REPRODUCTION
RESEARCH

Maternal exposure to an enriched environment promotes uterine vascular remodeling and prevents embryo loss in mice

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Abstract

Implantation-related events are crucial for pregnancy success. In particular, defects in vascular remodeling at the maternal–fetal interface are associated with spontaneous miscarriage and recurrent pregnancy loss. Physical activity and therapies oriented to reduce stress improve pregnancy outcomes. In animal models, environmental stimulation and enrichment are associated with enhanced well-being, cognitive function and stress resilience. Here, we studied whether the exposure of BALB/c mice to an enriched environment (EE) regulates crucial events during early gestation at the maternal–fetal interface. Pregnant BALB/c mice were exposed to the EE that combines non-invasive stimuli from the sensory pathway with voluntary physical activity. The pregnancy rate was evaluated. Implantation sites were investigated microscopically and macroscopically. Vascular adaptation parameters at the maternal–fetal interface were analyzed. We found that exposure to the EE prevented pregnancy loss between gestational days 7 and 15. Also, it increased the diameter of the uterine artery and decreased the wall:lumen ratio of the mesometrial decidual vessels, suggesting that EE exposure promotes vascular remodeling. Moreover, it increased nitric oxide synthase activity and inducible nitric oxide synthase expression, as well as prostaglandin F_{2a} production and endoglin expression in the implantation sites. Exposure of pregnant females to the EE regulates uterine physiology, promoting vascular remodeling during early gestation. These adaptations might contribute to preventing embryo loss. Our results highlight the importance of the maternal environment for pregnancy success. The design of an 'EE-like' protocol for humans could be considered as a new non-pharmacologic strategy to prevent implantation failure and recurrent miscarriage.

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Introduction

Approximately 15% of couples worldwide struggle with infertility (Boivin *et al.* 2007). This percentage is exacerbated in developing countries as one in four couples are not fertile (WHO 2012). The impossibility of conceiving implies major economic costs for health facilities and patients, in addition to the serious emotional and psychological consequences. Implantation failure and pregnancy loss are the common and distressing complications of early pregnancy. Moreover, failed implantation is a limiting factor in assisted reproductive technologies (Patrizio & Silber 2017).

Proper embryo implantation is crucial for the progress of pregnancy since it establishes the first communication between the mother and fetus. This reciprocal interaction modulates decidualization and vascular remodeling of the uterus, which are essential for placentation. During vascular remodeling at the maternal–fetal interface, vessels become more permeable and spiral arteries lose endothelial and vascular smooth muscle cells. As a result, blood flow increases and ensures nutrient and oxygen supply to the developing embryo. Several growth factors, hormones, nitric oxide, cytokines and chemokines are involved in these vascular processes (Osol & Mandala 2009). Failure in implantation-related events has been associated with spontaneous miscarriage and recurrent pregnancy loss (Plaisier *et al.* 2009, Quenby *et al.* 2009, Windsperger *et al.* 2017).

Maternal lifestyle affects the physiology of the mother and therefore could modulate the uterine environment and pregnancy outcome. In this sense, the American College of Obstetricians and Gynecologists recommends pregnant women without medical complications to practice moderate aerobic exercise, as it has been associated with a lower incidence of pathological processes related to pregnancy (ACOG 2020). Moreover, the European Society of Human

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Reproduction and Embryology suggests that women with recurrent pregnancy loss should change their lifestyle as a good strategy to improve the performance of assisted reproduction techniques (Bender Atik *et al.* 2018).

Environmental stimulation and enrichment have been associated with enhanced well-being, improved cognitive function and stress resilience in animal models (Bakos *et al.* 2009, Langdon & Corbett 2012). In particular, environmental enrichment is an experimental strategy that consists of housing modifications that provide a complex set of physical, social and cognitive stimuli (Mering & Jolkkonen 2015). The enriched environment (EE) exerts beneficial effects in neurological pathologies and shows advantageous transgenerational effects on neurodevelopment (Caporali *et al.* 2015, Jungling *et al.* 2017, Balthazar *et al.* 2018). Furthermore, environmental enrichment allows the study of maternal housing conditions during pregnancy. Our group has previously reported that maternal EE prevents preterm birth in an inflammatory mouse model (Schander *et al.* 2020). However, the effect of environmental enrichment on early pregnancy has not been reported yet. Therefore, we aimed to investigate whether maternal periconceptional EE regulates crucial events at the maternal–fetal interface during early gestation in mice.

Materials and methods

Ethical statement

The present study was performed under the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the School of Medicine, University of Buenos Aires (CICUAL; permit number 2547/2019). Animals were obtained from the School of Pharmacy and Biochemistry of the University of Buenos Aires.

Animals

Six-week-old BALB/c female mice were randomly assigned to either control or EE cages. Mice in both conditions received food and water *ad libitum* and were subjected to a 12 h light:12 h darkness schedule at constant temperature (19–21°C) and humidity.

After 6 weeks in control or EE cages, the females were mated with fertile males of the same strain housed in control conditions (Fig. 1A). The morning when the vaginal plug was detected was considered day 0 of pregnancy (d0). Females were then returned to control or EE cages respectively.

Under the conditions of our animal care facility, implantation occurs in d3 and spontaneous term labor in d19.

Pregnant animals on d7 (early gestation) and d15 (late gestation) were euthanized by cervical dislocation. Uterine horns were extracted, and the implantation sites and pregnancy rates were evaluated. The macrovasculature analysis was performed on d7. Also, d7 implantation sites were fixed in Bouin solution for hematoxylin–eosin (H&E) staining or were

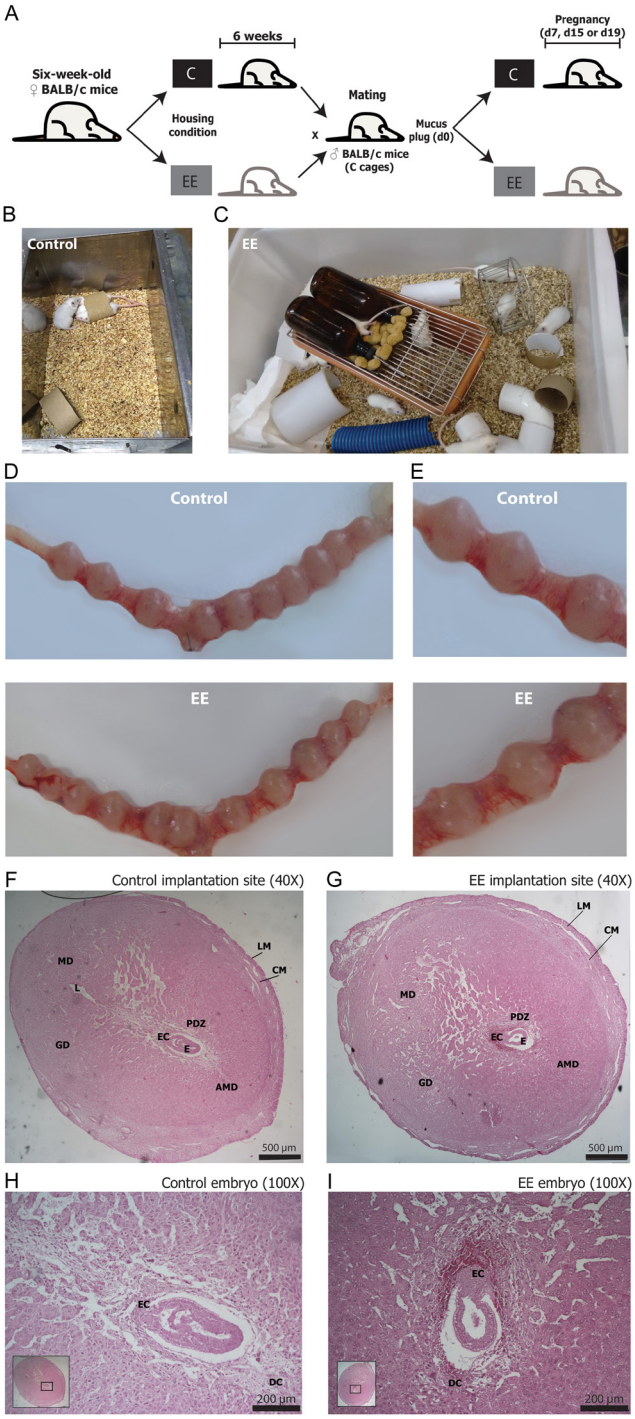

Figure 1 Effect of EE on implantation sites on d7. (A) Scheme of control and EE protocols. (B) Control housing condition. (C) EE condition. (D) Representative images of d7 implantation sites of control and EE mice. (E) Amplifications of d7 implantation sites of control and EE mice. Control (F and H) and EE (G and I) slices were stained with H&E. (C) and (D) scale bar = 500 µm; (E) and (F) scale bar = 200 µm. AMD, antimesometrial decidua; C, control; CM, circular muscle; DC, decidual crypt; d7, day 7 of gestation; E, embryo; EC, ectoplacental cone; EE, enriched environment; GD, glycogenic decidua; H&E, hematoxylin–eosin; L, lumen; LM, longitudinal muscle; MD, mesometrial decidua; PDZ, primary decidual zone.

stored at −70°C for western blot, nitric oxide synthase (NOS) enzyme assay and RIA studies.

Enriched environment

Mice exposed to the EE were housed in bigger cages (64 × 42 × 20 cm) in groups of 10 animals. EE cages contained different objects (running wheel, tunnels, shelter, stairs and toys) of different materials, textures and colors that were replaced weekly (Sano *et al.* 2019, Schander *et al.* 2020, Goldstein *et al.* 2021) (Fig. 1C). Once a week, the feeding boxes were moved to different cage points to encourage foraging and explorative behaviors. This protocol provides optimal conditions for further exploration, visual, cognitive, social interaction and voluntary exercise activity. Control conditions consisted of housing four females per standard laboratory cage (43 × 27 × 17 cm) containing bedding material and no objects (Fig. 1B).

Evaluation of the pregnancy rate

The pregnancy rate was quantified for d7, d15 and d19, as the number of pregnant mice on a specific day of gestation compared to the total number of females that presented a vaginal plug, expressed as percentage.

Macroscopical analysis of the implantation sites

Uterine horns were examined macroscopically. The number of implantation sites on d7 and d15 was recorded. The color, shape, size, wet weight and distribution of the implantation sites along the uterine horns were also assessed.

Uterine macrovasculature analysis

After euthanasia of d7 mice, uterine arteries were clamped, and the uterine horns with the associated vasculature were extracted and photographed. The number of vessels irrigating the implantation units was counted. Uterine artery diameter was analyzed using Image-Pro Plus version 4.5.0.29 (Scion Corporation, Media Cybernetics, Rockville, MD, USA), considering the cross-sectional length of the uterine artery in the image as the diameter of the vessel.

Histological structure analysis

Whole implantation sites were fixed in Bouin solution (Biopur, Buenos Aires, Argentina) overnight at room temperature, dehydrated in 70–100% ethyl alcohol gradient and embedded in paraffin. Tissues were oriented to be sectioned transversely using a microtome (Leica RM 2125). Sections of 5 µm were mounted on 2% xylan-coated slides and stained with H&E for 15 s. Slices were observed under a light microscope at 40×, 100× and 400× (Nikon Eclipse 200). The general tissue structure and cellular morphology were analyzed.

Microvasculature analysis

The microvasculature of d7 implantation sites was analyzed as previously described (Sordelli *et al.* 2017). One implantation site per animal was selected, and 10 fields per site were randomly photographed (400×). The number of intact vessels with defined boundaries was counted in the mesometrial decidua. The circumference, the vessel area and the lumen area were determined using the Image-Pro Plus version 4.5.0.29 (Scion Corporation). The wall area (vessel area – lumen area) and wall:lumen ratio (wall area/lumen area) were calculated.

Western blot

Protein levels of cyclooxygenase (COX) 1 and 2, inducible nitric oxide synthase (iNOS), endoglin, vascular endothelial growth factor (VEGF) and von Willebrand factor were analyzed.

One whole implantation site per animal was thawed, homogenized in lysis buffer (Tris buffer 50 mM pH 7.4, sodium deoxycholate 0.25% (v/v), NaCl 150 mM and proteinase cocktail inhibitors), sonicated for 10 s and centrifuged at 9000 ***g*** for 10 min at 4°C. Supernatants were isolated, and total proteins were quantified by the Bradford method.

Then, 75 µg of protein were loaded in each lane, separated in 7% (w/v) SDS-PAGE (100 V for 90 min at room temperature) and transferred to a nitrocellulose membrane (100 V for 90 min at 4°C). Membranes were blocked using dried skim milk 5% (w/v) in PBS and incubated overnight at 4°C with primary antibodies. These antibodies were used as follows: anti-COX1 (1:200) (Cayman Chemical Company, catalog #160109), anti-COX2 (1:200) (Cayman Chemical Company, catalog #160126), anti-iNOS (1:200) (Cayman, Chemical Company, catalog #160862), anti-endoglin (1:200) (Santa Cruz Biotechnologies), anti-VEGF (1:200) (Abcam, catalog ab46154), anti-von Willebrand factor (1:200) (Dako, A0082) and anti-β-actin (1:3000) (Sigma Chemical). Nitrocellulose membranes were then incubated with goat anti-rabbit HRP-conjugated antibody (1:3000, Sigma Chemical) for 1 h at room temperature. Membranes were washed three times after each incubation with PBS containing 0.1% (v/v) Tween-20 to remove non-specifically bound antibodies. Protein bands were detected using an enhanced chemiluminescence western blot system. Images were obtained using GeneGnome XRQ NM (Syngene) and analyzed using the ImageJ software package (free access). Results were expressed as the relative optical density to β-actin.

NOS enzyme assay

One whole implantation site per animal was used, and NOS activity was quantified by the modified method of Bredt & Snyder as previously described (Sordelli *et al.* 2012). One whole implantation site per animal was weighed and homogenized in HEPES buffer (20 mM HEPES, 0.45 mM $CaCl_2$, 2.5 mM DTT and 25 mM L-valine). Homogenates were incubated at 37°C for 15 min with [^{14}C]-L-arginine and 0.5 mM NADPH. Samples were centrifuged for 15 min at 12,000 ***g*** and the supernatants were applied to a DOWEX AG50W-X8 column. [^{14}C]-L-citrulline was eluted in distilled water, and the radioactivity was measured using liquid scintillation counting. Enzyme activity was expressed as fmol [^{14}C]-L-citrulline/mg wet weight/15 min.

Radioimmunoassay

Two implantation sites per animal were used, and prostaglandin (PG) E_2 and F_{2a} production were measured by RIA. Implantation sites were thawed, weighed and incubated in Krebs–Ringer bicarbonate buffer for 1 h at 37°C in a 5% CO_2 atmosphere. Tissues were discarded and the medium was acidified with 1 N HCl. Afterward, 2 mL of ethyl acetate was added and the organic phase was collected. The extraction was repeated two more times. The organic solvent was evaporated in a vacuum stove. PGF_{2a} and PGE_2 antiserum were highly specific for PGF_{2a} and PGE_2 respectively and showed low cross-reactivity with related compounds. Sensitivity was 5–10 pg per tube and Ka = 1.5 × 10^{10}L/mol. Values were expressed as pg PG/mg wet weight.

Statistical analysis

Statistical analysis was performed using the InfoStat software (Faculdad de Ciencias Agropecuarias, University of Cordoba, Argentina). The approximate sample size was calculated *a priori* for all determinations using this software. Pregnant mice were considered the experimental unit. No pregnant mice were excluded from the analysis.

Comparisons between groups were performed using one-way ANOVA followed by Tukey's multiple comparisons test. Normality and homoscedasticity were tested by Shapiro–Wilk and Levene tests respectively. Statistical analysis was performed using a randomized block design when necessary.

The pregnancy rate was analyzed by an arrangement of the data in T contingency tables. The chi-square test was used to analyze the hypothesis of independence between the housing condition and pregnancy rate.

Results were expressed as mean ± S.E.M. Differences were considered significant when the *P*-value was 0.05 or less.

Results

EE regulates the pregnancy rate between d7 and d15

Control mice on d19 and d15 presented a reduced pregnancy rate compared to d7 control females (Table 1). Interestingly, the pregnancy rate of EE mice on d19, d15 and d7 was similar. No differences were detected between the groups on d7. However, d15 EE mice showed a higher pregnancy rate than d15 control mice. These results indicate that EE exposure prevents pregnancy loss between d7 and d15.

Regarding the number of implantation sites, no differences were found between the groups and between the days of gestation (10 ± 4 on d7 and 8 ± 2 on d15 of gestation, for both groups).

Based on these results, we investigated whether the maintenance in pregnancy rate due to EE exposure is modulated by an effect on early pregnancy-related processes on d7.

Table 1 Effect of EE on the pregnancy rate.

Group	Pregnancy rate on		
	d7	d15	d19
C	70% (14/20)	45% *(9/20)	45% *(9/20)
EE	75% (15/20)	85% $^{+}$(17/20)	85% $^{\#}$ (17/20)

Percentage of pregnancy rate of C and EE mice on days 7, 15 and 19 of gestation ($P = 0.03$); Chi-square test ($n = 20$ animals per group).
*Statistically significant differences compared to control d7;
$^{+}$Statistically significant differences compared to control d15;
$^{\#}$Statistically significant differences compared to control d19.
C, control; EE, enriched environment; d7, day 7 of gestation; d15, day 15 of gestation; d19, day 19 of gestation.

Control and EE implantation sites present similar appearance and architecture

First, the implantation sites were analyzed macroscopically. D7 pregnant mice from both housing conditions (Fig. 1B and C) presented well-developed implantation sites spaced evenly along the uterine horns (Fig. 1D). No differences in the size, color, shape and wet weight (26.22 ± 0.59 vs 27.35 ± 1.49 mg, $n = 10$ $P = 0.5$) of the implantation sites were detected between the control and EE mice (Fig. 1E).

Then, we evaluated the histological structure of the implantation sites on d7. Control and EE implantation sites exhibited a highly conserved architecture and no differences were found between the groups (Fig. 1F and G). Mesometrial and antimesometrial decidua, the uterine lumen containing the embryo and the longitudinal and circular smooth muscle layers were observed. The ectoplacental cone and the decidual crypt were also detected (Fig. 1H and I). Mesometrial decidual cells presented small nuclei, scarce cytoplasm and vacuoles. The glycogenic decidua exhibited cells with multiple vacuoles. Antimesometrial decidual cells showed bigger nuclei with abundant cytoplasm, and binucleated cells were also observed.

EE favors vascular remodeling in early pregnancy

The uterine macrovasculature was analyzed (Fig. 2A). EE mice presented an increase in the diameter of the uterine artery compared to control ($P = 0.0002$) (Fig. 2B). No differences in the number of vessels irrigating each implantation site were found between the groups (Fig. 2C).

When the microvasculature of the mesometrial decidua was analyzed (Fig. 3A), we observed that the number of vessels per field and their circumferences were similar between the control and EE females (Fig. 3B and C). To evaluate vessel remodeling, the wall:lumen ratio was quantified. A lower ratio was detected in mice exposed to EE compared to control ($P = 0.003$) (Fig. 3D). As we observed an uneven distribution of the data, we discriminated the analysis between vessels with low (<2) and high (>2) wall:lumen ratios. Those vessels with a high ratio corresponded to thick-wall vessels

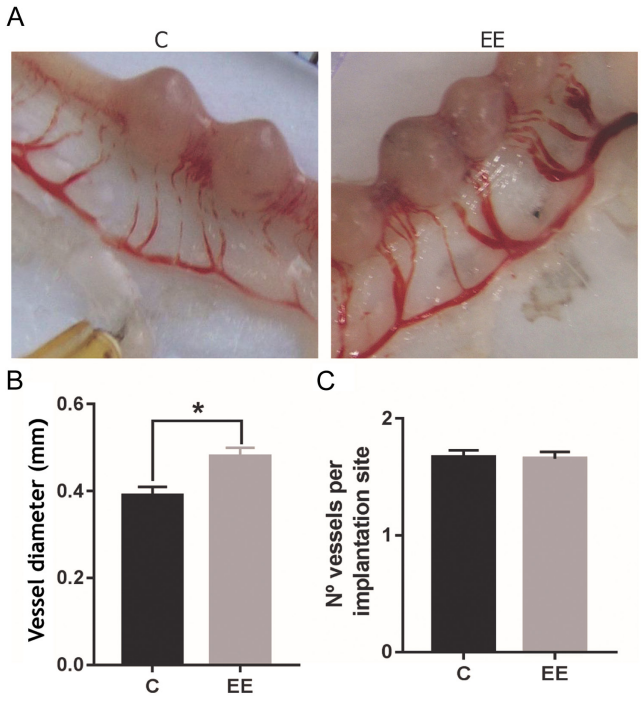

Figure 2 Effect of the EE on macrovascular remodeling in pregnant mice on d7. (A) Representative images of the main vessels supplying the uterine horns from control and EE mice. (B) Vessel diameter of the uterine artery on d7 (P = 0.0002). (C) Number of vessels irrigating each implantation site (P = 0.9). Results are expressed as means ± s.e.m. One-way ANOVA, Tukey's test (n = 4 animals per group), *Significant statistical differences. C, control; d7, day 7 of gestation; EE, enriched environment.

suggesting that they had not been remodeled. There was no difference between control and EE in this data set (Fig. 3E). Interestingly, EE mice presented decreased wall:lumen ratio in thin-wall vessels (<2) compared to control ($P = 0.001$) (Fig. 3F).

EE modulates vascular mediators

Vascular remodeling at the maternal–fetal interface is a process regulated by multiple mediators. Among these molecules, nitric oxide is a known vasodilator that mediates uterine angiogenesis. In the present work, we observed that d7 pregnant mice exposed to the EE presented higher NOS activity ($P = 0.04$) and iNOS protein levels compared to control ($P = 0.02$) (Fig. 4A and D).

PGs produced by COX are lipid hormones which regulate uterine contractions, decidualization and angiogenesis during early gestation. Control and EE mice presented similar levels of PGE_2 (Fig. 4B). Nonetheless, EE mice showed higher levels of PGF_{2a} compared to control ($P = 0.01$) (Fig. 4C). When COX1 and COX2 isoforms protein expression were analyzed, no differences were found between the experimental groups (Fig. 4E and F).

Finally, we investigated the expression of endoglin, VEGFA and von Willebrand factor, which are well-described mediators of vascular processes. We observed that EE mice presented higher protein levels of endoglin ($P = 0.01$) (Fig. 4G). No differences were detected when VEGFA and von Willebrand factor were studied (Fig. 4H and I).

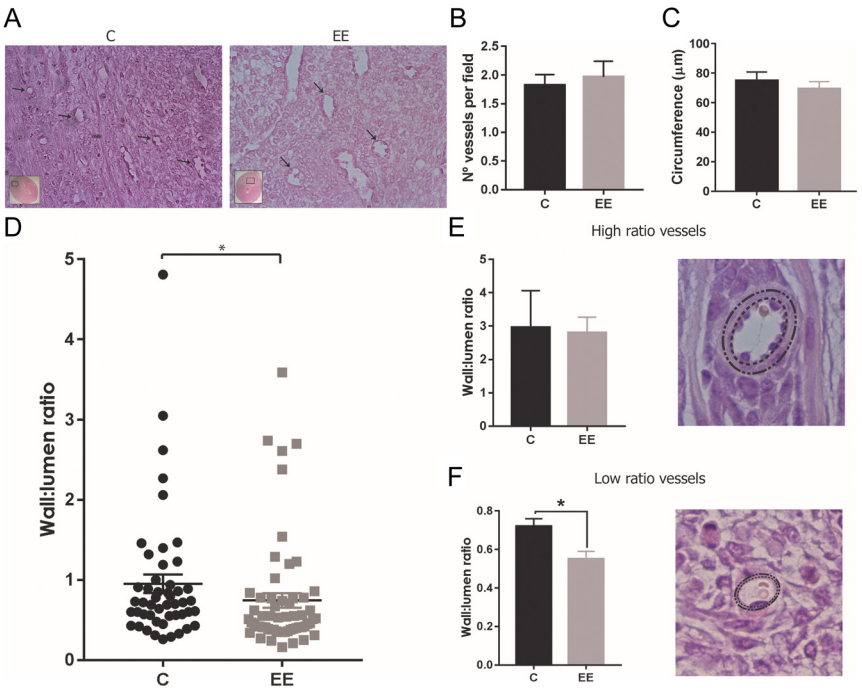

Figure 3 Effect of the enriched environment on microvasculature in pregnant mice on d7. (A) Representative images of mesometrial microvessels. Arrows indicate vessels that met the criteria. (B) Number of microvessels per field (P = 0.5). (C) Circumference of microvessels (P = 0.5). (D) Wall:lumen ratio of all microvessels (P = 0.003). (E) Wall:lumen ratio and a representative image of vessels with high ratio (P = 0.8). (F) Wall:lumen ratio and a representative image of vessels with low ratio (P = 0.001). Results are expressed as means ± s.e.m. One-way ANOVA, Tukey's test, (n = 4 animals per group), *Significant statistical differences.

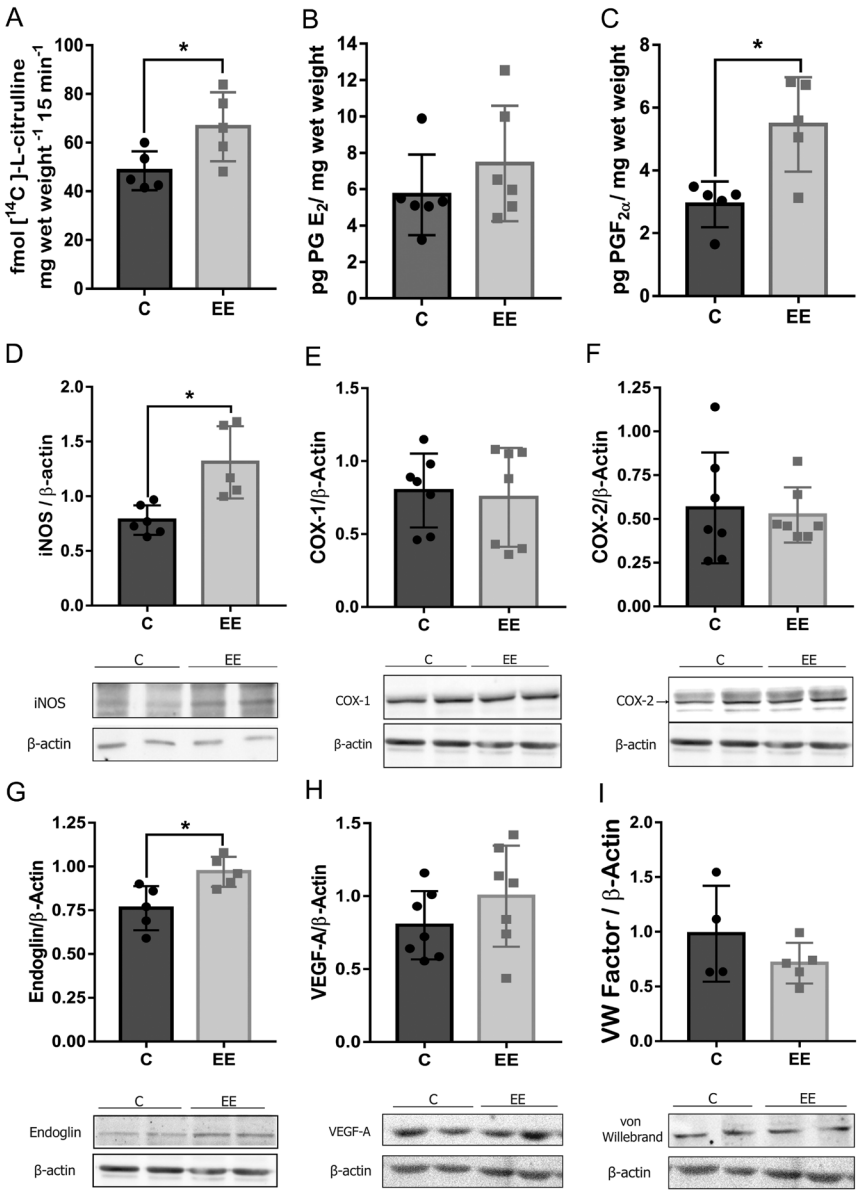

Figure 4 Effect of the EE on vascular mediators. (A) NOS activity was determined by the modified method of Bredt & Snyder ($P = 0.009$, n = 5 per group). (B and C) PGE_2 ($P = 0.2$, n = 6 per group) and PGF_{2a} ($P = 0.01$, n = 5 per group) levels were determined by RIA (D) iNOS ($P = 0.01$, n = 6 C and 5 EE), (E) COX1 ($P = 0.6$, n = 7), (F) COX2 ($P = 0.7$, n = 7), (G) endoglin ($P = 0.01$, n = 5), (H) VEGFA ($P = 0.2$, n = 7), (I) von Willebrand factor ($P = 0.4$, n = 5) protein levels were determined by western blot. Representative cropped blots are shown. Results are expressed as means ± S.E.M. One-way ANOVA, Tukey's test. *Significant statistical differences. C, control; EE, enhanced environment; iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase; PG, prostaglandin; VEGF, vascular endothelial growth factor.

Discussion

In the present work, we demonstrate that periconceptional exposure of females to the EE prevents embryo loss and promotes the vascular remodeling that takes place during early gestation.

Inadequate decidual angiogenesis contributes to early pregnancy loss (Plaisier *et al.* 2009, Quenby *et al.* 2009, Windsperger *et al.* 2017) and the development of obstetric pathologies at mid- and late gestation (Brosens *et al.* 2019*a*,*b*). Furthermore, it has been reported that insufficient decidual vascular development leads to embryo resorption and pregnancy loss by mid-gestation in mice (Douglas *et al.* 2009, Khankin *et al.* 2012, Kim *et al.* 2013). In recent years, the appearance of these obstetric pathologies has been associated with maternal lifestyle (Dunkel Schetter & Tanner 2012, Zhang *et al.* 2016, Sominsky *et al.* 2017). Indeed, maternal stress affects placental angiogenesis (Ozmen *et al.* 2017) and is a factor that might influence the performance of *in vitro* fertilization protocols (Massey *et al.* 2016). On the contrary, physical activity and relaxation techniques appear to have beneficial effects on the physical and emotional health during gestation and potentially improve pregnancy outcomes (Hinman *et al.* 2015, Matvienko-Sikar *et al.* 2016). In animal models, it has been observed that mice subjected to stress show fewer implantation sites (Burkuš *et al.* 2015). Interestingly, we previously reported that the exposure of pregnant females to the EE decreases the percentage of preterm delivery and perinatal death of the offspring in a mice model of inflammation (Schander *et al.* 2020). Moreover, pups born from EE mothers are healthy and present a normal development during lactation (Schander *et al.* 2021).

In this work, we showed that the pregnancy rate of control mice dropped between d7 and 15 of gestation, indicating that there is postimplantation embryo loss that occurs under control conditions. Exposure of pregnant mice to the EE maintains gestation, and this is evidenced by the finding that the pregnancy rate of EE mice remained the same. EE exposure also modulates vascular remodeling on d7, and these vascular adaptations could be involved in its protective effect. Our results, together with the data published by other authors, support the hypothesis that maternal lifestyle influences the quality and the progress of gestation and the well-being of the offspring.

The uterine artery is the main vessel that supplies blood to the uterine horns and branches to the spiral arteries that irrigate the endometrium. Pregnant females exposed to the EE showed an increase in the diameter of the uterine artery, suggesting an increase in blood flow to the implantation sites. The loss of smooth muscle layers during maternal vessel remodeling transforms the vascular walls allowing the increase in blood flow that occurs in response to the metabolic demands of the growing embryo and the placenta. In healthy pregnancies, this process proceeds without an excessive increase in the shear stress and the production of reactive oxygen species and pro-inflammatory cytokines (Cartwright *et al.* 2010, Mannaerts *et al.* 2018). In this sense, the microvessels of EE females have a lower wall:lumen ratio, suggesting that the exposure to the EE promotes vessel remodeling of the implantation sites. We hypothesize that the decrease in the wall:lumen ratio induced by the EE might be necessary to prevent embryo loss, as the number of vessels irrigating the implantation sites and their circumferences did not differ between treatments. Therefore, the EE might modulate maternal physiology protecting the implantation site from potential detrimental effects triggered by an increase in blood flow, which finally would lead to pregnancy loss.

The architecture of d7 implantation sites remains conserved between the control and EE females. This result suggests that the EE conditioned vascular remodeling without affecting other processes related to the structure of the implantation site.

We observed that the changes in the micro- and macrovasculature induced by EE are paralleled by the modulation of molecules involved in vascular processes.

Nitric oxide is a potent mediator of angiogenesis during gestation (Krause *et al.* 2011). The regulation of nitric oxide tone at embryo implantation sites is crucial to maintain early pregnancy (Ota *et al.* 1999, Purcell 1999, Novaro *et al.* 2001). We had previously reported that NOS activity is higher at rat implantation sites than at intersites, and it is differentially regulated by the blastocyst (Sordelli *et al.* 2011). We recently described that exposure to the EE augments NOS activity at mid-gestation in the mice uterus (Schander *et al.* 2020). Additionally, we reported that iNOS isoform is involved in the increase of interleukin-10, a vascular marker, in the rat uterus (Beltrame *et al.* 2013) and participates in the acquisition of the endovascular phenotype by the first-trimester trophoblast (Beltrame *et al.* 2018). Interestingly, iNOS is the main isoform of NOS expressed in the uterine endometrium in early gestation (Ali 1997). In the present work, EE females showed an increase in iNOS expression compared to control mice. This result suggests that the EE induces an increase in NOS activity at the implantation sites by an increase in iNOS expression, which in turn would promote vessel remodeling.

PGs play an essential role at the site of implantation. Different authors have proposed that whereas PGE_2 contributes to maternal decidualization and angiogenesis, PGF_{2a} regulates uterine contractions (Hamilton & Kennedy 1994, Stocco & Deis 1998, Callegari *et al.* 2005). Recently, a novel role has been described for PGF_{2a} as a regulator of the vascular processes at the endometrium (Kaczynski *et al.* 2016, 2020, Baryla *et al.* 2019). Therefore, we propose that the increase in PGF_{2a} favors the macro- and microvascular changes described in EE females. Although PGE_2 is associated with vascular physiology, high levels of PGE_2 are related to inflammation. It is then possible that the EE exerts a protective effect by preventing an increase in PGE_2 at the implantation site. A potential mechanism that might explain the increase in PGF_{2a} is the conversion of PGE_2 to PGF_{2a} by the PGE2-9-ketoreductase enzyme. However, more studies are needed to confirm this conjecture.

Within the wide range of mediators involved in the vascular process of early pregnancy, different authors have shown the importance of endoglin, von Willebrand factor and VEGF. Endoglin is expressed in the apical face of the luminal epithelium of the endometrium and the primary and secondary decidua in mice (Chadchan *et al.* 2016, Yuan *et al.* 2019). It has been shown that endoglin participates in the adhesion of the blastocyst to the uterus and modulates uterine receptivity during the window of implantation. Also, endoglin regulates angiogenesis through different signaling pathways (Lebrin *et al.* 2004, Mano *et al.* 2011, Kapur *et al.* 2013). In the present work, we observed an increase in endoglin expression at the implantation sites of females exposed to the EE. This increase might be related to the reception of signals released by the embryo (Chadchan *et al.* 2016, Yuan *et al.* 2019), which stimulate vascular remodeling of the maternal–fetal interface. On the other hand, the EE action on vascular remodeling seems not to be mediated by VEGFA or von Willebrand factor as we did not observe any differences between control and EE mice. Although EE has proven to have positive effects on the vascular adaptations in the brain after ischemia through changes in VEGF (Zhang *et al.* 2017, Zhan *et al.* 2020), we did not observe the regulation of this molecule at the maternal–fetal interface. However, we

could not rule out the involvement of other members of the VEGF family, such as VEGF receptors among others.

Neurotrophins are expressed in the placenta and stimulate trophoblast cell growth and survival (Kawamura *et al.* 2009), playing an important role during pregnancy and placental angiogenesis (Sahay *et al.* 2017). Also, neurotrophins induce nitric oxide production in endothelial cells (Meuchel *et al.* 2011, Marie *et al.* 2018) and cooperate with the TGF-β pathway to protect vessels and injured tissues (Schlecht *et al.* 2021). Besides, it has been reported that EE exposure modulates neurotrophins levels, like brain-derived neurotrophic factor, in the CNS and immune system (Cao *et al.* 2010, Mansour *et al.* 2021). Therefore, we postulate that one of the mechanisms modulated by EE exposure might be neurotrophin levels in the uterus and its triggered pathways stimulating vascular remodeling during early gestation. Nevertheless, more studies are required to elucidate the mechanisms underlying EE positive effects during gestation.

In conclusion, we observed that the exposure of pregnant females to the EE regulates the physiology of the uterus, preventing embryo loss and promoting the vascular remodeling that takes place during early gestation. Our findings support the notion that the well-being of mothers improves early pregnancy by mechanisms associated with the remodeling of the uterine vasculature. Even taking into account the limitations of animal models, the positive effects of non-pharmacological interventions in humans support the idea that an 'EE-like' protocol could be designed for women seeking pregnancy. This protocol would include relaxing, exercise and anti-stress therapies. This treatment might be advantageous for fertile women, but especially for those women who have to overcome a fertility treatment. The non-invasive nature of maternal environmental enrichment makes this tool particularly interesting when considering new strategies to prevent implantation failure and recurrent miscarriage.

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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Data availability

Data available upon reasonable request.

Author contribution statement

F L D B conducted the experiments, acquired and analyzed the data. J A S and J S B contributed to performing experiments and data analysis. M C contributed to performing experiments. A M F and M L R designed the experiments and contributed financially to this project. F L D B, J A S, J S B and M L R wrote the manuscript. A M F and M L R contributed equally.

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