Decidual vascularization during organogenesis after perigestational alcohol ingestion

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Abstract

Perigestational alcohol consumption up to early organogenesis can produce abnormal maternal vascularization via altered decidual VEGF/receptor expression. CF-1 female mice were administered with 10% ethanol in drinking water for 17 days prior to and up to day 10 of gestation. Control females received water without ethanol. Treated females had reduced frequency of implantation sites with expanded vascular lumen ($P < 0.05$), α -SMA-immunoreactive spiral arteries in proximal mesometrial decidua, reduced PCNA-positive endothelial cells ($P < 0.01$) and diminished uterine NK cell numbers ($P < 0.05$) in proximal decidua compared to controls. The VEGF expression (laser capture microscopy, RT-PCR, western blot and immunohistochemistry) was reduced in decidual tissue after perigestational alcohol consumption ($P < 0.05$). The uNK-DBA+ cells of treated females had reduced VEGF immunoexpression compared to controls ($P < 0.01$). Very low decidual and endothelial cell KDR immunoreactivity and reduced decidual gene and protein KDR expression was found in treated females compared to controls ($P < 0.001$). Instead, strong FLT-1 immunoexpression was detected in decidual and uNK cells ($P < 0.05$) in the proximal decidua from treated females compared to controls. In conclusion, perigestational alcohol ingestion induces the reduction of lumen expansion of spiral arteries, concomitant with reduced endothelial cell proliferation and uNK cell population, and uncompleted remodeling of the artery smooth muscle. These effects were supported by low decidual VEGF and KDR gene and protein expression and increased FLT-1 expression, suggesting that VEGF and KDR reduction may contribute, in part, to mechanisms involved in deficient decidual angiogenesis after perigestational alcohol consumption in mouse.

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Introduction

Fetal alcohol spectrum disorder (FASD) is a worldwide public health problem characterized by a wide range of physical, physiological, behavioral deficits, altered fetal outcomes and birth anomalies caused by maternal alcohol consumption during pregnancy (Hoyme *et al.* 2016, Roozen *et al.* 2016). The most severe form of FASD, the fetal alcohol syndrome (FAS), presents multiple defects such as intrauterine growth retardation (IUGR), craniofacial malformations, physical and mental retardation, behavioral and neurocognitive disabilities, delayed prenatal and postnatal growth and defective development of heart, limbs and central neural system (Barr & Streissguth 2001, May & Gossage 2011). Aside

from FAS, prenatal alcohol exposure (PAE) is a disorder that can include neurodevelopmental impairment without facial features (Chaudhuri 2000, O'Leary *et al.* 2010). Some animal models suggested a relation between abnormal placentation and a risk for adverse embryo development, intrauterine growth restriction (IUGR) and congenital defects (Chaudhuri 2000, Bada *et al.* 2005, Davis-Anderson *et al.* 2017). However, the knowledge of effects caused by maternal alcohol consumption on placental tissues is still incomplete. Alcohol can produce multiple alterations on systemic hemodynamic, vascular resistance and blood flow of maternal uterine tissue (Ramadoss & Magness 2012a). Moderate alcohol exposure impairs uterine artery vasodilation (Burd & Hofer 2008). Once-daily binge alcohol exposure

(4.5 g/kg body weight) for gestational day 7–17, in a pregnant rat alcohol model (with a peak blood alcohol concentration of 216 mg/dL) produces uterine vascular dysfunction (Ramadoss & Magness 2012a), although no gross deficits in either maternal or fetal body weights, fetal crown-rump length and placental weight were observed (Subramanian *et al.* 2014). Given that most alcoholic women often continue to drink alcohol before learning that they are pregnant (at least or during the organogenic 10–12 weeks of gestation) (Li *et al.* 2017), we have been interested in analyzing the alcohol effects on the embryo-placental development produced by perigestational alcohol intake, from previous to gestation and up to early organogenesis. Previously we showed that perigestational moderate alcohol ingestion prevents proper blastocyst implantation into the uterus (Perez-Tito *et al.* 2014), induces delayed embryo development and dysmorphogenesis and increases early resorption at organogenesis (Cebral *et al.* 2007, Coll *et al.* 2011, 2018). However, at present very little is known if maternal alcohol ingestion up to organogenesis disrupts the vascularization of decidual tissue and the mechanisms involved.

During early organogenesis, a profound angiogenesis-neovascularization, involving vascular remodeling of uterine arteries, branching, enlargement and network formation (Kim *et al.* 2013), is occurring in the maternal tissue. Decidual angiogenesis is a pivotal event to provide the uterine vascular elongation necessary for successful pregnancy. Maternal neovascularization is promoted by decidual and immune cells, including uterine natural killer cells (uNKs) (Blois *et al.* 2011), which have vital roles in spiral artery remodeling, in limiting trophoblast invasion (Ashkar & Croy 2001, Ashkar *et al.* 2003, Croy *et al.* 2003, Felker & Croy 2017, Meyer *et al.* 2017a,b) and in maintenance of decidual integrity (Greenwood *et al.* 2000, Wallace *et al.* 2012).

Decidual angiogenesis depends on the expression of the vascular endothelial growth factor (VEGF) and its receptors (Zygmunt *et al.* 2003, Coultas *et al.* 2005). In human and mouse placenta, VEGF is expressed in trophoblast, uNKs and decidual stromal cells (Zhang *et al.* 2011, Li *et al.* 2014). The effects of VEGF are exerted mainly by its receptor KDR (VEGFR2) and FLT-1 (VEGFR1). KDR seems to be the responsible for all physiological effects of VEGF, whereas FLT-1 modulates VEGF signaling via ligand sequestration (Chung & Ferrara 2011). In pathological conditions such as preeclampsia, VEGF expression is reduced whereas the soluble form of FLT-1 is elevated, generating an imbalance in the placental angiogenesis (Li *et al.* 2014). The pattern and extent of the expression of VEGF and its receptors in the maternal tissue during organogenesis after perigestational alcohol consumption was not studied. We hypothesized that moderate alcohol consumption previous to and up to day 10 of gestation in mouse produces detrimental effects on decidual angiogenesis by altering the VEGF

and receptor expression in maternal tissue. We aimed to analyze the vascularization of mesometrial decidua, focusing on the potential changes of maternal VEGF and receptor expression after perigestational alcohol exposure in the outbred mouse model.

Materials and methods

Animals

Conventional sexually mature stock of outbred CF-1 mice (CrlFcen:CF1, Mouse Genome Informatic (MGI)), from FCEN (School of Exact and Natural Sciences) of the University of Buenos Aires (Buenos Aires, Argentina) were used. Animals were housed in groups of three to five female mice in separate same-sex communal cages, and kept in controlled room temperature (RT: 22°C) and light cycle (14 h light:10 h darkness). They were fed with commercial mouse chow (Alimento 'Balanceado Cooperación Rata-Ratón' from the Asociación Cooperativa de Alimentos S.A. Buenos Aires, Argentina) and hyperchlorinated tap water, *ad libitum*. CF-1 female mice were 60 days old and average body weight was 27–30 g at the outset of ethanol treatment.

Experimental alcohol treatment

Experiments were carried out in compliance with regulations and ethical standards of the Institutional Animal Care and Use Committee (IACUC, protocol No. 57), of the FCEN, Universidad de Buenos Aires, and in compliance with the guidelines of the National Institute of Health.

Adult female mice were orally exposed to 10% (w/v) ethanol in drinking water for 17 days previous to mating and up to day 10 of gestation. Control CF-1 females received ethanol-free drinking water *ad libitum*. This perigestational ethanol model produced a blood alcohol concentration (BAC) of 24 mg/dL by day 10 of gestation and significantly increased embryo resorption, delayed embryo development and high frequency of organogenically abnormal embryos (Coll *et al.* 2011, 2017). In this mouse model, to synchronize mating of control and treated groups, females were superovulated on day 15 of treatment with an intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG; Novormon, Syntex S.A., Argentina) at 13:00 h followed by an i.p. injection of 5 IU of human chorionic gonadotrophin (hCG; Sigma Chemical Co.), 48 h later (treatment day 17) (Coll *et al.* 2011). Following the hCG injection, females were caged individually with a male overnight and checked for presence of vaginal plug on the following morning when day 1 of pregnancy was assumed. Then, CF-1-mated females were housed again with administration of 10% ethanol, and gestation continued up to day 10. CF-1-control females received ethanol-free drinking water for the same period as the ethanol treatment were mated and allowed to continue gestation up to day 10 of gestation.

Control (CF) and ethanol-treated pregnant (TF) female mice were weighed at the beginning and at the end of ethanol treatment. Every day in the morning, volume of liquid drunk and quantity of food consumed were recorded to monitor amounts of daily liquid, food, and calorie intake (estimated

by calorie value of the diet used (3976 kcal/kg) and calories derived from ethanol (estimated as 7.1 kcal/g). From these data, mean calorie intake and percentage of ethanol-derived calories (% EDC) were estimated for each experimental group. At least five pregnant mice per experimental group in each study were used.

Tissue collection and processing

On the morning of day 10 of gestation, control and treated females were killed by cervical dislocation, the abdominal cavity was surgically opened and uteri were quickly removed and placed in Petri dishes with Krebs–Ringer solution (11.0 mM glucose, 118 mM NaCl, 2.2 mM CaCl_2 , 4.7 mM KCl, 1.2 mM MgSO_4 , 23.8 mM Na_2HCO_3 , and 1.2 mM KH_2PO_4). Then, isolated implantation sites were immediately fixed in 4% paraformaldehyde (PF)/phosphate buffer saline (PBS) for 18 h at 4°C or in methacarn mixture (methanol 60%, chloroform 30% and glacial acetic acid 10%) for 3 h at 4°C or placed in Cryoplast freezing montage medium and promptly frozen at –70°C. To obtain the decidual tissue and the developing embryo contained in the cavity, the implantation sites were dissected under a stereomicroscope (Wild Heerbrugg Photomakroskop M400, 2×). After dissection of the yolk sac and extraembryonic membranes, the stage of developed embryo in the implantation site was determined by the Theiler system (Coll *et al.* 2011). Only the decidual tissue from implantation sites containing an embryo staged at E10 (with 14–20 somites and closed neural tube) was collected and immediately stored at –70°C for further determinations. Decidual tissues dissected from implantation sites with signs of embryo resorption (with embryo staged at E7 to E8.5 and necrotic and/or hemorrhagic tissues) or decidual tissues from implantation sites with retarded embryo (staged at E9 to E9.5) were not included in the study.

Histology and morphometrical analysis

Fixed implantation sites were dehydrated, immersed in xylene and embedded in paraffin. Transverse sections (7 µm) of implantation sites, cut perpendicularly to the longitudinal axis of the uterus, were adhered onto glass slides using 0.1% poly-L-Lysine (Sigma). According to the histological characteristics of the neural tube, somites and heart of the embryo contained in the cavity (Coll *et al.* 2011), and the area extension of mesometrial decidua, the implantation site slices were classified as E10-implantation sites (IS) when an embryo in E10 stage was contained into the cavity. E10-ISs were staining for hematoxylin and eosin (H-E) or PAS stain, following the standard protocols. Then, the relative expansion of maternal blood lumen was analyzed morphologically at both lateral sides of the vascular region of proximal decidua. To corroborate the morphological criterion, three 40× images of each left and right sides of decidual vascular region were photographed by Axiophot microscope (Carl Zeiss, Inc.) equipped with a camera driven by Olympus DP73 and image analyzer Olympus cellSens software (Olympus), and the lumen expansion area was measured by *Image J* software. The results were expressed as the mean area of vascular lumen

(mm²) over the area field (mm²), in a total of six fields per IS, each derived from five control and five treated females. Then, the frequency of E10-IS with expanded maternal blood lumen was recorded in a total of 38 implantation sites derived from 19 control and 19 treated females.

In the present experiments, a total of 247 implantation sites derived from 19 control females and 228 implantation sites from 19 treated females were used. Taken the total number of implantation sites used in each group, the frequency of resorption was 6.8% and 19.7% in control and treated females, respectively; the frequency of retarded embryo was 14.8% in control and 25.0% in treated females and the frequency of E10-embryos was 78.5 and 55.2% in control and treated females, respectively.

Uterine natural killer (uNK) cells

The uNK cells were identified by PAS histochemistry and peroxidase staining for *Dolichos biflorus* agglutinin (DBA) lectin (Lectin Kit BK 1000; Vector Laboratories, Inc., Burlingame, CA, USA). Briefly, for the later, deparaffinized and rehydrated E10-IS slices were washed (PBS) and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in absolute methanol (v/v) for 30 min at 4°C. After washing (PBS, three times for 5 min each), slices were blocked in BSA 0.1% (Sigma) in PBS for 1 h in humid chamber at RT and incubated in biotinylated DBA lectin (30 µg/mL in 0.1% BSA) overnight in humid chamber at 4°C. Then, they were washed and labeled with streptavidin binding complex (Dako LSAB2 System-HRP) for 20 min. After rinsing in PBS, slides were incubated in 3-3'-diaminobenzidine in chromogen solution (Cell Marque, Sigma) for 2 min, at RT, and counterstained with hematoxylin and mounted in DPX Mountant (Sigma). Negative control was performed by omitting biotinylated DBA lectin.

The uNK-PAS (cells with round nucleus, visible nucleolus and fuchsia-colored PAS+ granules) or DBA lectin-positive cells were identified in mesometrial distal (dMD) and proximal decidua (pMD). Data of DBA-positive uNK cells were expressed as the mean uNK cell number (and standard deviation, s.d.) in a total of 10 100×-area sections of distal decidua and left plus right areas of proximal decidua of five implantation site sections from five control and five treated females.

Immunohistochemistry

Retrieval of antigens in deparaffinized and rehydrated sections of E10-IS, the antigen retrieval was performed for KDR and FLT-1. The slices were heated in 10 mM sodium citrate in PBS (pH 6.0) for 10 min, at boiling temperature. Then, slices were permeabilized (except against VEGF) by incubating with 0.25% PBS-Triton X-100 for 15 min at RT, and after three 5 min washes with PBS, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 30 min at RT in methanol for 30 min at 4°C (for VEGF). After rinsing in PBS, the sections were blocked with normal goat serum or normal horse serum in PBS (1:40) for 1 h at RT in humid chamber. Then, slices were incubated overnight with primary antibody rabbit anti-VEGF (Santa Cruz Biotechnology), mouse

Table 1 Immunohistochemistry data specifications.

	α-SMA	PCNA	VEGF	KDR	FLT-1
Fixation	4% PF	Methacarn	4% PF	4% PF	4% PF
Permeabilization	0.25% PBST, 15 min	–	–	0.25% PBST, 15 min	0.25% PBST, 15 min
Endogenous peroxidase activity blocking	3% H ₂ O ₂ /PBS, 30 min-RT	3% H ₂ O ₂ /PBS, 30 min-RT	3% H ₂ O ₂ /methanol, 30 min-4°C	3% H ₂ O ₂ /PBS, 30 min, RT	3% H ₂ O ₂ /PBS, 30 min, RT
Non-specific blocking	NHS (1:40), 1 h, RT	NGS/PBS-10% BSA (1:1) 1 h, RT	NGS (1:40), 1 h, RT	NGS (1:40), 1 h, RT	NGS (1:40), 1 h, RT
Primary antibody	Mouse anti- α -SMA (1:50/NHS)	Rabbit anti-PCNA (1:500/PBS-Tween20-0.05%)	Rabbit anti-VEGF (1:150/NGS)	Rabbit anti-KDR (1:50/NGS)	Rabbit anti-FLT-1 (1:50/PBS)
Secondary antibody	Biotinylated horse anti-mouse (1:500/NHS)	Biotinylated goat anti-rabbit (1:2000/PBS)	Biotinylated goat anti-rabbit (1:500/NGS)	Biotinylated goat anti-rabbit (1:100/NGS)	Biotinylated goat anti-rabbit (1:100/PBS)
Conjugated complex (SHP)	(1:300/PBS)	(1:300/PBS)	(1:300/PBS)	(1:200/NGS)	(1:200/PBS)

α -SMA, smooth muscle actin; BSA, bovine serum albumin; BT, boiling temperature; H₂O₂, hydrogen peroxide; NGS, normal goat serum; NHS, normal horse serum; PBST, phosphate-buffered saline (PBS)-Triton-X100; PCNA, proliferating cell nuclear antigen; PF, paraformaldehyde; RT, room temperature; SHP, streptavidin-horseradish peroxidase-conjugate.

anti- α -smooth muscle actin (α -SMA) (Sigma), rabbit anti-proliferating cell nuclear antigen (PCNA) (Abcam), rabbit anti-KDR (Cell Signaling Technology) or rabbit anti-FLT-1 (Santa Cruz Biotech.) in at 4°C in humid chamber. For negative controls, primary antibody was omitted and PBS was added. After washing, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Inc.) for 1 h at RT. Then, slides were washed in PBS and incubated for 1 h in streptavidin-horseradish peroxidase conjugated (Invitrogen) at RT. Finally, samples were developed with 3-3'-diaminobenzidine (DAB; Dako Laboratories), slides were counterstained with hematoxylin or PAS staining, for KDR and FLT-1 immunoexpression and mounted in DPX (Sigma). Specific immunohistochemistry conditions are summarized in Table 1. Slices were photographed with an Anxiophot Zeiss light microscope (Carl Zeiss).

The endothelial proliferation index was calculated as the mean PCNA-positive endothelial cells over the total number of endothelial cells, in 6–10 40 $\times$ -areas of the vascular zone of proximal decidua, in a total of 12 implantation site slides derived from six control and six treated females. Qualitative analysis of α -SMA-positive reaction in the wall of blood vessels was performed in the non-decidualized endometrium, distal and proximal decidua, in a total of 12 implantation sites from six control and six treated females.

DBA lectin/VEGF double-fluorescence staining

Histological E10-IS slides were deparaffinized and rehydrated and blocked for 1 h at RT with normal goat serum (1:40). Samples were incubated with primary antibody rabbit anti-VEGF (1:10; Santa Cruz Biotech) overnight in humid chamber at 4°C. After rinsing in PBS, sections were incubated with FITC-goat anti-rabbit conjugated secondary antibody (1:50, Vector Lab) for 1 h at RT. After washing, they were blocked in 0.1% BSA in PBS for 1 h at RT. Then, sections were incubated overnight at 4°C with biotinylated DBA lectin (30 μ g/ml in 0.1% BSA in PBS) (Lectin Kit BK 1000, Vector Lab.) in humid chamber. Slides were washed and then incubated with Alexa

Fluor 647-streptavidin conjugated (1:150; Thermo Fisher Scientific Inc.). After washing, samples were mounted in PBS glycerol (1:1) to be observed and photographed in an Olympus FV300 confocal microscope.

VEGF fluorescence signal was semi-quantitatively analyzed in uNK cells (DBA-positive cells) by *ImageJ* software. In each slice, the area and mean fluorescence of VEGF signal of uNK cells was assessed, and background signal was measured in negative control (primary antibody omitted) slices by selecting ten random points and using their mean. Then, the corrected total cell fluorescence (CTCF) was calculated for each uNK cell as the following:

$$\text{CTCF} = \text{cell area} \times (\text{VEGF mean fluorescence} - \text{Background mean fluorescence})$$

Negative values were considered as scored 1 and data were transformed by natural logarithm to adjust normality and homoscedasticity.

Laser capture microdissection

Cryoplast embedded E10-implantation sites were cut into 10 μ m transversal sections using a Leica CM 1510S cryostat (Leica Biosystems) at –30°C. The sections placed on frosted microscope slides were fixed in 70% ethanol for 30 seconds, washed with RNase-free water, stained with Arcturus HistoGene staining solution (Applied Biosystems) for 20 s, and then washed with RNase-free water. Next, sections were dehydrated in 75, 95 and 100% ethanol for 30 s, and then incubated for 5 min in fresh xylene. Finally, the slides were air-dried for at least 5 min and immediately placed in vacuum desiccators (Bel-Art, NJ, USA). The slides were then placed in the Arcturus LCM system (Molecular Devices Corporation, CA, USA) to isolate the proximal mesometrial decidua. A single CapSure Macro LCM cap (Arcturus, CA, USA) was used for each tissue section. Each cap containing captured cells was tightly fitted to a microcentrifuge tube containing 50 μ L of extraction buffer and was placed in a vacuum oven (Sheldom Inc, Cornelius, OR, USA) for 30 min at 40°C. Cells dissected

from various tissue sections of a single sample were pooled in a single Eppendorf tube. After cell extraction, the mRNA levels of all genes (*Vegf*, *Kdr* and *Flt-1*) were analyzed by real-time reverse transcriptase–polymerase chain reaction (RT-PCR).

Real-time PCR

Total RNA from each tube containing cells isolated by LCM was extracted using the Arcturus PicoPure Frozen RNA Extraction Kit (Applied Biosystems). The RNA concentration was quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

cDNA was generated from RNA (1 µL (100 ng)) using the Superscript III first-strand synthesis system (Invitrogen) in a Mastercycler gradient thermal cycler (Eppendorf). Quantitative real-time RT-PCR was performed in a 20 µL total reaction volume containing cDNA, sense and antisense primers, 2× QuantiTect SYBR Green Master Mix and RNase-free water from the QuantiTect SYBR Green PCR Kit (Qiagen), and the reactions were run in a PTC-200 thermal cycler (MJ Research, Bio-Rad). Primer sources/sequences and the expected lengths of the resulting PCR products are shown in Table 2. The amount of each target DNA was normalized using the constitutively expressed gene hypoxanthine-guanine phosphoribosyl transferase 1 (*Hprt-1*), and the $2^{-\Delta\Delta C_t}$ method was employed to report this ratio as the relative mRNA expression level. Experiments for each analysis were run in triplicate. Values were expressed as the mean of $2^{-\Delta C_t} \pm$ standard deviation, from captured decidual area samples per implantation site derived from one female, with five females used per control and treated group.

Protein extraction and Western blotting

Decidual tissue samples were homogenized on ice in 300 µL of lysis buffer (10 mM Tris–HCl, pH 7.4, 1% NP-40, 2 mM EDTA, 1 mM EGTA, 5 mM NaVO₄, 50 mM NaF, 2 mM PMSF, 1% inhibitors cocktail (Sigma)). Homogenates were incubated on ice for 30 min and centrifuged at 16,000 g for 15 min at 4°C. Supernatant was collected and protein concentration was measured by Bradford Assay. Then, extracts were mixed with sample buffer (500 mM Tris–HCl, pH 6.8, 10% SDS, 30% glycerol, 0.5% β-mercaptoethanol and 0.5% bromophenol blue), boiled for 5 min and 40 µg of protein was added per lane. Molecular weight markers were loaded into one of the lanes. Proteins were resolved in 7.5% (for KDR and FLT-1) or 12% (for VEGF) SDS-PAGE and transferred to PVDF membranes at a constant voltage of 110V, for 1 h at 4°C. Transfer efficiency was checked by Ponceau Red staining. Following that, slices were incubated in blocking solution (5% non-fat powder

milk in TBS-Tween 0.5%) for 1 h at RT. Immunoblotting was done by incubating, overnight at 4°C with constant shaking, with primary antibodies: rabbit anti-VEGF (1:150; Santa Cruz Biotech.), rabbit anti-KDR (1:50; Cell Signaling Technology) or rabbit anti-FLT-1 (1:50; Santa Cruz Biotech.) and rabbit anti-β-actin (1:1000; Sigma) as load control. After four 5 min washes with 0.1% PBS Tween-20, the membrane was incubated with secondary biotinylated goat antibody anti-rabbit (1:1000 for VEGF, 1:100 for KDR, 1:1000 for FLT-1; Vector Lab) for 1 h at RT. After washing, streptavidin-horseradish-peroxidase conjugated was added (1:3000 for VEGF and 1:400 for KDR and FLT-1; Invitrogen) for 1 h at RT. Membranes were finally washed as previously described and reactive bands were detected using an ECL Western blotting system (GE Healthcare). Pre-stained protein standard (Bio-Rad), with a molecular weight range from 210 to 6.7 kDa, was used. The chemiluminescence intensities of specific protein bands were semiquantified by densitometry (*Scion Image* software) and relativized to the actin band. Data were expressed as the mean protein arbitrary units (AU) with standard deviation. Western blots were performed three times on a total of seven control and six treated samples derived from the same number of females.

Statistics

Data are expressed as mean $\pm$ s.d. Differences between the means of groups were statistically analyzed by two-tailed Student's *t*-test. Frequency values were compared by Fisher's exact test. uNK cell population was analyzed by Split-plot ANOVA, and *post hoc* analysis of simple main effects between treatments. For statistical analysis, we used the *InfoStat* 2015 version (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina (URL <http://www.infostat.com.ar>). Differences between groups were considered statistically significant when $P < 0.05$.

Results

After perigestational 10% alcohol oral treatment up to day 10 of gestation, the outbred CF-1 female mice consumed about 23.3 mL ethanol/kg/day or 0.5 g ethanol/mouse/day (18.3 g ethanol/kg/day), which represents the 20% ethanol-derived calories of the total daily calorie intake. Ethanol treatment produced neither differences in the total calorie intake between groups (controls: 785.4 ± 28.1 kcal/kg/day; treated: 741.1 ± 21.4 kcal/kg/day) nor in body weights between the initial and the final values after the period of ethanol administration, compared to controls.

Table 2 Primer references.

Gene	Ab number	Primer sequence		Product size (bp)
		Forward	Reverse	
<i>Flt-1</i>	NM_010228.3	5' CTGCGACCCTCTTTGGCTC 3'	5' TCAGTCTCTCCCGTGCAAAC 3'	180
<i>Kdr</i>	NM_010612.2	5' AGATGCCCATGACCCAAGAATG 3'	5' AGCATTGCCCATTCGATCCAG 3'	181
<i>Vegf</i>	NM_009505.4	5' GATTCTGTAGACACCCACC 3'	5' GACATCCTCTCCCAACACAAG 3'	180
<i>Hprt-1</i>	NM_0135656.2	5' TGGGCTTACCTACTGCTTTCC 3'	5' CCTGGTTTCATCATCGCTAATCACG 3'	139

Perigestational alcohol consumption induces morphological changes in vascularization of mesometrial decidua

On day 10 of gestation, the E10 implantation sites from control females had large area of expanded vascular lumen at both lateral sides of the proximal mesometrial decidua, showing the angiogenic decidual regions near to the trophoblast zone (Fig. 1A and inset in A). However, in treated females, the proximal mesometrial vascular zone of decidua was histologically compacted and had very narrow vascular lumens compared to controls (Fig. 1B and inset in B). The morphometric evaluation demonstrated that the mean vascular lumen area of morphological collapsed vessels was 0.064 mm^2 , while the mean vascular lumen area of expanded vessels was 0.41 mm^2 ($P < 0.05$), confirming the morphological vascular differences between implantation sites with expanded or reduced area lumen. Then, we assessed the number of implantation sites with expanded vascular lumen. Treated females had a significantly reduced frequency of implantation sites with expanded vascular lumen compared to those of control females ($P < 0.05$, Table 3).

Next, a detailed morphological analysis of the maternal blood vessels in the proximal decidua was performed. Control females had regular alignment of endothelial cells attached to the basement membrane (Fig. 1C). In contrast, the decidual vessels of implantation sites from treated females had a disorganized endothelium with few endothelial cells attached to basement membrane and many free cells into the lumen (Fig. 1D).

Given the potential decrease of endothelial cells in the maternal blood vessels, we assessed the endothelial cell proliferation in vessels of proximal decidua of control (Fig. 1E) and treated females (Fig. 1F). Treated females had a significantly reduced number of PCNA-positive endothelial cells compared to positive cells of controls ($P < 0.01$) (Fig. 1G).

In order to know whether the remodeling of smooth muscle wall of decidual arteries was also disrupted by alcohol treatment, the presence of smooth muscle cells in distal and proximal decidual vessels was analyzed by α -SMA immunohistochemistry. In non-decidualized endometrium of both control and treated females, the radial arteries had the typical smooth muscle actin-positive media lamina (Fig. 2A and B). In distal mesometrial decidua, the spiral arteries of control females had incomplete smooth muscle layer and few α -SMA-positive cells (Fig. 2C). In the same decidual region of treated females, positive α -SMA-immunoreaction was found surrounding the arteries (Fig. 2D). In proximal mesometrial decidua of treated females, α -SMA-positive cells were observed around the lumen of decidual spiral arteries (Fig. 2F), but none in spiral arteries of controls (Fig. 2E).

Figure 1 Histology of mesometrial decidua and vascular endothelial proliferation in control and treated females. Analysis of proximal mesometrial decidua in E10-implantation sites by hematoxylin-eosin (A and B), PAS-hematoxylin (C and D) and endothelial cell proliferation assessed by PCNA-immunohistochemistry (E, F and G). (A) Representative histological H-E-image section of E10-implantation site from control females (CF), showing lateral vascularized areas in proximal mesometrial decidua (pMD) and wide lumen of maternal blood vessels (asterisks) (inset in A). (B) Representative histological H-E image section of E10-implantation site from treated females (TF), showing reduced lateral lacunae of blood vessels (arrows and asterisks, inset B). (C) Representative histological PAS-stained image section of decidual vascular area from CF, showing regular alignment and attachment of endothelial cells (arrow) to the basement membrane, mature decidual cells (short arrows) and PAS-uNK-positive cells (arrowheads) located near the blood vascular lumen (asterisks). (D) Representative histological PAS-stained image section of decidual vascular area from TF, showing disorganized endothelium with few endothelial cells (arrow) attached to the basement membrane and many cells free in the lumen of maternal blood vessel (asterisk). (E and F) Representative image section of decidual vascular area from CF (E) and treated females (F), showing PCNA-positive cells (arrow) or PCNA-negative endothelial cells (arrowhead). AMD, antimesometrial decidua; dMD, distal mesometrial decidua; End, non-decidualized endometrium; pMD, proximal mesometrial decidua; TZ, trophoblastic zone. Scale bars: A and B: $500\text{ }\mu\text{m}$, C and F and insets A and B: $20\text{ }\mu\text{m}$. (G) Quantitative analysis of endothelial proliferation in decidual vascular area, expressed as the mean number of PCNA+ cells over the total endothelial cell number, and standard deviation. (** $P < 0.01$, Student's *t*-test, between groups. Number of implantation sites used for each control and treated group: 6.)

Table 3 Frequency of control and treated-derived implantation sites with expanded vascular lumen in mesometrial decidua.

Implantation sites	Proximal decidua	Distal decidua
Control derived	15/19 (78.9%)	12/19 (63.2%)
Treated derived	7/19 (36.8%)*	4/19 (21.1%)*

The number of implantation sites with expanded vascular lumen at proximal and distal mesometrial decidua was recorded in a total of 38 implantation sites derived from 19 females for each group.

* $P < 0.05$, compared to control, Fisher's exact test.

uNK cell population diminishes in proximal vascular decidua after perigestational alcohol consumption

Considering the reduced vascular lumen and that smooth muscle cells were still present in spiral arteries of proximal decidua from treated females, and given the role of uNK in vascular remodeling and decidual angiogenesis (reviewed in Wallace *et al.* 2012), in mouse models (Kim *et al.* 2013) and human placental cell culture (Robson *et al.* 2012), we postulated that alcohol treatment alters the uNK cell population in mesometrial decidua. In control females, abundant DBA-positive uNK cells were identified in endometrium and in distal and proximal decidua (Fig. 3A). However, in treated females, the DBA-positive uNK cell population diminished in decidual tissues (Fig. 3B). After uNK cell quantification in distal decidua, no significant differences between the uNK frequency of control and treated females were found. However, a significantly reduced number of uNK cells was observed in proximal decidua of treated females compared to controls ($P < 0.01$, Fig. 3C).

Expression of angiogenic markers in decidual and uNK cells following perigestational alcohol intake

In proximal decidua from control females, strong expression of VEGF was immunolocalized in endothelial and decidual cells (Fig. 4A). In contrast, in treated females, low VEGF immunoreactivity was observed in decidual cells while VEGF immunoexpression was negative in endothelial cells (Fig. 4B). Given these decidual VEGF differences between control and treated females, we assessed the total level of VEGF protein expression. A band of molecular weight around 21 kDa, corresponding to VEGF expression, was detected in control and treated-derived decidual samples (Fig. 4C). According to expectations, decidual tissue of treated females had a significantly reduced VEGF expression level as compared to controls ($P < 0.001$) (Fig. 4D). To elucidate whether the diminished protein expression in treated females was due to a change in the gene expression, we isolated the vascular area of the proximal decidua from control and treated females by laser capture microdissection and determined the mRNA levels of VEGF by RT-PCR. Treated females had a significantly reduced level of *Vegf* in the vascular

Figure 2 Smooth muscle remodeling in maternal vascular wall of control and treated females. Immunohistochemistry for α -smooth muscle actin (α -SMA) of smooth muscle wall of spiral arteries in non-decidualized endometrium (End), distal (dMD) and proximal decidua (pMD) from control (CF) (A, C and E) and treated females (TF) (B, D and F). (A) Representative image section of End from CF, showing arteries (asterisk) with the typical wall with smooth muscle actin-positive media lamina (arrow). (B) Representative image section of End from TF, showing α -SMA-positive immunoreactivity in the vascular arterial wall. (C) Representative image section of distal mesometrial decidua (dMD) from CF, showing spiral arteries with some few α -SMA-positive cells. (D) Representative image section of dMD from TF showing positive α -SMA-immunoreactive cells around the arteries. (E) Representative image section of proximal mesometrial decidua (pMD) from CF, without smooth muscle cells in spiral arteries. (F) Representative image section of pMD from TF showing α -SMA-positive cells (arrow) in decidual spiral arteries. Number of implantation sites analyzed in each group: 6 (derived from six control and six treated females). Scale bar: 50 μ m.

area of proximal decidua as compared to the value of controls ($P < 0.001$) (Fig. 4E). Next, we assumed that the reduced decidual VEGF expression after alcohol treatment could result from low VEGF expression of uNK cells. To elucidate this hypothesis, we examined the uNK cell-VEGF production by double fluorescent staining for VEGF and DBA lectin-uNK cells in control (Fig. 5A, B and C) and treated females (Fig. 5D and E). The VEGF immunofluorescence signal in uNK-DBA+ cells was significantly reduced in proximal decidua of treated females as compared to controls ($P < 0.01$, Fig. 5C and F, respectively, and Fig. 5G).

In the vascular area of proximal decidua from controls, KDR was strongly immunoexpressed in decidual cells but slightly in some endothelial cells (Fig. 6A). Treated females had low immunoreactivity of KDR in decidual

Figure 3 uNK cells in mesometrial decidua in control and treated females. DBA lectin-positive uNK cells in non-decidualized mesometrial endometrium (End), distal (dMD) and proximal (pMD) mesometrial decidua. (A) DBA lectin-positive uNK cells in implantation site from CF. (B) DBA lectin-positive uNK cells in implantation site from treated females. Inserts: implantation site without DBA lectin-cell reaction (negatives). dMD, distal decidua; End, endometrium; pMD, proximal decidua. Scale bars A, B: 500 μ m. (C) Quantitative analysis of DBA lectin-positive uNK cell between pMD areas from control (■) and treated (□) females (mean number and standard deviation). (** $P < 0.001$, Student's t -test, number of implantation sites used in each group: 5, derived from five control and five treated females.)

Figure 4 VEGF gene and protein expression in mesometrial decidua from control and treated females. VEGF expression in proximal mesometrial decidua assessed by immunohistochemistry, western blot and RT-PCR. (A) Representative image of VEGF immunoreactive area section of vascular decidua from control females, showing VEGF immunoreactivity in decidual (arrowhead) and endothelial cells (arrow). (B) VEGF immunoreactivity in proximal decidua of treated females, showing low VEGF immunoreactivity in decidual cells and negative VEGF immunoreactivity in endothelial cells. (Number of implantation sites used for each group: 6, derived from six females for each). Asterisk: lumen of decidual blood vessel. Scale bars: A, B: 20 μ m. (C) Representative immunoblot of VEGF expression in control and treated-derived decidua samples, showing VEGF-positive protein bands corresponding to molecular weight of 21 kDa. (D) Densitometric analysis of VEGF protein bands, normalized to β -actin and expressed as the mean AU and standard deviation, of three independent experiments performed with a total of seven samples for each groups, derived from seven control and seven treated females. (E) Quantitative analysis of *Vegf* mRNA levels assessed by laser capture microdissection of proximal decidual area per implantation site, and Real-Time-PCR. Values were calculated as the mean of $2^{-\Delta Ct}$ and standard deviation, and expressed as level of mRNA. (Number of implantation site used for each groups: 5, derived from five control and five treated females.) *** $P < 0.001$, Student's t -test, between control and treated females.

and endothelial cells (Fig. 6B). Both, uNK cells of control and treated females did not presented KDR immunoreactivity (Fig. 6C and D). The pattern of FLT-1 immunostaining was punctuated and slightly expressed in some endothelial, decidual (Fig. 6E) and uNK cells (Fig. 6G) from control females. In treated females, FLT-1 was strongly immunoreacted in many decidual cells,

Figure 5 VEGF expression in DBA-uNK cells from control and treated females. Uterine NK-VEGF expression was determined by confocal VEGF immunofluorescence analysis (A and D), DBA lectin-fluorescence staining (B and E) and merges (C and F). Upper panel: representative image section of control-derived proximal decidual area tissue. Lower panel: representative image section of treated-derived proximal decidual area tissue. Asterisk: lumen of decidual blood vessel. Scale bar: 50 μ m. Graphic represents the semiquantitative analysis of VEGF fluorescence signal in uNK cells (DBA-positive cells), expressed as the mean arbitrary units (AU) of CTCF Ln and standard deviation, in control and treated females. ** $P < 0.01$, Student's *t*-test, between control and treated females. Number of implantation sites used in each group: 5.

while all endothelial cells presented immunoexpression of FLT-1 (Fig. 6F). In addition, uNK cells had a generalized FLT-1 immunostaining (Fig. 6H) compared to those of controls (Fig. 6G).

Subsequently, we analyzed KDR and FLT-1 protein and gene expression in decidua from control and treated females. By western blot, bands of molecular weights of about 210 kDa corresponding to KDR expression, and of 180 kDa corresponding to FLT-1 expression, were detected in decidual samples from control and treated animals (Fig. 7A). Semiquantitative analyses of bands showed that KDR protein expression was significantly reduced ($P < 0.05$), but FLT-1 expression level was significantly increased in decidual tissue from treated females compared to controls ($P < 0.05$) (Fig. 7B). The mRNA level of *Kdr* in laser captured-vascular area of proximal decidua from treated females was significantly reduced compared to controls ($P < 0.001$) (Fig. 7C), while *Flt-1* mRNA level in decidua from treated females was similar to mRNA quantity of controls (Fig. 7C).

Discussion

As far as we know, this is the first *in vivo* study reporting deleterious effects of perigestational moderate alcohol consumption up to early mouse organogenesis on decidual vascular development and its association with disruption of the angiogenic markers, the VEGF and its receptors.

Perigestational alcohol consumption of 0.5 g ethanol/mouse/day (that generates a BAC of 24.5 mg/dL) produces retarded embryo development and

dysmorphic E10 embryos (Coll *et al.* 2011, 2017). In this mouse model, the ethanol intake quantities, that are equivalent to 200 mL of wine/day or ingestion of 18–27 g of ethanol/day (Gonzalez Martín *et al.* 2011), are achieved by consumption of two glasses of wine, containing 11% of ethanol, per day (Gonzalez Martín *et al.* 2011). Apart from consumption of more than 100 g of ethanol per week (14 g absolute ethanol/day) leads to a risk of FAS, a high frequency of babies with low birth weight after consumption of 14 or more drinks per week (two glasses of 11% wine per day or 18–30 g absolute ethanol/day) was reported. Moreover, high risk of placental abruption was observed after consumption of 7–21 drinks per week (a mean of two drinks per day and BAC of 5–100 mg/dL) (Burd *et al.* 2007). Taken account these human clinical studies, the present mouse model contribute to elucidate the deleterious effects of moderate maternal alcohol consumption on decidual development and its vascularization, potential cause for abnormal development of organogenic embryos.

We demonstrated that moderate perigestational oral alcohol intake up to organogenesis leads to low vascular lumen expansion of spiral vessels of mesometrial decidua, suggesting that this effect could be one cause of small implantation sites in treated females (Coll *et al.* 2018). A potential consequence of the negative effect of alcohol treatment on decidual lumen expansion may be the diminution of decidual blood flow to the placental zone. Regarding this, severe chronic ethanol consumption during gestation induces vascular resistance and umbilical cord vasoconstriction (Siler-Khodr *et al.* 2000, Acevedo *et al.* 2001, Roberts

Figure 6 KDR and FLT-1 immunoexpression in decidual and uNK cells in control and treated females. Immunohistochemical localization and expression of KDR and FLT-1 in proximal mesometrial decidua of control (A, C, E and G) and treated females (B, D, F and H). (A) Representative image section of decidual vascular area of control females, showing localization and immunoexpression of KDR in decidual cells (short arrows) and in some endothelial cells (large arrows) of maternal vessel (mv). (B) Representative image section of decidual vascular area of treated females, showing low or negative KDR immunoreaction in decidual cells and almost no KDR-positive endothelial cells. (C) Detailed representative image of KDR immunoreactive-PAS counterstained decidua of control females, showing punctuate and strong KDR immunoexpression in decidual cells (short arrow) and absence in uNK cells (arrowhead). (D) Detailed representative image of KDR-immunoreactive PAS counterstained from treated females, showing very low KDR immunoreactivity in decidual cells. (E) Representative image section of decidual vascular area of control females, showing slightly moderate FLT-1 immunoreactivity in decidual (short arrows) and endothelial cells (large arrows). (F) Representative image section of decidual vascular area of treated females, showing strong FLT-1 immunoreactivity in many decidual and endothelial cells. (G) Detailed representative image of FLT-1 immunoreactive-PAS counterstained decidual vascular area of control females, showing punctuate and slightly moderate FLT-1 immunoexpression in decidual cells (short arrows), uNK (arrowheads) and in some endothelial cells (large arrow). (H) Detailed representative image of FLT-1 immunoreactive-PAS counterstained decidual vascular area of treated females, showing FLT-1 immunoexpression in the same cells as controls. Number of implantation sites used in each group: 5, derived from five control and five treated females. Scale bars: 20 μ m.

& Cooper 2001, Reynolds *et al.* 2006, Ramadoss *et al.* 2011, Bosco & Diaz 2012).

The abnormal decidual vascularization of treated females seems to be associated with cellular alterations of decidual spiral arteries. Alcohol treatment conduced to abnormal endothelial organization and disruption of endothelial cell alignment and attachment to the basement membrane. In addition, perigestational alcohol ingestion impaired the endothelial cell proliferation, which may be one factor for low expansion and poor dilation of the maternal vascular bed. One proposed mechanism for explaining the proliferation reduction may be a deficit of 17- β estradiol and its metabolites, since this hormone has been suggested to induce proliferation of uterine endothelial cells during pregnancy (Magness *et al.* 2001, Jobe *et al.* 2010) and alcohol inhibits estrogen-induced ovine uterine arterial endothelial proliferation (Ramadoss *et al.* 2011).

Around 8.5–10 days of gestation and during initial placenta establishment, the smooth muscle layer of spiral arteries has normally largely disappeared, and thus, profound growth and vascular remodeling of mesometrial decidual vessels takes place to provide uterine vascular elongation (Kim *et al.* 2013). Perigestational alcohol ingestion disrupted the smooth muscle cell remodeling in the spiral arteries and thus leads to permanent presence of smooth muscle wall in vessels of proximal decidua, suggesting that this may be a factor of impairing the maternal lumen expansion. Similarly, an adverse effect on the muscular layer of spiral artery was observed after human chronic alcohol consumption of 37% of calorie content from gestational days 6 to 16 (Gundogan *et al.* 2008).

At present, it is unknown whether perigestational alcohol ingestion up to organogenesis modifies the uNK cell population in decidua. In normal mouse pregnancy, the decidual uNK cells reach a peak number at about gestational day 10 and decline from day 12 to 14.5 (Adamson *et al.* 2002, Kather *et al.* 2003). Since the uNK cells are involved in dilation and thinning of spiral artery wall (Adamson *et al.* 2002, Charalambous *et al.* 2012), the diminished uNK cell number in the vascular zone of mesometrial decidua after alcohol ingestion up to organogenesis suggests that the uNK deficiency is involved in the absence or low decidual vascular dilatation and in the lack of remodeling of the vascular smooth muscle coat. Concordantly, the importance of uNK cells in spiral artery diameter regulation and smooth muscle remodeling of decidual arteries was seen in NK cell-deficient mice (Croy *et al.* 2000, Rätsep *et al.* 2015). Mouse strains that are genetically ablated from uNK cells, not only fail to undergo smooth muscle spiral artery remodeling but also show abnormal artery branching of the blood vascular bed, which leads to implantation sites with anomalous vascularization features. Therefore, the number of uNK cells is not only involved in the onset and progression of angiogenesis but

Figure 7 KDR and FLT-1 gene and protein expression in decidua from control and treated females. Quantitative analysis of protein and mRNA expression of KDR and FLT-1 in decidual tissue. (A) Representative immunoblotting bands of KDR and FLT-1 protein expression of control and treated-derived decidual tissue samples, corresponding to the molecular weight 210-230 kDa and 180 kDa, respectively. (B) Densitometry analysis of KDR and FLT-1 protein bands normalized to β -actin and expressed as the mean arbitrary units (AU) and standard deviation, of three independent experiments performed with 12 tissue samples derived from six control and six treated females. (C) Quantitative analysis of *Kdr* and *Flt-1* mRNA levels, assessed by laser capture microdissection of proximal decidual area per implantation site and real-time PCR. Values were calculated as the mean of $2^{-\Delta C_t}$ and standard deviation, and expressed as level of mRNA. Number of samples used in each group: 5, derived from five implantation sites per each group. * $P < 0.05$, *** $P < 0.001$, Student's *t*-test, between control and treated females.

also in the quality of decidual vascularization (Hofmann *et al.* 2014, Lima *et al.* 2014). Given the decidual recruitment of uNK cell precursors from secondary lymphoid tissues (Chantakru *et al.* 2002, Manaster & Mandelboim 2012), and considering that the increment of proliferation of uNK cells in decidual tissue requires progesterone, we also think that perigestational alcohol ingestion up to day 10 of gestation probably alters both the recruitment of uNK cells and their proliferation in decidua, by a progesterone-dependent mechanism.

The endothelial proliferation reduction and abnormal vascular wall remodeling in decidua after perigestational alcohol consumption can be explained, at least in part, by the downregulation of both *VEGF* gene and protein expression. In this regard, a novel data showed significant detrimental alcohol effects on genes controlling angiogenesis and supported a mechanistic role for abnormal utero-placental vascular development in FASD (Ramadoss & Magness 2012b). Similarly, others studies showed that chick extraembryonic tissues exposed to moderate and heavy (30 or 50%) alcohol doses for 24 or 48 h presented impaired vascular development and downregulation of VEGF and its receptors (Tufan & Satioglu-Tufan 2003).

Decidual cells and uNK cells are robust sources of VEGF expression that guide angiogenesis in maternal tissue (Gargett *et al.* 2001, Wulff *et al.* 2002, Heryanto *et al.* 2004, Taylor 2004). In addition to diminution of uNK cell population in decidua of treated mice, perigestational alcohol ingestion also reduces the VEGF expression in these cells. Probably, this deficient VEGF production by uNKs is involved in the low endothelial proliferation and incomplete remodeling of vascular wall of maternal vessels in treated mice, because depletion of uNK cells (Li *et al.* 2001, Wang *et al.* 2002, 2003) and/or their VEGF expression are suggested to cause decidual reduction of blood vessel lumen (Felker *et al.* 2013, Kim *et al.* 2013, Hofmann *et al.* 2014, Kieckbusch *et al.* 2014). In our model of alcohol exposure, a possible mechanism for reduction of VEGF expression may be the oxidative stress, which was recently shown by us in decidua (Coll *et al.* 2018), probably generated by direct ethanol exposure and/or indirectly as a consequence of diminution of maternal blood vascular expansion. On the other hand, since progesterone is essential for decidual vascular development because of its role in the regulation of *Vegfa* transcription in uNK cells (Chen *et al.* 2012, Lima *et al.* 2012), perhaps a possible reduction of progesterone levels in the implantation sites of treated females may also lead to the reduction of *VEGF* gene and protein expression in these uterine cells.

VEGF receptors play a major role in decidual angiogenesis during early pregnancy (Kim *et al.* 2013). The reduced KDR expression in decidual and endothelial cells of treated females would probably be another factor related to abnormal angiogenesis of decidua. In this regard, a single injection of anti-KDR blocking

antibody at peri-implantation decreases decidual vascular density and stromal cell differentiation, thereby disrupting pregnancy development and leading to lost gestation (Douglas *et al.* 2009). Moreover, local sprouting of blood vessels is blocked by interfering with the recruitment and function of bone marrow-derived KDR-positive cells (Douglas *et al.* 2009). Therefore, our results suggest that disruption of KDR expression is operating in the abnormal angiogenesis of decidua after perigestational alcohol intake. Although it is unclear at present whether FLT-1 receptor has a specific role during decidual angiogenesis (Douglas *et al.* 2009), it was proposed that FLT-1 drives anti-angiogenic effects by its binding to VEGF (Lima *et al.* 2014). Here, we think that, although *Flt-1* mRNA level was unchanged, the increased FLT-1 protein expression in decidual and uNK cells of treated females, probably is one factor responsible for alcohol induction of abnormal decidual angiogenesis. Given that increased FLT-1 expression was associated with oxidative factors (Daikoku *et al.* 2003, Kim *et al.* 2013), we propose that the increase of this receptor may be due to the oxidative stress generated after perigestational alcohol intake (Coll *et al.* 2018).

In conclusion, perigestational alcohol ingestion up to early organogenesis in outbred mouse impairs maternal angiogenesis by reduction of vascular lumen expansion, diminishes endothelial proliferation and uNK cell population and produces deficient arterial smooth muscle remodeling. The reduction in VEGF and KDR gene and protein expression but increased FLT-1 in decidual tissue of treated females could be involved-mechanisms of altered decidual angiogenesis, similar to other pregnancy disorders such as preeclampsia (Levine *et al.* 2004, Karumanchi *et al.* 2008, Patten *et al.* 2012). Moreover, since KDR is the main signaling pathway of action of VEGF-induced mitogenesis and permeability (Li *et al.* 2002), the reduction of both VEGF and KDR after perigestational alcohol intake may be key causes for the deficient decidual angiogenesis in alcohol-treated female mice during organogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All co-authors have read, approved and concur with the submitted manuscript. The authors have ensured the integrity of the work.

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