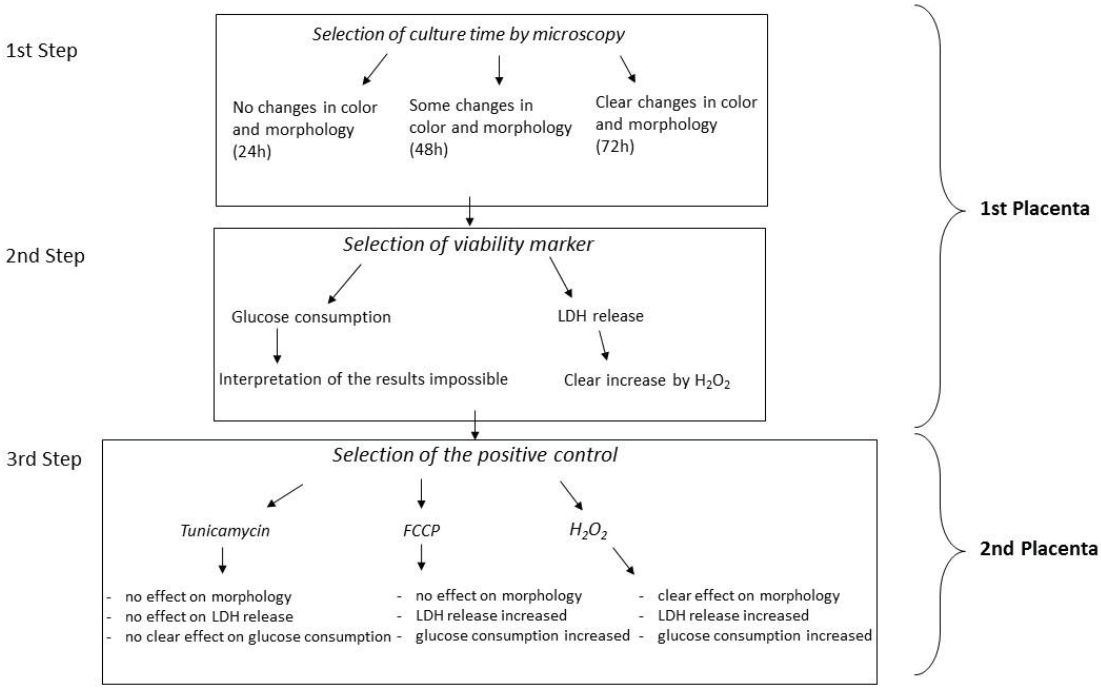


Figure 7. Comparison of culture times, viability markers and positive controls for term placental explant cultures. LDH = lactate dehydrogenase, FCCP = carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, H_2O_2 = hydrogen peroxide.

In addition to microscopy, the viability of term human placental villous explants was also assessed by glucose consumption and the release of lactate dehydrogenase (LDH) to culture medium (Figure 7; 2nd step). With respect to glucose consumption, the results were very different between first trimester and in term placental explants. In first trimester explants, the glucose concentration in medium actually increased from 48 to 72 hours (Figure 8A). The most likely explanation is the explants experience decreased glucose consumption when they start to deteriorate. In contrast, in term placental explants, the glucose concentration in the medium slightly decreased from 48 to 72 hours (Figure 8B), despite the clear changes when the villous' morphology under the microscope showed clear evidence of cell death.

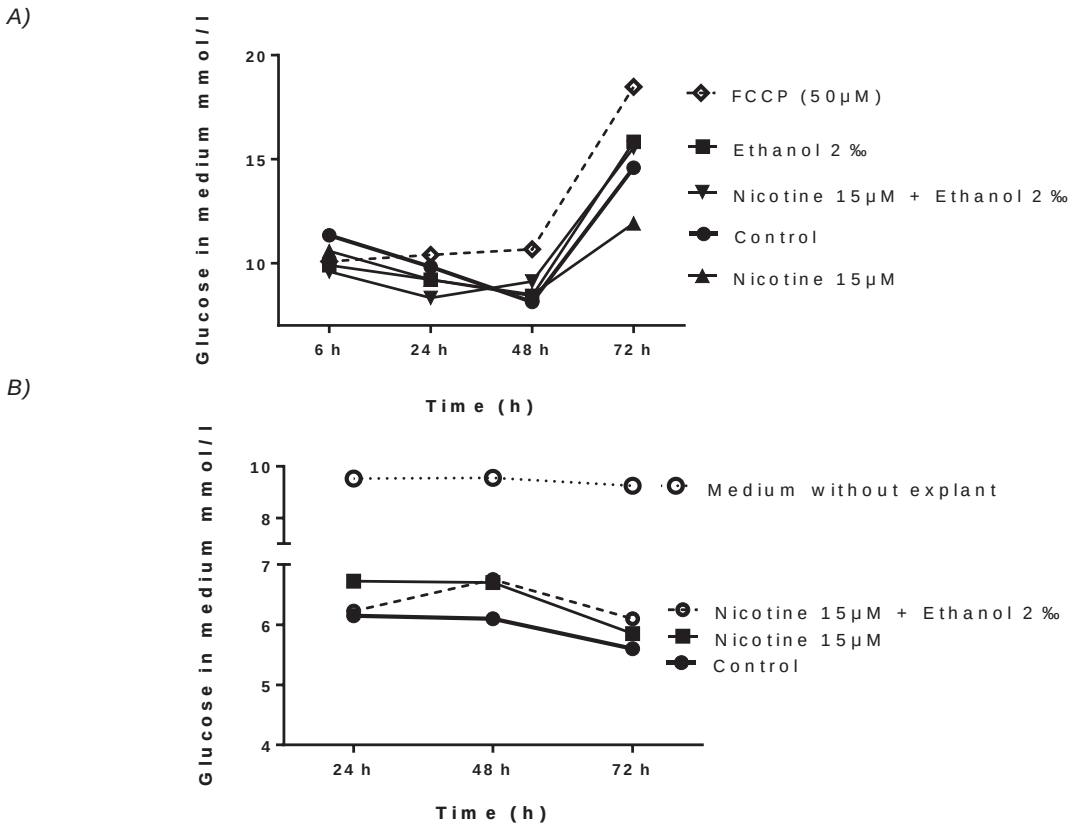


Figure 8. Glucose in the medium of human placental villous explants. A) First trimester explants, 6h (n=5), 24h (n=5), 48h (n=5) and 72h (n=3). B) Term explants, 24h (n=2), 48h (n=1), 72h (n=1). FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, positive control).

In first trimester explants, regardless of treatment, the glucose concentration in the medium was statistically significantly elevated when compared to that at 72h with the respective amount at 0h and 72h (Figure 9A-D), indicating reduced glucose consumption of the explants. A minor decrease in glucose concentration in the medium was also seen at 24h and 48h when compared to the beginning of the experiment, but the difference was not statistically significant, except in the case of the control at 48h (Figure 9A).

In medium, activity of LDH was not higher at 24 hours than at 0 time point, except in explants exposed to the positive control, H_2O_2, indicating that term placental explants were viable (Figure 7; 2nd step, Table 6). The method to culture human first trimester villous explants had been already established by Bechi et al. (2006) and Ietta et al. (2006, 2007) and FCCP was used as the positive control. Thus FCCP was also used as positive control in the experiments of first trimester explants in this study. In the application of the method to

culture term placental villi, some generally recognized and previously used cytotoxic agents in cell culture studies were compared. The effects of the ER stress inducer, tunicamycin (2µM), the mitochondrial uncoupler FCCP (50µM) and the oxidative stress inducer H_2O_2 (20mM) were compared to determine which would be the best positive control (Figure 7; 3rd step). Although both FCCP and H_2O_2 increased the release of LDH and glucose consumption, H_2O_2 (20mM) changed clearly the color and morphology of the explants in microscopy, unlike FCCP. In addition, H_2O_2 is a known and more widely used cytotoxic substance than FCCP in other cell culture studies (Cantoni et al. 1989, Kharfi Aris et al. 2007, Lehtonen et al. 2008). Thus, H_2O_2 was selected as the cytotoxic positive control for term placental explant cultures.

Because the activity of the released LDH in the culture medium varied extensively between experiments and placentas, the only way to present and compare LDH values was to analyze them as the ratio of treated to the corresponding control value of the same placenta and culture plate. The positive controls (FCCP in first trimester explants and H_2O_2 in term placental explants) increased the LDH release on average by more than 1.6 fold when compared to control (Table 6). The mean released LDH ratio (treated/control) of ethanol (2‰), nicotine (15µM) or their combination varied from 0.48 to 1.03. If the LDH ratio is more than one, there is the possibility that the treatment has decreased the viability of the explant. In this study, an LDH ratio of 1.5 was used to separate viable and non-viable explants. However, more studies will be needed to confirm the validity of this ratio for other compounds.

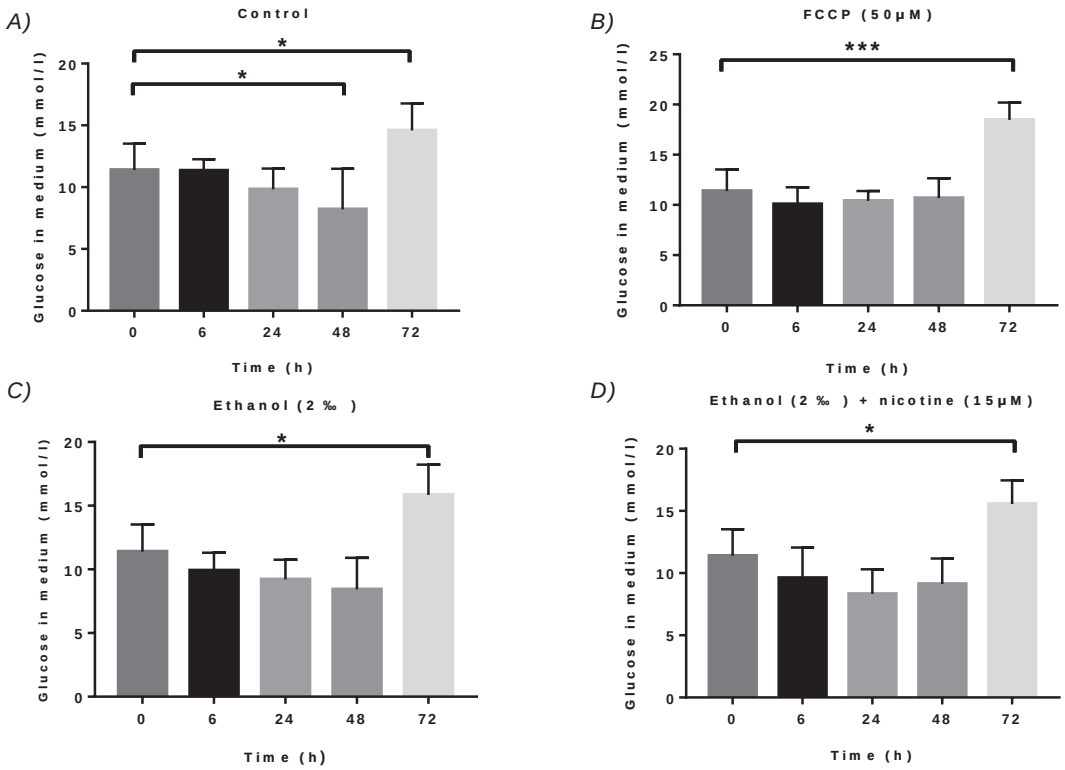


*Figure 9. Glucose in the medium of first trimester human placental villous explants, 6h (n=5), 24h (n=5), 48h (n=5) and 72h (n=3). A) control (no treatment), B) FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, positive control), C) ethanol (2 ‰), D) ethanol (2 ‰) + nicotine (15µM). One-way ANOVA followed by multiple comparison test, only statistical significance compared to 0h are shown. *=p<0.05, ***=p<0.001*

Table 6. LDH in culture medium of first trimester and term human placental explants expressed as a ratio of treated/control.

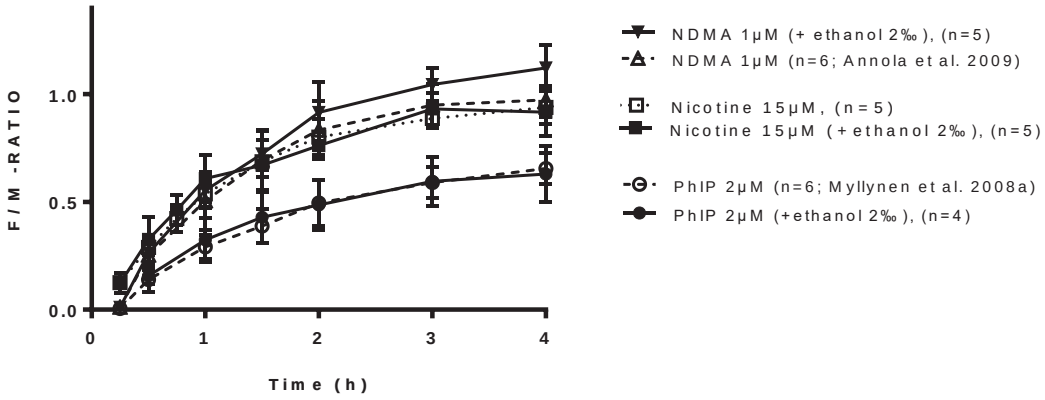
Treatment		24 h	
FIRST TRIMESTER	*n*	mean	SD
FCCP (50µM)	3	1,63	0.15
Ethanol 2 ‰	3	0.58	0.17
Nicotine 15µM	3	0.72	0.18
Ethanol + nicotine	3	0.95	0.23
TERM			
H_2O_2 (20mM)	4	2.45	0.66
Ethanol 2 ‰	4	1.03	0.10
Nicotine 15µM	4	0.48	0.15
Ethanol + nicotine	4	0.63	0.32

SD= standard deviation, FCCP= carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, H_2O_2 = hydrogen peroxide

5.2 TRANSFER OF COMPOUNDS THROUGH HUMAN PLACENTA (I)

Nicotine crossed human placenta easily and was detected in fetal circulation already within 15 minutes (Figure 10). Nicotine also reached equilibrium between maternal and fetal circulations in 3 hours with a mean feto-maternal-ratio (FM-ratio) of 0.94 ± 0.08 (n=5). The mean FM-ratio of nicotine in perfusions with ethanol (2‰) was 0.92 ± 0.11 (n=5) at 4 hours, with no statistically significant difference when compared to perfusions with nicotine only. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) passed through human placenta to some extent (the mean FM-ratio 0.65 ± 0.07, n=6), but the FM-ratio did not reach 1, even with 6 hours of perfusion (data published earlier by Myllynen et al. 2008a). The mean FM-ratio of PhIP in perfusions with ethanol (2‰) was 0.63 ± 0.13 (n=4) at 4 hours, with no statistically significant difference when compared to PhIP only perfusions. NDMA (n-nitrosodimethylamine) crossed human placenta easily (data published earlier by Annola et al. 2009), with a mean FM-ratio of 0.97 ± 0.08 (n=6) at 4 hours. Ethanol increased the transfer of NDMA through human placenta (mean FM-ratio of NDMA with ethanol 1.12 ± 0.11, n=5) at 4 hours, but the difference without and with ethanol was not statistically significant. In all perfusions, the reference compound antipyrine was added at the same time with the studied substances (Figure 10B). The FM-ratio of antipyrine was 0.94 ± 0.09 at 3 h (n=31).

A) TRANSFER OF STUDY COMPOUNDS



B) TRANSFER OF ANTIPYRINE

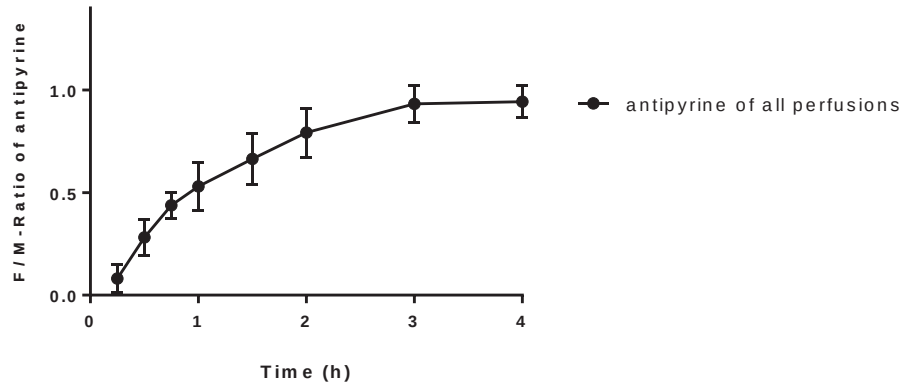


Figure 10. Transfer of NDMA (Annola et al. 2009), PhIP (Myllynen et al. 2008a) and nicotine with or without ethanol in human placental perfusion (A), compared to antipyrine in the same perfusions (B) (n=31). Transfer presented as Feto-Maternal-ratio (F/M-Ratio). PhIP= 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, NDMA=n-nitrosodimethylamine.

5.3 CELL STRESS IN HUMAN PLACENTA (II, III)

Nicotine (15µM) alone and together with ethanol (2‰) increased the expression of factors related to cell stress in human placental BeWo cells (Table 7). Ethanol (2‰) alone did not evoke statistically significant changes in any of the studied proteins related to cell stress in BeWo cells, but when administered together with nicotine, ethanol increased the amount of reactive oxygen species (ROS). Ethanol, nicotine or their combination did not decrease cell viability in BeWo cells at any of the studied time points (24h, 48h and 72h). However, nicotine alone (24h; $p<0.01$; n=4 and 72h; $p<0.05$; n=4) and the combination of ethanol and nicotine (24h; $p<0.01$, n=4) decreased the relative cell number in a statistically significant manner. These results suggest that nicotine alone or together with ethanol reduces the proliferation of BeWo cells.

Table 7. Proteins and other factors related to cell stress in human placental BeWo cells. Only results statistically significantly different from controls (p<0.05) are shown. Statistical significance was determined by one-way ANOVA followed by Dunnett`s test.

Protein / factor	Effect	Substance	Time	*n*
GRP78/Bip	Expression increased	Nicotine (15µM)	72 h	n=4
JNK (Thr 183, Tyr 185)	Phosphorylation decreased	Nicotine (15µM)	24h	n=3
ROS	Increased	Nicotine (15µM) & Ethanol (2 ‰)	24h and 48h	n=4
Relative Cell Number	Decreased	Nicotine (15µM)	24h and 72h	n=4
	Decreased	Nicotine (15µM) & Ethanol (2 ‰)	24h	n=4

GRP78/BiP=Glucose Regulated Protein (BiP), JNK=Jun amino-terminal kinases, Thr=Threonine, Tyr=Tyrosine, ROS=Reactive Oxygen Species

In human placental Bewo cells, nicotine increased the expression of GRP78/BiP and a similar effect of nicotine on this ER stress related protein was observed in human placental explants (Figure 11). Nicotine (15µM) increased the expression of GRP78/BiP in all explants by about 1.5 fold when compared to the non-treated control explants. The effect of nicotine on GRP78/BiP was not statistically significant in term human placental explants. However, in term human placental explants, ethanol (2‰) increased the expression of the ER stress marker GRP78/BiP statistically significantly when compared to non-treated control at 24 hours (n=4, p<0.05). Nicotine (15µM), ethanol (2‰) and nicotine (15µM) + ethanol (2‰) had very similar effects on the expression of GRP78/BiP in both early and late placental explants (study III). Thus, nicotine alone was more toxic than ethanol. There were no synergistic effects evoked by nicotine and ethanol in any of the studied parameters.

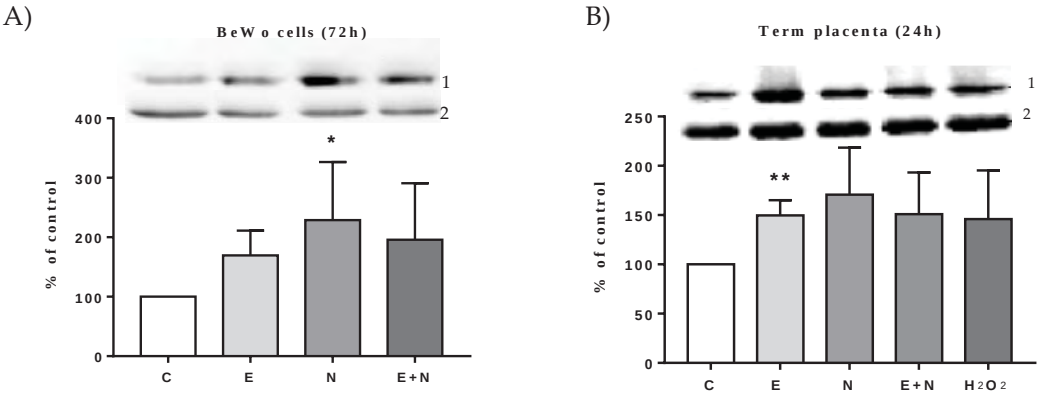


*Figure 11. Expression of GRP78/BiP protein (glucose regulated protein 78) in A) human trophoblastic BeWo cells (mean±SD, n=4) and B) in human term placental explants (mean±SD, n=5). Densitometric values in immunoblotting (20µg proteins of BeWo cells and 30µg of explants) are shown as percent of control. The results were first normalized individually according to the loading control (beta-actin) and then the ratio of GRP78/BiP from treated cells/tissue to the corresponding control (untreated) was calculated. Examples of blots GRP78/BiP (1) and corresponding beta-actin in the same blot (2) are shown. C= control, E= ethanol (2‰), N= nicotine (15µM), H_2O_2=hydrogen peroxide (20mM). One-way ANOVA followed by Dunnett´s test, * = p<0.05, ** = p<0.01*

In human placental BeWo cells, the expression of another ER stress marker that GRP78/BiP directly activates, inositol requiring enzyme 1 (IRE1α), was not changed after treatments of ethanol (2‰), nicotine (15μM) or their combination when compared to control (Study II, Table 8). In human placental villous explants, nicotine (15μM) or ethanol (2‰) increased the expression of IRE1α in both early and late human placental explants (study III). In first trimester explants, IRE1α was not expressed at all in non-treated (control) explants, but the expression increased to the densitometric mean value of 0.78 after treatments with ethanol, nicotine or their combination when compared to the control value (0.00). In term placentas, IRE1α expression was already seen in non-treated (control) villous explants, and the expression increased with ethanol (2‰) and nicotine (15μM) treatments. However, since the variation between the placentas was high, none of these changes reached statistical significance.

Table 8. Expression of inositol requiring enzyme 1 (IRE1α) in BeWo cells and human placental villous explants after no treatment (control) or treatment with ethanol, nicotine or their combination (studies II and III).

	Control	**Ethanol (2 ‰)**	**Nicotine (15μM)**	**Ethanol (2 ‰) + Nicotine (15μM)**
BeWo cells	yes	yes	yes	yes
First trimester human placental villous explants	no	increased *	increased *	increased *
Term human placental villous explants	yes	increased *	increased *	yes

* no statistical significance when compared to control

6 Discussion

6.1 CHEMICAL STRESS IN PLACENTA

6.1.1 Endoplasmic reticulum (ER) stress

Endoplasmic reticulum (ER) stress in human placenta has been linked to many complications in pregnancy, such as placental dysfunction (Yung et al. 2007) and preeclampsia (Yung et al. 2014, Fu et al. 2015). Before we published our paper (II), nicotine-induced ER stress had been studied only in human periodontal ligament cells (Lee et al. 2012). Our report was the first to observe that nicotine increases the expression of GRP78/BiP in a human placental model, indicating ER stress in placenta. GRP78/BiP is the main protein that regulates the unfolded protein response (UPR) in ER and can further activate three different pathways, inositol-requiring protein 1α (IRE1α), pancreatic endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (for a review, see Malhotra and Kaufman 2007). After the appearance of this report, Wong et al. (2015) showed that nicotine exposure increased the amount of GRP78/BiP also in Wistar rat placentas (Wong et al. 2015). Later Wong et al. (2016) have utilized a rat placental cancer cell line (Rcho-1) to reveal that nicotine increases the phosphorylation of PERK, a protein which is directly activated by GRP78/BiP. Recently, we have also shown that nicotine putatively increases the expression of IRE1α in both early and late placentas (study III). All this indicates that nicotine may induce ER stress in human placenta also *in vivo*. In addition to placenta, tobacco smoke extract and nicotine have been shown to trigger ER stress in mouse ocular tissues (Kunchithapautham et al. 2014). ER stress is elevated in preeclamptic placentas, as indicated by the increased expression of GRP78/BiP (Yung et al. 2014, Fu et al. 2015).

At present, there is relatively little information about placental toxicity and its associated mechanisms (Vähäkangas et al. 2014). Alcohol drinking and tobacco smoking are probably two of the most prevalent and preventable causes of toxicity during pregnancy (Stene-Larsen et al. 2013, Tan et al. 2015, Niemelä et al. 2016, Finnish National Institute for Health and Welfare 2016). Alcohol and many of the chemicals present in tobacco smoke are directly toxic to the developing fetus (for reviews, see Kuczkowski 2003, Lassen et al. 2010, Dorrie et al. 2014). However, some effects can be mediated via placental toxicity, although the detailed mechanisms are still not fully understood (for a review, see van Faassen and Niemelä 2011). Tobacco smoking has been linked to epigenetic mechanisms in placenta, related to downregulation of miR-16, miR-21 and miR-146a (Maccani et al. 2010), to placental DNA methylation of GTF2H2C and GTF2H2D genes (Chhabra et al. 2014), and to the over-expression of some nicotinic-acetylcholine receptor subtypes (Machaalani et al. 2014).

Ethanol has been shown to cause ER stress in many other tissues in addition to placenta. C57BL/6 mice were given ethanol subcutaneously and many ER stress related proteins, including GRP78/BiP were increased in fetal brain tissues (Ke et al. 2011). Ethanol induced ER stress has recently been detected in human prostate cancer cells (Kim et al. 2015), in pancreatic tissues of C57BL/6 mouse (Ren et al. 2016) and liver (Yi et al. 2015).

We utilized term human placental villous explants and demonstrated that ethanol induces the expression of the ER stress marker GRP78/BiP and putatively IRE1α too. Ethanol has been reported to activate apoptotic pathways in a dose-dependent manner in the human Jeg-3 placental cancer cell-line (Clave et al. 2014) and to inhibit taurine transfer in human placental BeWo cells and placental first trimester explants (Lui et al. 2014). We demonstrated that ethanol can also induce ER stress in placenta as one potential mechanism of toxicity. Furthermore, our study was carried out with normal human placental tissue, which should

mimic the *in vivo* situation better than can be achieved with cancer cell lines. Cancer cell lines differ from normal placental tissue with respect to the expression of some metabolic enzymes and transporter proteins (for reviews, see Orendi et al. 2011, Vähäkangas et al. 2011). For these reasons, cancer cell lines do not necessarily respond to chemical stress in an identical manner as primary cells or tissue.

6.1.2 Oxidative stress

We also observed in our study that simultaneous exposure to both nicotine and ethanol increased the amount of reactive oxygen species (ROS) in human placental BeWo cells. The statistically significant effect was not seen with individual exposures of these agents; only combined exposure to ethanol and nicotine caused oxidative stress in placenta. The exposure times (24h and 48h) reflected only acute exposure and thus molecular changes occurring after chronic exposure to ethanol and nicotine remain to be clarified. Recently, oxidative stress has been directly linked to the release of the preeclampsia biomarker 'soluble fms-like tyrosine kinase 1' sFLT1 (Huang et al. 2015). Protein sFLT1 together with 'soluble endoglin' (sEng) are both specific markers of preeclampsia, planned to be validated for clinical use in near future (for reviews, see Hod et al. 2015, Phipps et al. 2016).

The study by Kay and co-workers (2000) indicated that physiologically excessively high concentrations of ethanol (5–10‰) could increase the expression of nitrogen species in term placental villous explants after a 2 hours' exposure. As far as we are aware, this is the only study in the literature, which has linked ethanol exposure to nitrosative and/or oxidative stress in placenta. The lowest concentration (2.5‰) used in that previous experiment did not cause any changes in nitrogen species, indicating that with this concentration, oxidative stress is not increased in placenta. Our study is in line with this supposition, because ethanol (2‰) alone did not increase the amount of ROS in BeWo cells.

Both nicotine and ethanol separately are known to evoke oxidative stress in tissues other than placenta (for recent reviews, see Cederbaum et al. 2009, Das and Mukherjee 2010, Mishra et al. 2015). In particular, brain tissue seems to be very sensitive to ethanol-induced oxidative stress (Chen and Sulik 2000, Ramachandran et al. 2003, Heaton et al. 2006). In addition, nicotine has been linked to oxidative stress in other systems, e.g. in murine (MLE-12) and human (BEAS-2B) lung epithelial cells (Zanetti et al. 2014), in HIV-1 transgenic rat brain (Song et al. 2016) and in Wistar rat pancreas (Bhattacharjee et al. 2016).

There is one rat study (Dhouib et al. 2015) examining oxidative stress after the simultaneous exposure to both ethanol and nicotine. Wistar rats were injected subcutaneously with nicotine or the combination of nicotine and ethanol. Both of the treated groups showed an increase in several oxidative stress markers i.e. malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) in lung tissue as compared to control rats. In contrast to expectations, the level of ROS was not increased in our study in ethanol only – treated BeWo cells. Other markers of oxidative stress related to MDA, SOD and CAT, such as thioredoxin, glutaredoxin and peroxiredoxins should also be studied to obtain a more complete picture.

6.1.3 Links between endoplasmic reticulum (ER) and oxidative stress

ER stress and oxidative stress have been linked to each other in many tissues, including mouse liver (Joshi et al. 2015), pancreatic tissue of C57BL/6 mouse (Ren et al. 2016), mouse ocular tissue and human epithelial retinal pigment epithelial (ARPE-19) cells (Kunchithapautham et al. 2014). In placenta, ER stress and oxidative stress are also linked to each other (for reviews, see Burton et al. 2009, Burton and Jauniaux 2011). Furthermore, the MAPK proteins p38 and JNK, can be activated both in oxidative and ER stress (for reviews, see Malhotra and Kaufman 2007, Burton and Jauniaux 2011).

In contrast to expectations, in our study the phosphorylated JNK decreased statistically significantly after nicotine exposure in BeWo cells, even though nicotine exposure increased GRP78/BiP, an indication of ER stress. The phosphorylation and total expression of the p38 protein were increased, although the effect was not statistically significant. Thus, it remains unclear, which pathways finally lead to the decreased proliferation of BeWo cells after their exposure to nicotine. According to Burton and Jauniaux (2011), ER stress in placenta may be detected at lower levels of chemical insults than oxidative stress. Our study seems to be in line with this proposal, because the combined exposure to both ethanol and nicotine caused oxidative stress, but ER stress was seen already with individual exposures of ethanol and nicotine.

We have shown that both oxidative and ER stress are linked to ethanol and nicotine exposure in human placenta as their mechanism of toxicity. Oxidative stress has been shown to directly increase preeclampsia markers (Huang et al. 2015), but more studies with ethanol and nicotine will be needed to reveal their role in preeclampsia. There is evidence that preeclampsia is initiated during the very beginning of pregnancy when the placental cytotrophoblast fails to invade into myometrium (for a review, see Hod et al. 2015). Thus, the studies with ethanol and nicotine in relation to preeclampsia biomarkers need to be studied in early human placental tissues.

6.2 TRANSPLACENTAL TRANSFER OF XENOBIOTICS

6.2.1 Importance of placental transfer

Transplacental transfer of nutrients and fetal medication is needed for the growth, development and well-being of fetus (for a review, see Staud et al. 2012). On the other hand, the transfer of some xenobiotics (alcohol, chemicals in tobacco smoke, other teratogenic compounds) should to be minimized (for reviews, see Obican and Scialli 2011, Ross et al. 2015). Chemicals have to pass through the human placenta to evoke direct toxicity in fetus. Fetus and placenta have a very restricted xenobiotic metabolism and thus concentrations that might be tolerable to the mother, may well be toxic to her fetus (for reviews, see Syme et al. 2004, Vähäkangas and Myllynen 2009).

We found a very different transfer pattern for nicotine compared to PhIP (Myllynen et al. 2008a) through human placenta (Figure 10A), which is in line with the literature (Pastrakuljic et al. 1998, Sastry et al. 1998, Myllynen et al. 2008a). Nicotine transfer followed closely that of antipyrine indicating that passive diffusion was the transfer mechanism (I). This result should mimic the respective human situation better, because in the same perfusions, the reference compound antipyrine crossed placenta easily and very similarly in every perfusion. The transfer of antipyrine through human placenta is one of the most important factors for assessing the success of the perfusion (Mathiesen et al. 2010, Mose et al. 2012, Karttunen et al. 2015). Antipyrine is known to pass through human placenta via passive diffusion (Schneider et al. 1972, Challier et al. 1983) and its transfer is not dependent on concentration (Karttunen et al. 2015), but on the flow rates of the placental circulations (Challier et al. 1983). Transfer of antipyrine (FM-ratio more than 0.75) in the perfusions indicates that there is some necessary overlap between maternal and fetal circulations.

6.2.2 Effects of chemical characteristics on the transfer and metabolism

Molecular weight is one of the most important factors affecting transplacental transfer (Table 9, for a review, see Syme et al. 2004). Compounds that are smaller than 500Da (g/mol) can generally cross human placenta via passive diffusion. However, there are exceptions; in the

study by Woo and co-workers (2012) ochratoxin A (404Da) did not cross human placenta at all, even in a perfusion lasting 20 hours. Thus, every compound has to be experimentally studied, and no judgements can be made simply by examining the molecular weight of the compound.

In addition to molecular weight, pH, lipophilicity and protein binding are also important chemical characteristics affecting the transfer characteristics (for reviews, see Syme et al. 2004, Myllynen et al. 2007). Some chemicals may even accumulate in the fetal circulation or amniotic fluid, for example due to differences in pH between the maternal and fetal circulations. Weak bases and weak acids can cross membranes only in nonionized form. Changes in pH may lead to accumulation to fetal circulation. Some chemicals bind to proteins in maternal circulation, which affects the transfer (Mathiesen et al. 2009), while others may bind to placental tissue (Myllynen et al. 2008b, Soininen and Repo et al. 2015) and never reach fetal circulation. Genotoxic compounds may form DNA-adducts in placenta, if the active metabolites have been known to be formed in placenta through xenobiotic metabolsim (Karttunen et al. 2010). Nevertheless, most of the studied substances are able to pass through human placenta (e.g. Pienimäki et al. 1995, Annola et al. 2008, Karttunen et al. 2010, Partanen et al. 2010, for a recent review, see Myllynen and Vähäkangas 2013).

Table 9. Transplacental transfer of the compounds included in this thesis (I)

Compound	Molecular weight	Transfer from M to F	FM-Ratio	Reference
Antipyrine	188 g/mol	passive	1.00	(I)
Nicotine	162 g/mol	passive	1.00	(I)
NDMA	74 g/mol	passive	0.97	Annola et al. 2009
PhIP	224 g/mol	limited by transporter	0.60	Myllynen et al. 2008a

M=maternal circulation, F=fetal circulation, FM-Ratio= fetal concentration divided by maternal concentration in 4 hour

In this study (I), nicotine (162 Da, 0.5 mg) crossed human placenta in human placental perfusion easily and very similarly to antipyrine, reaching a plateau at 3 hours. Our result is an important addition to the existing literature. Pastrakuljic et al. (1998) and Sastry et al. (1998) also reported transplacental transfer of nicotine similarly to antipyrine (Pastrakuljic et al. 1998, Sastry et al. 1998). However, before we published our study (I), there was discrepant data on nicotine metabolism in human placenta. Sastry and co-workers (1998) had stated that less than 1% of cotinine was found in placental tissue after a 3h perfusion with nicotine (2 mg), indicating that placenta could metabolize nicotine, at least to a minor extent. In our study and also in that of Pastraculjic et al. (1998), no formation of metabolites was detected, which indicates that all of the nicotine present in the maternal circulation can pass through placenta without being subjected to placental metabolism. In addition, there is a lack of the nicotine metabolizing enzyme CYP2A6 in human placenta (Hakkola et al. 1996, Koskela et al. 1999), which supports our conclusion that human placenta is not able to metabolize nicotine. In our study, small and variable concentrations of nicotine were found in placental tissue after four hours of perfusion, but not in the non-perfused control placental tissue. Our study confirms that passive diffusion is the transfer mechanism of nicotine through human placenta and the absence of placental nicotine metabolism in human placental perfusion.

6.2.3 Combined exposure

Because ethanol is known to increase the permeability of biological membranes (Patra et al. 2006), it was deemed interesting to examine whether it could alter the placental barrier to

allow greater exposure of the fetus to nicotine. Against expectations, ethanol did not increase the transfer of nicotine through human placenta (I). Interestingly, in their birth cohort study, Patayova and co-workers (2013) reported that the cord blood samples of the mothers who had consumed ethanol during pregnancy had higher concentrations of polychlorinated biphenyls (PCB) than mothers who did not drink alcohol during pregnancy. Their results were expressed as the ratio of cord blood concentration to the corresponding maternal blood concentration, to exclude environmental factors (i.e different exposure levels of PCBs) and furthermore, the mothers had been exposed to ethanol chronically, throughout the pregnancy, whereas in our perfusion, the exposure to ethanol was short, only 4 hours. If we had had the possibility to access the placentas of alcohol consuming mothers, this could have provided a better understanding of the impacts of chronic exposure to ethanol on human placental tissue.

ABCG2/BCRP is a functionally active transporter in term placenta (Myllynen et al. 2008a). It is known to be sensitive to xenobiotics, because the expression of this transporter is decreased by exposure to metals and hormonal disrupters (Kummu et al. 2012, Sieppi et al. 2016). However, during the perfusion period (I), ethanol had no effect on the function of ABCG2/BCRP, as analysed by the accumulation of fumitremorgin in cells. In addition, ethanol exerted no statistically significant effect on the transfer of PhIP or NDMA in human placental perfusion. Interestingly, the food carcinogen, PhIP, showed a more restricted transfer in human placental perfusion than nicotine or antipyrine. The FM-ratio of PhIP was never more than 0.6, whereas with antipyrine, the mean FM-ratio was 1 at 3 and 4 hours. In the case of other compounds using passive transport for placental transfer, the mean FM-ratio is also known to be about 1 (Mathiesen et al. 2010, Karttunen et al. 2015). On the basis of our results and those by Myllynen et al. (2008a), it is speculated that PhIP may be a ligand for the ABCG2/BCRP transporter.

6.3 HUMAN PLACENTAL VILLOUS EXPLANT CULTURES

6.3.1 Models to compare early and late human placenta

We used human placental explants to compare placentas at different stages of pregnancy (III), because only late placentas can be modelled well with perfusion. Perfusion of first trimester placentas is virtually impossible, because of the fragility of these first trimester placentas. The earliest placentas that have been reported being perfused in human placental perfusion system have been 26–27 weeks old (Loibichler et al. 2002, Nanovskaya et al. 2002) and to perfuse even earlier placentas is most likely impossible. Trophoblastic cells may be isolated from both first trimester and term placentas, but cell-cell communication which is an essential component of transfer studies, is missing in isolated cell cultures (Brucher and Jamall 2014).

Human placenta develops and changes throughout the pregnancy, and thus placentas at different stages of pregnancy would need to be studied separately to collect a more comprehensive picture of the events. Basically, the technique to culture term and first trimester explants do not differ from each other, but the isolation and viability of term placental villous explants may be more challenging as the placenta is at the end of its lifespan (Benirschke et al. 2006). As earlier suggested by Miller and co-workers (2005), in this study, we did not notice any difference in the culture of first trimester and term placental explants.

We compared several known cytotoxic compounds in cell culture studies, such as the ER stress inducer, tunicamycin (Leleu et al. 2009), the mitochondrial uncoupler, FCCP (Keating et al. 2006, Mlejnek and Dolezel 2015) and the oxidative stress inducer, H_2O_2 (Cantoni et al. 1989, Kharfi Aris et al. 2007, Lehtonen et al. 2008) as potential cytotoxic positive controls in

term placental explants to ensure that the viability tests were working properly. Surprisingly, tunicamycin did not decrease the viability at 24 hours, even though both FCCP and H_2O_2 were toxic. H_2O_2 was the only compound that caused clear microscopically detectable changes in color (turning the explants whitish). Thus, H_2O_2 was selected as the positive control to be used in term explant culture studies; in fact, there is no consensus in the literature on what represents the best positive control.

6.3.2 Viability markers in villous explant culture

In human placental villous explant cultures, the isolation of explants may cause severe changes which can be detected in the microscope (for a review, see Miller et al. 2005). For optimal results, it is crucial to exclude non-viable explants before starting any exposures. Thus, only the explants that look viable under the microscope are selected for further experiments. However, for more subtle changes, viability markers are needed to estimate the success of explant cultures. In toxicology studies, a viability marker that can be measured from the medium is often preferred, because normally all the tissue of a small explant (~100 mg) is needed for the actual experiments. At present, the human placental villous explant method has not been validated, and thus no consensus exists about what represents the best viability marker.

Our study (III) indicated that glucose consumption was very difficult to interpret as a viability marker, because it seemed to change due to culture time and the developmental stage of placenta. There are four studies in the literature, where glucose consumption has been used as a viability marker in term human placental explants (Chen and Lin 1998, Di Santo et al. 2003, Reti et al. 2007, Hulme et al. 2012). In all these studies, regardless of the treatment, a decrease in the glucose consumption rate was detected at 12–24 h. In our study, glucose consumption decreased in first trimester explants and increased in term explants at 72 hours. It was very difficult to interpret whether an increase in glucose consumption (and at which level) was a sign of toxicity or a sign of viability. The tissue displays increased glucose consumption as a result of oxidative stress before glucose stores are totally consumed (Kozlovsky et al. 1997). This complicates the use of glucose consumption as a viability marker.

In the published reports in the literature, if viability markers have been used at all, LDH has been the most common viability marker in placental explants and in isolated placental trophoblast cells (Siman et al. 2001, Reti et al. 2007, Audette et al. 2010, Messerli et al. 2010, Sengupta et al. 2011, Vaidya et al. 2011). LDH release has been a widely exploited viability marker in many cell-lines (Legrand et al. 1992), but its use in human placental villous explants has not been thoroughly investigated. In our study (III), treatments with ethanol, nicotine or their combination did not clearly increase the LDH activity in the medium (Table 6), whereas positive controls induced an over 1.6 fold increase in LDH activity. In the literature, LDH activity has been reported in many ways, which naturally makes the comparison of the results very difficult. Units of LDH per number of cells (Sengupta et al. 2011), LDH as a mass (μg) (Reti et al. 2007), LDH in units/L/mg of wet tissue (Vaidya et al. 2011) and percentages (%) of LDH when compared to control explant (Messerli et al. 2010) have been used when reporting LDH values. In our study (III), the LDH-values varied extensively between experiments and thus the only valid way to make comparisons between experiments was to compare the exposure values to the corresponding control values (LDH-ratio). Many laboratories utilize a LDH-cytotoxicity kit, which is based on the comparison of the absorbance results to positive and negative controls but the kit does not provide a reference compound for a standard curve. Without a standard curve, it is impossible to convert absorbance into exact units. Thus, according to our results, of the two viability markers studied, glucose consumption and LDH release, LDH seemed to be a promising viability marker with the ratio treated/control being the best way to describe it numerically.

In the future, it would be of interest to seek other viability markers for human placental villous explants, such as secretion of hormones and the placenta protein 13 (Huppertz et al.

2011). Placenta protein 13 is a relatively new factor associated with cytotoxicity that can be measured from the medium. Recently, it has been also linked to preeclampsia (Huppertz et al. 2013, De Muro et al. 2016). The decreased urinary levels of PP13 at the beginning of pregnancy have shown a correlation with pregnancy related complications, such as preeclampsia, later in pregnancy (De Muro et al. 2016).

The use of viability markers is crucial in explants studies, because it is necessary to decide whether the observed toxicity is due to specific toxicity evoked by the study chemical or is it due to an overall reduced viability of the explants. Isolation of villous explants is a stress-causing factor, and some explants do not survive the isolation procedure (for a review, see Miller et al. 2005). Thus, it is not sufficient to include control explants for each experiment, instead the viability of all explants needs to be evaluated as well.

6.4 COMPARISON OF THE USED MODELS TO MIMIC TOXIC EFFECTS IN HUMAN PLACENTA AND STUDY LIMITATIONS

Most papers in the literature using human placental models have used the tissue taken from term placenta (Contractor and Krakauer 1976, Orendi et al. 2011, DuBois et al. 2012), even though human placenta develops throughout the pregnancy (for a review, see Benirschke et al. 2006). In this work, the human trophoblastic cancer cell line BeWo (II), human placental villous explant cultures of first trimester and term placentas (III) and perfusion of human term placenta (I) were used to study toxic effects and transplacental transfer of ethanol and nicotine in human placenta. In the literature, there are only a few studies that have compared first and third trimester placentas (Sooranna et al. 1999, D'Elia et al. 2012, Lye et al. 2015), but no studies have been published on the effects of ethanol or nicotine in early and late placentas.

Human placental perfusion mimics in vivo conditions of placenta very closely (for a review, see Vähäkangas and Myllynen 2006), but obviously it lacks the influence of the surrounding tissues and body, and thus cannot fully reflect the physiological situation. In this work, ethanol and nicotine crossed human placenta with ease in human placental perfusion (study I) and our results are in line with the literature (Idänpään-Heikkilä 1972, Pastrakuljic et al. 1998, Sastry et al. 1998). Due to the practical difficulties inherent in placental perfusion (Karttunen et al. 2015), it is reasonable to apply the simplest model that can answer to the study question. For instance, protein expression can be studied as well with human placental villous explants that are also normal placental tissue and express the same ABC transporters and xenobiotic metabolizing enzymes as the placental tissue used for human placental perfusion (Table 10). However, the stage of the pregnancy determines the protein expression of ABC transporter proteins and metabolizing enzymes (for reviews, see Vähäkangas and Myllynen 2006, Vähäkangas et al. 2011). The expression of ABC transporter proteins seems to be decreased by xenobiotics (Sieppi et al. 2016), which potentially has been a confounding factor in many studies showing different expression levels of certain ABC transporter proteins at different stages of pregnancy (Mathias et al. 2005, Meyer zu Schwabedissen et al. 2006, Yeboah et al. 2006).

Here, we observed some differences in the expression of stress proteins between the BeWo cell line and human placental villous explants after treatment with ethanol and nicotine. Since we did not detect any change in the expression of IRE1α in BeWo cells (II), instead there was a clear treatment-induced increase of the same protein in both first trimester and term placental explants with ethanol or nicotine (III). Therefore, we conclude that cancer cell lines, such as BeWo cells, are not the best model with which to study stress responses in placenta. Most probably, the discrepant results are due to the fact that ER stress is upregulated in

cancer cells in general (for a review, see Yadav et al. 2014). However, the upregulation of stress proteins has not been confirmed in human placental cell lines. Because the human trophoblastic cancer cell line BeWo, as with many other cancer cell lines, may change due to long culture time, many authors recommend short culture times to minimize the genetic drift (Capes-Davis et al. 2013, Kleensang et al. 2016). Furthermore, in this study, an immunoblotting method was used to analyze the proteins, which is at best, only a semi-quantitative method. This will obviously affect the reliability of the results. More quantitative techniques, such as mass spectrometry -based metabolomics, are planned to be used in the future.

Table 10. Comparison of human placental models and their features used in this thesis (data from Vähäkangas and Myllynen 2006, Orendi et al.2011, Vähäkangas et al. 2011, Vähäkangas et al. 2014).

	BeWo cell line	**Villous explants**	**Placental perfusion**
Origin	trophoblastic cancer cells	normal placental tissue	normal placental tissue
Stage of pregnancy	not defined	first trimester and term[a]	third trimester / term
Expression of proteins			
ABC-transporter proteins	ABCB1/P-gp[b] ABCC1/MRP1 ABCC5/MRP5 ABCG2/BCRP	ABCA1 ABCB1/P-gp ABCB4/MDR3 ABCB5 ABCG2/BCRP ABCG1 ABCC1/MRP1 ABCC2/MRP2 ABCC3/MRP3 ABCC5/MRP5	ABCA1 ABCB1/P-gp ABCB4/MDR3 ABCB5 ABCG2/BCRP ABCG1 ABCC1/MRP1 ABCC2/MRP2 ABCC3/MRP3 ABCC5/MRP5
Xenobiotic metabolizing enzyme proteins	no expression of CYP3A4 or CYP2C9; other CYPs not studied[d]	CYP19A1[c] CYP1A1[c] CYP11A1 CYP2J2 CYP2R1 CYP27B1 CYP24A1	CYP19A1[c] CYP1A1[c] CYP11A1 CYP2J2 CYP2R1 CYP27B1 CYP24A1
Transplacental transfer studies possible	only with the clone b30 in a Transwell system	no	yes
Intact placental barrier	no	no	yes

a) *also second trimester possible, but rarely used;* b) *very low or nonexistent (Vähäkangas et al. 2014); c) CYP1A1 and CYP19A1 (aromatase) are functional throughout the pregnancy; d) data from Pavek et al. 2007, expression of enzymes may vary between BeWo cells from different laboratories.*

The models of this work represent only acute exposure from a couple of hours to a maximum of three days. In the future, longer exposures or other methods will be needed to clarify the chronic effects of the studied substances. In vivo studies using placentas of mothers who smoked or drank alcohol during their pregnancy would make it possible to gain insights into the impacts of more chronic exposures (for a review, see Vähäkangas and Myllynen 2006). In this work, human placental toxicity was studied in first trimester and term placentas, but no information from mid-gestational placentas was gathered. That would require placentas

from spontaneous abortions, these would be difficult to acquire and would probably face insurmountable ethical problems.

The models used in this work, represent only placental toxicity and no direct conclusions about fetal toxicity can be drawn. However, human placenta is mostly of fetal origin (for a review, see Benirschke et al. 2006); it is reasonable to hypothesize that placental tissue and cells may be taken as a surrogate for toxicity in the fetus. Usually it is not possible to study fetal tissues due to ethical reasons, but cord blood and other samples taken after birth do represent the fetal period and thus can be exploited to evaluate fetal exposure and toxicity. Furthermore, if placenta is exposed, the fetus is also exposed, because most chemicals are able to pass through the human placenta (Pienimaki et al. 1995, Annola et al. 2008, Karttunen et al. 2010, Partanen et al. 2010). In addition, any toxicity in placental tissue will obviously harm the fetus, because the normal function of placenta is crucial for fetal development and growth. For all of these reasons, it is very important to develop models utilizing human placenta to estimate better whether fetal toxicity can be evaluated using human placental experimental models.

7 Summary and Conclusions

1) No agreement exists on the best viability markers for human placental villous explant cultures. In our study, microscopy, glucose consumption and lactate dehydrogenase (LDH) were used. When viewed under the microscope, the color and morphology of villous explants changed in conjunction with the decrease in their viability. Glucose consumption was very difficult to interpret as a viability marker, because it seemed to change more due to culture time rather than to the observed signs of toxicity. The released LDH in the medium increased statistically significantly among the increased toxicity, analysed by the MTT test and microscopy. Thus, LDH was regarded as a consistent and exploitable marker with which to estimate the viability of explants. The positive controls H_2O_2 (used in term explants) and FCCP (used in first trimester explants) caused a clear increase in the LDH released into the medium. The viability of term human placental villous explants did not differ from the culture of first trimester explants, as analyzed by microscopy and LDH release. To estimate subtler changes in decreased viability, more investigations will be needed to screen functional markers such as production of hormones. In addition, a change of the culture medium in relation to viability should also be studied in the future.

2) Although ethanol is known to damage cell membranes, ethanol added concurrently did not affect the transfer of nicotine, PhIP or NDMA in human placental perfusion. Nicotine alone crossed human placenta with the same kinetics as the reference compound antipyrine, an indication that passive transfer was its transfer mechanism. Both antipyrine and nicotine reached a feto-maternal ratio of 1 within 3–4 hours. On the other hand, the feto-maternal ratio of the food carcinogen, PhIP, equilibrated between the fetal and maternal circulations only to value of 0.6 in 3 hours and did not change even in perfusions of 4–6 hours. Ethanol did not have any statistically significant effect on the transfer of nicotine, PhIP or NDMA in human placental perfusions of 4–6 hours. There is a difference between our results and the literature, because chronic exposure to ethanol has been shown to increase the fetal exposure of PCBs, *in vivo,* as analyzed by cord blood. Our studies were conducted for only a short period of time (about 4–6h) and with term placentas, which probably explains the differences. In addition, the effect of ethanol was only studied in combination with nicotine, PhIP and NDMA. The effect of ethanol on the transfer of other compounds should also be examined.

3) Nicotine increased the expression of GRP78/BiP in BeWo cells statistically significantly, indicative of the presence of ER stress. In addition, ethanol and ethanol+nicotine increased the expression by about 1.7 fold, but the difference was not statistically significant. In addition, the relative cell number, but not the viability of BeWo cells, was decreased with the combination of nicotine and ethanol. This indicates decreased proliferation, most probably due to the observed ER stress. Nicotine and ethanol concurrently increased the amount of reactive oxygen species (ROS) in BeWo cells, further evidence of oxidative stress in placenta. The effects in primary tissue were evaluated by exposing early and late human placental villous explants to ethanol, nicotine and their combination. All treatments increased the

expression of GRP78/BiP at least by 1.5 fold. However, the difference was statistically significant only with ethanol treatment. There was no statistically significant difference in another ER stress marker, IRE1α, in term villous explant cultures and in BeWo cells, treated with ethanol, nicotine or their combination when compared to control. In conclusion, results obtained in BeWo cells and placental villous explants seem to correlate well in the studies related to oxidative and ER stress when these preparations are exposed to ethanol, nicotine or their combination. In the future, other compounds should also be evaluated with these methods in order to estimate the applicability of BeWo cells in relation to human placental explants.

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JENNI REPO

Many pregnant women both smoke and drink alcohol during pregnancy. This thesis confirms the transplacental transfer of ethanol and nicotine through human placenta and provides new information about the mechanisms of toxicity of these substances in placenta. Thus, exposure to both ethanol and nicotine during pregnancy pose a health risk to the developing fetus not only due to transplacental transfer but also by direct interference with normal placental function. This study also compares different experimental models allowing human placenta to be exploited as a research tool.

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