Toxicology Letters 225 (2014) 216–221

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Ethanol cytotoxic effect on trophoblast cells

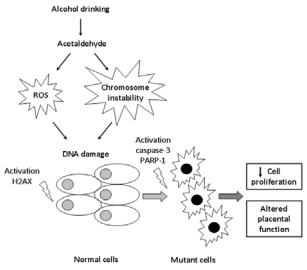
S. Clave[a,b,1], X. Joya[a,b,1], J. Salat-Batlle[a,b], O. Garcia-Algar[a,b,c,*], O. Vall[a,b,c]

[a] Unitat de Recerca Infància i Entorn (URIE), Institut Hospital del Mar d'Investigacions Mèdiques (IMIM), PRBB, C/ Dr. Aiguader 88, 08003 Barcelona, Spain
[b] Red de Salud Materno Infantil y del Desarrollo (SAMID), Programa RETIC, Instituto Carlos III, C/ Sinesio Delgado 4, 28029 Madrid, Spain
[c] Departament de Pediatria, Obstetricia i Ginecologia, i Medicina Preventiva, Universitat Autònoma de Barcelona (UAB), Campus M, 08193 Bellaterra, Spain

HIGHLIGHTS

- Sustained ethanol exposure causes cytotoxicity in JEG3 trophoblast cells.
- The mechanism is by activating apoptotic pathways as a result of DNA damage.
- This activation of apoptosis represents an induction of cellular damage.
- This damage can be related in part to functional alterations in placenta.
- These results may explain the consequences in other tissues, such as neurons.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 November 2013
Received in revised form
18 December 2013
Accepted 19 December 2013
Available online 27 December 2013

Keywords:
Alcohol
Ethanol
Placenta
Apoptosis
Cytotoxic effect
Trophoblast

ABSTRACT

Prenatal ethanol exposure may cause both, altered fetal neurodevelopment and impaired placental function. These disturbances can lead to growth retardation, which is one of the most prevalent features in Fetal Alcohol Syndrome (FAS). It is not known whether there is a specific pattern of cytotoxicity caused by ethanol that can be extrapolated to other cell types. The aim of this study was to determine the cytotoxic effects caused by sustained exposure of trophoblast cells to ethanol. The cytotoxic effect of sustained exposure to standard doses of ethanol on an *in vitro* human trophoblast cell line, JEG3, was examined. Viable cell count by exclusion method, total protein concentration, lactate dehydrogenase (LDH) activity and activation of apoptotic markers (P-H2AX, caspase-3 and PARP-1) were determined. Sustained exposure to ethanol decreased viable cell count and total protein concentration. LDH activity did not increased in exposed cells but apoptotic markers were detected. In addition, there was a dose-dependent relationship between ethanol concentration and apoptotic pathways activation. Sustained ethanol exposure causes cellular cytotoxicity by apoptotic pathways induction as a result of DNA damage. This apoptotic induction may partially explain the altered function of placental cells and the damage previously detected in other tissues.

1. Introduction

Chronic alcohol consumption can cause damage to several organs, resulting in certain disturbances (Benassi-Evans and Fenech, 2011). Most importantly, prenatal chronic exposure to ethanol can produce severe deleterious effects on neurodevelopment. The exact mechanism of this damage is unknown and it depends on gestational age of the exposed fetus and the level and characteristics of sustained consumption. There are different hypotheses and placental damage due to ethanol is one of them. One of the health consequences of alcohol consumption in pregnant

* Corresponding author at: Unitat de Recerca Infància i Entorn (URIE), Institut Hospital del Mar d'Investigacions Mèdiques (IMIM) – Parc de Salut Mar, Parc de Recerca Biomèdica de Barcelona (PRBB), C/ Dr. Aiguader 88, 08003 Barcelona, Spain. Tel.: +34 932483551; fax: +34 932483254.
E-mail address: 90458@hospitaldelmar.cat (O. Garcia-Algar).
[1] These authors contributed equally to this work.

0378-4274/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2013.12.018

women is Fetal Alcohol Syndrome (FAS), a condition induced by the exposure of the developing embryo to ethanol (Jones and Smith, 1973). The clinical features of FAS can be broadly divided into: morphological malformations, especially craniofacial defects, central nervous system impairment, neuropsychological traits, and growth retardation (Moore et al., 2007; Rostand et al., 1990). Offsprings of mothers who drink heavily during pregnancy can develop FAS with all the symptoms described above, but in some cases there is no physical or morphological evidence of prenatal alcohol effects at birth, which is explained by variations in the extent of exposure and continuity (Gemma et al., 2006). On the other hand, current estimates suggest that at least 9.1/1000 of the pediatric population has Fetal Alcohol Syndrome Disorder (FASD) (Sampson et al., 1997).

Of the three clinical features of FAS, growth retardation is the most prevalent (Greene et al., 1991; Hellstrom et al., 1996; Pennington et al., 1983). Specifically, intrauterine growth retardation has been partly associated with a placenta that cannot fulfill all its functions during pregnancy (Gundogan et al., 2008; Salihu et al., 2010). Since placenta acts as a barrier to protect the fetus from toxic chemicals coming from the maternal circulation, there is a possible accumulation of these compounds which can induce changes in placental cells (Kuczkowski, 2007; Ortigosa et al., 2012). It has been shown previously that placental function can be altered by ethanol exposure (Burd et al., 2012).

Furthermore, considering other cell types, previous studies have described apoptotic induction in neurons located in the developing brain (Luo, 2012). It has been also described that prenatal ethanol exposure causes damage to endocrine and hypothalamic neurons (Hellemans et al., 2008; Sarkar et al., 2007) and that, upon ethanol exposure during fetal development, a large number of these neurons undergo cell death by apoptotic pathways (Chen et al., 2006).

Apoptotic *in vitro* studies should include viable cell counts to see how the total number of cells varies. Assays such as Trypan Blue dye exclusion method and total protein concentration measurement are common (Rodriguez-Gonzalez et al., 2013). The necrosis levels should be quantified as well by calculating the amount of cells which died by plasma membrane ruptures. It is therefore common to use intracellular markers, such as lactate dehydrogenase (LDH), which can be released to the medium due to this cause (Krysko et al., 2008). Finally, programmed cell death markers, such as histone H2AX phosphorylation (P-H2AX) (Svetlova et al., 2010), caspase-3 activation (Porter and Janicke, 1999) and poly-ADP-ribose polymerase 1 cleavage (PARP-1) (Soldani and Scovassi, 2002) should be determined.

In this sense, prenatal ethanol exposure disrupts placental function, possibly causing intrauterine growth retardation (Gundogan et al., 2008; Salihu et al., 2010). By the other hand, it is also known that ethanol can cause apoptosis in several types of neurons (Chen et al., 2006; Hellemans et al., 2008; Luo, 2012; Sarkar et al., 2007). Knowing ethanol's cytotoxic effects on trophoblast cells, may allow to extrapolate the consequences to other cell types which underwent the same alcohol exposure pattern.

The aim of this study was to determine the cytotoxic effects of sustained ethanol exposure in an *in vitro* system of human trophoblast cells as a surrogate marker of damage due to prenatal exposure to ethanol.

2. Materials and methods

2.1. Cell culture

The aim was to use specifically placental cells previously used in the literature. Human placental choriocarcinoma cell line was purchased from the American Type Culture Collection (ATCC): JEG3 (HTB-36, Manassas, USA). Cells were maintained in Minimum Essential Media (MEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 20 mM L-glutamine, 10 mM sodium pyruvate, 100 mg/mL streptomycin and 100 U/mL penicillin; all get from Gibco, Montreal, CA. Cell cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

2.2. Experimental design

In this study, a comparison between non-exposed (Group C or control) and exposed placental cells to standard concentrations of ethanol was done (Karl et al., 1998; Karl and Fisher, 1993). It was decided to expose every 72 h two groups of placental cells to concentrations of 25 mM (Group A) and 50 mM ethanol (Group B) (Eckardt et al., 1998; Jones and Sternebring, 1992) as a two growing concentrations identified as comparables to moderate and heavy chronic consumption of ethanol in humans. To prevent evaporation of ethanol during this incubation period, we used culture dishes wrapped in Parafilm (Eysseric et al., 1997). This system almost completely stabilized the ethanol concentration in the culture medium for 3 days with an acceptable loss of 20% ethanol (Eysseric et al., 1997; Ramadoss et al., 2011; Signorini-Allibe et al., 2005). Control cells were cultured in the same conditions in the absence of ethanol. These three groups were grown in separate flasks during the sustained exposure. Cells from each group were subcultured every two passages to perform the experiments. Each sustained experimental was replicated five times at the beginning period and five more at the end.

Cytotoxic effects studied were the reduction of cell count and total protein concentration as biomarkers of cellular necrosis, and the increase of LDH, caspase-3, P-H2AX and PARP-1 as biomarkers of apoptosis.

2.3. Viable cell count by exclusion method

Cells of the three groups were subcultured at a seeding density of 0.1×10^6 viable cells/mL in 100 mm plates (Corning Life Sciences, Amsterdam, The Netherlands). Cell viability was determined at the moment when these plates were trypsinized, using Trypan blue/haemocytometer exclusion method (Rodriguez-Gonzalez et al., 2013).

2.4. LDH activity measurements in supernatants

Supernatants were collected from the three groups subcultured in 100 mm plates, centrifuged at 12 000 rpm and stored at −80 °C until assayed. The assessment of LDH activity was performed using the Lactate Dehydrogenase Activity Assay Kit (Sigma–Aldrich, St. Louis, USA) following the manufacturer's instructions. The conversion of NAD to NADH was detected colorimetrically at 450 nm (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

2.5. Caspase-3 immunocytochemistry

Cells of the three groups were subcultured in pretreated slides (Thermo Fisher Scientific, Rockford, USA) at a seeding density of 0.1×10^6 viable cells/mL. Cells were rinsed with Phosphate Buffer Saline (PBS) (Gibco, Montreal, CA), fixed with 4% (v/v) paraformaldehyde (Sigma–Aldrich, St. Louis, USA and permeabilized with Triton X-100 and blocked joints with 5% (v/v) bovine serum albumin (BSA), both obtained from Sigma–Aldrich, St. Louis, USA. Cells were incubated with anti-cleaved caspase-3 1:100 (9664, Cell Signaling, Danvers, USA) for 1 h at mild agitation. After the incubation, the cells were washed with PBS and incubated with a secondary antibody, 1:700 Alexa555 (A-31851, Molecular Probes, Montreal, CA). Nuclei were counterstained with DAPI 1:10 000 Mowiol content. Both products were purchased from Sigma–Aldrich, St. Louis, USA.

2.6. Total protein concentration and P-H2AX and PARP-1 Western blotting

Cell extracts were prepared from 100 mm plates by adding 100 μL of cold lysis buffer (50 mM Tris–HCl, 150 mM $NaCl_2$, 2 mM EDTA, 50 mM NaF, pH 7.4) containing proteases and phosphatase inhibitors. All chemicals were purchased from Sigma–Aldrich, St. Louis, USA. Samples were maintained for 10 min on ice and centrifuged at 12 000 rpm. Protein level was determined with DC Protein Assay (Bio-Rad, Hercules, USA). Protein lysates (50 μg) were run in a SDS/PAGE electrophoresis (90 min, 100 mV) and transferred to nitrocellulose membranes (Whatman, Kent, UK). The membranes were blocked with 4% dehydrated milk and blotted with anti-PARP-1 1:1000 (supplied by J. Yelamos laboratory) (Saenz et al., 2008) and anti-P-H2AX 1:1000 (Ab-139, Sigma–Aldrich, St. Louis, USA). Subsequently, the membranes were washed in TBS-Tween and incubated for 60 min with a goat anti-mouse IgG-HRP (1:10 000) (Santa Cruz Biotech; sc-2005) washed with TBS-Tween and developed with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, USA) according to manufacturer's instructions.

2.7. Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) or Student's *t*-test (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean ± SD of the five biological replicates at the beginning period and five more at the end period. A p value of less than 0.05 was considered statistically significant.