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Effects of Ethanol Sustained Exposure on Human Trophoblast Cell Hormonal Production

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Abstract

Foetal Alcohol Syndrome Disorder (FASD) is a complex condition resulting from the consumption of alcohol during pregnancy. Diagnosis of prenatal exposure to alcohol depends on questionnaire about consumption, biomarkers in neonatal alternative matrices as maternal hair and neonatal meconium, and postnatal clinical data as facial features. However, biomarkers of cellular damage due to exposure to alcohol during pregnancy are lacking. Prenatal alcohol exposure can lead to altered placental cellular function, resulting in changes in hormone production. Herein, the alteration in the synthesis of this hormonal output was studied using a cultured human trophoblast cell line (JEG3). The production of insulin-like grow factor 2 (IGF2), human chorionic gonadotrophin (hCG), human placental lactogen (hPL) and pregnancy specific glycoprotein 1 (PSG1) was analyzed. Sustained ethanol exposure significantly increased the cellular production and release of IGF2 and hCG in a dose-dependent manner related to the ethanol input. Moreover, ethanol exposure also caused a loss in cell viability and a significant decrease in total protein production. This hormonal alteration may be used in future studies as preliminary candidate to validate surrogate biomarkers of damage.

Keywords: Alcohol; Trophoblast cell; IGF2; hCG; Biomarkers of damage; Ethanol

Introduction

Chronic alcohol consumption can cause damage to various organs, resulting in different disturbances [1]. One of the health consequences of alcohol consumption in pregnant women is Foetal Alcohol Syndrome (FAS), a condition resulting from the exposure of the developing embryo to ethanol [2,3]. The clinical features of FAS can be broadly divided into: morphological malformations, especially craniofacial defects, central nervous system impairment, neuropsychological traits, and growth retardation [4,5]. Offspring of mothers who drink heavily during pregnancy can develop FAS with all the symptoms described above, but some cases show no physical or morphological evidences of prenatal alcohol effects at birth [6]. On the other hand, current estimates suggest that at least 9.1/1000 of the paediatric population has Foetal Alcohol Syndrome Disorder (FASD). A large majority of this group is characterized by adverse neurobehavioral consequences that may be misdiagnosed for years, diminishing the beneficial prospect of earlier interventional opportunities [7,8].

Neonatal FAS can be properly diagnosed based on its facial features, but for the majority of FASD cases, strategies for diagnosis are lacking. The detection of these newborns and children has been focused in questionnaire about consumption and biomarkers of exposure as direct ethanol, ethanol metabolites or compounds that chemically interact with ethanol in neonatal alternative matrices [9].

Up to date, the determination of Fatty Acid Ethyl Esters (FAEEs) in meconium or maternal hair is the best procedure to identify exposed newborns, but with a cut off value in the range of heavy drinking population. In addition, there are not biomarkers that offer reliable information about the injury in the foetus [10]. In this sense, only a few number of papers have proposed potential biomarkers of damage associated to prenatal ethanol exposure [11,12].

Placenta is the most accessible foetal-maternal tissue after birth and provides valuable information about the course of pregnancy [13]. As known previously, placental function can be altered by ethanol exposure, resulting in an increased risk for a wide range of adverse pregnancy outcomes [14]. For these reasons, placenta could be considered a target tissue to find biomarkers of damage.

Herein, as a preliminary *in vitro* study, we used JEG3 trophoblast cell line which is specifically present in the syncytiotrophoblast and is the major hormone-producing cell line, expressing a high number of hormones and growth factors [15,16]. Of all these, insulin-like grow factor 2 (IGF2), human chorionic gonadotrophin (hCG), human placental lactogen (hPL) and pregnancy specific glycoprotein 1 (PSG1) are the most relevant based on previous published research [17-19].

So, the main objective of this study was to evaluate the effect of sustained ethanol exposure on the hormonal production of JEG3 cultured trophoblasts to propose preliminary surrogate biomarkers of damage.

Materials and Methods

Cell culture

Human placental choriocarcinoma cell line [20,21] was purchased from the American Type Culture Collection (ATCC): JEG3 (HTB-36, Manassas, USA). Cells were maintained in Minimum Essential Media

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(MEM) supplemented with 10% (*v/v*) Foetal 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 37ºC in humidified 5% CO_2 atmosphere.

Experimental design

Similarly to previous studies [17,22], a comparison between placental cells non-exposed and exposed to standard concentrations of ethanol was done. It was decided to expose every 72 h two groups of cells at concentrations of 25 mM and 50 mM ethanol (VWR, Radnor, USA). These concentrations are equivalent to the expected in the human placental tissue from moderate ethanol consumers (5-90 mg/dL; approximately 20 mM) [23] and in alcohol-depending consumers (386 mg/dL; approximately 85 mM) [24]. To prevent evaporation of ethanol during this incubation period, we used culture dishes wrapped in parafilm [25]. This system almost completely stabilized the ethanol concentration in the culture medium for 3 days with an acceptable loss of 20% of ethanol [25-27]. Control cells were cultured in the same conditions in the absence of ethanol. These three groups were grown in separated flasks during all the sustained exposure. Every five passages we obtained three subculture replicates of each group to carry out the experiments, at a seeding density of 0.1×10^6 viable cells/mL in 100 mm-plate (Corning Life Sciences, Amsterdam, The Netherlands). Cells and culture medium of these replicates were harvested after 1 h, 24 h and 72 h of the last ethanol exposure [28,29] (Figure 1).

Western blotting

Cell extracts were prepared from 100 mm-plate cell culture 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 protein and phosphatase inhibitors; all purchased from Sigma-Aldrich, St. Louis, USA. Samples were maintained 10min on ice and centrifuged at 12000rpm. Protein level was determined with DC Protein Assay (Bio-Rad, Hercules, USA). Protein lysates (50 µg) were run in a SDS/PAGE and transferred (90 min, 100 mV) to nitrocellulose membranes (Whatman, Kent, UK). Membranes were blocked with 4% dehydrated milk and blotted with the indicated primary antibodies O/N at 4ºC (Table 1). Membranes were washed in TBS-Tween and incubated for 60min with a goat anti-mouse IgG-HRP (1:10000) (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. To quantify, ImageJ software (National Institute of Health, USA) was used. The values of 25 mM and 50 mM groups were normalized against the control group.

ELISA assays

Cell medium of every 100 mm-plate were recovered and centrifuged at 1200 rpm. These mediums were used as samples for ELISA kits for testing IGF2 and hCG (Biovendor, Modrice, Czech Republic). The procedure was carried out according to manufacturer's instructions. Measurement of absorbance was done at the established wavelength of 450 nm (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Results were plotted with the standard curve and normalized by the number of viable cells counted at the moment of harvesting.

Viability assays

To extrapolate the ELISA results in relation to the number of cells alive, cell viability was determined at the moment of harvesting 100 mm-plates of the three lineages using trypan blue/haemocytometer

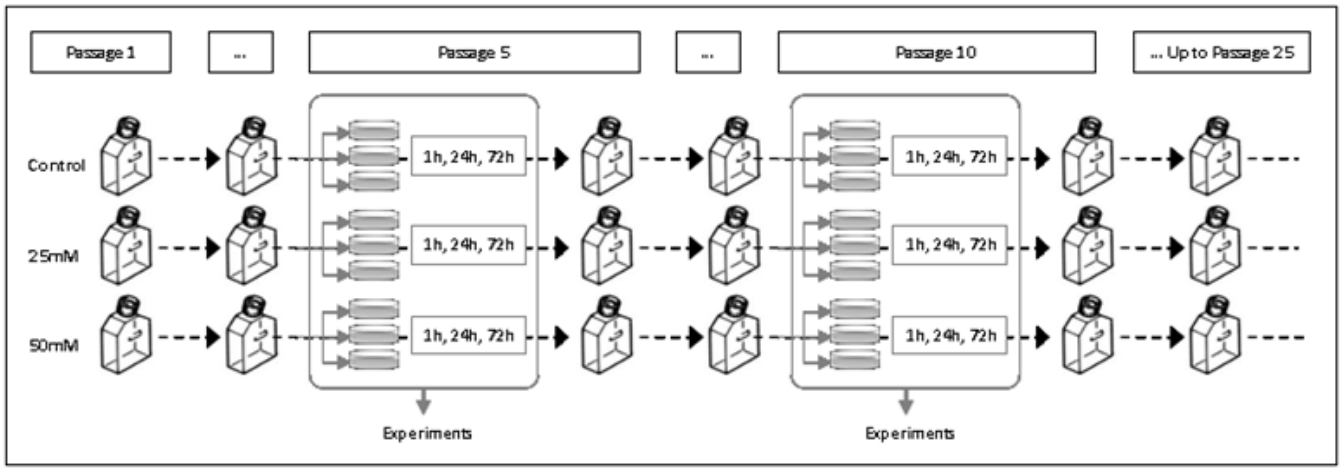


Figure 1: Experimental design: Control, 25 mM and 50 mM groups were groups were grown separated flasks during all the sustained exposure. Every five passages we obtained three subculture replicates of each group to carry out the experiments, at a seeding density of 0.1×10^6 viable cells/ml in 100 mm- plate. Cells and culture medium of these replicates were harvested after 1 h, 24 h and 72 h of the last ethanol exposure.

Target	Source	Species	Size(kDa)	Dilution
IGF2	Santa Cruz Biotech.; sc-74119	Mouse	23	1 : 1000
hCG	R&D Systems; MAB4169	Mouse	28	1 : 2000
hPL	Abcam; EPR8264	Rabbit	25	1 : 3000
PSG1	Abcam; ab114940	Mouse	69	1 : 1000
B-Actin	Sigma-Aldrich; A3859	Mouse	42	1 : 10000

Table 1: Antibodies used for Western blot detection.

exclusion method. Moreover, protein levels were determined with DC Protein Assay.

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 ± standard deviation of five biological replicates. A p value less than 0.005 was considered statistically significant.

Results

Effects of ethanol exposure in JEG3 cells IGF2 production

Alcohol treated trophoblast cells showed an important increase in the expression of IGF2 (Figure 2). The 25 mM and 50 mM groups showed stronger bands in Western blot analyses when compared with the control group (Figure 2A). Quantified bands showed an increasing trend in IGF2 release depending in the exposure time and dosage (Figure 2B). To validate these results, an ELISA test was performed. ELISA results confirmed increased IGF2 release due to ethanol exposure in a dose depending manner (Figure 2C). The 25 mM group showed significant increases in IGF2 values after 72 h exposure time (p<0.005 vs. control). On the other hand, the 50 mM group, showed significant increases values after 1h, 24h and 72h exposure times (p<0.005 vs. control) (Figure 2D).

Effects of ethanol exposure in JEG3 cells hCG production

Trophoblast cells treated with ethanol showed an increase in the expression of hCG (Figure 3). The 25 mM and 50 mM groups showed stronger bands in Western blot analyses when we compared with the control group. This fact was more evident in 50 mM group (Figures 3A and 3B). The 50 mM group increased hCG release after 1h, 24h and 72h exposure times (p<0.005 vs. control) (Figures 3B and 3D). In the ELISA test, 50 mM group showed significant increases after all exposure times (p<0.005 vs. control) (Figures 3C and 3D).

Effects of ethanol exposure in JEG3 cells hPL and PSG1 production

Ethanol exposure did not change hPL and PSG1 levels significantly. In Western blot analyses, an increment of hPL was observed in each 25 mM group exposure times, but it decreased to the control group level when the cells were treated with 50 mM. PSG1 levels were lower in both exposed groups when compared with the control group, but these differences were not significant.

Cell viability and protein levels in JEG3 culture cells

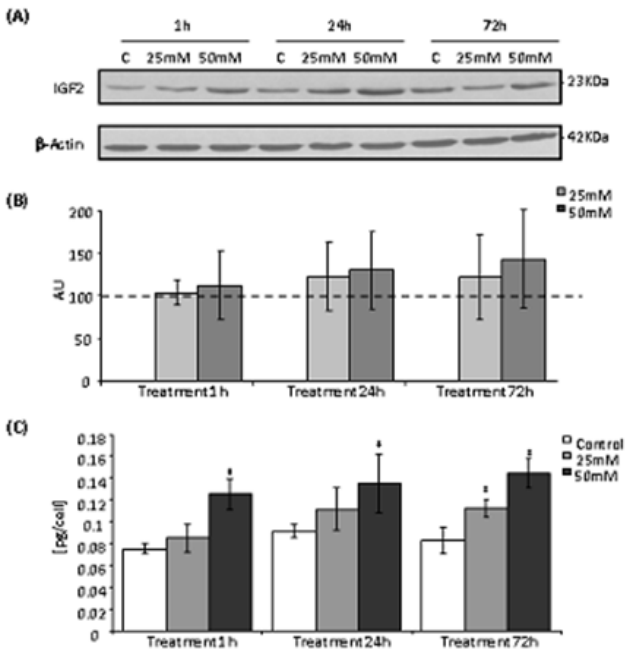
Ethanol input decreased viability of JEG3 cell line resulting significant in 50 mM group after 72h exposure time (p<0.005 vs. control) (Figures 4A and 4C). Similar to the viable count, a reduction in total protein levels was observed. The 50 mM group showed significant decreased levels (p<0.005 vs. control) after 24 h and 72 h exposure times (p<0.005 vs. control) (Figures 4B and 4C).

Discussion

The first finding of this *in vitro* study was the detection of a high expression of IGF2 in cell lysates by Western blot, in agreement with subsequent ELISA results, in a significant dose-dependent manner. This effect was observed when cells were exposed to increasing concentrations of ethanol. The IGF system activates PI3 kinase-Akt and it is related with prenatal and postnatal growth and this pathway is involved in processes such as growth, survival and metabolic signal transmission [30]. A reduced expression of placental IGF2 has been associated with intrauterine growth restriction [31]. Thus, it is likely that higher levels of IGF2 allow catch-up growth in children with FASD [18].

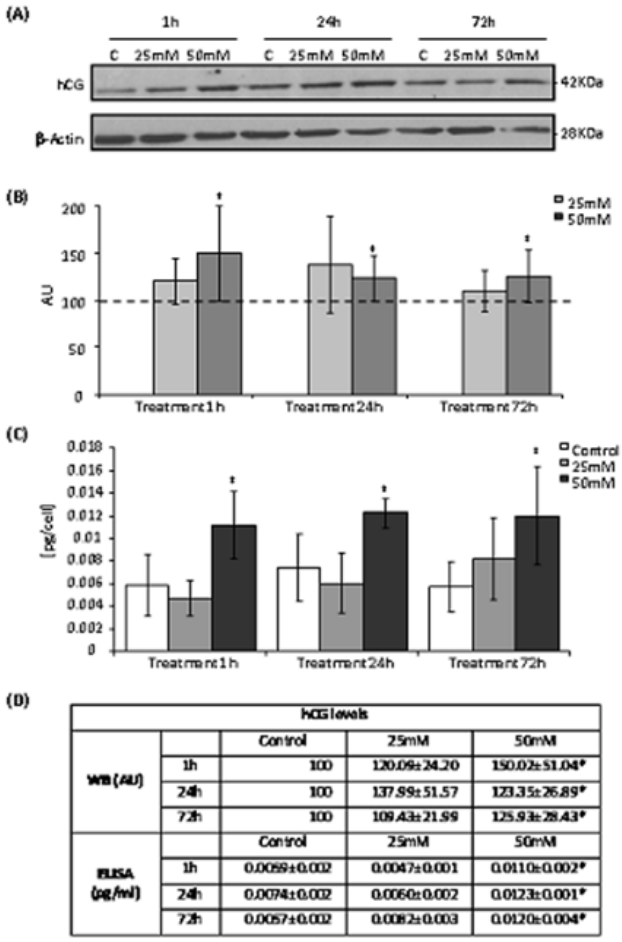
Previous studies, using rodents as an *in vivo* model, demonstrated that only continuous ethanol exposure induced an increase in placental *igf2* mRNA levels [31]. The ethanol blood levels of these animals were approximately 50-60 mM during all their pregnancy. These rodents blood ethanol concentration and those found in our cell culture supernatant are comparable at cellular level. Thus, our results showed that IGF2 levels increased similarly when cells were exposed during short periods (1 h) and long periods (72 h) of time. Therefore, the levels were also modified with occasional exposure to alcohol.

Recently, other authors observed increased IGF2 serum levels in children diagnosed with FAS [18]. This study only associated the increase in the expression of IGF2 to ethanol exposure but it does not



IGF2 levels				
WB (AU)		Control	25mM	50mM
	1h	100	103.82±15.03	111.68±39.48
	24h	100	122.64±39.50	129.78±45.16
	72h	100	122.52±49.65	143.43±57.65
ELISA (pg/ml)		Control	25mM	50mM
	1h	0.075±0.005	0.085±0.013	0.130±0.014*
	24h	0.091±0.006	0.110±0.019	0.140±0.03*
	72h	0.082±0.012	0.110±0.008*	0.150±0.013*

Figure 2: Effects of ethanol treatments in JEG3 cells IGF2 production. {A} Representative Western blot detecting IGF2. {B} Columns show the mean ± SD of 25 mM and 50 mM groups normalized against the control group, represented as a discontinuous line {100AU}. Results are expressed as arbitrary units {AU}. {C} columns show the mean±SD IGF2 content in JEG3 cells culture medium from the three groups measured by ELISA. Results are expressed as pg/cell. {D} Western blot and ELISA results for each group. *p<0.005 vs. control.



hCG levels				
WB (AU)		Control	25mM	50mM
	1h	100	120.09±24.20	150.02±51.04*
	24h	100	137.89±51.57	123.35±26.89*
	72h	100	109.43±21.99	125.93±28.43*
ELISA (pg/ml)		Control	25mM	50mM
	1h	0.0069±0.002	0.0047±0.001	0.0110±0.002*
	24h	0.0074±0.002	0.0060±0.002	0.0123±0.001*
	72h	0.0057±0.002	0.0082±0.003	0.0120±0.004*

Figure 3: Effects of ethanol treatments in JEG3 cells HOG production. {A} Representative Western blot detecting IGF2. {B} Columns show the mean ± SD of 25 mM and 50 mM groups normalized against the control group, represented as a discontinuous line {100AU}. Results are expressed as arbitrary units {AU}. {C} columns show the mean ± SD HOG content in JEG3 cells culture medium from the three groups measured by ELISA. Results are expressed as pg/cell. {D} Western blot and ELISA results for each group. *p<0.005 vs. control.

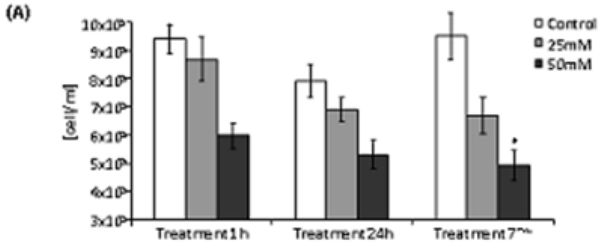
described whether there was a dose- or time-dependent correlation. Our data proved that the increased levels of IGF2 in our preliminary *in vitro* model were dependent on the dose of ethanol received. This fact is relevant because it shows correspondence between dose and response, behaviour which is essential for being candidate molecules to biomarkers of damage. Although, as seen in the literature review, IGF2 was already postulated to this use, our study refills a gap of evidence about cellular behaviour under controlled conditions of alcohol exposure.

The second observation about placental hormone production was the detection of a high expression of hCG in cell lysates by Western blot, in agreement with subsequent ELISA results, in a significant dose-dependent manner. hCG is a glycoprotein with similar biological activity to luteinizing hormone. hCG has multiple biological functions, the best known being the corpus luteum stimulation to maintain progesterone production [32]. A previous *in vitro* report showed an increase in hCG production in trophoblast cells exposed to ethanol [17,22]. However, they used an experimental design that exposed cells to a daily peak concentration of 0-150 mM of ethanol. Using these amounts, they observed a peak hCG level at 65 mM. This dose of ethanol is far for being similar to the standard human levels of ethanol [33]. In our study, we extended these observations and analyzed whether there was a constant release pattern according to the exposure time and the ethanol dosage. Our results proved that the ethanol dosage is significant and that hCG released by trophoblast cells increased in an amount-depending manner.

Finally, we observed a significant decrease in the number of viable cells related to ethanol exposure times. This was observed in other *in vitro* culture cell types exposed to ethanol [29,34,35]. Therefore, our study showed that alcohol causes cell damage and thus, interferences in hormone release levels, could be used as primary targets for the research of possible biomarkers of damage.

Our study showed the different behavior of two molecules depending on the concentration of ethanol they were exposed to. This was proved on a specific trophoblast cell line and with a coherent design, comparing groups exposed to standard concentrations of ethanol. These results should be considered preliminary and future studies will be needed to validate these findings in clinical human placental samples. Finally, it will be necessary to check these potential biomarkers in a cohort of pregnant women with alcohol use, primarily due to physiological variability in hormones.

In conclusion, the present results showed that ethanol induced



Cell viability				
Viable count (cell/ml)		Control	25mM	50mM
	1h	888750±52967	812500±75277	547500±43770
	24h	741250±58925	638750±41810	480000±47140
	72h	900000±81819	620000±64334	442500±52388*
Protein levels				
Concentration (mg/ml)		Control	25mM	50mM
	1h	4.04±1.05	4.13±0.13	3.53±0.68
	24h	4.54±0.66	3.54±0.72	3.96±0.72*
	72h	4.73±0.61	3.79±0.8	2.92±0.31*

Figure 4: Viable cell count after treatment and total protein quantification in JEG3 cells exposed to ethanol. {A} Cell number {mean ± SD} assessed by trypan blue exclusion method in the three groups. Results are expressed as arbitrary units cell/ml. {B} Total protein {mean ± SD} measured with DC protein Assay in the three groups. Results are expressed as mg/ml. {C} viable count and protein quantification results. *p<0.005 vs. control.

changes in the hormonal secretory JEG3 trophoblast function. In our study, we observed a significant increase in IGF2 and hCG production, which could be considered in future studies as preliminarily surrogate biomarkers of damage induced by prenatal ethanol exposure.

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