RESEARCH ARTICLE

Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mouse placentas

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Abstract

Background

During pregnancy, the placenta is an extremely important organ as it secretes its own hormones, e.g. insulin-like growth factor 1 (IGF-1), to ensure proper intrauterine fetal growth and development. Ethanol, an addictive and widely used drug, has numerous adverse effects during pregnancy, including fetal growth restriction (FGR). To date, the molecular mechanisms by which ethanol triggers its toxic effects during pregnancy, particularly in the placenta, are not entirely known. For this reason, a murine model of partial IGF-1 deficiency was used to determine ethanol alterations in placental morphology and aspartyl/asparaginyl β-hydroxylase (AAH) expression.

Methods

Wild type (WT, Igf1 <sup>+</sup>/<sup>+</sup>) and heterozygous (HZ, Igf1 <sup>+</sup>/<sup>-</sup>) female mice were given 10% ethanol in water during 14 days as an acclimation
period and throughout pregnancy. WT and HZ female mice given water were used as controls. At gestational day 19, pregnant dams were sacrificed, placentas were collected and genotyped for subsequent studies.

Results

IGF-1 deficiency and ethanol consumption during pregnancy altered placental morphology, and decreased placental efficiency and AAH expression in placentas from all genotypes. No differences were found in Igf1, Igf2, Igf1r and Igf2r mRNA expression in placentas from all groups.

Conclusions

IGF-1 deficiency and ethanol consumption throughout gestation altered placental development, suggesting the crucial role of IGF-1 in the establishment of an adequate intrauterine environment that allows fetal growth. However, more studies are needed to study the precise mechanism to establish the relation between both insults.

Keywords
placenta, IGF-1 deficiency, fetal growth restriction, ethanol.
**Abbreviations**

AAH: aspartyl/asparaginyl β-hydroxylase  
AKT: protein kinase B  
cDNA: complementary DNA  
DAB: diaminobenzidine  
EDTA: ethylenediaminetetraacetic acid  
ELISA: enzyme-linked immunosorbent assay  
eNOS: endothelial nitric oxide synthase  
FASD: fetal alcohol spectrum disorders  
FGR: fetal growth restriction  
HRP: horseradish peroxidase  
HZ: heterozygous  
IGF-1: insulin-like growth factor 1  
IGF-2: insulin-like growth factor 2  
IGFBP-1: IGF-1 binding protein 1  
IGFBP-3: IGF-1 binding protein 3  
IGF1R: IGF-1 receptor  
IGF2R: IGF-2 receptor  
INSR: insulin receptor  
KO: knock-out  
MAPK: mitogen-activated protein kinase  
PBS: phosphate buffered saline  
PCR: polymerase chain reaction  
P3K: phosphoinositide-3-kinase  
RT-qPCR: reverse transcription coupled to polymerase chain reaction  
SD: standard deviation  
WT: wild type

**1. Introduction**

Throughout pregnancy, the placenta is an essential organ for both mother and fetus; being the major determinant of intrauterine growth and serving as a protective barrier against external and internal insults.

Insulin-like growth factor 1 (IGF-1) is a pleiotropic hormone with several functions: mitochondrial protection, cell proliferation and survival, tissular growth and development. It regulates placental morphogenesis and hormone secretion into umbilical and maternal circulations, processes that are indispensable for fetal development. Furthermore, IGF-1, as well as insulin-like growth factor 2 (IGF-2), are key providers of placental resource allocation either for development or for response to external and environmental insults.

To this day, numerous IGF-1 deficiency conditions have been described in humans, e.g. fetal growth restriction (FGR). FGR is a disorder where reduced levels in both IGF-1 and IGF-1 binding protein 3 (IGFBP-3) are observed, suggesting that reduced IGF-1 concentration in the fetus, mother and/or placenta may contribute to growth restriction. Also, FGR, among other harmful consequences, such as fetal death, miscarriage, low birth weight, premature birth and fetal alcohol spectrum disorders (FASD), can be a result of ethanol consumption during pregnancy.
Current experimental studies in rodents and sheepes have shown that chronic exposure to high levels of ethanol during pregnancy reduces fetal weight\textsuperscript{12} as well as maternal and fetal plasmatic levels of both IGF-1 and IGFBP-3.\textsuperscript{13} Also, ethanol increases both IGF-1 binding protein 1 (IGFBP-1, an inhibitory protein for IGF-1) and IGF-2 levels,\textsuperscript{13} inhibits insulin, IGF-1 and IGF-2 placental gene expression and/or secretion\textsuperscript{12,14} and reduces activities of both insulin and IGF-1 receptors\textsuperscript{15,16}; thus, altering IGF-1 bioavailability and its downstream signaling.

The molecular mechanism for ethanol toxic effects during pregnancy, specially in the placenta, is not totally understood. Several studies in rodent models of ethanol consumption suggest that this molecule alters the insulin and IGF-1 signaling pathway, impairing cell viability, metabolism, homeostasis, and hence, normal placental growth and development.\textsuperscript{9,14,17}

Aspartyl-(asparaginyl) β-hydroxylase (AAH), a type 2 transmembrane protein that hydroxylates epidermal growth factor-like domains of proteins that have a functional role in cell motility and invasion, is regulated by the IGF-1 signaling pathway.\textsuperscript{18} AAH is highly expressed in trophoblast cells, which are motile and invasive epithelial cells that mediate the appropriate development of placenta and implantation.\textsuperscript{19} Experimental studies in murine models with chronic ethanol exposure, showed a reduced expression of IGF-1 in placentas, being associated with decreased expression of AAH in trophoblastic cells, suggesting a role of the IGF-1 signaling pathway in placentation and fetal development.\textsuperscript{9,14}

For this reason, as either IGF-1 deficiency or ethanol consumption during pregnancy produces FGR, the aim of the present study was to determine whether IGF-1 partial deficiency is responsible for placental alterations in morphology and AAH expression, as well as if chronic ethanol exposure during gestation contributed to these placental changes.

2. Methods

2.1 Animals and experimental design

As previously reported, IGF-1 heterozygous mice were obtained by cross-breeding transgenic mice, line 129SV and Igf1\textsuperscript{tm1Ts/ImJ} (003258, Jackson Laboratory, Maine, USA) and CD1 (non-consanguineous, Circulo A.D.N S. A de C.V., Ciudad de Mexico, Mexico).\textsuperscript{20}

Animals were housed in cages in a room with 12-hour light/dark cycle, constant humidity (50–55%) and temperature (20–22°C). Food (PicoLab\textsuperscript{90} Rodent Diet 20 5053*, Missouri, USA) and water were given ad libitum. All experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), the NORMA Oficial Mexicana (NOM-062-ZOO-1999) for technical specifications to produce, care and use of laboratory animals and the ARRIVE guidelines. Also, all experimental procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals of Tecnologico de Monterrey (Protocol 2018-006). All efforts were made to ameliorate any suffering of animals, such as minimal handling, adequate space for living and no other experimentation or pain inducing procedures.

The sample size was calculated using the resource equation method,\textsuperscript{21} where the value of E is calculated and should lie between 10 and 20 to be considered adequate: 

\[
E = \frac{\text{Total number of animals}}{\text{Total number of groups}} = \frac{7 \times 2 - 2}{2} = 12.
\]

Wild-type (WT, \textit{Igf1}\textsuperscript{+/+}) and heterozygous (HZ, \textit{Igf1}\textsuperscript{+/−}) female mice 16 ± 8 weeks old were randomly included into four experimental groups: two control groups given water (WT-Control, \textit{n} = 6; and HZ-Control, \textit{n} = 6), and two other groups given food grade ethanol in water (WT-Ethanol, \textit{n} = 4; and HZ-Ethanol, \textit{n} = 9) (purity: 96%; La Fe, Nuevo Leon, Mexico).

An adaptation period to ethanol (14 days) was performed, where WT and HZ female mice were given increasing concentrations of 2%, 5% and 10% of ethanol in water, each introduced after a 48-hour acclimation to the previous concentration, according to Kleiber et al.\textsuperscript{22} During this period, control WT and HZ female mice were provided water (Figure 1).

After the acclimation period to ethanol, WT and HZ male mice were mated overnight with WT and HZ female mice. To avoid ethanol consumption from males during mating, water was given to the ethanol groups. Once the vaginal plug was observed, it was considered gestational day one and males were removed. Throughout gestation, 10% ethanol or water was given to pregnant dams (WT-Ethanol or HZ-Ethanol, and WT-Control or HZ-Control; respectively), food and beverage consumption and weight gain were monitored throughout the experimental protocol. Nonpregnant females from WT-Ethanol and HZ-Ethanol groups were given 10% ethanol during 24 hours and then mated again with male mice. If vaginal plug was not observed after this second mating, ethanol exposed female mice were sacrificed by cervical dislocation. On the other hand, nonpregnant females from WT-Control and HZ-Control groups were mated again until the presence of the vaginal plug was observed.
At gestational day 19 (before the end of the gestational period in mice), pregnant females were sacrificed by cervical dislocation. Subsequently, blood was collected by cardiac puncture and a caesarean section was performed to obtain fetuses and placentas (from HZ x HZ matings), which were measured and weighted. Tails from fetuses were cut for genotype determinations. Fetuses and placentas were stored randomly in either paraformaldehyde 4%, for histology and immunohistochemistry analyses, or liquid nitrogen, for reverse transcription coupled to polymerase chain reaction (RT-qPCR) determinations.

2.2 Genotyping of animals
DNA was extracted from tails of fetuses using the Wizard Genomic DNA Purification Kit (A1125, Promega, Wisconsin, USA) following manufacturer’s instructions, and stored at –20°C until analysis. End-point polymerase chain reaction (PCR) (Veriti 96 well Thermal Cycler, Applied Biosystems, California, USA) was carried out for genotyping using the following set of primers for \textit{Igf1} gene: WT forward 5'-TTCATGCCACACTGCTCTTC-3'; common 5'-AGAGGGGATGGGAGAGCTAC-3'; and mutant forward 5'-GCCAGAGGCCACCTTGTGTAG-3'. All primers were acquired from IDT (Iowa, USA). Secondly, conventional PCR analysis was achieved using the GoTaq Green Master Mix (M712C, Promega, Wisconsin, USA) following manufacturer’s instructions.

2.3 Serum IGF-1 circulating levels at gestational day 19
In order to evaluate serum IGF-1 circulating levels in HZ dams at gestational day 19, sera from control (WT) mice treated both with water (n = 5) or ethanol (n = 5) were also used. Serum IGF-1 levels were determined by enzyme-linked immunosorbent assay (ELISA) using the Mouse/Rat IGF-1 commercial kit (22-IG1MS-E01, Alpco, New Hampshire, USA), following manufacturer’s instructions. The signal was measured using a spectrophotometer Synergy HT (Biotek, Vermont, USA) and data were interpreted using Gen5 Data Analysis Software (Biotek, Vermont, USA).

2.4 Placental histological study
For histopathological analysis, placental samples from all experimental groups were fixed in 4% paraformaldehyde diluted in 10 mM phosphate buffered saline (PBS) solution for 24 hours. Once samples were properly fixed, they were dehydrated in increasing ethanol concentrations and were embedded in liquid paraffin using the automated equipment Leica TP 1020 (Leica, Wetzlar, Germany). Sections 4 μm thick were cut using a Reichert Jung Leica BC2030 Histocut Rotary Microtome (Leica, Hesse, Germany) and subsequently stained with hematoxylin-eosin (HHS32 and 2853, Sigma-Aldrich, Missouri, USA). Finally, all histological preparations were evaluated independently by three observers (double-blind) in a Zeiss Axio Imager M2 microscope (Zeiss, Baden-Württemberg, Germany) to establish morphology alterations in the placenta due to ethanol consumption throughout gestation.

\begin{center}
\textbf{Figure 1. Diagram of the experimental procedure.} D: day; GD: gestational day.
\end{center}
All images were analysed using the processing package ImageJ (National Institutes of Health, Maryland, USA) (RRID: SCR_003070). The area of the junctional zone of the placenta was evaluated according to the presence of trophoblasts in this placental zone. Three images were taken per sample of at least three individuals from each group and genotype.

2.5 Immunohistochemical staining
Paraffin-embedded placental tissues were deparaffinized and rehydrated in decreasing ethanol concentrations. Sample sections were incubated with 3% hydrogen peroxide at room temperature in darkness for 30 minutes to inactivate endogenous peroxidases. Retrieval of antigen was induced with 2 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 and 50 mM Tris–HCl pH 9.0 by microwave heating at 100°C for 8 minutes. Sections were incubated overnight at 4°C with specific primary antibodies: rabbit anti-ASPH (362200, USBiological Life Sciences, Massachusetts, USA) diluted at 1:200; or rabbit anti-β actin (ab8227, Abcam, Cambridgeshire, United Kingdom) diluted at 1:1,000, the latter being an endogenous control. Afterwards, primary antibodies sections were incubated with goat anti-rabbit IgG H&L (horseradish peroxidase, HRP) (ab205718, Abcam, Cambridgeshire, United Kingdom) diluted at 1:5,000 for one hour at room temperature. Staining was developed using Steady Diaminobenzidine (DAB)/Plus kit (ab103723, Abcam, Cambridgeshire, United Kingdom) following manufacturer’s instructions. Negative controls were conducted by omitting the primary antibody.

Digital images of tissue sections (five images per placenta) were captured and analyzed by three observers (double-blind) using a Zeiss Axio Imager M2 microscope. All images were analysed using the processing package ImageJ (National Institutes of Health, Maryland, USA).

2.6 Gene expression studies in placentas via RT-qPCR
RNA was isolated from placentas using Trizol reagent (15596026, Invitrogen, California, USA). Quality was checked by the A260/A280 ratio using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Massachusetts, USA), whereas integrity was determined by electrophoresis in 1% agarose gel. Samples were stored at −80°C until analysis. Subsequently, the SuperScript III First-Strand Synthesis System (Invitrogen, California, USA) was utilized to prepare complementary DNA (cDNA) from 500 ng of the total RNA, according to the manufacturer’s instructions.

For the PCR analyses, TaqMan Universal PCR Master Mix (Thermofisher, Massachusetts, USA) was used in a total volume of 20 µL containing 400 nM of each oligonucleotide and 200 mM of specific Taqman® probes for particular genes, supplied by Applied Biosystems (California, USA) (Supplementary table 1). The assays were performed in a 96-well reaction plate on a Quant Studio 3.0 thermocycler (Applied Biosystems, California, USA). Ribosomal 18S RNA expression was used as an endogenous control (Applied Biosystems, California, USA).

Each sample was run in triplicate, and negative controls were included in the same plate. Results were analyzed using the comparative threshold cycle method for relative gene expression.25

2.7 Statistical analysis
All data are represented by mean±standard deviation (SD). Significance was estimated with non parametric (Mann–Whitney U test and Kruskal–Wallis test) or parametric (Student T test and ANOVA) statistical tests, using Mann–Whitney U test or Student T test to compare between two groups (for example between HZ-Control and HZ-Ethanol groups), whereas comparisons among more than two groups were carried out using the Kruskal–Wallis test or ANOVA (for example, comparisons between WT, HZ and knock-out (KO) placentas). To evaluate the effect of the two independent variables in the present study (ethanol consumption and IGF-1 deficiency) a general linear model was performed. Differences were considered significant at a level of p < 0.05. Statistical analyses were performed on SPSS 26 (IBM, New York, USA) (RRID:SCR_019096) and graphs were generated on Prism 8.2.1 (GraphPad Software, California, USA) (RRID:SCR_002798).

3. Results
3.1 IGF-1 serum levels in pregnant dams chronically exposed to ethanol at gestational day 19
To validate IGF-1 serum levels in the present experimental model, control (WT) pregnant female mice treated with water or ethanol were used. At gestational day 19, as expected, IGF-1 serum levels in HZ pregnant dams (HZ-Control, 454 ± 58 ng/mL, and HZ-Ethanol, 422 ± 43 ng/mL) were decreased compared to WT female mice (WT-Control, 739 ± 48 ng/mL, and WT-Ethanol, 795 ± 147 ng/mL) (p < 0.001). HZ-Control and HZ-Ethanol dams had significant lower IGF-1 serum levels than WT-Control female mice (p < 0.05 and p < 0.01, respectively). Additionally, ethanol consumption decreased even more IGF-1 serum levels than WT-Control (p < 0.01) (Table 2).
3.2 Alterations in placental morphology due to ethanol consumption during gestation

The placenta is conformed of several strata, such as myometrium, decidua, junctional zone and labyrinth (Extended data: figure 1). In the WT placentas from the control group (from HZ x HZ matings) all morphology and structure that characterizes these layers were well defined (Figure 2A). It was observed that IGF-1 deficiency promoted a disorganization in placental strata, especially in the junctional zone, where trophoblast cells were observed (Figure 2B). Remarkably, an abnormal placental development was observed in KO placentas from the control group, where trophoblasts were arrested in islets (Figure 2C). Ethanol consumption throughout gestation exacerbated placental layer

![Image of Figure 2](image)

**Figure 2.** Effect of gestational ethanol consumption on placental morphology on gestational day 19. Placentas from WT, HZ and KO fetuses from pregnant dams given water (A, n = 5; B, n = 6; and C, n = 6) or ethanol (D, n = 7; E, n = 6; and F, n = 5) were stained with hematoxylin & eosin and examined by light microscopy. Representative images from one placenta of fetuses from each group are included. Black arrows indicate trophoblast islets, suggesting an abnormal migration. IGF-1 deficiency promoted placental disorganization, especially in the junctional zone; ethanol consumption during gestation exacerbated such disorganization. Original magnification 100×. Scale bar = 100 μm.
disorganization and promoted trophoblast arrest in islets in all groups (WT, HZ and KO) (Figures 2D, 2E and 2F). Additionally, ethanol use altered trophoblast migration (Figure 2D) particularly in HZ and KO placentas (Figures 2E and 2F). Due to the variability between all experimental groups, no significant differences were observed in the area of the junctional zone of the placenta (data not shown); in this area a clear disorganization is detected with both IGF-1 deficiency and ethanol consumption.

3.3 Impairments in placental efficiency due to ethanol consumption and IGF-1 deficiency

Placental efficiency is frequently defined as the grams of fetus produced per gram of placenta, being an indicator of the ability of this organ to maintain an adequate nutrient supply to the fetus. The chronic exposure to ethanol during gestation resulted in a significant reduction in placental efficiency (p < 0.001) compared to control groups. Subsequently, genotype offspring outcome was evaluated, showing that IGF-1 deficiency significantly decreased placental efficiency in KO placentas compared to WT placentas (p < 0.05) and HZ placentas (p < 0.01) from control group (Figure 3). Also, ethanol consumption during gestation significantly reduced placental efficiency in KO placentas compared to WT and HZ placentas from ethanol treated group (p < 0.001 in both cases) (Figure 3). Markedly, there was a significant diminution in 0.75-fold in placental efficiency in KO ethanol treated placentas compared to KO placentas from control group (p < 0.05) (Figure 3).

3.4 Immunohistochemical analysis of AAH placental expression

To determine ethanol’s harmful effects on placental development, protein expression levels of AAH were determined by immunohistochemistry. AAH was predominantly expressed in the junctional zone, as shown in WT placentas from control group (Figure 4A). IGF-1 deficiency revealed a decrease in AAH expression within the junctional zone in both
HZ and KO placentas compared with WT placentas from control group (Figures 4B and 4C). Ethanol consumption during gestation also decreased AAH expression, especially in HZ and KO placentas, denoting the aforementioned alterations in placental morphology, particularly in the junctional zone, where trophoblasts are arrested in islets, avoiding their correct migration towards the junctional zone for an appropriate placental development (Figures 4D, 4E and 4F).

3.5 The effect of ethanol consumption during gestation in placental expression of the IGF-1 signaling pathway

The placental expression of components from the IGF-1 signaling pathway (Insulin-like growth factor 1 (Igf1), Insulin-like growth factor 2 (Igf2), Insulin-like growth factor 1 receptor (Igf1r), Insulin-like growth factor 2 receptor (Igf2r) and Aspartyl/asparaginyl β-hydroxylase (Asph)) was analyzed by RT-qPCR. No significant differences were found in Igf1

Figure 4. Effect of ethanol consumption during gestation in AAH levels analyzed by immunohistochemistry in placentas from WT, HZ and KO mice given water (A, n = 5; B, n = 6; and C, n = 6) or ethanol (D, n = 7; E, n = 6; and F, n = 5). Original magnification 100x. Scale bar = 100 μm. Representative images from one mice of each group are included. Red arrows denote AAH expression in the junctional zone of the placenta, where trophoblast cells are expressed. Blue arrows indicate trophoblast islets. G) Effect of ethanol consumption during gestation in Asph gene expression in placentas from WT, HZ and KO fetuses from pregnant dams given water or ethanol (n = 4 per group). The statistical tests used were Mann-Whitney U to compare between two groups, ANOVA or Kruskal-Wallis test to compare between more than two groups, and general linear model to evaluate the effect of the two independent variables in the present study (ethanol consumption and IGF-1 deficiency). Differences were considered significant at a level of p < 0.05.

HZ and KO placentas compared with WT placentas from control group (Figures 4B and 4C). Ethanol consumption during gestation also decreased AAH expression, especially in HZ and KO placentas, denoting the aforementioned alterations in placental morphology, particularly in the junctional zone, where trophoblasts are arrested in islets, avoiding their correct migration towards the junctional zone for an appropriate placental development (Figures 4D, 4E and 4F).
Table 1. Reverse transcription coupled to polymerase chain reaction analyses revealing gene expression of Insulin-like growth factor 1 signaling pathway related proteins.

| Gene description                        | Gene name | Control | Ethanol |
|-----------------------------------------|-----------|---------|---------|
|                                          |           | WT      | KO      | WT      | KO      |
|                                          |           | Fold change (SD) | Fold change (SD) | Fold change (SD) | Fold change (SD) |
| Insulin-like growth factor 1            | Igf1      | 1.00 (0.75) | 0.70 (0.52) | 2.34 (2.23) | 0.38 (0.21) | 0.92 (1.40) | 0.66 (0.56) |
| Insulin-like growth factor 2            | Igf2      | 1.00 (2.02) | 2.11 (3.99) | 0.79 (1.98) | 1.87 (4.03) | 1.49 (2.32) | 2.39 (3.69) |
| Insulin-like growth factor 1 receptor   | Igf1r     | 1.00 (0.56) | 1.45 (0.55) | 3.51 (1.52) | 5.44 (3.45) | 3.52 (0.77) | 5.71 (1.86) |
| Insulin-like growth factor 2 receptor   | Igf2r     | 1.00 (0.38) | 0.95 (0.25) | 1.57 (0.46) | 1.79 (0.43) | 1.88 (0.59) | 1.82 (0.40) |
| Aspartyl/asparaginyl β-hydroxylase       | Asph      | 1.00 (0.42) | 1.13 (0.45) | 3.62 (0.82) | 2.84 (1.57) | 4.00 (1.74) | 3.40 (0.05) |

The statistical tests used were Mann-Whitney U or Student T test to compare between two groups, and general linear model to evaluate the effect of the two independent variables in the present study (ethanol consumption and IGF-1 deficiency). Differences were considered significant at a level of $p < 0.05$. The letters showed the significant differences founded: $^a p < 0.05$ KO vs WT, $^b p < 0.05$ KO vs HZ.
and Igf2 expression level between both treatments (control and ethanol). When grouped by genotype, no significant differences were found (Table 1).

Regarding Igf1r and Igf2r expression, a significant increase in Igf1r was observed with ethanol consumption throughout gestation ($p < 0.05$). When grouped by genotype, no significant differences were observed (Table 1).

When Asph expression levels were evaluated, no significant differences were shown between both treatments (water or control, and ethanol). When grouped by genotype, a significant increase in Asph expression was observed in KO placentas compared to WT ($p < 0.05$) and HZ ($p < 0.05$) placentas from control group (Figure 4G).

4. Discussion

The placenta is an exciting organ, as it performs crucial roles during intrauterine development: nutrient and oxygen supply to the fetus, exchange of waste products between mother and fetus, metabolism of various molecules, and hormone production and secretion. Also, this organ can operate as a barrier to avoid infections, maternal diseases and propagation of xenobiotics, contributing to a suitable fetal development.29 However, the placenta continues to be an mysterious organ, as there are still many questions to be solved.

Gestation is an intricate process where, additionally to the reorganization of the mother’s organs, numerous adaptations in the endocrinological axis occur to prepare fetal development. For example, IGF-1 serum levels increase during pregnancy, especially in the first trimester, and continue to rise throughout this period30 in order to provide a suitable intrauterine environment for fetal and placental growth.31 Results in this project showed that ethanol consumption during gestation significantly affects IGF-1 serum levels in HZ pregnant dams, according to existing literature.32

The placenta is composed of by several strata, such as decidua, junctional zone (cytotrophoblast and syncytiotrophoblast layers) and labyrinth, each presenting certain characteristics that allow the placenta to achieve its functions properly.33 The major source of placental hormones is the syncytiotrophoblast layer, e.g., IGF-1 is produced and secreted in this stratum.34,35 Recently, reduced IGF-1 levels have been associated with several pathologies, such as FGR,8,36,37 a consequence of ethanol consumption.38 Results herein revealed that both IGF-1 deficiency and ethanol consumption during gestation promoted placental disorganization, particularly in the junctional zone, where trophoblasts are arrested in islets, highlighting the crucial role of IGF-1 in placental development. It is important to notice that also IGF-2 has an important role in fetal growth due to its direct anabolic actions within the fetus, and by its modulation of placental supply capacity, which may be a compensatory mechanism for IGF-1 deficiency.39

According to these results, ethanol consumption during gestation in the present experimental model aggravated placental disorganization, particularly in IGF-1 deficient placentas, suggesting a synergic effect between both issues, that could lead to poor placental development and, hence, an abnormal fetal growth. One of the main determinants of placental formation and function are trophoblasts, located in junctional and labyrinth zones of the placenta, the major cell type affected by ethanol consumption. Ethanol consumption during gestation can lead to an altered trophoblast motility and invasion, resulting in a distorted placental barrier thickness and abnormal development that can promote an aberrant nutrient exchange, producing impairments in fetal growth and development.40,41

Several studies conducted in mice, pigs and sheeps have disclosed that IGF-1 exogenous treatment during gestation improves nutrient transport and waste exchange between mother and fetus, enhancing fetal growth and development.26 In this context, lower placental efficiency results in reduced birth weight, which translates as an inefficient placenta that failed to adapt its nutrient supply to meet the demands of the rapidly growing fetus.27,28 Results herein showed that IGF-1 deficiency decreased placental efficiency especially in KO placentas, being these fetuses the smallest ones, highlighting the crucial role of this hormone in placental development.42

Results herein reported showed that ethanol decreased placental efficiency specially in KO placentas, suggesting a key role of IGF-1 in placental formation and function necessary for a suitable fetal development. In this sense, ethanol consumption during gestation also reduces placental efficiency due to reductions in angiogenesis-related proteins.13,14 However, the results suggest that ethanol consumption during gestation did not gravely affect the development of HZ embryo-placenta units. This could be due to the intricate genetics of the placenta, being noted that it is an organ conformed of a fetal part and a maternal part, which are in constant communication. Both parts are necessary for the appropriate development of this organ, being able to be affected in the same way by external factors, such as IGF-1 deficiency and/or ethanol consumption.
In vivo experimental studies of ethanol consumption during gestation have revealed that ethanol use diminishes AAH expression (both mRNA and protein levels) and, hence, trophoblast survival, motility and invasion; resulting in distorted vascular remodeling and placentation. In vitro studies with human trophoblastic cells have revealed that small interfering RNA inhibition of AAH reduced Notch signaling, impairing trophoblastic cell motility, both effects related to altered fetal growth. Our results suggested a decrease in AAH protein expression due to IGF-1 deficiency, mainly in the junctional zone, where trophoblast cells are located. Ethanol consumption throughout gestation seemed to promote the arrest of trophoblasts in islets and also reduced AAH expression in the junctional zone of HZ and KO placentas, thus altering placental morphology. Regarding to mRNA expression levels, IGF-1 deficiency showed a significant increase in Asph expression in KO placentas from control group, while ethanol consumption during gestation also exhibited an increase in Asph in WT and HZ placentas, suggesting that a mechanism is taking place in order to promote IGF-1 downstream signaling expression to try to stimulate placentation development despite IGF-1 deficiency and ethanol consumption. Also of note, although there is no information on other proteins that regulate AAH in the placenta, there are other protein entities that modulate its expression and would be interesting to evaluate.

IGF-1 plays a crucial role in placental and fetal development through binding to its putative receptor (IGF1R). Also, IGF-1 can bind to IGF-2 receptor (IGF2R) or to insulin receptor (INSR), but with lower affinity. The majority of these receptors are members of the tyrosine kinase family, activating two main downstream signaling pathways: the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) cascades, both related to cell survival, growth and proliferation, e.g. trophoblast cell migration to establish the maternal–fetal interface that allows nutrient exchange between mother and fetus across the placenta. Several studies conducted in human FGR placentas have shown an increase in Igf2 and Igf1r expression levels and activated INSR, and a reduction in IGF1R protein content, with no change in Igf1, Insr and Igf2r expression levels, suggesting a placental compensatory mechanism in response to growth retardation. In contrast, experimental studies in rats have shown reduced Igf1r expression in FGR placentas, with no change in Insr expression, suggesting a decreased nutrient delivery across the placenta that would reduce the growth-promoting effects of insulin-like growth factors. In this way results herein disclosed that no significant differences were observed in Igf1, Igf2, Igf1r and Igf2r placental expression levels due to IGF-1 deficiency. These differing results suggest that more studies are needed to elucidate the role of IGF-1 downstream molecules in intrauterine growth.

The present study had some limitations. RT-qPCR analyses have revealed that the regulation of the IGF-1 signaling pathway in the placenta is very complex, especially when there are two independent variables (IGF-1 deficiency and ethanol consumption) to analyze. More studies are needed to investigate the regulation of this cascade at protein level, such as the phosphorylation level of the molecular components of the IGF-1 signaling pathway or caspase levels to observe apoptosis. Additionally, mouse placentas have a small size, so it would be more convenient to use rat placentas, which are even more similar to human placentas, allowing a better separation and analysis of placental layers. The present study showed that IGF-1 deficiency and ethanol consumption during pregnancy impaired AAH expression in the placenta. However, more studies are needed to determine the crucial role of this enzyme in trophoblastic cell migration to promote placentation. These future experiments would allow detailed study the placenta as a valuable organ to serve as a diagnostic tool to identify novel biomarkers for detecting the outcome of IGF-1 deficiency and/or ethanol’s teratogenicity.

5. Conclusions
To conclude, the present study discloses that both IGF-1 deficiency and ethanol consumption during pregnancy promote placental disorganization, particularly in the junctional zone where trophoblast cells are expressed, and decrease placental efficiency, especially in KO fetuses, thus altering placentation development and fetal growth. In addition, both parameters alter AAH protein expression in the placenta, suggesting an abnormal placental development.

Data availability
Underlying data
Zenodo: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mice. https://doi.org/10.5281/zenodo.5750155

This project contains the following underlying data:
- Figure 2A_H&E Image Placenta WT (Control) 10×.tif
- Figure 2B_H&E Image Placenta HZ (Control) 10×.tif
Extended data
Zenodo: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mice. [https://doi.org/10.5281/zenodo.5750155](https://doi.org/10.5281/zenodo.5750155)

This project contains the following extended data:

- Extended data Figure 1_H&E Image Placenta 1.25×.tif

Reporting guidelines
Zenodo: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mice. [https://doi.org/10.5281/zenodo.5750155](https://doi.org/10.5281/zenodo.5750155)

- ARRIVE checklist.pdf (The ARRIVE guidelines 2.0)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0).

Author’s contributions
M-EI conceived, designed, developed and performed the experiments, manuscript writing, generated the first draft and successive versions of the manuscript; F-ROR designed the experiments and data analysis; BLM resource acquisition, discussion and manuscript edition; Z-MC, G-R M and LLA developed the experiments; RMD histological analysis; G-AP reviewed bibliography; C-CI conceived and designed the study and experimental model, data analysis and manuscript writing; C-TF conceived and designed the experiments, data analysis, contributed with reagents/materials/analysis tools, generated the first draft and successive versions of the manuscript. All authors revised and approved manuscript’s final version.

Acknowledgements
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Version 3

Reviewer Report 04 December 2024

https://doi.org/10.5256/f1000research.167655.r336632

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✔ Johannes van der Merwe

KU Leuven, University Hospitals Leuven, Leuven, Belgium

No further comments. The authors responded to all my comments/queries and amended the manuscript where necessary.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Fetal medicine, placental development, preterm birth

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 08 June 2024

https://doi.org/10.5256/f1000research.163402.r278020

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❓ Johannes van der Merwe

1 KU Leuven, University Hospitals Leuven, Leuven, Belgium
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Comments for the Authors:
This preclinical study aimed to evaluate the effect ethanol consumption during pregnancy in a mice model with/without IGF-1 deficiency. Overall, the topic is very relevant, the manuscript is well structured. However, there are some minor results reservations and multiple conclusions in the discussion is not supported by the results.

Specific comments:

1) Abstract

Please define AAH in background section and then use abbreviation.

2) Results:

Section 3.1. Is it possible to give the IGF-1 serum levels data in table format. As it is unclear whether there are any differences between the relative control and ethanol groups.

Section 3.2. Please give the data of the junctional zone comparisons.

Section 3.3 Please give the absolute weight of the fetuses.

3) Discussion:

Paragraph 2 “Results in this project showed that ethanol consumption during gestation significantly affects IGF-1 serum levels in HZ pregnant dams, according to existing literature.” This statement is not supported by the results in 3.1 as there is no direct comparison between the HZ-controls and HZ-Ethanol. Please clarify.

Paragraph 3. “The placenta is composed of by several strata, such as myometrium,...” The myometrium is not part of the placenta. Please rewrite.

Paragraph 7. “Our results showed a decrease in AAH protein expression due to IGF-1 deficiency, mainly in the junctional zone...” and “Ethanol consumption throughout gestation promoted the arrest of trophoblasts in islets and also reduced AAH expression in the junctional zone of HZ and KO placentas” This statement is not supported by results in 3.4 as no quantification of the AAH expression is given in this section. Only representative images are given in Figure 4. Can the authors quantify the expression of AAH in order to make direct statistical comparisons?

4) Conclusions:

“...ethanol consumption during pregnancy promote placental disorganization, particularly in the junctional zone where trophoblast cells are expressed, and decrease placental efficiency, thus altering placental development and fetal growth.”

The above statement does not seem to be supported by the results as shown. Since there is no quantification of junctional zone data and it was stated that there was no difference. Moreover, placental efficiently was only decreased in the KO group. It seems that this conclusion is overstated.
The authors chose to quote the link to AAH protein expression but there is no quantifiable data given to support this statement. Furthermore, there was an increase in the mRNA expression for this protein. Please clarify or rewrite.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Fetal medicine, placental development, preterm birth

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Jun 2024

**Fabiola Castorena Torres**

Monterrey, June 17th 2024

Dear Editorial Board members,

First of all, I would like to thank you for your kind answer as for carefully revising the manuscript, that has greatly aimed us to improve our manuscript. Herein we will outline any changes made to the manuscript following reviewers' advice (changes are shown in green in the manuscript) or, to the contrary, giving a rationale when a suggestion was not implemented.

**Reviewer 4:**
This preclinical study aimed to evaluate the effect ethanol consumption during pregnancy in a mice model with/without IGF-1 deficiency. Overall, the topic is very
relevant, the manuscript is well structured. However, there are some minor results reservations and multiple conclusions in the discussion is not supported by the results.
We would like to thank the reviewer for his/her comments, which have helped us to improve the manuscript.

Specific comments:

1. **Abstract:**
   - Please define AAH in background section and then use abbreviation.
   
   The reviewer is right. Sorry for this mistake, we have defined the abbreviation AAH.

2. **Results:**
   - Section 3.1. Is it possible to give the IGF-1 serum levels data in table format. As it is unclear whether there are any differences between the relative control and ethanol groups.
   
   The reviewer is right. We have included IGF-1 serum levels data in the Table.

   - Section 3.2. Please give the data of the junctional zone comparisons.
   
   As mentioned in section 3.2, we have included one figure as extended data (Figure 1) which shows all the placental zones analyzed in the present study: decidua, junctional zone and labyrinth. This figure is included in the following repository Zenodo: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mice.
   
   https://doi.org/10.5281/zenodo.5750155

   - Section 3.3. Please give the absolute weight of the fetuses.
   
   The information about fetal weight is presented in the following article of our research group: “Martín-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1- Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.

3. **Discussion:**
   - Paragraph 2. “Results in this project showed that ethanol consumption during gestation significantly affects IGF-1 serum levels in HZ pregnant dams, according to existing literature.” This statement is not supported by the results in 3.1 as there is no direct comparison between the HZ-controls and HZ-Ethanol. Please clarify.

   In the present manuscript, serum from pregnant dams from the four experimental groups (WT-Control, WT-Ethanol, HZ-Control and HZ-Ethanol) was used to determine IGF-1 serum levels. As shown in Table 2 mentioned above, as expected, HZ-Control pregnant dams have lower IGF-1 serum levels compared to WT-Control dams. Additionally, ethanol consumption decreased even more IGF-1 serum levels than WT-Control. To obtain these results, all groups were compared (Mann-Whitney U test was used to compare between two groups, and Kruskall-Wallis test was used to compare between all experimental groups).

   - Paragraph 3. “The placenta is composed of by several strata, such as myometrium,...” The myometrium is not part of the placenta. Please rewrite.

   The reviewer is right. We have rewritten that sentence.

   - Paragraph 7. “Our results showed a decrease in AAH protein expression due to IGF-1 deficiency, mainly in the junctional zone...” and “Ethanol consumption throughout gestation promoted the arrest of trophoblasts in islets and also
reduced AAH expression in the junctional zone of HZ and KO placentas. This statement is not supported by results in 3.4 as no quantification of the AAH expression is given in this section. Only representative images are given in Figure 4. Can the authors quantify the expression of AAH in order to make direct statistical comparisons?

Thanks to the reviewer for this suggestion. We tried to measure quantitatively AAH expression in immunohistochemistry images, but unfortunately results were not conclusive. The results herein reported are only qualitative data. We have corrected these sentences as follows: “Our results suggested a decrease in AAH protein expression due to IGF-1 deficiency, mainly in the junctional zone, where trophoblast cells are located. Ethanol consumption throughout gestation seemed to promote the arrest of trophoblasts in islets and also reduced AAH expression in the junctional zone of HZ and KO placentas”.

Conclusions:

1. Conclusions:
   - “…ethanol consumption during pregnancy promote placental disorganization, particularly in the junctional zone where trophoblast cells are expressed, and decrease placental efficiency, thus altering placental development and fetal growth.” The above statement does not seem to be supported by the results as shown. Since there is no quantification of junctional zone data and it was stated that there was no difference. Moreover, placental efficiently was only decreased in the KO group. It seems that this conclusion is overstated.

The reviewer is right. We have corrected the sentence as follows: “ethanol consumption during pregnancy promote placental disorganization, particularly in the junctional zone where trophoblast cells are expressed, and decrease placental efficiency, especially in KO fetuses, thus altering placental development and fetal growth.”
   - The authors chose to quote the link to AAH protein expression but there is no quantifiable data given to support this statement. Furthermore, there was an increase in the mRNA expression for this protein. Please clarify or rewrite.

The reviewer is right. Thanks for this suggestion. In spite of IGF-1 deficiency showed a significant increase in Asph expression in KO placentas from control group, while ethanol consumption during gestation also exhibited an increase in Asph in WT and HZ placentas; results for AAH protein expression in immunohistochemistry analyses suggested that both insults (IGF-1 deficiency and ethanol consumption) decreased AAH protein expression. However, immunohistochemistry analyses could not be quantified.

This difference between mRNA and protein expression seems to point to the existence of a mechanism is taking place in order to promote IGF-1 downstream signaling expression to try to stimulate placental development despite IGF-1 deficiency and ethanol consumption. Nevertheless, more studies are needed in order to study this hypothesis, as suggested in the limitations section of the present manuscript.

Yours faithfully,

Inma Castilla de Cortázar Larrea, MD, PhD.
Professor of Medical Physiology
HM Velazquez Cabinet
Velazquez 25, 28001 Madrid, Spain
Tel.: +34 686652710
General comments:
This study used a mouse model of maternal/paternal IGF1 partial deficiency (+/-) to investigate the effects of prenatal alcohol exposure on placental morphology and gene expression. This resulted in the full complement of genotypes (HZ +/-, KO -/- and WT +/-) for IGF1 in the fetal/placental pairs from each dam. The rationale for using this deficiency model was that alterations in IGF are proposed to be the underlying mechanism for alcohol-induced alterations in the placenta and subsequent growth restriction in the fetus. However, it is unclear how using a transgenic model with perturbed IGF1 expression in combination with alcohol exposure is more useful at determining mechanism than just measuring IGF1 protein/gene expression levels in an alcohol exposure model in WT mice. Indeed, the latter has already been done. While this study reports some potentially interesting observations regarding effects on placental morphology and gene expression, this is not supported by quantitative data. We also note that many of the comments raised by a previous reviewer have not been adequately addressed in the second version of the manuscript.

Specific comments:
- The previous reviewer requested that data for placental and fetal weights, as well as the fetal/placental ratio, be presented. The authors mention that this data is previously published (DOI: 10.3390/biology11091264) and yet there is no mention anywhere in the
manuscript of this publication (although it has been tacked on at the end of the reference list with no citation in the text). This needs to be mentioned in the introduction to provide context for the current study.

- This previous study should also be mentioned in the methods when describing the animal treatments to provide important maternal/pregnancy information such as food and liquid intake, weight gain over gestation, average number of pups, sex ratio, as well as the fetal weight and placental weight mentioned above. This could simply be a description with a reference to the study with the original data.

- While the number of HZ dams that were ethanol vs control is provided in the methods (n=7 per group) it is unclear how many placentas were selected per dam for subsequent experiments. We reiterate the concerns made by the previous reviewer that the sample size for the WT control group is surprisingly small based on the expected ratio of HZ/WT/KO offspring. Also, the sample size indicated in the legend of Figure 3 for the KO control group was 22 and yet the supplemental file with raw data (and the subsequent study) only had a sample size of 19. The sample size calculation is also not optimal and a power analysis should have been used, particularly given that there has been a previous similar study done by the authors to estimate SD for this calculation.

- One of the major concerns of this study is that placental sex is not mentioned anywhere in the paper and is not factored into the analysis. There is a large body of evidence from both preclinical and clinical studies that the sex of the placenta influences adaptations to protect the fetus from maternal exposures, such as alcohol consumption. The responses to the previous reviewer indicate that fetal/placental sex was determined by the researchers. Indeed, the authors reveal that they only used male placentas in their analysis of gene expression to “reduce variability between groups”. They also reveal that a greater number of females were used for the HZ ethanol group in the data for figure 3. None of this is justified or mentioned in the methods for the paper.

- Another major concern is that representative images for immunohistochemistry of AAH and morphology of placentas are provided but there is no quantification to justify the claims in the text. Immunohistochemistry is best used to demonstrate localisation of protein in the tissue while Western Blot is required for quantification. The authors provide no evidence for the claims of decreases in AAH in the ethanol-exposed tissues. In fact, in response to a similar question by a previous reviewer, they mention that they did do quantification of their immunohistochemical staining but there were no significant differences, so data is not shown.

- Similarly, with respect to Figure 2, the authors claim in the text there is “…altered trophoblast migration and promoted placental hypoplasia, particularly in HZ and KO placentas”. However, there is no quantitative data to support this. The description of the tissues and cell types are also incorrect, as pointed out by the previous reviewer.

- The authors also claim that there were differences in gene expression due to ethanol treatment, but this is not justified by the data presented.

- The statistical analysis in Figure 3 is incorrect. The figure legend mentions that individual t-tests were used, but this should be analysed using a 2-way ANOVA with an appropriate post-hoc test (although the authors also mention that a GLIM was used but it is unclear if the P values come from this or the t-tests). There is also duplication of data from Figure 4G in Table 1, making the figure redundant.

- An interesting point was raised by the previous reviewer the different genotype for IGF-1 in decidua (maternal tissue) versus the fetus (i.e. that there is always partial deficiency in the decidua). The authors respond by suggesting a potential compensatory role of IGF2 but
none of these points are discussed in the paper.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Animal models of prenatal alcohol exposure examining physiological, molecular and morphological outcomes in the placenta and offspring.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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**Author Response 17 Jun 2024**

**Fabiola Castorena Torres**

Monterrey, June 17th 2024

Dear Editorial Board members,

First of all, I would like to thank you for your kind answer as for carefully revising the manuscript, that has greatly aimed us to improve our manuscript. Herein we will outline any changes made to the manuscript following reviewers’ advice (changes are shown in green in the manuscript) or, to the contrary, giving a rationale when a suggestion was not implemented.

**Reviewer 3:**
This study used a mouse model of maternal/paternal IGF1 partial deficiency (+/-) to investigate the effects of prenatal alcohol exposure on placental morphology and gene expression. This resulted in the full complement of genotypes (HZ +/-, KO -/- and
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We would like to thank the reviewer for his/her comments, which have helped us to improve the manuscript. Herein we will outline any changes made to the manuscript following reviewers' advice or, to the contrary, giving a rationale when a suggestion was not implemented.

Specific comments:

○ The previous reviewer requested that data for placental and fetal weights, as well as the fetal/placental ratio, be presented. The authors mention that this data is previously published (DOI: 10.3390/biology11091264) and yet there is no mention anywhere in the manuscript of this publication (although it has been tacked on at the end of the reference list with no citation in the text). This needs to be mentioned in the introduction to provide context for the current study.

The reviewer is right. It should be noted that the present manuscript belongs to a series of results of a larger project and it was first published in F1000 Research in December 2021. The second part of the results obtained from this project was published in August 2022 (DOI: 10.3390/biology11091264), the reason why there was no information about the data requested by the reviewers in the present manuscript. In this sense, we believe it would be inappropriate to cite an article that was published later than the present manuscript.

○ This previous study should also be mentioned in the methods when describing the animal treatments to provide important maternal/pregnancy information such as food and liquid intake, weight gain over gestation, average number of pups, sex ratio, as well as the fetal weight and placental weight mentioned above. This could simply be a description with a reference to the study with the original data.

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○ While the number of HZ dams that were ethanol vs control is provided in the methods (n=7 per group) it is unclear how many placentas were selected per dam for subsequent experiments. We reiterate the concerns made by the previous reviewer that the sample size for the WT control group is surprisingly
small based on the expected ratio of HZ/WT/KO offspring. Also, the sample size indicated in the legend of Figure 3 for the KO control group was 22 and yet the supplemental file with raw data (and the subsequent study) only had a sample size of 19. The sample size calculation is also not optimal and a power analysis should have been used, particularly given that there has been a previous similar study done by the authors to estimate SD for this calculation.

In the present study, the following four experimental groups were analyzed:
- WT-Control, \( n = 6 \) pregnant dams.
- WT-Ethanol, \( n = 4 \) pregnant dams.
- HZ-Control, \( n = 6 \) pregnant dams.
- HZ-Ethanol, \( n = 9 \) pregnant dams.

In the HZ-Ethanol group, a greater number of pregnant dams was used to decrease the risk of death or early delivery due to ethanol treatment. As mentioned by the reviewer, a low number of WT individuals were obtained in the HZ-Control group of pregnant dams, but nevertheless, Mendelian genetics is fulfilled.

In the supplemental file with raw data only 4 placentas from each group were used to analyze gene expression by RT-PCR analysis.

One of the major concerns of this study is that placental sex is not mentioned anywhere in the paper and is not factored into the analysis. There is a large body of evidence from both preclinical and clinical studies that the sex of the placenta influences adaptations to protect the fetus from maternal exposures, such as alcohol consumption. The responses to the previous reviewer indicate that fetal/placental sex was determined by the researchers. Indeed, the authors reveal that they only used male placentas in their analysis of gene expression to “reduce variability between groups”. They also reveal that a greater number of females were used for the HZ ethanol group in the data for figure 3. None of this is justified or mentioned in the methods for the paper.

Fetal sex was determined in the present study. However, only males were used to determine placental morphological alterations and gene expressions. As aforementioned, in the HZ-Ethanol group, a greater number of pregnant dams was used to decrease the risk of death or early delivery due to ethanol treatment.

Another major concern is that representative images for immunohistochemistry of AAH and morphology of placentas are provided but there is no quantification to justify the claims in the text. Immunohistochemistry is best used to demonstrate localisation of protein in the tissue while Western Blot is required for quantification. The authors provide no evidence for the claims of decreases in AAH in the ethanol-exposed tissues. In fact, in response to a similar question by a previous reviewer, they mention that they did do quantification of their immunohistochemical staining but there were no significant differences, so data is not shown.

Thanks to the reviewer for this suggestion. First of all, we tried to measure AAH protein expression in placenta by Western Blot analysis, but no results were obtained, because the amount of sample was not sufficient to obtain results. Also, we tried to measure quantitatively AAH expression in immunohistochemistry images, but unfortunately results were not conclusively. The results herein reported are only qualitative data. We have corrected the discussion section as follows: “Our results suggested a decrease in AAH
protein expression due to IGF-1 deficiency, mainly in the junctional zone, where trophoblast cells are located. Ethanol consumption throughout gestation seemed to promote the arrest of trophoblasts in islets and also reduced AAH expression in the junctional zone of HZ and KO placentas”.

- Similarly, with respect to Figure 2, the authors claim in the text there is “...altered trophoblast migration and promoted placental hypoplasia, particularly in HZ and KO placentas”. However, there is no quantitative data to support this. The description of the tissues and cell types are also incorrect, as pointed out by the previous reviewer.

The reviewer is right. Although there are findings that could indicate the presence of placental hypoplasia (e.g., placental weight), more studies are needed to reach this conclusion. We removed this sentence.

- The authors also claim that there were differences in gene expression due to ethanol treatment, but this is not justified by the data presented.

The reviewer is right. In the present study, when statistical analysis was performed, first we grouped the results by treatment (Water -control- or ethanol), and significant results were obtained ($p < 0.05$). However, when the results were grouped by genotype (WT, HZ and KO), no significant differences were observed.

- The statistical analysis in Figure 3 is incorrect. The figure legend mentions that individual t-tests were used, but this should be analysed using a 2-way ANOVA with an appropriate post-hoc test (although the authors also mention that a GLIM was used but it is unclear if the P values come from this or the t-tests). There is also duplication of data from Figure 4G in Table 1, making the figure redundant.

Thanks to the reviewer for his/her suggestion. ANOVA analyzes the magnitude of the effect, while general linear model analyzes both the magnitude and the direction of the effect. In this case, to better evaluate the effect of the two independent variables in the present study (ethanol consumption and IGF-1 deficiency) a general linear model was performed. In this sense, the p-values obtained belong to both statistical analyses, considering significant differences at a level of $p < 0.05$.

In Table 1, we present the results from RT-PCR analyses, having Asph expression statistical significance. For this reason, these results were the only ones that were represented graphically, in order to better visualize the results.

- An interesting point was raised by the previous reviewer the different genotype for IGF-1 in decidua (maternal tissue) versus the fetus (i.e. that there is always partial deficiency in the decidua). The authors respond by suggesting a potential compensatory role of IGF2 but none of these points are discussed in the paper.

The reviewer is right, thanks for his/her suggestion. The present manuscript focuses mainly on IGF-1. However, we have included the following sentence: “Although the decidua, which is in contact with the maternal side, is always partially deficient in IGF-1, the rest of the placenta depends on the fetus; that is, if the fetus is WT, the placenta will be of the same genotype. Also, IGF-2 has an important role in fetal growth due to its direct anabolic actions within the fetus, and by its modulation of placental supply capacity, which may be a compensatory mechanism for IGF-1 deficiency (10.1016/j.placenta.2018.01.005).”

Yours faithfully,
The article of Martín-Estal et al. presents data on the effect of ethanol consumption during pregnancy in the mouse. In parallel, the authors knocked-out IGF-1 in the dams to generate WT, HZ and KO fetuses and placentas. This is an interesting study looking into the double effect of IGF-1 deficiency and ethanol consumption. This reviewer has identified quite a number of specific concerns, see below for details.

General concerns
Please add the species to the respective presentation of published data throughout the manuscript, e.g. page 4, 2nd paragraph.

Specific concerns
Title: Please provide the species in the title. This always helps the reader to directly assign the study to the correct field. E.g.: “… in IGF-1 deficient MOUSE PLACENTAS”
Abstract, background: Please define the abbreviation AAH here.
Abstract, methods: Please better define what is meant with “… were given 10% ethanol …”. Was this
10% ethanol in water or in food or both? Please specify.
P4, 4th paragraph: Here the authors state that “AAH is overexpressed in trophoblast cells”. For this reviewer the word “overexpressed” points to a non-physiologically high expression of the protein. Since this is not the case, this reviewer would recommend to use a different wording, such as “AAH is HIGHLY expressed in trophoblast cells”.
P4, 9th paragraph: The 10% ethanol were only given in water? If so, was water changed every day? Did the authors took the evaporation of ethanol over time into account? Have the authors monitored how much water was used? If so, were there differences between water and 10% ethanol?
P5, 2nd paragraph: Here the authors state: “a caesarean section was performed to obtain fetuses and placenta, which were measured and weighted (Figure 1).” However, fetuses, measurements and weights cannot be found in figure 1. Figure 3 shows ratios, while the original values are missing.
P5, 2rd paragraph: What is meant with “Fetuses and placenta were stored randomly blind”? Please explain. The authors should know to which group the fetuses/placenta belong and what the fetus-placenta pairs are.
P5, 2.3: Only here the authors tell the reader that WT mice were treated with ethanol as well. Please extend chapter 2.1 and clearly describe the groups and treatments. Also, it does not become clear whether the WT mice were pregnant as well. Please specify.
P5, 2.3: Please clearly describe which sera were used for IGF-1 Elisas. In the moment, the description is unintelligible (first sentence of 2.3).
P6, 1st paragraph: Here the authors describe that “All images were analysed using the processing package ImageJ …”. Please describe the way images were taken (Randomly? Systematic randomly? Magnification? Number of images per placenta? etc.)
P6, 3rd paragraph: Here the authors describe that “Digital images of tissue sections (five images per placenta) were captured by three observers (double-blind) …”. What does this mean? Was there a total of 15 images per slide or only 5? How did the observers choose the images? What was the magnification of the images?
P6, 3.1: This reviewer does not understand the last sentence of this chapter: “Interestingly, a possible synergic effect, that decreased IGF-1 serum levels, was observed by the partial IGF-1 deficiency and ethanol consumption (HZ-Ethanol) during gestation compared to WT-Control group (p < 0.01).” The IGF-1 values for the HZ groups are not different and the IGF-1 values for the WT groups are also not different (each w and w/o ethanol). And of course, the IGF-1 values in the HZ groups are significantly reduced compared to the WT groups. Where do the authors see a synergistic effect of IGF-1 deficiency and ethanol? What about the serum IGF-1 values in the KO groups?
P7, 3.2: In the first sentence, the authors refer to figure 1 for extended data on the strata of the placenta. However, this information is not given in figure 1.
P7, 3.2: Only now the authors provide the information that there were pregnant WT animals. Please add their numbers, age, placental and fetal weights, number of pups etc. to the methods section. Best would be to provide a table with the HZ and WT animals and their numbers, weights (fetal and placental), etc.
P7, 3.2 & figure 2: The text reads as if figure 2A is derived from a WT placenta of a WT mouse (“In the WT placentas from the control group ...”). If this is the case, this needs to be clarified in the caption of figure 2. Then, figure 2 needs an additional row of images with the WT placentas from HZ dams.
P7, 3.2: If the wording above (“In the WT placentas from the control group ...”) points to WT dams with WT fetuses, then he question arises what the following animals are: “KO placentas from the control group”. This is hard to understand. Please explain.
P7-8, 3.2: Here the authors state that ethanol consumption “promoted trophoblast arrest in islets in
all groups”. How do the authors know there is arrest of trophoblast cells? The “islets” may well be finger-like structures of the junctional zone reaching into the labyrinth, pointing to a less organized layering of labyrinth and junctional zone.

P8, 3.2: Here the authors state that “ethanol use altered trophoblast migration (Figure 2D) and promoted placental hypoplasia”. Questions: (1) How did the authors assess differences in trophoblast migration from these images? (2) Where did the authors identify placental hypoplasia? From the images in figure 2, it looks as if the area of the labyrinth is increased with ethanol, at least in WT and HZ.

P8, 3.3: Here the authors state that “The chronic exposure to ethanol during gestation resulted in a significant reduction in placental efficiency (p < 0.001) compared to control groups.”. Please add any value to support this statement. As can be seen from figure 3, only in the KO group there was a significant (<0.05) reduction in the fetal/placental weight ratio.

P8, 3.3: Idea: It looks as if the placenta tried to counteract the reduced fetal growth under ethanol. The increased area of the labyrinth points to this. Hence, could the authors check volumes/areas of the labyrinth between groups?

P8, 3.4: Here the authors state that “IGF-1 deficiency revealed a decrease in AAH expression within the junctional zone …”. Such a statement should only be made if there is a respective analysis with a p<0.05. However, there are only a few images that have not been quantitatively analyzed.

P9, 3.4, last sentence (“Ethanol consumption …”): The statement that ethanol decreased AAH expression is not supported by respective data. Protein has not been quantified and RNA does not show differences. The rest of the sentence is mere speculation without supporting data, so please remove or rephrase.

P10, 3rd paragraph of discussion: The junctional zone of the mouse placenta is NOT composed of syncytiotrophoblast and cytotrophoblast, this is only true for the labyrinth. Please correct.

P10, 3rd paragraph of discussion: Since the authors have wrongly allocated the syncytiotrophoblast (as major source for IGF-1) to the junctional zone, their concept of reduced expression of IGF-1 in the junctional zone has lost its scientific basis. See also p12, lines 5-8.

P10, 4th paragraph of discussion: The authors do not show data to support placental hypoplasia or changes in trophoblast motility and trophoblast invasion. Hence, the respective discussion should be written with great caution.

Figure 1: This is a nice representation of the experimental procedure. This could be further improved by adding the following: (1) Add the number of pregnant animals per group. (2) Since the HZ animals were given ethanol during pregnancy 8 or not), this could be indicated as well. (3) The WT animals need to be added as well. (4) The number of placentas in the three groups can be added.

Figure 2: The comparison of images in A and D (WT w and w/o ethanol) shows a massively increased area of the labyrinth. Is this only present in this one case or has this been a general feature of ethanol in the WT mice? A similar difference can be found in the HZ/HZ mice (2B/E).

Figure 4: Just a comment: The data from the analysis of the AAH protein (A-F) do not fit the data from the analysis of the AAH RNA (G). This is not surprising as differences between RNA and protein expression are regularly found.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Partly
Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Placenta; trophoblast; cell biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Author Response 06 Jun 2024**

**Fabiola Castorena Torres**

Monterrey, June 5th 2024

Dear Editorial Board members,

First of all, I would like to thank you for your kind answer as for carefully revising the manuscript, that has greatly aimed us to improve our manuscript. Herein we will outline any changes made to the manuscript following reviewers’ advice (changes are shown in green in the manuscript) or, to the contrary, giving a rationale when a suggestion was not implemented.

**Reviewer 2:**
The article of Martín-Estal et al. presents data on the effect of ethanol consumption during pregnancy in the mouse. In parallel, the authors knocked-out IGF-1 in the dams to generate WT, HZ and KO fetuses and placentas. This is an interesting study looking into the double effect of IGF-1 deficiency and ethanol consumption. This reviewer has identified quite a number of specific concerns, see below for details.

We would like to thank the reviewer for his/her comments, which have helped us to improve the manuscript.

**General concerns:**
Please add the species to the respective presentation of published data throughout the manuscript, e.g. page 4, 2nd paragraph.
The reviewer is right. We added this information.
Specific concerns:
  ○ **Title:** Please provide the species in the title. This always helps the reader to directly assign the study to the correct field. E.g.: “… in IGF-1 deficient MOUSE PLACENTAS”.
    The reviewer is right. We have changed the title as follows: “Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mouse placenta”.
  ○ **Abstract, background:** Please define the abbreviation AAH here.
    The reviewer is right. Sorry for this mistake, we have defined the abbreviation AAH.
  ○ **Abstract, methods:** Please better define what is meant with “… were given 10% ethanol ...”. Was this 10% ethanol in water or in food or both? Please specify.
    The reviewer is right. Mice were given 10% ethanol in water during the experimental protocol. We have added this information in the abstract section. In the methods sections, this information was specified: “An adaptation period to ethanol (14 days) was performed, where HZ female mice were given increasing concentrations of 2%, 5% and 10% of ethanol in water” (Kleiber ML, Wright E, Singh SM: Maternal voluntary drinking in C57BL/6j mice: Advancing a model for fetal alcohol spectrum disorders. Behav. Brain Res. 2011;223:376–387. Elsevier B.V. 21601595 10.1016/j.bbr.2011.05.005).
  ○ **P4, 4th paragraph:** Here the authors state that “AAH is overexpressed in trophoblast cells”. For this reviewer the word “overexpressed” points to a non-physiologically high expression of the protein. Since this is not the case, this reviewer would recommend to use a different wording, such as “AAH is HIGHLY expressed in trophoblast cells”.
    The reviewer is right. Sorry for this mistake, we have changed this word as suggested.
  ○ **P4, 9th paragraph:** The 10% ethanol were only given in water? If so, was water changed every day? Did the authors took the evaporation of ethanol over time into account? Have the authors monitored how much water was used? If so, were there differences between water and 10% ethanol?
    The 10% ethanol was only given in water, and this water was changed every day for all mice included in the ethanol group. We have considered the evaporation of ethanol over time, but no significant differences were observed between ethanol groups and control groups (water).
  ○ **P5, 2nd paragraph:** Here the authors state: “a caesarean section was performed to obtain fetuses and placenta, which were measured and weighted (Figure 1).” However, fetuses, measurements and weights cannot be found in figure 1. Figure 3 shows ratios, while the original values are missing.
    The reviewer is right. We removed “Figure 1” from this statement. This information about fetal parameters (fetal weight, fetal height and placental weight) is presented in the following article of our research group: “Martín-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1- Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.
  ○ **P5, 2nd paragraph:** What is meant with “Fetuses and placenta were stored randomly blind”? Please explain. The authors should know to which group the fetuses/placentas belong and what the fetus-placenta pairs are.
After cesarean section, fetuses an placentas from pregnant dams from different groups were stored in paraformaldehyde 4% or liquid nitrogen. The determination of which set of fetuses and placentas from a pregnant dam from an experimental group was determined randomly for each assay, but we know which group the fetuses/placentas belong and what the fetus-placenta pairs are. We removed “blind” from this statement.

**P5, 2.3:** Only here the authors tell the reader that WT mice were treated with ethanol as well. Please extend chapter 2.1 and clearly describe the groups and treatments. Also, it does not become clear whether the WT mice were pregnant as well. Please specify.

The reviewer is right. We are sorry for this mistake. In the present project, four experimental groups of pregnant dams were analyzed: two groups given water (WT-Control and HZ-Control), and two groups given 10% ethanol in water (WT-Ethanol and HZ-Ethanol). For subsequent analysis, only fetuses and placentas from HZ x HZ matings were analyzed. We have included this information in the manuscript.

**P5, 2.3:** Please clearly describe which sera were used for IGF-1 Elisas. In the moment, the description is unintelligible (first sentence of 2.3).

The reviewer is right. We are sorry for this mistake. As mentioned above, in the present project, four experimental groups of pregnant dams were analyzed: two groups given water (WT-Control and HZ-Control), and two groups given 10% ethanol in water (WT-Ethanol and HZ-Ethanol). Serum from pregnant dams from these four experimental groups were used to determine IGF-1 values.

**P6, 1st paragraph:** Here the authors describe that “All images were analysed using the processing package ImageJ ...”. Please describe the way images were taken (Randomly? Systematic randomly? Magnification? Number of images per placenta? etc.).

In the placental histological study section, this information is included in the present manuscript: “Finally, all histological preparations were evaluated independently by three observers (double-blind) in a Zeiss Axio Imager M2 microscope (Zeiss, Baden-Württemberg, Germany) to establish morphology alterations in the placenta due to ethanol consumption throughout gestation.” Also, this statement is included: “Three images were taken per sample of at least three individuals from each group and genotype.”

In Figure 2, this information is included in the figure legend: “Original magnification 100x. Scale bar = 100 μm.”

In the immunohistochemical staining section, this information is included in the present manuscript: “Digital images of tissue sections (five images per placenta) were analyzed by three observers (double-blind) using a Zeiss Axio Imager M2 microscope. All images were analysed using the processing package ImageJ (National Institutes of Health, Maryland, USA).”

In Figure 4, this information is included in the figure legend: “Original magnification 100x.”

We have included the following information: “Scale bar = 100 μm.”

**P6, 3rd paragraph:** Here the authors describe that “Digital images of tissue sections (five images per placenta) were captured by three observers (double-blind) ...”. What does this mean? Was there a total of 15 images per slide or only 5? How did the observers choose the images? What was the magnification of the images?

In the immunohistochemical staining section, five images per placenta were captured and then were analyzed by three observers (double-blind). The reviewer is right, we changed the...
The magnification of the images is included in the legend of figure 4.

○ **P6, 3.1**: This reviewer does not understand the last sentence of this chapter: “Interestingly, a possible synergic effect, that decreased IGF-1 serum levels, was observed by the partial IGF-1 deficiency and ethanol consumption (HZ-Ethanol) during gestation compared to WT-Control group (p < 0.01).” The IGF-1 values for the HZ groups are not different and the IGF-1 values for the WT groups are also not different (each w and w/o ethanol). And of course, the IGF-1 values in the HZ groups are significantly reduced compared to the WT groups. Where do the authors see a synergistic effect of IGF-1 deficiency and ethanol? What about the serum IGF-1 values in the KO groups?

To validate IGF-1 serum levels in the present experimental model, serum from pregnant dams from the four experimental groups were used (WT-Control, WT-Ethanol, HZ-Control and HZ-Ethanol). As shown in the following table, as expected, HZ-Control pregnant dams have lower IGF-1 serum levels compared to WT-Control dams. Ethanol consumption decreased even more IGF-1 serum levels than WT-Control.

| Group             | IGF-1 Serum Levels |
|-------------------|--------------------|
| WT-Control        | 739 ± 48 ng/mL     |
| WT-Ethanol        | 795 ± 147 ng/mL    |
| HZ-Control        | 454 ± 58*, a ng/mL |
| HZ-Ethanol        | 422 ± 43*, b ng/mL |

* p < 0.001 HZ vs WT.

a p < 0.005 HZ-Control vs WT-Control.

b p < 0.01 HZ-Ethanol vs WT-Control.

○ **P7, 3.2**: In the first sentence, the authors refer to figure 1 for extended data on the strata of the placenta. However, this information is not given in figure 1.

We have included one figure as extended data (Figure 1) which shows all the placental zone analyzed in the present study. This figure is included in the following repository Zenodo: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient mice. https://doi.org/10.5281/zenodo.5750155 52.

○ **P7, 3.2**: Only now the authors provide the information that there were pregnant WT animals. Please add their numbers, age, placental and fetal weights, number of pups etc. to the methods section. Best would be to provide a table with the HZ and WT animals and their numbers, weights (fetal and placental), etc.

The reviewer is right. We are sorry for this mistake. In the present project, four experimental groups of pregnant dams were analyzed: two groups given water (WT-Control and HZ-Control), and two groups given 10% ethanol in water (WT-Ethanol and HZ-Ethanol). For subsequent analysis, only fetuses and placentas from HZ x HZ matings were analyzed. We have included this information in the manuscript nad in Figure 1. The other data suggested by the reviewer can be found in the following article from our research group: “Martin-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1-Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.

○ **P7, 3.2 & figure 2**: The text reads as if figure 2A is derived from a WT placenta of a WT mouse (“In the WT placentas from the control group ...”). If this is the case, this needs to be clarified in the caption of figure 2. Then, figure 2 needs an
additional row of images with the WT placentas from HZ dams.

The images shown in figure 2 belong to placentas from HZ-Control and HZ-Ethanol groups. These groups were mated with HZ male mice (HZ x HZ), resulting in three different fetal genotypes: WT, HZ and KO. In this case, WT, HZ and KO placentas correspond to fetal placentas from HZ-Control and HZ-Ethanol groups.

- **P7. 3.2:** If the wording above (“In the WT placentas from the control group ...”) points to WT dams with WT fetuses, then he question arises what the following animals are: “KO placentas from the control group”. This is hard to understand. Please explain.

The wording “In the WT placentas from the control group...” belongs to placentas from HZ-Control group. Sorry for these misunderstandings due to the nomenclature.

- **P7-8. 3.2:** Here the authors state that ethanol consumption “promoted trophoblast arrest in islets in all groups”. How do the authors know there is arrest of trophoblast cells? The “islets” may well be finger-like structures of the junctional zone reaching into the labyrinth, pointing to a less organized layering of labyrinth and junctional zone.

The reviewer is right. However, as it can be seen in Figure 2, trophoblast are arrested in island-like forms, denoting poor organization and development of the placenta.

- **P8. 3.2:** Here the authors state that “ethanol use altered trophoblast migration (Figure 2D) and promoted placental hypoplasia”. Questions: (1) How did the authors assess differences in trophoblast migration from these images? (2) Where did the authors identify placental hypoplasia? From the images in figure 2, it looks as if the area of the labyrinth is increased with ethanol, at least in WT and HZ.

The reviewer is right. Although there are findings that could indicate the presence of placental hypoplasia (e.g., placental weight), more studies are needed to reach this conclusion. We removed this sentence.

- **P8. 3.3:** Here the authors state that “The chronic exposure to ethanol during gestation resulted in a significant reduction in placental efficiency (p < 0.001) compared to control groups.”. Please add any value to support this statement. As can be seen from figure 3, only in the KO group there was a significant (<0.05) reduction in the fetal/placental weight ratio.

This statement corresponds to the comparison between both ethanol and control groups, proving that ethanol first reduces placental efficiency. Then, a multiple comparison (general linear model) between all genotypes from all experimental groups were performed, in order to validate if IGF-1 deficiency promotes this alteration in placental efficiency.

- **P8. 3.3:** Idea: It looks as if the placenta tried to counteract the reduced fetal growth under ethanol. The increased area of the labyrinth points to this. Hence, could the authors check volumes/areas of the labyrinth between groups?

The reviewer is right. This is an interesting suggestion, but we could not be able to measure the volumes/areas of the placentas. This measurement would be very useful to understand and explain the ethanol's teratogenic effects during gestation.

- **P8. 3.4:** Here the authors state that “IGF-1 deficiency revealed a decrease in AAH expression within the junctional zone ...”. Such a statement should only be made if there is a respective analysis with a p<0.05. However, there are only a few images that have not been quantitatively analyzed.

We tried to measure quantitatively AAH expression in immunohistochemistry images, but
The results herein reported are only qualitative data.

- **P9, 3.4, last sentence (“Ethanol consumption …”):** The statement that ethanol decreased AAH expression is not supported by respective data. Protein has not been quantified and RNA does not show differences. The rest of the sentence is mere speculation without supporting data, so please remove or rephrase.

  The reviewer is right. We have removed this sentence.

- **P10, 3rd paragraph of discussion:** The junctional zone of the mouse placenta is NOT composed of syncytiotrophoblast and cytotrophoblast, this is only true for the labyrinth. Please correct.

  Thanks for the reviewer's comments. Trophoblast giant cells, the invasive cells responsible for invading maternal vasculature to promote placental development, are located in the labyrinth. However, spongiotrophoblast cells and glycogen trophoblast cells are found in the labyrinth and in the junctional zone (10.3390/nu15163564).

- **P10, 3rd paragraph of discussion:** Since the authors have wrongly allocated the syncytiotrophoblast (as major source for IGF-1) to the junctional zone, their concept of reduced expression of IGF-1 in the junctional zone has lost its scientific basis. See also p12, lines 5-8.

  Thanks for the reviewer's comments. As aforementioned, trophoblast giant cells, the invasive cells responsible for invading maternal vasculature to promote placental development, are located in the labyrinth. However, spongiotrophoblast cells and glycogen trophoblast cells are found in the labyrinth and in the junctional zone (10.3390/nu15163564). Throphoblast invasion is observed in the junctional zone, in order to develop a normal placenta. Alterations in this trophoblast migration can lead to adverse pregnancy consequences due to a limit blood flow, such as fetal growth restriction and preeclampsia (10.1067/mob.2002.127305).

- **P10, 4th paragraph of discussion:** The authors do not show data to support placental hypoplasia or changes in trophoblast motility and trophoblast invasion. Hence, the respective discussion should be written with great caution.

  The reviewer is right. Although there are findings that could indicate the presence of placental hypoplasia (e.g., placental weight), more studies are needed to reach this conclusion. We removed this sentence.

- **Figure 1:** This is a nice representation of the experimental procedure. This could be further improved by adding the following: (1) Add the number of pregnant animals per group. (2) Since the HZ animals were given ethanol during pregnancy 8 or not), this could be indicated as well. (3) The WT animals need to be added as well. (4) The number of placentas in the three groups can be added.

  The reviewer is right. We have updated Figure 1 as suggested.

- **Figure 2:** The comparison of images in A and D (WT w and w/o ethanol) shows a massively increased area of the labyrinth. Is this only present in this one case or has this been a general feature of ethanol in the WT mice? A similar difference can be found in the HZ/HZ mice (2B/E).

  This increased area of the labyrinth was presented in most of the cases, suggesting that this increase in the area may be due to placental disorganization caused by ethanol consumption.

- **Figure 4:** Just a comment: The data from the analysis of the AAH protein (A-F) do not fit the data from the analysis of the AAH RNA (G). This is not surprising as
differences between RNA and protein expression are regularly found.
The reviewer is right. One of the limitations of the study is that we could not measure AAH protein expression levels in placenta, results that can be useful to explain the ethanol teratogenic effects during gestation and their relation with IGF-1 deficiency.

Yours faithfully,

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Competing Interests: The authors declare no competing interests.
development. They notably showed that total IGF-1 deficiency and ethanol consumption both led to structural anomalies in the placental layers. They also showed a reduced placental efficiency in IGF-1 KO placentas, that was further reduced by ethanol consumption. They also evaluated the mRNA and protein expression of AAH and the mRNA expression of a few other genes.

While the data are interesting, their description and analysis is not sufficient. The fetal weight is an important element and thus should be presented. Fetal growth retardation/restriction is indeed a key aspect of IGF-1 signaling and ethanol consumption, as it is well described in the introduction. I would suggest adding 2 panels to Figure 3 with the fetal and placental weight (in addition to the ratio), this is especially important as the authors mentioned placental hypoplasia that is not visible in the presented data. The placental morphology should be analyzed more thoroughly with for example a quantification of the length ratio between JZ/labyrinth and/or the number of abnormal islets in the labyrinth (the quality of the image should also be improved as the text within the image is hardly readable). The AAH immunohistochemistry would also benefit for a quantification analysis (in specific layers). For all the quantitative data, the statistical analysis effectively used should be described with the indication of the number of analyzed samples per group. Post hoc test should be performed after two-way ANOVA or Kruskal Wallis test, to compare the genotypes within the water or ethanol group, and compare the effect of ethanol within each genotype. Only with these additional analyses will it be possible to conclude more clearly on the results.

More specific comments:

In Figure 3, there are Control: WT, n = 9; HZ, n = 32; KO, n = 22; Ethanol: WT, n = 23; HZ, n = 44; KO, n = 16. This does not fit with the expected ratio of 25/50/25% of WT/HZ/KO. It seems that there are some missing control WT. Could the authors explain this?
And for the RT-qPCR, it does not seem that all these placentas were analyzed. How were the one included chosen?

Syncytiotrophoblast are in the labyrinth layer and not the junctional zone (see for example 10.3389/fimmu.2021.771054). The myometrium is not part of the placenta.

For clarity, I would suggest to use Igf+/+, Igf +/- and Igf-/- rather than WT, HZ and KO throughout the manuscript.

The author should also discuss the fact that the decidua is always partially deficient in IGF1 which may influence placental establishment, as well as the disparity of mice age that could also influence the results.

Did the ethanol consumption influence the weight gain of the mice throughout the study?
How many HZ-ethanol female were actually mated?

The title should be: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient placentas

Is the fetal weight further reduced in Ethanol KO compared to water-KO?

Is the work clearly and accurately presented and does it cite the current literature?
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Reproductive biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Author Response 04 Mar 2024**

**Fabiola Castorena Torres**

Monterrey, March 4th 2024

Dear Editorial Board members,

First of all, I would like to thank you for your kind answer as for carefully revising the manuscript, that has greatly aimed us to improve our manuscript. Herein we will outline any changes made to the manuscript following reviewers’ advice (changes are shown in green in the manuscript) or, to the contrary, giving a rationale when a suggestion was not implemented.

**Reviewer 1:**
Martín-Estal et al. performed a well-planned study to evaluate the effect of ethanol consumption on placental development (and fetal growth) in mice partially deficient for IGF1, as well as a potential synergy between (partial or total) IGF-1 deficiency and ethanol consumption on placental development. They notably showed that total IGF-1 deficiency and ethanol consumption both led to structural anomalies in the placental layers. They also showed a reduced placental efficiency in IGF-1 KO placentas, that was further reduced by ethanol consumption. They also evaluated the mRNA and protein
expression of AAH and the mRNA expression of a few other genes. We would like to thank the reviewer for his/her comments, which have helped us to improve the manuscript.

While the data are interesting, their description and analysis is not sufficient.
- The fetal weight is an important element and thus should be presented. Fetal growth retardation/restriction is indeed a key aspect of IGF-1 signaling and ethanol consumption, as it is well described in the introduction.

The reviewer is right. This information about fetal parameters (fetal weight, fetal height and placental weight) is presented in the following article of our research group: “Martín-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1-Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.

- I would suggest adding 2 panels to Figure 3 with the fetal and placental weight (in addition to the ratio), this is especially important as the authors mentioned placental hypoplasia that is not visible in the presented data.

The reviewer is right. Information is presented in the following article of our research group: “Martín-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1-Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.

- The placental morphology should be analyzed more thoroughly with for example a quantification of the length ratio between JZ/labyrinth and/or the number of abnormal islets in the labyrinth (the quality of the image should also be improved as the text within the image is hardly readable).

Thanks for the reviewer’s comments. Nevertheless, the quantification of the length ratio between JZ/labyrinth was performed, but no significant results were obtained (data not shown). The layout of images in Figure 2 was changed to make it more readable.

- The AAH immunohistochemistry would also benefit for a quantification analysis (in specific layers). For all the quantitative data, the statistical analysis effectively used should be described with the indication of the number of analyzed samples per group.

The reviewer is right. However, a quantification analysis of the immunohistochemistry images was accomplished, but no significant results were obtained (data not shown).

- Post hoc test should be performed after two-way ANOVA or Kruskal Wallis test, to compare the genotypes within the water or ethanol group, and compare the effect of ethanol within each genotype. Only with these additional analyses will it be possible to conclude more clearly on the results.

After two-way ANOVA, two post-hoc tests were executed with SPSS software: Bonferroni and Tukey tests.

More specific comments:
1. In Figure 3, there are Control: WT, n = 9; HZ, n = 32; KO, n = 22; Ethanol: WT, n = 23; HZ, n = 44; KO, n = 16. This does not fit with the expected ratio of 25/50/25% of WT/HZ/KO. It seems that there are some missing control WT. Could the authors explain this?

Because of the treatment scheme being used in the present study, a greater number of females in the HZ-Ethanol group was used to decrease the risk of death or early delivery
due to ethanol treatment.

1. **And for the RT-qPCR, it does not seem that all these placentas were analyzed. How were the one included chosen?**
   To reduce variability between groups, only male fetuses were selected for RT-PCR analysis. In addition, the animal model could not be continued due Biotherium issues.

1. **Syncytiotrophoblast are in the labyrinth layer and not the junctional zone (see for example 10.3389/fimmu.2021.771054). The myometrium is not part of the placenta.**
   Thanks for your comments. Trophoblast giant cells, the invasive cells responsible for invading maternal vasculature to promote placental development, are located in the labyrinth. However, spongiotrophoblast cells and glycogen trophoblast cells are found in the labyrinth and in the junctional zone (10.3390/nu15163564).
   Throphoblast invasion is observed in the junctional zone, in order to develop a normal placenta. Alterations in this trophoblast migration can lead to adverse pregnancy consequences due to a limit blood flow, such as fetal growth restriction and preeclampsia (10.1067/mob.2002.127305).

1. **For clarity, I would suggest to use Igf+/+, Igf +/- and Igf-/- rather than WT, HZ and KO throughout the manuscript.**
   Thanks for the reviewer’s suggesting. The “Animals and experimental design” section explains the nomenclature used: WT (Igf1+/+); HZ (Igf1+/-) and KO (Igf1-/-). We select that nomenclature because it was used in previous studies carried out by the research group with the present experimental model.

1. **The author should also discuss the fact that the decidua is always partially deficient in IGF1 which may influence placental establishment, as well as the disparity of mice age that could also influence the results.**
   Although the decidua, which is in contact with the maternal side, is always partially deficient in IGF-1, the rest of the placenta depends on the fetus; that is, if the fetus is WT, the placenta will be of the same genotype. Also, IGF-2 has an important role in fetal growth due to its direct anabolic actions within the fetus, and by its modulation of placental supply capacity, which may be a compensatory mechanism for IGF-1 deficiency (10.1016/j.placenta.2018.01.005) (Lines: 385-388).
   On the other hand, the difference in age is not a large period, since it is a difference of 8 weeks, which is equivalent to 6.6 years in humans, a period in which the mice are still reproductively young (10.1016/j.lfs.2015.10.025).

1. **Did the ethanol consumption influence the weight gain of the mice throughout the study?**
   Maternal parameters such as weight gain of pregnant dams, average of food and beverage consumption where measured throughout gestation. It was found that both HZ groups (HZ-Control and HZ-Ethanol) weighed less than the WT groups (WT-Control and WT-Ethanol), and this parameters was not affected by ethanol treatment. These results were reported in the following article: “Martín-Estal, I., et al.(2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1-Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”.

1. **How many HZ-ethanol female were actually mated?**
In HZ-Ethanol group, 9 female mice were mated with HZ male mice.

1. The title should be: Ethanol consumption during gestation promotes placental alterations in IGF-1 deficient placentas.

The reviewer is right. We will change the title as suggested, thanks.

1. Is the fetal weight further reduced in Ethanol KO compared to water-KO?

This result was reported in the following article: “Martín-Estal, I., et al. (2022). Effect of Ethanol Consumption on the Placenta and Liver of Partially IGF-1-Deficient Mice: The Role of Metabolism via CYP2E1 and the Antioxidant Enzyme System. Biology, 11(9), 1264. https://doi.org/10.3390/biology11091264”. In this article, the presence of KO genotype and ethanol consumption affected fetal height and weight when compared to the WT and HZ genotypes.

Yours faithfully,

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Competing Interests: No competing interests were disclosed.