



The effect of ethanol and nicotine on ER stress in human placental villous explants

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ABSTRACT

Pregnant mothers continue smoking and drinking during pregnancy. To clarify the mechanisms of nicotine and ethanol toxicity during development, we have examined their effects on endoplasmic reticulum (ER) stress in human first trimester and term placental explants. First trimester and term human placental explants were treated with ethanol (2 %) or nicotine (15 μ M), or their combination. The ER stress markers glucose regulated protein 78 (GRP78/BiP) and inositol requiring enzyme 1 α (IRE1 α) were analyzed by immunoblotting. A statistically significant increase ($p < 0.05$) of GRP78/BiP by nicotine was noted in first trimester placental explants at 48 h, and in term placental explants at 24 h. Ethanol did not change protein expression of GRP78/BiP in either first trimester or term placental explants. IRE1 α increased, although not statistically significantly, by all treatments in both first trimester and term placental explants. Thus, regardless of the known structural and functional differences in early and late placenta, both responded very similarly to the toxic compounds studied. These data support our earlier results in BeWo cells (Repo et al., 2014) implicating that nicotine induces ER stress in human placenta and may interfere with placental functions potentially disrupting fetal growth and development.

Introduction

Despite the known adverse effects of ethanol and nicotine, in Europe the prevalence of smoking and drinking during pregnancy is around 8 % and 25 %, respectively (Popova et al., 2017; Lange et al., 2018). Ethanol has long been known to cause many types of developmental disorders collectively known as Fetal Alcohol Spectrum Disorders, the most conspicuous of which is fetal alcohol syndrome (FAS). At cellular level ethanol can induce both oxidative and endoplasmic reticulum (ER)-stress in many tissues, mainly shown in animals (for reviews see Burton and Jauniaux, 2011; Dorrie et al., 2014). The effects of nicotine on placental physiology, e.g. on placental blood flow have been studied in animals (Birnbaum et al., 1994) and in human placental perfusion (Bainbridge and Smith, 2006), but molecular toxicity in human placental tissue has not yet been studied. Nicotine serum concentrations when smoking cigarette or using nicotine replacement therapy may vary between 25 nm and 25 μ M (DeVeugh-Geiss et al., 2010; Massadeh et al., 2009; Oncken et al., 1997) and 2% ethanol corresponds to state where clinical symptoms include e.g., confusion, impaired balance, and slurred speech. In this study we used dose levels of nicotine (15 μ M) and

ethanol (2%) that corresponds to the worst-case scenario of tobacco smoking and alcohol use (Skurtveit et al., 2002; Massadeh et al., 2009).

Placental villous trees are finger-shaped functional units of placenta, and they consist of syncytiotrophoblast that forms the surface of the villous trees. Beneath the syncytiotrophoblast are cytotrophoblasts, a heterogenous and mononucleated stem cell population, that by fusion give rise to syncytiotrophoblast, and fetal endothelial cells (Benirschke et al. 2006). Human placental villous explant cultures have been rarely used in toxicology studies, even though explants are primary tissue. In addition, human placental models are superior because of the differences between species as to placental structure and physiology (Benirschke et al., 2006). Human placenta goes through extensive development throughout pregnancy, with significant differences between early and late placentas (Benirschke et al., 2006; Huppertz et al., 2014). It has been shown that during the nine months of development the expression of transporters (Walker et al., 2017), enzymes (Myllynen et al., 2007) and proteins (Sarvestani et al., 2021) are significantly changed. Thus, it is feasible to anticipate that the effects of chemicals may vary between early and term placenta.

We found earlier (Repo et al., 2014) in human trophoblastic cancer

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cells (BeWo) an increase in GRP78/BiP protein by nicotine indicating ER stress in placenta. In this study we used human placental villous explant cultures from first trimester and term placentas using the same concentrations of ethanol and nicotine as in BeWo study, allowing comparison of the effects in primary human placental tissue with BeWo cells. Similar responses as in BeWo cells were found in villous explants supporting ER stress response in human placenta by nicotine.

Materials and methods

Chemicals

Ethanol was from Altia Corporation (Finland) and hydrogen peroxide (H₂O₂) was from Fisher Scientific (UK). (-)-Nicotine was from Sigma-Aldrich, St. Louis, MO (USA). RPMI-1640 without phenol-red and Dulbecco's Phosphate Buffered Saline (DBPS) were from Lonza, Verviers (Belgium).

Culture of first trimester human placental villous explants

The culture method of first trimester explants has been published before (Caniggia et al., 1997; Jetta et al., 2007; Sieppi et al., 2016). Eleven first trimester placentas were collected (6–10 weeks of gestation, from the Hospital of Campostaggia, Siena, Italy) but only five showed structural integrity and therefore used for experiments at the University of Siena (Department of Life Sciences). Placental tissues were obtained after voluntary elective termination of pregnancies and the local ethics committee approved the study (VITRO-RIP 2013). Volunteering healthy women signed a consent after given written and oral information. It is not known whether the women used tobacco or alcohol. Placental samples were anonymized (no links between the code and the person were retained). Placentas were handled as previously described (Caniggia et al. 1997) with minor modifications. Briefly, pieces of the placentas were rinsed in a tube with 10 ml of ice-cold PBS. Villous trees were isolated under a stereomicroscope. Villi were placed onto 96-well polystyrene culture plates, one explant per well and cultured in 200 µl of RPMI-1640 without phenol-red, but with 1% of penicillin–streptomycin. A floating culture technique that is described by Miller et al. (2005) was used. The number of used placentas for different experiments are given in the figure legends.

Culture of term placental explants

The culture method of first trimester explants was applied to term human placental villous explants. Altogether five term placentas were obtained after normal delivery (n = 1) or Caesarean section (n = 4) (>36 weeks of gestation, from the Kuopio University Hospital, Finland). The local official Ethics Committee of the Northern Savo Hospital District in Kuopio, Finland approved the study (No. 54/2007, 30.5.2007). Non-smoking, not heavy drinking, healthy women (no signs of pre-eclampsia or no major diseases) were asked to participate in the study, and volunteering women signed a consent after given written and oral information. Placental samples were anonymized. Preparation of tissue and the culture method of term placentas was the same as the one used for first trimester explants, except that 24-well plates and a volume of 500 µl of culture medium was used in each well because term villi were naturally larger than first trimester villi. The number of used placentas for different experiments are given in the figure legends.

Treatment of explant cultures

After the establishment of the cultures in 24-well plates, they were incubated at 37 °C for 24 h. Next day, medium was replaced by a freshly prepared medium containing the study compound and treated for 24 and 48 h. Each treatment including the controls was done in triplicate with explants from the same placenta and on the same plate,

constituting one experiment. Compounds were diluted in culture medium. Control wells (three in each plate) were untreated.

Analysis of lactate dehydrogenase

Lactate dehydrogenase (LDH) was analysed with the in vitro Toxicology Assay Kit (Sigma-Aldrich, St. Louis). A medium sample (30 µl) was pipetted in duplicates or triplicates into the wells of a 96-well plate. LDH assay mixture (60 µl) was added in each well. After 30 min of incubation (at room temperature), the absorbance at 490 nm was analyzed and the background at 690 nm was subtracted from the primary values. LDH-values were expressed as a ratio (treated/control). H₂O₂ was used to show that toxicity releases LDH in term placenta explants.

Tissue lysis and protein extraction for immunoblotting

The first trimester explants were lysed by sonication (three times 10 s for 20 Hz + 130 V) in 30 µl of RIPA lysis buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton x-100; 1 % Na-Deoxycholate; 0.1 % SDS, pH 7.5) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich) and 0.1 mM Na-orthovanadate (Sigma-Aldrich). Term explants were lysed by sonication as above in 45 µl per explant of RIPA-like lysis buffer (50 mM Tris-HCl; 250 mM NaCl; 0.1 % SDS; 0.5 % NP40, pH 7.4) with 1 % protease inhibitors (Complete Mini Protease Inhibitor cocktail tablets, Roche) and 0.1% phosphatase inhibitors (Halt Phosphatase inhibitor cocktail, Thermo Scientific, Rockford, USA). The explants were kept on ice during the lysis. Lysates were stored at –80 °C. Protein concentration of the lysates was determined using the Bradford Protein assay (Bio Rad Microscience, Germany).

Immunoblotting

Protein extractions and antibody incubations (for antibodies and used dilutions see Table 1) were carried out essentially the same way for all explants. Protein bands were visualized with ECL select or ECL Prime system (Immunoblotting detection system, Amersham BioSciences, Buckinghamshire, UK) according to the instructions by the manufacturer. Protein bands of the first trimester explants (Bhattacharjee et al., 2010) were visualised in blue nitrocellulose membranes after processing in dark room, and protein bands of term placentas were visualised with the help of ImageQuantTM-RT ECL (Amersham, UK). All protein bands were analysed by densitometry using the Quantity One Software (1-D Analysis Software, version 4.6.3, Bio-Rad Laboratories Inc., USA).

Statistical analysis

The one-way ANOVA followed by the Tukey's multiple comparison post hoc test was used to analyse the statistical significance with the help of Graphpad Prism 7.0 (GraphPad Prism software, USA). The p-value < 0.05 was regarded as statistically significant.

Results and discussion

In this study we used the in vivo worst-case scenario concentrations of nicotine (15 µM) and ethanol (2‰) that were not causing placental explant cell death according to LDH release (data not shown). Chen et al. (2020) used several nicotine concentrations (0.1–10 µM) at three time points, that did not cause cell death in HTR-8/SVneo cells supporting our viability results. However, a two times higher concentration of ethanol (4 mg/ml), compared to the one used in this study (2‰ that equals to 2 mg/ml), has been shown to cause cell death in HTR-8/SVneo cells at 48 h (Shanmugam et al., 2019). Based on the viability studies and our previous studies in BeWo cells, the used concentrations of nicotine and ethanol were considered relevant and not affecting the molecular markers studied in the explants. An increase in the expression of GRP78/BiP was observed after the treatment with ethanol, nicotine or

Table 1
Antibodies and their dilutions used in immunoblotting experiments.

Primary antibody	Company	Dilution	HRP-labeled Secondary antibody	Company	Dilution
GRP78/BiP (C50B12)	Cell Signaling	1:1000	Anti-Rabbit IgG	Calbiochem	1:2000
IRE1 α (14C10)	Cell Signaling	1:1000	Anti-Rabbit IgG	Calbiochem	1:2000
Nrf-2 (D1Z9C)	Cell Signaling	1:1000	Anti-Rabbit IgG	Calbiochem	1:2000
nAChR α 7 (sc-5544)	Santa Cruz Biotechnology	1:1000	Anti-Rabbit IgG	Calbiochem	1:2000
Heme oxygenase 1 (sc-136256)	Santa Cruz Biotechnology	1:500	Anti-Mouse IgG	Amersham	1:2000
β -actin	Sigma-Aldrich	1:2 \times 10 ⁻⁵	Anti-Mouse IgG	Amersham	1:2000

HRP = horseradish peroxidase; GRP78/BiP = glucose regulated protein 78; IRE1 α = inositol requiring enzyme 1 α ; Nrf-2 = nuclear factor-like 2; nAChR α 7 = nicotinic acetylcholine receptor subtype α 7.

their combination. However, the effect was statistically significant only in the case of nicotine in first trimester placental villous explants at 48 h and in term villous explants at 24 h (Fig. 1A and B). These results are in line with our previous study with BeWo cells (Repo et al., 2014) and support the hypothesis that nicotine can cause ER-stress in human placenta thus potentially disturbing placental functions and harming the fetal development. Also, an in vivo study with rat placenta supports these findings (Wong et al., 2015).

Changes in GRP78/BiP expression are functionally important in placenta as indicated by the data showing that GRP78/BiP is overexpressed and involved in trophoblast invasion at the beginning of pregnancy (Arnaudeau et al., 2009; Ghahesi-Fard et al., 2015). Furthermore, GRP78/BiP interacts with the tumor suppressor protein p53 in trophoblastic cells during invasion by inactivating and stabilizing the function of p53 (Arnaudeau et al., 2009). In addition to early placenta, there are two studies in the literature which link overexpression of GRP78/BiP protein in term placenta to preeclampsia (Yung et al., 2014; Fu et al., 2015). Thus GRP78/BiP seems to be an important protein in placenta throughout the pregnancy, and nicotine seems to be able to change its level, with possible detrimental effects on placental development and function. However, it must be highlighted that the way of birth of the placenta (vaginal delivery vs. Caesarian section) influences ER stress markers by increasing the expression of e.g. GRP78/BiP (Veerbeek et al., 2015). In our study, from five term placentas one was from vaginal delivery that may have affected the basic level of GRP78/BiP.

Clearly measurable amounts of IRE1 α were found in first trimester placental explants after treatment with ethanol, nicotine or their combination, while extremely low level of IRE1 α were found in untreated explants (Fig. 2A). However, none of the changes reached statistical significance. In term placental explants neither ethanol, nicotine nor their combination induced statistically significant changes. (Fig. 2B). Both mRNA and protein expression of IRE1 α has been reported by Fu and coworkers (2015) who showed it in similar levels in preeclamptic and normal placentas (third trimester or term placentas). According to our knowledge, there are no other studies about IRE1 α expression in early human placenta. It must be considered that total IRE α was measured in this study, not the phosphorylated form which is the active form of the protein. The consistent increase, although not statistically significant, in the expression of total IRE1 α compared to control levels after all treatments suggests a biologically significant effect.

Neither ethanol nor nicotine caused any significant changes in the expression of the two studied oxidative stress markers in preliminary experiments (Nrf2, HOX1, Fig. 3) speaking against oxidative stress being behind ER stress. Protein expression of the nicotinic acetylcholine receptor subtype α 7 (nAChR α 7) in human term placenta was confirmed (Kwon et al., 2007). No consistent changes in the expression of nAChR α 7 by ethanol or nicotine were seen in the experiments carried out (Fig. 3). Interestingly, Chen et al. (2020) have shown that immortalized first trimester extravillous trophoblast (HTR-8/SVneo) cells also express nAChR α 7 both at mRNA and protein level.

There are two reasons to pursue human placental villous explant cultures. Firstly, there are significant differences in placental structure and physiology between the species (Benirschke et al., 2006; Heinonen, 2015). Secondly, placental villous culture is normal tissue and thus

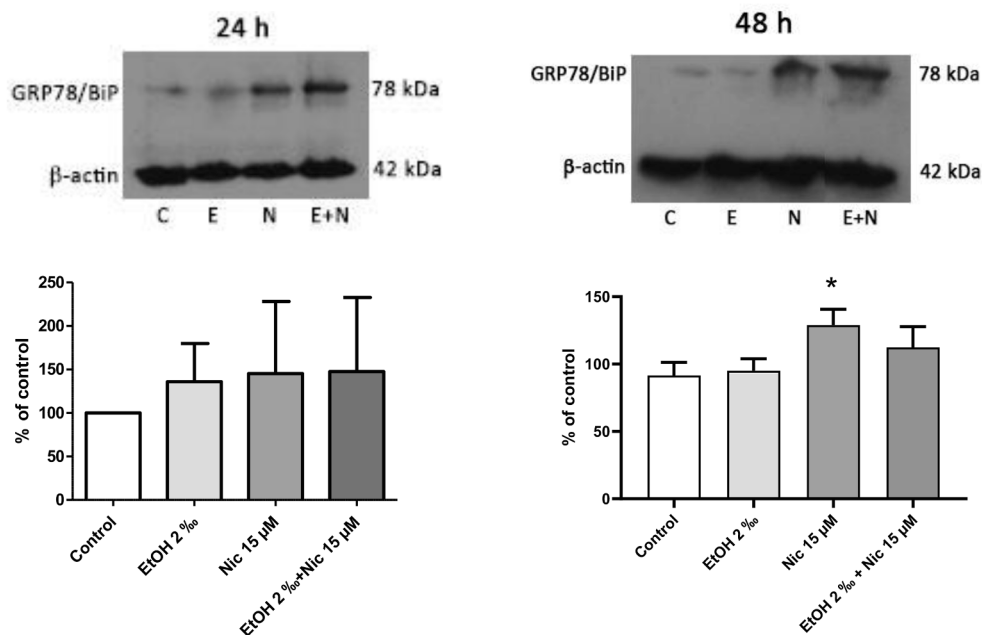
preferable to placental trophoblastic cancer cells lines such as BeWo, to study toxicity of chemical compounds in the placenta (for a review see Gohner et al., 2014). However, endogenous and exogenous factors can change the function of the tissue causing interindividual differences between placentas (Vähäkangas and Myllynen, 2006). Also, the culture conditions of the villi may have an influence on the results. Lye et al. (2013) showed that different oxygen level has an impact on the gene and protein expression of transporters. Although Miller and co-workers (2005) suggest no difference in the culture of early and term human placental explants, culture at term may be more challenging because placental tissue is at the end of its lifespan and viability may be restricted (Benirschke et al., 2006).

There are limitations in this study which may have affected the results. The number of placentas is limited and the way of delivery of term placentas may have influenced responses to stressors. In addition, endogenous and exogenous factors can increase interindividual differences between placentas (Vähäkangas and Myllynen, 2006). Detailed demographic information (smoking, drinking, medicine use) of the mothers is missing and they can influence the results. This needs to be taken into account in the future studies using human placental villous explants. The culture conditions of the villi may have an influence on the results. However, in all cases control villi from the same placenta were used. Also, the culture conditions were the same for all experiments. The size of villi between different cultures may vary somewhat but the relation between syncytiotrophoblast and cytotrophoblasts are quite the same because the used placentas represent restricted periods of pregnancy (first 6–10 weeks and term > 36 weeks). In this work we used only single doses of nicotine and ethanol, not allowing dose-responses evaluation of the effect. Finally, only two shorth time-points were used, and the results thus reflect acute effects only.

Compounds in the tobacco smoke, such as benzo(a)pyrene (Karttunen et al., 2010) and nicotine (Veid et al., 2011), as well as ethanol (Veid et al. 2011) go easily through human placenta and may be toxic in placental tissue (for a review, see Karttunen et al., 2017). In a recent review about the toxic effects of nicotine (Suter and Aagaard, 2020) the authors point out that “no amount of nicotine is known to be safe in pregnancy”. In this study nicotine induced ER stress in human placenta as indicated by the increased expression of GRP78/BiP. Also, the data showing no changes in cell viability suggests that ER stress activation is a cell response aimed to restore placental homeostasis. Further studies are, however, needed to investigate other molecular responses induced by nicotine and/or ethanol in placenta. In the future, organoid technology with new in vitro models of placenta, e.g. trophoblast organoids (Turco et al., 2018, Sheridan et al., 2020), can be used in studies on placental and fetotoxicity. Also, a recent study (Kupper et al., 2021) on placental villus explants showed better integrity of the tissue in flow culture compared to traditional static culture, the culture method we have used in this study.

In conclusion, regardless of the known structural and functional differences in early and late placentas, both responded very similarly to the toxic compounds studied. These results support our earlier results in BeWo cells (Repo et al., 2014) implicating that nicotine induces ER stress in human placenta and may interfere with placental functions potentially disrupting fetal growth and development.

A) First trimester placental explants



B) Term placental explants

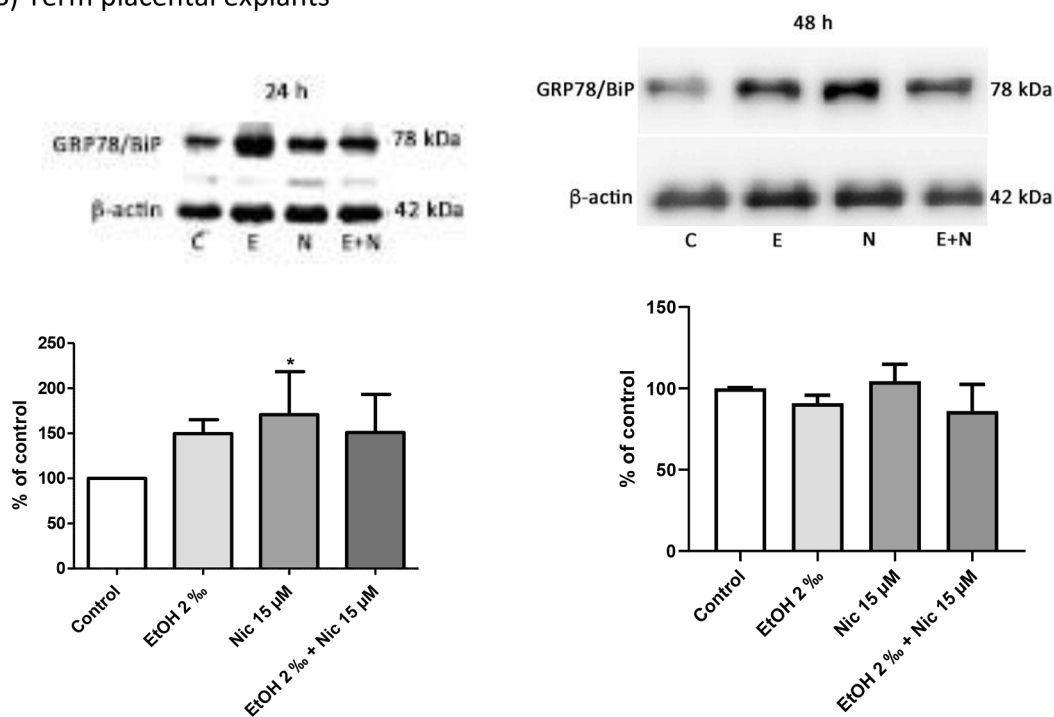


Fig. 1. Protein expression of the glucose regulated protein 78 (GRP78/BiP) in human placental villous explants 24 h and 48 h after treatments analyzed by immunoblotting. Each treatment was done in triplicate with explants from the same placenta and on the same plate constituting one experiment. The results were first normalized individually by the loading control beta-actin. (A) Columns: mean \pm SD, n = 5. (B) Columns: mean \pm SD, n = 5 in 24 h, n = 3 in 48 h. A representative immunoblot of GRP78/BiP and beta-actin in the same blot are shown in each case. The bands were quantified by densitometry and expressed as percent of control. C = control E = ethanol (2 ‰), N = nicotine (15 μ M). Statistical significance was analyzed by the One-way ANOVA followed by the Tukey's multiple comparison post hoc test. * p < 0.05 compared to control.

CRediT authorship contribution statement

M. Huovinen: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **F. Ietta:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing, Visualization. **J.K. Repo:** Methodology,

Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **L. Paulesu:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **K.H. Vähäkangas:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

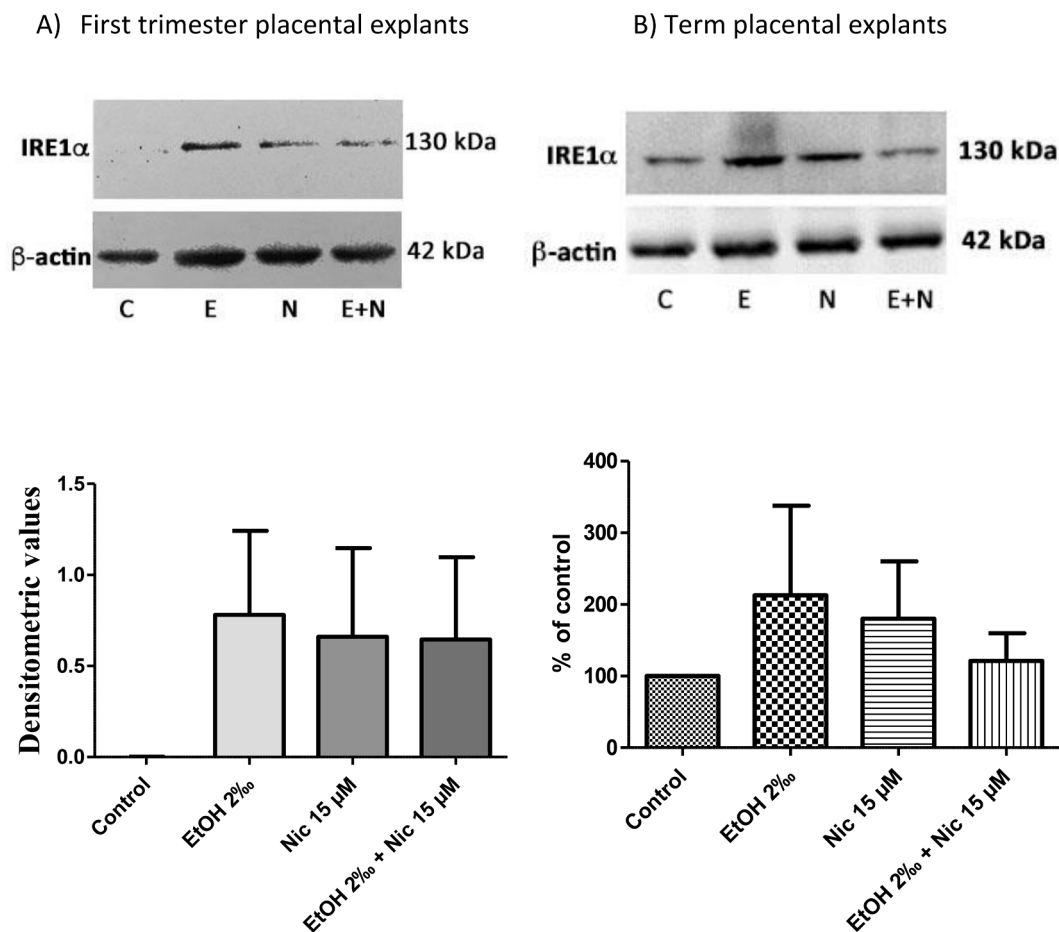


Fig. 2. Protein expression of the inositol requiring enzyme 1 α (IRE1 α) in human placental villous explants at 24 h after various treatments analyzed by immunoblotting. Each treatment was done in triplicate with explants from the same placenta and on the same plate constituting one experiment. The results were first normalized individually by the loading control beta-actin. (A) Columns: mean \pm SD, n = 3. Because the control did not express IRE1 α , absolute densitometric values are given. (B) Columns: mean \pm SD, n = 5. A representative immunoblot of IRE1 α , rehybridized for beta-actin are shown in each case. The bands were quantified by densitometry and expressed as percent of control. C = control, E = ethanol (2‰), N = nicotine (15 μ M).

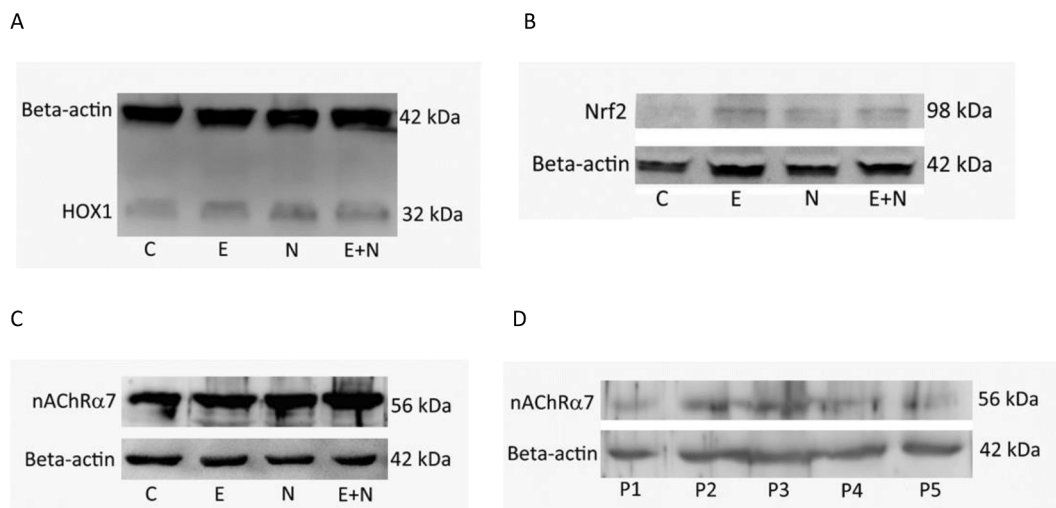


Fig. 3. Protein expression of heme-oxygenase 1 (HOX-1), nuclear factor-like 2 (Nrf-2) and nicotinic acetylcholine receptor subtype α 7 (nAChR α 7) in term human placental explants. Expression of A) HOX-1, B) Nrf2 or C) nAChR α 7 in control (no treatment) or after various treatments and corresponding beta-actin (in the same blot) at 24 h. D) Expression of nAChR α 7 in the control samples (no treatment) of five term placentas and corresponding beta-actin (in the same blot). P = placenta, C = control, E = ethanol (2‰), N = nicotine (15 μ M), E + N = ethanol (2‰) and nicotine (15 μ M).

Declaration of Competing Interest

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

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