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Short communication

Alcohol exposure impairs trophoblast survival and alters subtype-specific gene expression *in vitro*

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ABSTRACT

Maternal alcohol consumption is common prior to pregnancy recognition and in the rat results in altered placental development and fetal growth restriction. To assess the effect of ethanol (EtOH) exposure on the differentiation of trophoblast stem (TS) cells, mouse TS lines were differentiated *in vitro* for 6 days in 0%, 0.2% or 1% EtOH. This reduced both trophoblast survival and expression of labyrinth and junctional zone trophoblast subtype-specific genes. This suggests that fetal growth restriction and altered placental development associated with maternal alcohol consumption in the periconceptional period could be mediated in part by direct effects on trophoblast development.

1. Introduction

Maternal perturbations during early pregnancy, including a low protein diet [1–3], undernutrition [4,5], or alcohol (EtOH) exposure [6,7], can result in fetal growth restriction and programming of adult disease. Alcohol is a common exposure during pregnancy, with current statistics being 47–58% of all pregnancies [11,12]. Consumption of 5+ standard drinks has also been reported in the period prior to pregnancy recognition (14%) [11,12]. *In vivo* rodent models of EtOH exposure during the periconception period [6] and intermittently throughout gestation [13,14], have shown alterations to placental structure and expression of metabolic transporters. This is of interest as normal formation and function of the placenta is a critical determinant of fetal growth. Derived from the trophectoderm of the pre-implantation embryo, trophoblast cells contribute the majority of cells within the mature placenta and consist of a number of unique cell types with diverse morphologies and functions [8,9]. The definitive chorioallantoic placenta is organised into two zones containing specialised trophoblast cell types; the junctional zone which has a structural and endocrine role, while the labyrinth zone contains the fetal and maternal vasculature and is the location of nutrient exchange [10]. Perturbations during pregnancy often result in modifications to placental growth [2,5] and zonal allocation [6] in late gestation, however few studies have determined whether alterations to trophoblast differentiation in early pregnancy may be mediating these effects. We have previously reported that periconceptional EtOH exposure prior to implantation in the rat causes an increase in glycogen trophoblasts (GlyT) in the junctional zone during late gestation [6]. In addition, high dose (18–37% vol/vol) EtOH exposure from implantation until close to term (E6-E18) reduced invasion of trophoblast cells into the maternal decidua and caused labyrinth disorganisation [14]. However, it is unknown if placental defects are due to direct exposure of the trophoblast stem cells of the trophectoderm to EtOH within the uterine cavity, or via other indirect mechanisms such as interactions with altered uterine cells. Here we utilise an *in vitro* model of differentiating mouse trophoblast stem (TS) cells to examine the direct effects of EtOH on proliferation and differentiation.

2. Methods

2.1. TS cell culture

Murine TS cells (EGFP line) were maintained as previously described [15]. Cells were seeded at 5×10^4, and differentiated in 0% (control), 0.2%, or 1% EtOH in TS media. To assess cell proliferation, cells were lifted off the culture plates and counted using a hemocytometer on days 2, 4 and 6 of culture ($N = 3$/treatment).

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Gene expression analysed on day 6 ($N = 9$/treatment, 3 technical replicates per set). Media was changed every 2 days including the addition of fresh EtOH. The 0.2% EtOH dose was based on the maximum blood alcohol concentration from 12.5% v/v EtOH exposure in Sprague Dawley dams [6] whilst the 1% EtOH dose has been used previously in culture [24]. In culture, as much as 50% evaporation occurs by 8 h and 80–90% evaporation by 24 h in tissue culture plates [18].

2.2. RNA extraction and qRT-PCR

RNA isolation and qRT-PCR were carried out as previously described [24]. Markers specific to labyrinth or junctional zone trophoblasts were analysed for gene expression relative to *Rn18s* using the ΔΔCt method [17] (primer sequences in Supplemental Table 1).

2.3. Histological assessment of trophoblast number

Fluorescent *in situ* hybridisation (FISH) localised *Tpbpa* and *Prl7a2* positive trophoblasts ($N = 3$–4/treatment) from day 6 cultures [8], see supplemental file for more detail. Fluorescein or DIG-labelled probes were made following manufacturer's instructions (Roche) with primer sequences as described previously [8,20]. Prior to use on cultured cells, RNA probes were tested on either E12.5 or E14.5 mouse placenta fixed in 4% paraformaldehyde overnight to confirm subtype-specific expression. Coverslips were imaged on an inverted microscope (Leica) in 20 × 20 frames, 20× magnification, with all cells being utilised within this area for calculation. Cells were counted using ImageJ (NIH).

2.4. Statistical analyses

Mean relative gene expression of treatments were standardised to the control group for each line. Expression data, proliferation and FISH counts were analysed by one-way ANOVAs to compare treatments. Tukey's post-hoc tests were used where appropriate, and when data was not normally distributed, a non-parametric Kruskal-Wallis test was used. Statistical differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results and discussion

To determine the mechanism by which EtOH exposure during early pregnancy may be mediating altered placental growth and function, we explored the direct impact of EtOH on TS cell proliferation and differentiation. To investigate EtOH exposure at a physiological level, 0.2% EtOH was chosen as this was the peak blood alcohol content found in a model of *in vivo* alcohol exposure in the rat at 30 min after initial consumption [6]. In addition, 0.2% EtOH is also the maximum dose that the TS cells would be exposed to when the media is applied, and due to alcohol evaporation [18], would not be sustained at this level over the entire 6 days of treatment. TS cells express a well-validated set of genetic markers enabling identification of individual placental trophoblast cell types in the mouse [8]. This presents an opportunity to relate alterations in gene expression to potential changes in differentiation.

3.1. Cell counts

Proliferation rates were first characterised to determine the effects of EtOH over the course of TS cell differentiation. While no differences in cell count were found between treatments on day 2 or 4, marked reductions in cell counts in both the 0.2% (48% reduction) and 1% EtOH groups (44% reduction) were observed on day 6 (Fig. 1A). This time point coincides with terminal differentiation of trophoblasts, as expression of stem cell markers are rapidly lost by day 2 of culture and expression of markers of mature cell types is just commencing on day 4 [19]. This result is consistent with studies that demonstrate reduced proliferation in response to 20–40 mM (0.09–0.18%) EtOH in cultured human cytotrophoblasts (first trimester villous explants and BeWo cells) [16]. However, our observation of decreased cell numbers at day 6 of differentiation is unlikely the result of decreased proliferation, but more likely represents a loss of cell viability. This is supported by the observation of significant floating cells in the treatment but not control media on day 6 (data not shown). In addition, human cytotrophoblasts undergo apoptosis when exposed to EtOH [21,22]. To determine whether this also occurs in mouse trophoblasts exposed to EtOH, we analysed the expression profiles of pro- and anti-apoptotic markers (*Bax* and *Bcl2* respectively) from days 2–6 of culture. No changes were found to either gene on days 2 or 4 (data not shown). However, on day 6, whist no change was found to *Bax* expression, *Bcl2* expression was reduced by EtOH (see Supp. Fig. 1A,B), suggesting a perturbed balance between pro- and anti-apoptotic signals. This may extend to an increase in apoptosis, however, to determine this definitively would require histological analysis. Collectively, these data indicate that placental trophoblasts are highly sensitive to EtOH during terminal differentiation.

3.2. Junctional zone cell markers

EtOH exposure caused dose-dependent decreases in gene expression of *Tpbpa* (Fig. 1B), a marker of both fully differentiated junctional zone trophoblasts and their earlier ecto-placental cone progenitors [23]. Additionally, both *Prl7a2,* a marker of spongiotrophoblast (SpT) and parietal trophoblast giant cells (P-TGCs), and *Prl7b1* a marker of invasive glycogen trophoblast (GlyT) cells and spiral artery-associated TGCs (SpA-TGCs) were decreased by EtOH exposure (Fig. 1C,D). Curiously, this suggestion of reduced GlyTs *in vitro* conflicts with the observation of increased area occupied by this cell type in the junctional zone of periconceptionally exposed placentas *in vivo* [6]. It is tempting to speculate that altered GlyT differentiation may also have affected their invasive behaviour as seen in other EtOH exposure models [13]. No differences were found in the 0.2% EtOH groups for *Prl7b1* or *Prl7a2* relative to control or 1% groups.

No alterations were found for *Prl3d1* (*Pl1*, Fig. 1E), or *Prl2c (Plf,* Fig. 1F) which are expressed by secondary TGCs *in vivo*, indicating that EtOH exposure does not alter expression of all trophoblast subtype specific gene markers. When this same experiment was performed on another cell line – RS26, similar expression profiles were seen at day 6 for all junctional zone trophoblast markers with the exception of *Tpbpa*, which did not change (Supp. Fig. 2A–F). This difference between the TS lines may be either due to clonal differences between cell lines, or alternately a sex-specific result, as the RS26 line is male and the EGFP line is female (data not shown). Maternal perturbations commonly result in sexually dimorphic impacts on placental structure, function, and differentiation - evidenced by GlyT cell accumulation in the junctional zone of females only following periconceptional EtOH exposure [6]. Further examination of multiple male and female cell lines would be required to determine this possibility.

The trophoblast subtype markers in this study have been used previously to infer effects of differentiation of specific trophoblast subtypes. However, it is also possible that EtOH may be directly altering gene expression levels, rather than the differentiation of the trophoblast subtypes per se. Therefore to confirm whether EtOH reduces the number of differentiated trophoblast subtypes, FISH was carried out on cells collected from day 6 cultures to