REPRODUCTION
RESEARCH

The enrichment of maternal environment prevents pre-term birth in a mice model

Julieta Aylen Schander[1], Julieta Aisemberg[1], Fernando Correa[1], Manuel Luis Wolfson[1], Lorena Juriol[2], Cora Cymeryng[3], Federico Jensen[2] and Ana María Franchi[1]

[1]*Laboratorio de Fisiopatología de la Preñez y el Parto, Centro de Estudios Farmacológicos y Botánicos, CONICET-UBA, Buenos Aires, Argentina,* [2]*Laboratorio de Inmunología de la Reproducción, Centro de Estudios Farmacológicos y Botánicos, CONICET-UBA, Buenos Aires, Argentina and* [3]*Laboratorio de Endocrinología Molecular, Centro de Estudios Farmacológicos y Botánicos, CONICET-UBA, Buenos Aires, Argentina*

Correspondence should be addressed to J A Schander; Email: juli.schander@gmail.com

Abstract

Maternal lifestyle affects both mother health and pregnancy outcome in humans. Several studies have demonstrated that interventions oriented toward reducing stress and anxiety have positive effects on pregnancy complications such as preeclampsia, excessive gestational weight, gestational diabetes and preterm birth. In this work, we showed that the environmental enrichment (EE), defined as a noninvasive and biologically significant stimulus of the sensory pathway combined with voluntary physical activity, prevented preterm birth (PTB) rate by 40% in an inflammatory mouse model induced by the systemic administration of bacterial lipopolysaccharide (LPS). Furthermore, we found that EE modulates maternal metabolism and produces an anti-inflammatory environment that contributes to pregnancy maintenance. In pregnant mice uterus, EE reduces the expression of TLR4 and CD14 (the LPS receptor and its coactivator protein), preventing the LPS-induced increase in PGE2 and PGF2α release and nitric oxide synthase (NOS) activity. In cervical tissue, EE inhibits cervical ripening events, such as PGE2 release, matrix metalloproteinase (MMP)-9 increased activity and neutrophil recruitment, therefore conserving cervical function. It seems that EE exposure could mimic the stress and anxiety-reducing techniques mentioned above, explaining, at least partially, the beneficial effects of having a healthy lifestyle before and during gestation. Furthermore, we propose that designing an EE protocol for humans could be a noninvasive and preventive therapy for pregnancy complications, averting pre-term birth occurrence and dreaded sequelae that are present in the offspring born too soon.

Reproduction (2020) **159** 479–492

Introduction

Preterm birth (PTB), defined as birth occurring before 37 weeks of gestation in humans, is a major determinant of neonatal morbi-mortality. PTB is considered a syndrome caused by multiple pathological processes (Romero *et al.* 2014) and of all putative causes, intra-amniotic infection is the only process for which a causal link has been established (Romero *et al.* 1994, 2006*a*). Indeed, infection accounts for 30% of PTBs (Romero *et al.* 2006*b*). It is well accepted that infection causes a transition from an anti-inflammatory to a pro-inflammatory state in the mother, which involves infiltration of innate immune cells and increased expression of inflammatory mediators at the maternal–fetal interface, with the subsequent release of prostaglandins, endocannabinoids, nitrogen and oxygen reactive species and metalloproteinases that triggers preterm parturition (Romero *et al.* 2006*a*, Gomez-Lopez *et al.* 2014). Current therapeutic approaches are directed toward stopping or delaying premature labor when it has already started, which are generally unsuccessful.

Maternal lifestyle affects pregnancy outcome and can further adversely impact the offspring's health in adulthood. Indeed, different studies have shown that chronic maternal stress and depression increases the risk of PTB and intrauterine growth restriction (Latendresse 2009, Grote *et al.* 2010). In keeping with this, it is known that leisure-time physical activity (Hegaard *et al.* 2007, 2008) and anti-stress treatments (such as psychological accompaniment, midwife-led continuity of care or relaxation therapy) (Narendran *et al.* 2005, Khianman *et al.* 2012, Deshpande *et al.* 2013, Sandall *et al.* 2016, Cunningham *et al.* 2019) improve maternal health and reduce gestational diseases in humans.

In animal models, the effect of environmental stimulation and enrichment is typically associated with enhanced well-being, improved cognitive function and stress resilience, which are driving by molecular,

© 2020 Society for Reproduction and Fertility
ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-19-0572
Online version via https://rep.bioscientifica.com
Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

cellular and physiological changes (Bakos *et al.* 2009, Langdon & Corbett 2012). Environmental enrichment (EE) strategy has been found to be beneficial in animal models of Parkinson disease (Jungling *et al.* 2017, Wi *et al.* 2018) and to exert neuroprotective effects to harmful stimuli (Horvath *et al.* 2013) and retinal damage (Dorfman *et al.* 2014, González Fleitas *et al.* 2019). Furthermore, it may also prevent age-related cognitive impairment and adverse consequences of prenatal stress (Patten *et al.* 2015). Hence, EE appears to be associated with an overall improvement in the psychological and physical wellbeing of animals.

EE is described as a mixture of complex social and inanimate motivations, which provides enhanced sensory, motor, cognitive, and social opportunities. Animals are housed in large cages containing different interacting objects such as tunnels, balls, nesting material, running wheels and usually a high number of subjects per cage, so they receive a higher and continuous sensory, cognitive and motor stimulation, compared to control animals housed in standard conditions.

Therefore, the aim of this work was to evaluate the possible beneficial effects of the exposure to an enriched environment on the triggering of preterm labor and the deleterious consequences on pregnancy provoked by an immunological challenge. Furthermore, we studied the molecular and physiological changes in the mother associated with EE exposure.

Materials and methods

Reagents

LPS from *Escherichia coli* (serotype 05:B55), gelatin, anti-β-actin antibody, secondary horse radish peroxidase (HRP)-conjugated antibody, HEPES, CaCl2, DTT, citrulline and NADPH were purchased from Sigma Chemical Co. Valine was obtained from USB (Mundra, India). [14C]-L-arginine was purchased from Perkin Elmer and DOWEX AG50W-X8 columns from Bio-Rad.

Western blotting reagents were obtained from Bio-Rad and Sigma Chemical Co. The anti-COX-2 and anti-iNOS antibodies were purchased from Cayman while the anti-CD14 and anti-TLR4 antibodies were obtained from Santa Cruz Biotechnology Inc..

Quick-Zol reagent for total RNA extraction from tissues was obtained from Kalium Technologies (Bernal, Argentina). Reagents used for mRNA retro-transcription (RT) (ultrapure H2O, RNAse-free DNAse I, DNAse buffer, random primers, Tris 5X Buffer, DTT) were purchased from Invitrogen, while the RNAse inhibitor and dNTPs were supplied by Genbiotech (Buenos Aires, Argentina). Reverse transcriptase M-MLV, green GoTaq reaction buffer 5X and DNA polymerase used for PCR were obtained from Promega. All other chemicals were analytical grade.

Animals

BALB/c mice were purchased from the Instituto Nacional de TecnologíaAgropecuaria (INTA) and bred in our animal care facility at the School of Medicine of Buenos Aires University (CABA, Argentina). All mice were kept under a circadian cycle (light:dark = 12:12 h). Food and water were provided *ad libitum*. Twelve-week-old females were mated with males of the same phenotype. Female mice were checked daily for the appearance of a mucus plug, which indicated zero days post coitum (dpc).

The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFyBO–CONICET) and by the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine (University of Buenos Aires) (CD Nº 900/2016) and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH).

The animals were assigned to each experimental group in a randomized manner.

Enriched environment (EE) protocol

The EE protocol consisted in housing ten animals (6-week-old female BALB/c mice) in bigger cages (640 × 420 × 200 mm) than those of standard environment. The cage contained running wheels and objects of different shapes, textures and colors (toys, tunnels, shelters and stairs) that were completely changed once a week. Every 3–4 days, one of the objects was removed, moved or a new object was added to the cage. This protocol provides optimal conditions for further exploration, visual, cognitive, social interaction and voluntary exercise activity. Standard conditions (control environment, CE) consisted of standard laboratory cages that housed four animals (Fig. 1A and B).

Animals stayed in EE for 6 weeks before being mated with males in regular cages. Pregnant females were returned to EE cages till day 15th of pregnancy, when LPS (or saline solution) was administered. A group of animals were killed and several tissues were collected whereas a different set of animals were followed up to evaluate the percentage of PTB (Fig. 1C).

Treatments

Pregnant mice from EE or CE received two injections of sterile saline solution (SS) or bacterial lipopolysaccharide (LPS) on day 15 of gestation: the first one (0.13 mg/kg LPS) at 09:00 h and the second at 12:00 h (0.39 mg/kg) intraperitoneally (i.p.). This protocol induced delivery between 8 and 12 h after the first dose. Animals were killed by cervical dislocation and different tissues were collected (Fig. 1C). For RT-PCR analyses, mice were killed 3 h after the second LPS injection. For immunohistochemistry, Western blot, zymography and RIA assays, mice were killed 5 h after the second LPS injection. Four experimental groups were included in this study: Group 1: Pregnant females (CE) + vehicle; Group 2: Pregnant females (CE) + LPS; Group 3: Pregnant females (EE) + vehicle; Group 4: Pregnant females (EE) + LPS. Mice were monitored for evidence of delivery to evaluate the percentage of PTB (delivery before day 18 of gestation).

Blood glucose levels measurement and oral glucose tolerance test

Blood glucose levels were determined using an Accu-Chek® Performa glucometer (Roche S.A.Q. e I.). Oral glucose tolerance

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

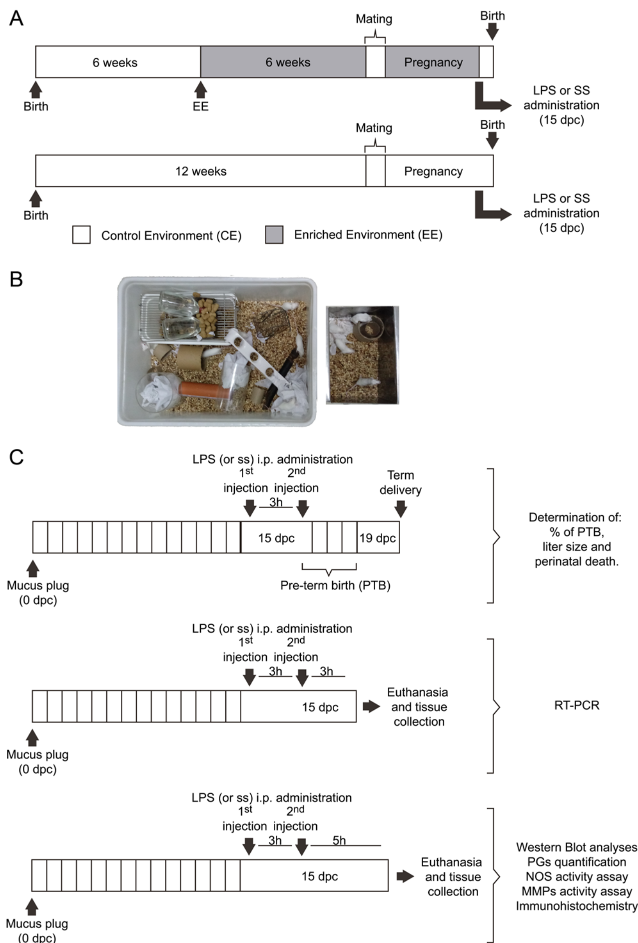

Figure 1 Enriched environment and preterm birth (PTB) experimental design. (A) Enriched environment (EE) and control environment (CE) protocol. (B) Examples of EE (left) and regular cages (CE, right). (C) Scheme showing the timing of LPS or vehicle (saline solution, SS) administration and tissue collection.

test (OGTT) was performed to control (SS administered) females from CE or EE protocol after 8 h of fasting, following an intragastric administration of 2 g/kg of dextrose on 15th day of pregnancy. Blood was drawn from the tail vein and glucose levels were monitored at 0, 30, 60 and 120 min after dextrose administration (t0, t30, t60 and t120 respectively).

Determination of total nitric oxide synthase (NOS) activity

NOS enzyme activity was quantified in uterine and cervical strips using the modified method of Bredt and Snyder, which measures the conversion of [14C]-L-arginine to [14C]-L-citrulline (Bredt & Snyder 1989). Briefly, samples were weighed and homogenized in a buffer containing 20 mM HEPES, 4.5 μM $CaCl_2$, 100 mM DTT and 25 mM valine. After homogenization, 10 μM [14C]-arginine (0.3 μCi) and 0.12 mM NADPH were added. Samples were incubated for 15 min in a 5% CO_2 atmosphere at 37°C and immediately centrifuged at 18,000 ***g*** for 15 min (4°C). Then the supernatants were applied to 1 mL DOWEX AG50W-X8 columns (Na+ form) equilibrated with HEPES medium and citrulline. Finally, [14C]-citrulline was eluted in 3 mL of water. The radioactivity was measured by liquid scintillation counting. Enzyme activity is reported in fmol of [14C]-citrulline produced by 1 mg of tissue in 15 min.

Measurement of cervical MMP-2 and MMP-9 activities by gelatin zymography

Zymography was performed to evaluate the presence of gelatinase activity in cervical tissue as described by Pustovrh *et al.* (2005). Both MMPs and pro-MMPs were analyzed by zymography since the exposure to SDS induces changes in pro-MMPs conformation which are associated with their activation. Briefly, cervical tissue was homogenized in 50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100 and 40 μg of protein from cervical homogenates were mixed with loading buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM Tris–HCl, pH 6.8) and subjected to a 7.5% sodium dodecyl sulfate (SDS)-PAGE, in which 1 mg/mL gelatin (type A from porcine skin) was incorporated. Following electrophoresis, gels were washed in 2.5% Triton X-100 for 60 min to remove SDS. Next, gels were incubated for 24 h in 50 mM Tris Buffer pH 7.4, containing 150 mM NaCl and 10 mM $CaCl_2$, at 37°C. Gels were stained with Coomassie blue, and then de-stained with 10% acetic acid and 30% methanol in water. The areas of proteolytic activity appeared as negatively stained bands in the dark background. The identities of MMPs were based on their molecular weights. The enzymatic activity was quantified using the ImageJ software package and expressed as arbitrary densitometric units, which were normalized to the corresponding control MMP activity (CE+SS group). Data are shown as relative to a value of 1 assigned to the mean values for MMP-9, MMP-2 and pro-MMP-2 respectively, in control cervixes.

RT-PCR

Cervixes were isolated from pregnant females 3 h after the second LPS (or SS) administration and 1 mL of Quick-Zol reagent was added to each sample which were kept at −70°C till used. Total RNA was isolated according to manufacturer's recommendations. RNA concentration was determined using a micro-volume spectrophotometer (Eppendorf). cDNA was generated from 2 μg of total RNA pretreated with DNAse. RT was performed by incubating the samples with M-MLV enzyme, random primers and triphosphate deoxyribonucleotides in the presence of a recombinant ribonuclease inhibitor for 10 min at 25°C, 50 min at 37°C and 15 min at 70°C. cDNA amplification was done using specific primers designed with Primer-Blast program (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers sequences and PCR conditions are shown in Table 1. Products were loaded onto 4% agarose gel, stained with ethidium bromide, recorded with a digital camera (Olympus C-5060) and analyzed using the ImageJ software package. Data were expressed as the relative amount of each PCR product versus β-actin.

Western blot analysis

Uteri and cervix from pregnant animals were collected 5 h after second LPS (or SS) administration and were kept

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

Table 1 RT-PCR primers and product size.

cDNA	Foward primer	Reverse primer	Product size (bp)
Cxcl-15	GGTGAAGGCTACTGTTGGC	TATTCTCTTGTTCTCAGGTCTCC	151
Ccl5	CCTCACCATCATCCTCACTG	CACTTGGCGGTTCCTTCG	196
Ccl3	CCGACTGCCTGCTGCTTCTCCTAC	CGTTCCTCGCTGCCTCCAAGACTC	213
Ccl2	CTCACCTGCTGCTACTCATTCAC	ATGTCTGGACCCATTCCTTCTTG	163
Cxcl-10	ATCCCTGCGAGCCTATCC	AATTCTTGATGGTCTTAGATTCCG	103
Mmp8	TGGTGATTTCTTGCTAACCCC	TACACTCCAGACGTGAAAAGC	139
β-actin	TGTTACCAACTGGGACGACA	TCTCAGCTGTGGTGGTGAAG	392

at −70°C till used. Tissues were homogenized in lysis buffer (0.02% sodium azide, 0.1% SDS, Deoxycholate 0.5%, Nonidet P40 1%, inhibitors cocktail, and PBS), sonicated and centrifuged at 9000 ***g*** for 10 min. Supernatants were isolated and total proteins were quantified by Bradford method (Bradford 1976). Samples were then processed for subsequent electrophoretic separation. 100 µg of protein for uteri and 40 µg for cervixes, were mixed with loading buffer and loaded in each lane. Samples were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-COX-2 (1:200), anti-iNOS (1:200) anti-TLR4 (1:200), anti-CD14 (1:200) or anti-actin (1:4000) antibodies. Blots were washed with T-PBS (PBS and 0.1% (v/v) Tween 20, pH 7.5) followed by 1-h incubation with horse radish peroxidase-conjugated anti-rabbit (or anti-goat in case of anti-CD14 antibody) secondary antibody (1:5000) and reveled using the enhanced chemiluminescence Western blot system. Images of immunoreactive bands were acquired using the ImageQuant System (GE Healthcare Life Sciences) and analyzed using the ImageJ software package (free access). Relative protein levels were normalized to β-actin and results were expressed as relative optical density.

Radioimmunoassay (RIA)

Prostaglandins F2α (PGF2α) and E2 (PGE2) concentration were determined by RIA (Cambell & Ojeda 1987). Tissues from pregnant mice were isolated and stored at −70°C until used. To measure uterine and cervical prostaglandins production, tissues were weighed, homogenized and incubated in Krebs–Ringer–Bicarbonate buffer (KRB: 118 mM NaCl, 4.7 mMKCl, 1.18 mM KH_2PO_4, 1.22 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 11.1 mM glucose) at 37°C for 60′ with 5% CO_2. Tissues were discarded and the KRB was acidified to pH 3 with 1 N HCl and then 2 mL of ethyl acetate was added. The organic phase, containing PGs, was collected, and the extraction was repeated two more times. The organic solvent was evaporated in a vacuum stove. PGF2α and PGE2 antiserum were highly specific for PGF2α and PGE2 respectively and showed low cross reactivity with related compounds. Sensitivity was 5–10 pg per tube and $Ka = 1.5 \times 10^{10}$ L/mol. Values are expressed as pg PG/ total protein (µg/mL).

Corticosterone levels were determined in serum samples by RIA after dichloromethane extraction, as previously described (Cymeryng *et al.* 1998).

Immunohistochemical studies

Cervixes from pregnant females were collected 5 h after LPS (or SS) administration and fixed in formaldehyde 10% in PBS. Tissues were dehydrated in increasing gradient of alcohol and embedded in paraffin. Sections of 4 µm were performed with a microtome and mounted on silane-coated slides. Antigen retrieval was performed by heating slices at 90°C for 30 min in citrate buffer (pH 6.3). Sections were immersed in 0.1% Triton X-100 (Roche Diagnostics GmbH) in 0.1 M PBS for 20 min for permeabilization. Sections were preincubated with 5% normal horse serum for 1 h and then were incubated overnight at 4°C with primary antibody (1:200 anti-Ly6G). After several washings, secondary antibody (1:200 of a goat anti-rat IgG secondary antibody conjugated to Alexa 488) was added, and sections were incubated for 2 h at room temperature. Regularly, some sections were treated without the primary antibodies to confirm specificity. Nuclei were stained with Hoechst (1 µg/mL; Sigma Chemical Co.), mounted with fluorescent mounting medium and observed under an epifluorescence microscope (Nikon Eclipse 200). Comparative digital images from different samples were grabbed using identical time exposition, brightness and contrast settings. Neutrophils were identified analyzing Ly6G+ mark and characteristic nuclei form. Ten fields were analyzed in the whole section and the results showed total number of neutrophils in ten fields. Neutrophils were classified according to the area of the tissue in which the cells were located: in the epithelium, in the interstitial tissue or in the lumen of blood vessels.

Statistical analysis

Results were analyzed by one or two-way ANOVA in a completely randomized design as appropriate. Comparisons were made by Tukey's or DGC test (Rienzo *et al.* 2002). Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene tests, respectively. In the case of data that did not meet the assumptions, normal scores transformation was applied and continued in the same way as with real data. Statistical analysis was performed using a randomized block design when necessary. Data were expressed as mean ± s.e.m. Differences were considered significant when P was less than 0.05. When two-way ANOVA was used, significance of the interaction between the two factors (housing condition and treatment) was tested first. If the interactive effect was not statistically significant ($P > 0.05$), the main effects were evaluated. All statistical analyses were performed using the statistical program Infostat (FCA, University of Córdoba, Argentina). The approximate sample size was calculated

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

a priori for all the determinations using the statistical software Infostat, as required by the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine (University of Buenos Aires). This program allows to calculate the minimum sample size to detect the difference between group means specified by the user taking into account the number of treatments, the variance (obtained from bibliography and previous laboratory experiments), the level of significance (0.05) and the minimum difference to be detected, associated with power values, taking into account that the latter are always greater than 80%.The incidence of preterm birth was analyzed by making an arrangement of the data in contingency tables T. The chi-square test was used to analyze the hypothesis of independence between the administration of LPS and the occurrence of preterm birth. When the independence hypothesis was rejected with a P value <0.05, it was concluded that there were significant differences in the percentages of PTB between the groups.

Results

Enriched environment exposure reduces body weight gain in non-pregnant mice while lowers cholesterol and triglycerides serum levels in pregnant animals

We began evaluating physiological parameters, such as body weight gain in virgin females mice housed in EE cages or under control conditions (Fig. 1A and B). We observed that females housed in EE cages presented a reduced rate of body weight gain compared to CE exposed mice (Fig. 2A). However, these differences disappeared once females became pregnant, as body weight was similar in EE and CE pregnant animals on day 15 of pregnancy (Fig. 2B). We evaluated the adipose tissue weight on those pregnant females and found that in day 15 of pregnancy all the adipose deposits evaluated were higher in the EE exposed mice when compared to controls (Fig. 2C, D and F), suggesting that they gain more weight because of the adipose tissue. Next, we wondered whether EE exposition might modulate maternal metabolism during pregnancy. Indeed, oral glucose tolerance test performed on day 15 of pregnancy to EE or CE-exposed pregnant mice depicted no significant differences among the groups (Fig. 2F). However, we observed that EE exposure led to a significant reduction in cholesterol as well as triglycerides serum levels as compared to CE-control pregnant mice (Fig. 2G and H).

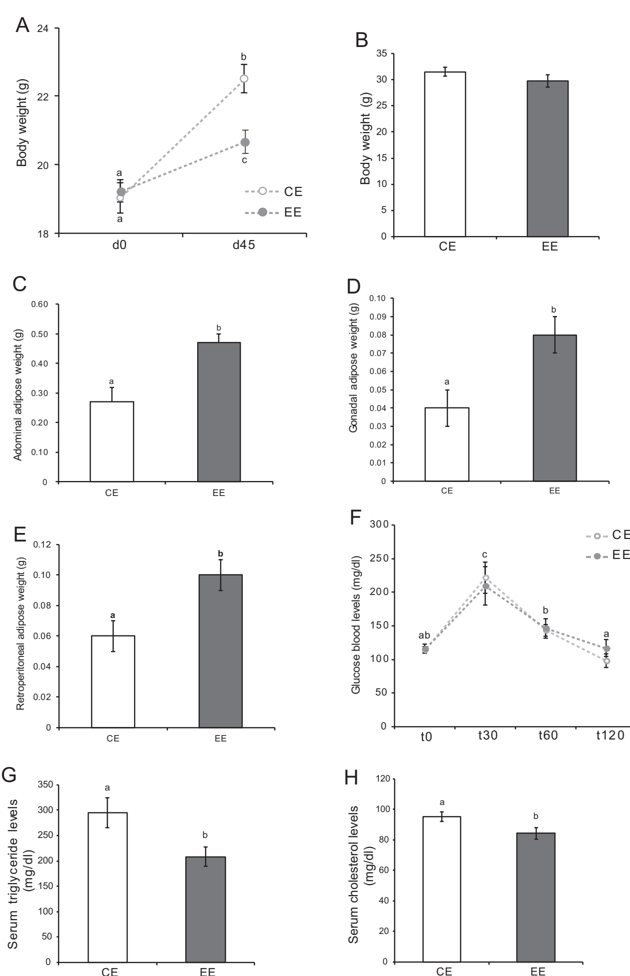

Figure 2 EE exposure effects on preconceptional body weight and metabolic parameters on day 15 of pregnancy. (A) Body weight before (day zero, d0) and after (day 45, d45) EE or CE housing. Two-way repeated measures ANOVA. $n=10$ animals per group. (B) Body weight on day 15 of pregnancy after EE or CE exposure. One-way ANOVA followed by Tukey's test, $n=16$ animals per group. Weight of abdominal (C), gonadal (D) and retroperitoneal (E) adipose tissue on day 15 of pregnancy, One-way ANOVA followed by Tukey's test, $n=6$ animals per group. Different letters indicate significant statistical differences given by an interaction effect between time and housing conditions ($P<0.05$). Oral glucose tolerance test of females on day 15 of pregnancy after EE or CE protocol. Two-way repeated measures ANOVA. $n=6$ animals per group. Different letters indicate significant statistical differences between times ($P<0.05$). Neither interaction effect, nor housing condition effect was found. Serum levels of triglycerides (D) and cholesterol (E) in pregnant females on day 15 of pregnancy. One-way ANOVA followed by Tukey's test, $n=15$ animals per group. Different letters indicate statistically significant differences ($P<0.05$).

Enriched environment exposure significantly reduces LPS-induced preterm birth rate and offspring perinatal death

Next, we wanted to evaluate whether EE exposure might exert a protective effect on inflammation-induced PTB in mice. To address this, virgin female mice were maintained on EE (or CE) cages for 6 weeks and then mated with males in regular cages. Following vaginal plug detection, animals were returned to EE or CE cages. Pregnant mice were systemically challenged on day 15 of pregnancy with LPS. Expulsion of a pup before day 18 was considered PTB (Fig. 1C). As expected, based on previous work from our laboratory using this mouse model (Cella *et al.* 2010, Domínguez Rubio *et al.* 2014,

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

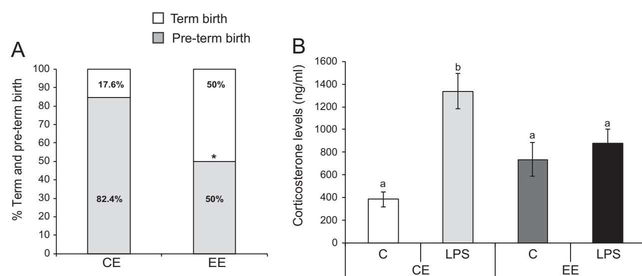

Figure 3 The exposure to an EE prevents preterm delivery and LPS-induced corticosterone serum surge. (A) Percentage of females that had PTB after i.p. administration of LPS. * indicates statistically significant differences ($P < 0.05$). Chi-squared test, $n = 16$ animals per group. (B) Corticosterone serum levels from pregnant females (day 15 of gestation) housed in EE or CE following LPS (or saline) administration. Two-way ANOVA followed by DGC test, $n =$ five animals per group. Different letters indicate statistically significant differences between groups, given by an interaction effect ($P < 0.05$). CE = Control environment, EE = Enriched Environment.

Bariani *et al.* 2015), 82.4% of control animals challenged with LPS delivered preterm pups. Remarkably, EE exposure significantly reduced LPS-induced PTB up to 40% (Fig. 3A). It is worth noting that 0% of control animals (mice treated with saline solution) either in CE or EE presented PTB. In those mothers that presented PTB (CE-LPS and EE-LPS groups) the severity was similar, meaning that fetuses died in the uterus or shortly after being born due to their prematurity, whereas the mother did not present clinical sequelae the following days to the preterm delivery.

Next, in those mothers that delivered at term, we compared the litter size, the percentage of mothers that delivered at least one dead pup (percentage of mothers that presented perinatal death) and the percentage of dead pups within the litter among control mice (both CE and EE-exposed) and LPS-treated mice (both CE and EE-exposed) that delivered at term (percentage of dead pups). This parameter includes the stillbirths and the offspring that were born alive but died between post-natal days 1 to 7. Noteworthy, 100% of control animals (both CE and EE) delivered at term, whereas only 15% of CE-exposed mice and 50% of EE-exposed mice delivered at term after LPS treatment (Fig. 3A). As shown in Table 2, the litter size was similar in EE and CE animals. However, CE mice depicted significantly higher percentages of mothers that presented perinatal death and a higher percentage of dead pups upon LPS challenge when compared to EE mice (Table 2).

Exposure to an enriched environment prevents LPS-induced increase in corticosterone serum levels

In an effort to understand the physiological meaning of LPS-induced PTB protection mediated by EE exposure and because inflammatory response is associated with stress mediators release (Gądek-Michalska & Bugajski 2004, Vakharia & Hinson 2005, Mohn *et al.* 2011, Wang *et al.* 2017), we evaluated the concentration of corticosterone in the serum of LPS-challenged EE and CE control mice. As shown in Fig. 3B, mice that were exposed to an enriched environment (EE) condition showed significantly lower levels of serum corticosterone surge following an LPS challenge when compared to animals in CE (Fig. 3B).

Enriched environment exposure lowers the expression of TLR4 and CD14 in the uterus of LPS-challenged mice

It is well known that activation of pattern recognition receptors, specifically toll like receptors (TLRs), by bacteria or bacteria components such as LPS promotes, in reproductive tissues during pregnancy, the initiation of molecular events leading to delivery. We therefore evaluated the expression of TLR4 and its co-activator protein CD14 in reproductive tissues and observed that both, TLR4 and CD14 protein levels were significantly lower in uterine tissue from EE-exposed mice compared to CE control animals. Even though it did not reach statistically significance, we observed a trend to decrease in both proteins in cervix from EE exposed mice when compared to CE ones (Fig. 4).

Enriched environment exposure induces molecular changes in uterus and cervix that preclude LPS-induced PTB

We have previously demonstrated that a large production of NO and PGs upon an LPS challenge precedes the onset of PTB (Cella *et al.* 2010, Domínguez Rubio *et al.* 2014). Here, albeit we did not find differences in the protein expression levels of iNOS and cyclooxygenase 2

Table 2 Comparison of the litter size, percentage of mothers that delivered at least one dead pup (percentage mothers that presented perinatal death) and percentage of dead pups within the litter among control mice (both CE and EE-exposed) and LPS-treated mice (both CE and EE-exposed) that delivered at term (% of death pups).

	Littersize	% of mothers that presented perinatal death	% deadpups
CE			
C	7 ± 1	33.3	24.3
LPS	7 ± 1	66.7	78.1
EE			
C	9 ± 1	16.7	16.7
LPS	8 ± 1	20.0	16.7

This parameter includes the offspring born death, and the offspring that born alive but died between days 1 to 7 post-natal. Litter size was similar among groups (considering alive and dead pups). LPS increased the percentage of mothers that presented perinatal death and percentage of dead pups within the litter in CE group when compared to the EE group. $n = 5$–6 mothers per group. CE, control environment, EE, enriched environment.

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

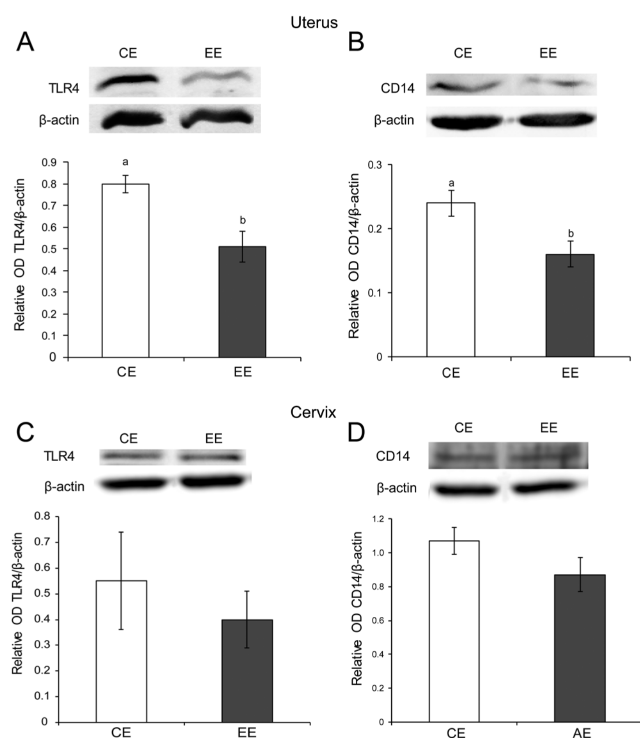

Figure 4 EE exposure modulates the expression of toll like receptor 4 (TLR4) and its co-activator protein CD14 in the uterus of pregnant mice. Protein levels of (A) TLR4 and (B) CD14 in uterus; (C) TLR4 and (D) CD14 in cervix, from pregnant females (day 15 of gestation) housed in EE or CE. One-way ANOVA followed by Tukey's test, $n=8$ animals per group. Different letters indicate statistically significant differences between housing conditions ($P<0.05$). CE, control environment, EE, enriched environment.

(COX-2) in uterine tissue, the enzymatic activity of NOS was significantly lower in EE compared to CE mice after an LPS challenge (Fig. 5E). Furthermore LPS-induced PGs increase in uterus was not observed in EE-exposed mice (Fig. 5C and D).

Similarly, we observed that even though LPS significantly increased protein expression levels of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in cervical tissue of EE and CE mice (Fig. 6A and C), NOS enzymatic activity as well as PGE2 levels were significantly lower in EE mice when compared to CE animals after a challenge with LPS (Fig. 6B and D).

Enriched environment exposure prevents LPS-induced neutrophil infiltration into the cervix as well as metalloprotease activity in this tissue

Premature cervical remodeling/ripening is believed to contribute to preterm birth. Thus, we evaluated cervical neutrophil infiltration as an early event of cervical ripening. We observed that, as expected, LPS induced an increase of neutrophil infiltration in EE as well as CE mice as compared to vehicle-treated mice. However, EE-exposed mice displayed a significantly lower number of infiltrating neutrophils when compared to CE animals (Fig. 7). Since neutrophil infiltration in the cervix was increased in LPS-treated mice, we next analyzed several chemokine mRNA levels this tissue. We found that even though LPS administration increased cervical Ccl5,Ccl3, Cxcl10 and Ccl2 mRNA expression, there were no differences between EE and CE-exposed animals (Fig. 8).

Another critical event associated to cervical ripening is the increase in metalloproteinase (MMP) activity. In this regard, we did not observe differences in Mmp8 mRNA expression in the cervix (Fig. 8). However, we did observe that EE-exposed mice showed a significantly lower MMP9 activity when compared to CE mice (Fig. 9A and B) with no differences observed regarding MMP2 activity (Fig. 9C).

Discussion

The positive effects of an enriched environment (EE) on brain morphology, chemistry and function under physiological and pathological conditions have been extensively studied (reviewed by Sale *et al.* 2014). However, little is known whether a similar strategy could be beneficial in reproductive tissues and their pathophysiological processes. This is, to the best of our knowledge, the first study showing that enriched environment exposure, a non-invasive biologically significant stimulus of the sensory and motor pathway, improves pregnancy outcome after an LPS challenge during gestation. Using an experimental model of LPS-induced maternal inflammation that mimics the main aspects of infection-induced preterm delivery in humans (Domínguez Rubio *et al.* 2014, 2017, Bariani *et al.* 2015, 2017), here we demonstrated that exposing pregnant mice to an EE resulted in metabolic and molecular changes in the mother such as reduced blood cholesterol and triglyceride levels as well as lower expression of TLR4 and CD14 in the uterus. Furthermore, EE exposure conferred protection against LPS-induced inflammation with a diminished corticosterone surge, lower uterine PGs production and both, uterine and cervical NOS activity, a reduced recruitment of neutrophil to the cervix, lower cervical MMP-9 activity and a diminished PGE2 production. Overall, these molecular and metabolic changes resulted in a significantly lower LPS-induced PTB rate when compared to pregnant mice exposed to a standard environment. Furthermore, we observed a protective effect of the maternal enrichment of the environment on the offspring health. LPS administration increased perinatal death in the CE-exposed mice, which was not observed in the EE-LPS exposed mothers. The harmful effects of the exposure to an inflammatory stimuli during uterine life has been largely reported by our group and others (Domínguez Rubio *et al.* 2014, Pujol Lopez *et al.* 2015, Bariani *et al.* 2017, Shi *et al.* 2018). In addition, maternal behavior is one

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

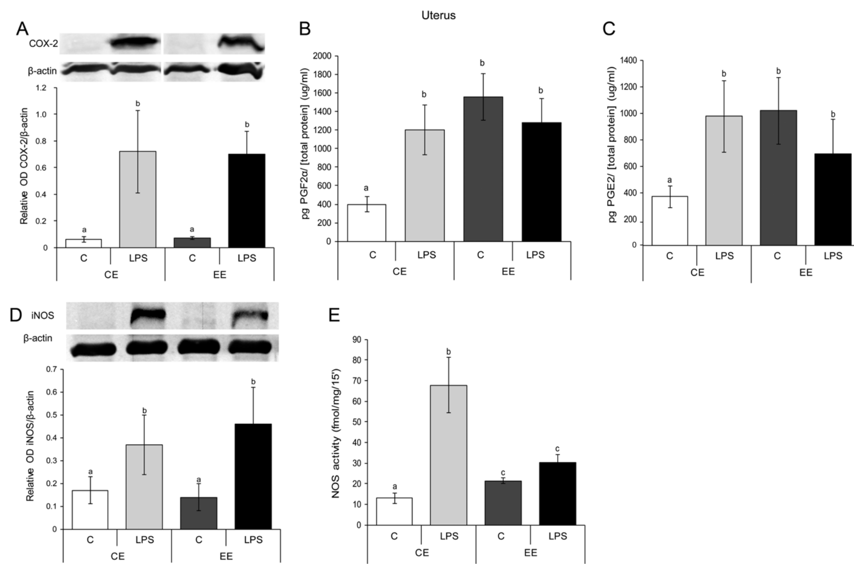

Figure 5 EE exposure has no effects on LPS-induced upregulation of uterine COX-2 and iNOS protein expression but prevents the increase of PGs synthesis and NOS activity. (A) Representative blot and quantification of COX-2 protein levels. (B) Prostaglandin F2 alpha (PGF2α) and (C) Prostaglandin E2 (PGE2) measurement; (D) Representative blot and quantification of iNOS protein levels; and (E) NOS activity. All analyses were performed in uterus from pregnant mice on day 15 of gestation housed in EE or CE following LPS (or saline) administration. Two-way ANOVA followed by Tukey's test, $n=8$ animals per group. Different letters indicate statistically significant differences ($P<0.05$). In figures A and D, there was no interaction effect of factors and the differences were given by the treatment (C vs LPS). In figures B, C and E different letters denote significant statistical differences given by an interaction effect. CE, control environment; EE, enriched environment.

of the factors involved in the survival of the offspring. Mendes-Lima *et al.* (2020) have recently demonstrated that the LPS administration during lactation decreased maternal behavior and slightly increased predatory behavior, without inducing sickness behavior. There are several factors that contribute to the increase of perinatal death induced by the LPS administration and the beneficial effects of the EE could interfere with several of them. More experiments are needed to elucidate the mechanisms underlying in these protective effects of the EE on the offspring.

The beneficial effects of EE exposure in our inflammation-elicited preterm delivery appear to be mediated by a modulation of the innate immune response. Similarly, Aranda *et al.* (2019) reported that EE housing prevented the LPS-induced damage of the optic nerve by reducing the protein expression of iNOS, COX-2 as well as the mRNA expression of IL-1β and TNFα in rats in a model of experimental optic neuritis. Comparably, Williamson and co-authors (Williamson *et al.* 2012) showed that hippocampal mRNA expression of Ccl2, Ccl3 and Cxcl2, Tnfα and Il-1β were all significantly decreased upon LPS administration in EE-housed rats when compared to standard housed animals (Williamson *et al.* 2012). These reports, together with our results, suggest that EE exerts anti-inflammatory effects and contributes to an immunoprotective status. Although the exact mechanisms by which EE protects from endotoxin-induced inflammation remain to be fully explored, we provided evidence here that the downregulation of the LPS-binding receptors TLR4 and CD14 in the uterus could account at least partially for this protective effect. Interestingly, it has been recently reported that EE-exposed mice showed an improved capacity to clear systemic microbial infection in a

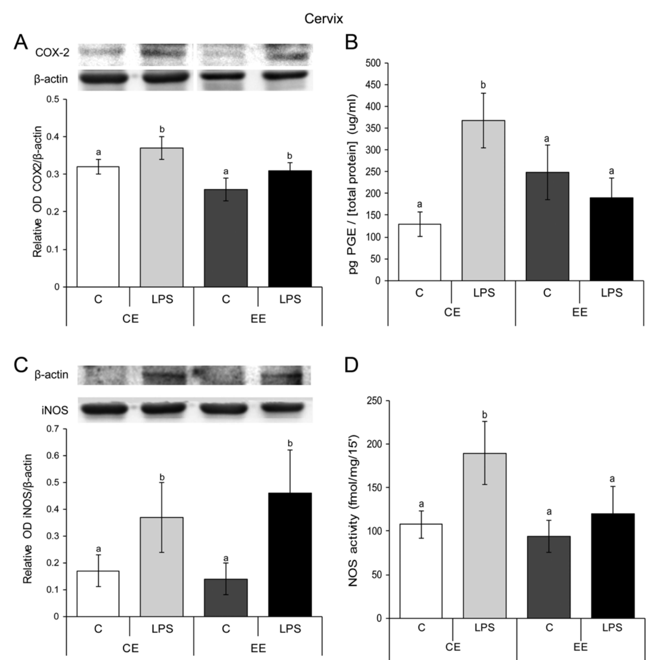

Figure 6 EE exposure has no effects on LPS-induced upregulation of cervical COX-2 and iNOS protein expression but prevents the increase of PGE2 synthesis and NOS activity. (A) Representative blot and quantification of COX-2 protein levels; (B) prostaglandin E2 (PGE2) measurement; (C) representative blot and quantification of iNOS protein levels; and (D) NOS activity. All analyses were performed in the cervix from pregnant mice on day 15 of gestation housed in EE or CE and following LPS (or saline) administration. Two-way ANOVA followed by Tukey's test, $n=8$ per group. In figures A and C, there was not interaction effect and different letters indicate statistically significant differences given by the treatment (C vs LPS) ($P<0.05$). In figures B and D different letters denote significant statistical differences given by an interaction effect ($P<0.05$). CE, control environment; EE, enriched environment.

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

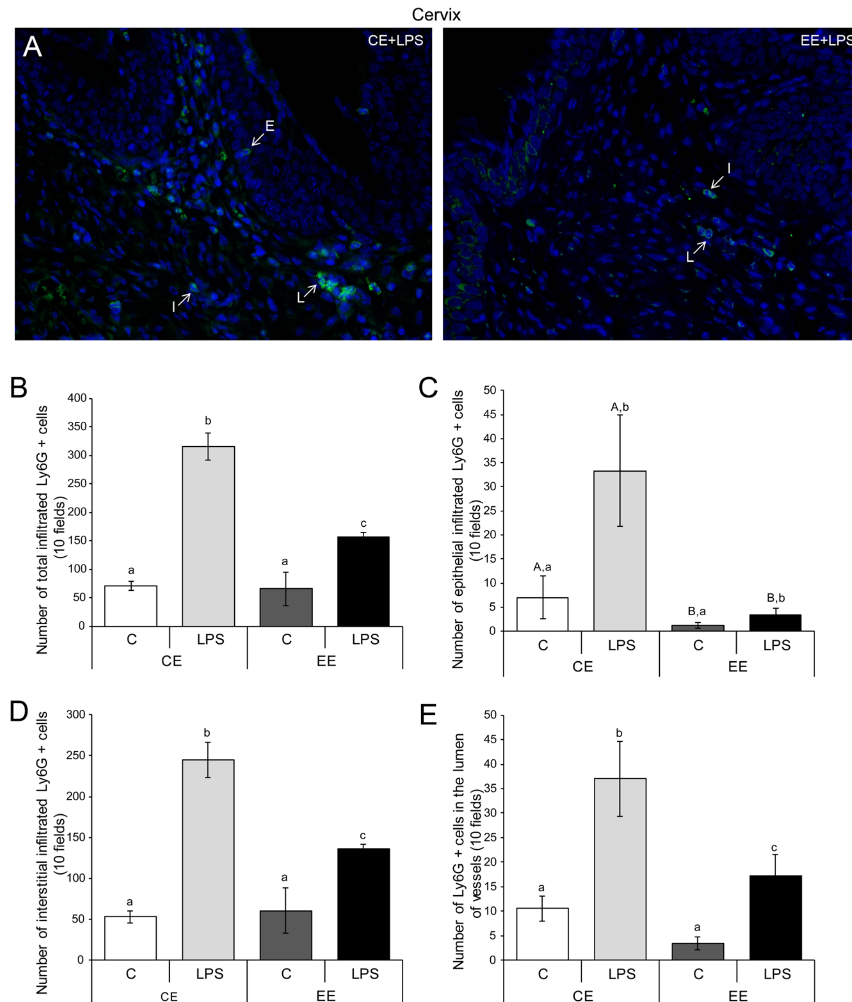

Figure 7 EE exposure partially prevents LPS-induced increase in neutrophils (Ly6G+ cells) infiltration in the cervix. (A) Cervical immunohistochemistry of Ly6G (green). White arrows show Ly6G+ cells and the letters indicate the classification used according to the area of the tissue in which the cells were located: E, epithelial; I, interstitial and L, luminar cells. Quantification of (B) total of Ly6G+ cells. (C) Epithelial Ly6G+ cells; (D) interstitial Ly6G+ cells and (E) Ly6G+ cells in the lumen of vessels (luminal cells). Two-way ANOVA followed by Tukey's test, $n = 8$. In figures B, D and E, different letters denote significant statistical differences given by an interaction effect ($P < 0.05$). In figure (C) there was no interaction effect and main effects were evaluated: capital letters show statistically significant differences between housing conditions, whereas lower case letters show statistically significant differences between treatments. CE, control environment; EE, enriched environment.

cecal ligation and puncture model of sepsis (Brod *et al.* 2017). Comparably, peritoneal macrophages obtained from EE-housed mice showed increased phagocytic activity and chemokine production *in vitro* (Otaki *et al.* 2018). Conversely, we found that EE exposure neither prevented LPS-induced increase either in COX-2 or in iNOS protein expression in the uterus. However, both endotoxin-induced increase in PGs production and NOS activity were hampered in EE exposed mice. Although these are seemingly contradictory results, protein levels of the enzymes not always correspond with its biological effect. We believe that even the fact of the LPS increase protein levels of both COX-2 and iNOS, its activity is diminished in EE exposed mice and that is related to PTB prevention. Even though vehicle-treated mice housed in EE had increased basal uterine content of PGF2α and PGE2 when compared to vehicle-treated mice housed in standard conditions, the fact that the former showed protection against LPS induction of PTB suggests that they still managed to maintain uterine quiescence. Previous results from our laboratory have shown that there is a crosstalk between NO and PGs pathways in a murine embryonic resorption model induced by LPS (Aisemberg *et al.* 2007). Here we show that a NO donor increased uterine levels of PGE_2 and $PGF_{2\alpha}$. On the other hand, selective inhibitors of COX-2 inhibit the increase on NOS activity induced by LPS in uterus. Furthermore, selective inhibitor of iNOS modulated basal amounts of PGs in uterus and decidua. Since NOS activity is hampered in the uterus of LPS-treated mice exposed to EE, this could explain the lack of LPS-induced increase of PGs synthesis despite

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

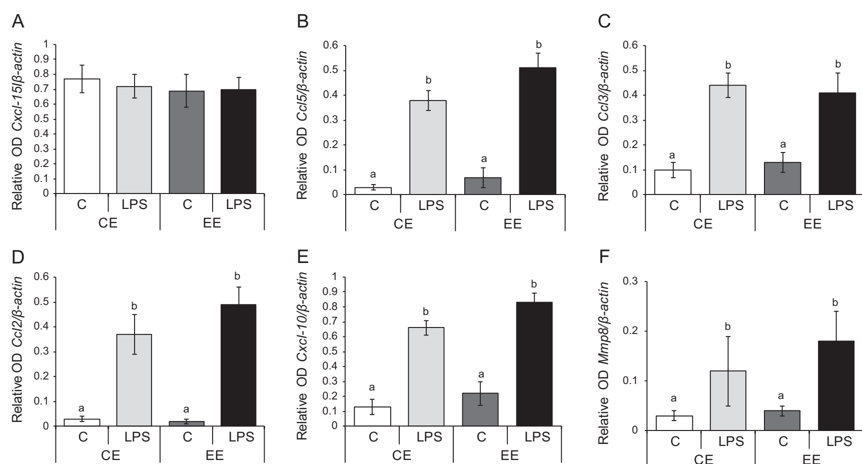

Figure 8 LPS modulates chemokine and MMP8 mRNA expression in cervix in both EE and CE mice. Relative mRNA levels of (A) Cxcl15, (B) Ccl5, (C) Ccl3, (D) Mcp-1, (E) Cxcl-10 and (F) *Mmp8* in cervix from pregnant females (15dpc) after EE or CE protocol and LPS (or SS) administration. Two-way ANOVA, followed by Tukey's test. $n = 6$ animal per group. No interaction effect was found so main effects were evaluated: different letters indicate statistically significant differences between treatments (C vs LPS) ($P < 0.05$). CE, control environment; EE, enriched environment.

the fact that uterine protein expression of COX-2 is upregulated. We propose that the EE modulates NO and PGs pathways, modulating basal levels of PGs in uterus and cervix, avoiding NOS activity increase induced by LPS and preventing the LPS-induced increase of PGs when compared to basal conditions. Nevertheless, further experiments are needed in order to elucidate the mechanisms involved in these events. A proper cervical function is essential to prevent preterm passage of the fetus through the birth canal. Inflammation has been linked to preterm delivery by inducing untimely cervical softening and ripening. In a murine model of LPS-induced inflammation, premature cervical ripening has been shown to be associated with an influx of neutrophils in the cervix, increased expression of COX-2 and PGE2 production (Holt *et al.* 2011, Timmons *et al.* 2014). Furthermore, endotoxin-induced activation of the complement system has been shown to increase MMP-9 expression and activity (Gonzalez *et al.* 2011a) which leads to an increased tissue distensibility of the cervix. In accordance to these observations, our results showed that LPS-induced maternal inflammation is associated with an increased cervical COX-2 expression and PGE2 synthesis. Moreover, we observed that EE exposure reduced the effects of LPS on cervical PGE2 production, thus contributing to the prevention of pathological cervical softening and preterm labor. We also observed an LPS-induced increase of cervical iNOS expression in our model, consistent with previous reports regarding the participation of this pro-inflammatory mediator in preterm birth (Törnblom *et al.* 2005, Nold *et al.* 2018). Similarly to previous reports (Aranda *et al.* 2019), we observed that EE exposure prevented LPS induction of cervical NOS activity in our model. Although the molecular mechanisms by which nitric oxide contributes to cervical ripening remain unclear, it can be argue that as it is a potent vasodilator agent, it might favor the invasion of macrophages and neutrophils into the cervix, with subsequent production of inflammatory mediators and metalloproteases activators capable of digesting the extracellular matrix (Gonzalez *et al.* 2011*a*, *b*). In this sense, it has been shown that LPS-mediated cervical ripening is associated with an increased neutrophil chemotaxis (Holt *et al.* 2011, Nallasamy *et al.* 2018). In agreement with these reports, we found that different chemokines mRNA levels were increased in the cervix of LPS-treated pregnant mice, resulting in an increased neutrophil infiltration that was partially prevented in EE exposed group. Although LPS effect on mRNA expression was similar in both CE and EE groups, we do not know how the protein production is and its biological effect, but we observed a diminished neutrophil recruitment in EE-LPS-treated group when compared to CE-LPS. However, the final biological significance of this invasion remains to be elucidated, since the depletion of neutrophils in LPS-treated mice is unable to prevent LPS-induced preterm birth (Rinaldi *et al.* 2014, Filipovich *et al.* 2015). LPS-induced invading macrophages may play an important role in the secretion of MMPs. Particularly; it has been shown that MMP-9 is involved in the premature cervical remodeling observed in LPS-treated mice (Gonzalez *et al.* 2011*b*) since it digests not only gelatin but also collagen I, III and IV (Bigg *et al.* 2007). Similarly, we observed that LPS induced an increase in cervical MMP-9 but not MMP-2 activity, which was prevented in mice housed in an EE. Taken together, our results suggest that EE exposure prevent the deleterious effects of LPS on cervical function, therefore preventing the endotoxin-induced PTB.

The majority of the studies on EE and prenatal stress have focused on the prevention of the negative impact on fetal brain development and long-lasting effects on the offspring (Charil *et al.* 2010, Sale *et al.* 2014). However, little attention has been given to the effects of EE on pregnancy outcome and maternal health. Our results point toward preconceptionally EE exposure having beneficial effects on maternal well-being and metabolism during pregnancy. Thus, pregnant mice exposed to EE showed lower blood triglyceride and cholesterol levels and a diminished corticosterone

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

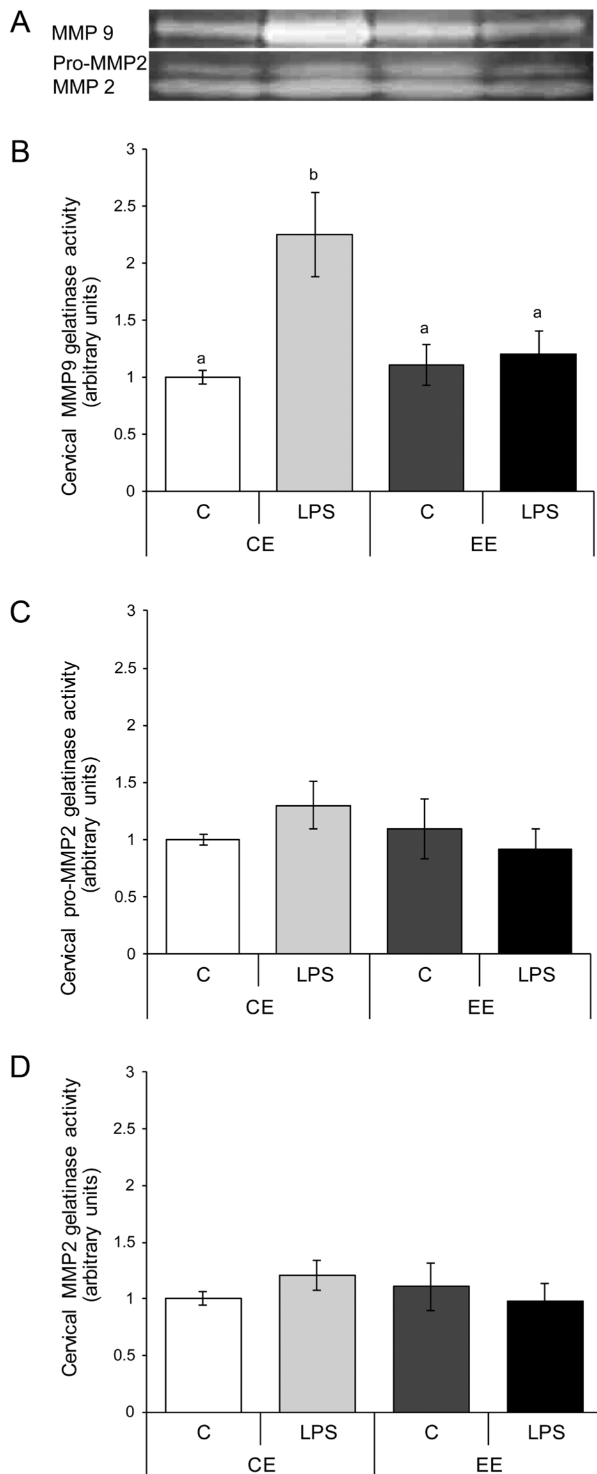

Figure 9 EE exposure prevents LPS-induced increase in cervical MMP-9 gelatinase activity. Cervical metalloproteinase 2 (MMP-2) and 9 (MMP-9) activity. (A) Representative image of a gelatinase activity zymogram gel. (B) MMP9 activity, (C) pro-MMP2 activity, and (D) MMP2 activity in cervical tissue from mice on day 15 of gestation housed in EE or CE following LPS (or saline) administration. Two-way ANOVA followed by Tukey's test, $n = 6$ animals per group. Different letters indicate statistically significant differences given by an interaction effect on MMP9 gelatinase activity ($P < 0.05$).

surge response to LPS when compared to pregnant mice housed in standard conditions. It is well established that LPS administration results in the activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in increased blood levels of adrenal glucocorticoids (Beishuizen & Thijs 2003, Vakharia & Hinson 2005, Mohn *et al.* 2011). Psychoneuroimmunological stressors and/or high levels of anxiety have been associated with chronic activation of the HPA axis together with an increased incidence of PTB (Latendresse 2009, Wadhwa *et al.* 2011, Vollrath *et al.* 2016) and low birth weight (Vrekoussis *et al.* 2010, Liou *et al.* 2016) in humans. In accordance to our results, Mlynarik *et al.* (2004) reported that adult rats housed in an EE showed a blunted HPA response to a challenge with LPS. Pre- and periconceptional maternal mental health has been the focus of numerous studies with certain proposed interventions oriented toward reducing stress and anxiety, such as mild-to-moderate leisure-time physical activity (reviewed by Aune *et al.* 2017 and Wen *et al.* 2017), relaxation techniques (reviewed by Khianman *et al.* 2012), midwife-led continuity care (reviewed by Sandall *et al.* 2016), and others (Kieffer *et al.* 2013). Interestingly, an inverse association between physical activity and preterm birth has been reported since it has positive effects on pregnancy complications such as preeclampsia (Sorensen *et al.* 2003, Meher & Duley 2006), excessive gestational weight (Löf *et al.* 2008, Stuebe *et al.* 2009) and gestational diabetes (Aune *et al.* 2017). Although physical exercise is not the main purpose of our EE paradigm, the presence of one or more running wheels provided the opportunity to our mice to engage in voluntary physical activity. Therefore, we cannot rule out the possibility that some of the beneficial effects of maternal exposure to EE on pregnancy outcome could be in part due to an increased physical activity. Current data remain incapable of addressing which components of EE (motor, sensory, cognitive, or social stimulation) are responsible for the pregnancy protection against the proinflammatory environment induced by LPS-administration, but ongoing studies by our group are in progress to analyze this issue. Despite that more studies are required to define the mechanism responsible for the pregnancy protection of animals in enriched conditions; the non-invasive nature of EE makes this tool particularly worthy of further examination.

Up to now EE has been proposed as an 'endogenous pharmacotherapy' for diseases related to the nervous system, in which neural plasticity is not obtained by external administration of active substances (Sale *et al.* 2014, González Fleitas *et al.* 2019) but using the environmental stimulation to enhance the spontaneous reparative potential held by the brain and also to influence the development and dynamics of the immune response (Brod *et al.* 2017). Our results suggest, by the first time, that EE could modulate maternal physiology and prevent PTB. In this line, the further elucidation of

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

the mechanisms underlying the beneficial effects of EE could help in implementing successful interventions aimed at protecting pregnancy outcome.

Overall, our results point toward the pre- and periconceptional benefits of EE exposure on pregnancy outcome and prevention of LPS-induced PTB. Even when taking into account the limitations of our animal model of inflammation-induced PTB, our results are in agreement with the 'healthy mother effect' hypothesis that propose that the long-term effects of a healthy physical and mental lifestyle both pre- and periconceptionally exert an important role in pregnancy outcome (Guendelman *et al.* 2017). Ideally, all women seeking pregnancy should have easy access to preconceptionally counseling to manage risk factors in lifestyle and nutrition as well as stress and mental health issues. Our results contribute to the discussion on the need of designing public policies that implement programs to improve the quality of life of mothers in order to prevent complications during pregnancy.

Furthermore, we propose that the design of enrichment of the maternal environment protocol for humans is feasible and could be a non-invasive and preventive therapy for pregnancy complications averting pre-term birth occurrence and dreaded sequelae that are present in the offspring born too soon. Consulting medical specialists on the area, we propose that the protocol could include relaxing and antistressor therapies, psychological accompaniment, suggest to control the quantity and quality of working hours and practicing voluntary physical activity among others.

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.

Funding

This work was supported by Agencia Nacional para la Promoción Científica y Tecnológica (PICT 2013/0097) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2012/0061).

Author contribution statement

J A S conducted the experiments, acquired and analyzed data. J A, M L W and L J contributed to performing experiments and data acquisition. C C and F C provided reagents. F J and A M F designed the experiments and discussed data. A M F contributed financially to this project. J A S and F C wrote the manuscript.

Acknowledgments

The authors thank Anabel Cecilia Rodríguez and Maximiliano Cella for their excellent technical support. Finally, they would like to thank the animal care technicians Marcela Márquez and Daniel González, for their excellent care of the animals used in this study.

References

Aisemberg J, Vercelli C, Billi S, Ribeiro ML, Ogando D, Meiss R, McCann SM, Rettori V & Franchi AM 2007 Nitric oxide mediates prostaglandins' deleterious effect on lipopolysaccharide-triggered murine fetal resorption. *PNAS* **104** 7534–7539. (https://doi.org/10.1073/pnas.0702279104)

Aranda ML, González Fleitas MF, Dieguez HH, Milne GA, Devouassoux JD, Keller Sarmiento MI, Chianelli M, Sande PH, Dorfman D & Rosenstein RE 2019 Therapeutic benefit of environmental enrichment on optic neuritis. *Neuropharmacology* **145** 87–98. (https://doi.org/10.1016/j.neuropharm.2017.12.017)

Aune D, Schlesinger S, Henriksen T, Saugstad OD & Tonstad S 2017 Physical activity and the risk of preterm birth: a systematic review and meta-analysis of epidemiological studies. *BJOG* **124** 1816–1826. (https://doi.org/10.1111/1471-0528.14672)

Bakos J, Hlavacova N, Rajman M, Ondicova K, Koros C, Kitraki E, Steinbusch HWM & Jezova D 2009 Enriched environment influences hormonal status and hippocampal brain derived neurotrophic factor in a sex dependent manner. *Neuroscience* **164** 788–797. (https://doi.org/10.1016/j.neuroscience.2009.08.054)

Bariani MV, Domínguez Rubio AP, Cella M, Burdet J, Franchi AM & Aisemberg J 2015 Role of the endocannabinoid system in the mechanisms involved in the LPS-induced preterm labor. *Reproduction* **150** 463–472. (https://doi.org/10.1530/REP-15-0211)

Bariani MV, Correa F, Leishman E, Domínguez Rubio AP, Arias A, Stern A, Bradshaw HB & Franchi AM 2017 Resveratrol protects from lipopolysaccharide-induced inflammation in the uterus and prevents experimental preterm birth. *Molecular Human Reproduction* **23** 571–581. (https://doi.org/10.1093/molehr/gax036)

Beishuizen A & Thijs LG 2003 Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *Journal of Endotoxin Research* **9** 3–24. (https://doi.org/10.1179/096805103125001298)

Bigg HF, Rowan AD, Barker MD & Cawston TE 2007 Activity of matrix metalloproteinase-9 against native collagen types I and III. *FEBS Journal* **274** 1246–1255. (https://doi.org/10.1111/j.1742-4658.2007.05669.x)

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254. (https://doi.org/10.1006/abio.1976.9999)

Bredt DS & Snyder SH 1989 Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *PNAS* **86** 9030–9033. (https://doi.org/10.1073/pnas.86.22.9030)

Brod S, Gobbetti T, Gittens B, Ono M, Perretti M & D'Acquisto F 2017 The impact of environmental enrichment on the murine inflammatory immune response. *JCI Insight* **2** e90723. (https://doi.org/10.1172/jci.insight.90723)

Cambell WB & Ojeda SR 1987 Measurement of prostaglandins by radioimmunoassay. *Methods in Enzymology* **141** 323–341. (https://doi.org/10.1016/0076-6879(87)41080-x)

Cella M, Farina MG, Dominguez Rubio AP, Di Girolamo G, Ribeiro ML & Franchi AM 2010 Dual effect of nitric oxide on uterine prostaglandin synthesis in a murine model of preterm labour. *British Journal of Pharmacology* **161** 844–855. (https://doi.org/10.1111/j.1476-5381.2010.00911.x)

Charil A, Laplante DP, Vaillancourt C & King S 2010 Prenatal stress and brain development. *Brain Research Reviews* **65** 56–79. (https://doi.org/10.1016/j.brainresrev.2010.06.002)

Cunningham SD, Lewis JB, Shebl FM, Boyd LM, Robinson MA, Grilo SA, Lewis SM, Pruett AL & Ickovics JR 2019 Group prenatal care reduces risk of preterm birth and low birth weight: a matched cohort study. *Journal of Women's Health* **28** 17–22. (https://doi.org/10.1089/jwh.2017.6817)

Cymeryng CB, Dada LA & Podestá EJ 1998 Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *Journal of Endocrinology* **158** 197–203. (https://doi.org/10.1677/joe.0.1580197)

Deshpande CS, Rakhshani A, Nagarathna R, Ganpat TS, Kurpad A, Maskar R, Nagendra HR, Sudheer DC, Abbas R, Raghuram N *et al.*

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

2013 Yoga for high-risk pregnancy: a randomized controlled trial. *Annals of Medical and Health Sciences Research* **3** 341–344. (https://doi.org/10.4103/2141-9248.117933)

Domínguez Rubio AP, Sordelli MS, Salazar AI, Aisemberg J, Bariani MV, Cella M, Rosenstein RE & Franchi AM 2014 Melatonin prevents experimental preterm labor and increases offspring survival. *Journal of Pineal Research* **56** 154–162. (https://doi.org/10.1111/jpi.12108)

Domínguez Rubio AP, Correa F, Aisemberg J, Dorfman D, Bariani MV, Rosenstein RE, Zorrilla Zubilete M & Franchi AM 2017 Maternal administration of melatonin exerts short- and long-term neuroprotective effects on the offspring from lipopolysaccharide-treated mice. *Journal of Pineal Research* **63**. (https://doi.org/10.1111/jpi.12439)

Dorfman D, Aranda ML, González Fleitas MF, Chianelli MS, Fernandez DC, Sande PH & Rosenstein RE 2014 Environmental enrichment protects the retina from early diabetic damage in adult rats. *PLoS ONE* **9** e101829. (https://doi.org/10.1371/journal.pone.0101829)

Filipovich Y, Agrawal V, Crawford SE, Fitchev P, Qu X, Klein J & Hirsch E 2015 Depletion of polymorphonuclear leukocytes has no effect on preterm delivery in a mouse model of Escherichia coli-induced labor. *American Journal of Obstetrics and Gynecology* **213** 697.e1–697.10. (https://doi.org/10.1016/j.ajog.2015.07.025)

Gądek-Michalska A & Bugajski J 2004 Role of prostaglandins and nitric oxide in the lipopolysaccharide-induced ACTH and corticosterone response. *Journal of Physiology and Pharmacology* **55** 663–675.

Gomez-Lopez N, StLouis D, Lehr MA, Sanchez-Rodriguez EN & Arenas-Hernandez M 2014 Immune cells in term and preterm labor. *Cellular and Molecular Immunology* **11** 571–581. (https://doi.org/10.1038/cmi.2014.46)

González Fleitas MF, Aranda ML, Dieguez HH, Devouassoux JD, Chianelli MS, Dorfman D & Rosenstein RE 2019 Pre-ischemic enriched environment increases retinal resilience to acute ischemic damage in adult rats. *Experimental Eye Research* **178** 198–211. (https://doi.org/10.1016/j.exer.2018.10.007)

Gonzalez JM, Franzke CW, Yang F, Romero R & Girardi G 2011*a* Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. *American Journal of Pathology* **179** 838–849. (https://doi.org/10.1016/j.ajpath.2011.04.024)

Gonzalez JM, Dong Z, Romero R & Girardi G 2011*b* Cervical remodeling/ripening at term and preterm delivery: the same mechanism initiated by different mediators and different effector cells. *PLoS ONE* **6** e26877. (https://doi.org/10.1371/journal.pone.0026877)

Grote NK, Bridge JA, Gavin AR, Melville JL, Iyengar S & Katon WJ 2010 A meta-analysis of depression during pregnancy and the risk of preterm birth, low birth weight, and intrauterine growth restriction. *Archives of General Psychiatry* **67** 1012–1024. (https://doi.org/10.1001/archgenpsychiatry.2010.111)

Guendelman S, Broderick A, Mlo H, Gemmill A & Lindeman D 2017 Listening to communities: mixed-method study of the engagement of disadvantaged mothers and pregnant women With digital health technologies. *Journal of Medical Internet Research* **19** e240. (https://doi.org/10.2196/jmir.7736)

Hegaard HK, Pedersen BK, Nielsen BB & Damm P 2007 Leisure time physical activity during pregnancy and impact on gestational diabetes mellitus, pre-eclampsia, preterm delivery and birth weight: a review. *Acta Obstetricia et Gynecologica Scandinavica* **86** 1290–1296. (https://doi.org/10.1080/00016340701647341)

Hegaard HK, Hedegaard M, Damm P, Ottesen B, Petersson K & Henriksen TB 2008 Leisure time physical activity is associated with a reduced risk of preterm delivery. *American Journal of Obstetrics and Gynecology* **198** 180.e1–180.e5. (https://doi.org/10.1016/j.ajog.2007.08.038)

Holt R, Timmons BC, Akgul Y, Akins ML & Mahendroo M 2011 The molecular mechanisms of cervical ripening differ between term and preterm birth. *Endocrinology* **152** 1036–1046. (https://doi.org/10.1210/en.2010-1105)

Horvath G, Reglodi D, Vadasz G, Farkas J & Kiss P 2013 Exposure to enriched environment decreases neurobehavioral deficits induced by neonatal glutamate toxicity. *International Journal of Molecular Sciences* **14** 19054–19066. (https://doi.org/10.3390/ijms140919054)

Jungling A, Reglodi D, Karadi ZN, Horvath G, Farkas J, Gaszner B & Tamas A 2017 Effects of postnatal enriched environment in a model of Parkinson's disease in adult rats. *International Journal of Molecular Sciences* **18** E406. (https://doi.org/10.3390/ijms18020406)

Khianman B, Pattanittum P, Thinkhamrop J & Lumbiganon P 2012 Relaxation therapy for preventing and treating preterm labour. *Cochrane Database of Systematic Reviews* CD007426. (https://doi.org/10.1002/14651858.CD007426.pub2)

Kieffer EC, Caldwell CH, Welmerink DB, Welch KB, Sinco BR & Guzmán JR 2013 Effect of the healthy MOMs lifestyle intervention on reducing depressive symptoms among pregnant latinas. *American Journal of Community Psychology* **51** 76–89. (https://doi.org/10.1007/s10464-012-9523-9)

Langdon KD & Corbett D 2012 Improved working memory following novel combinations of physical and cognitive activity. *Neurorehabilitation and Neural Repair* **26** 523–532. (https://doi.org/10.1177/1545968311425919)

Latendresse G 2009 The interaction between chronic stress and pregnancy: preterm birth from a biobehavioral perspective. *Journal of Midwifery and Women's Health* **54** 8–17. (https://doi.org/10.1016/j.jmwh.2008.08.001)

Liou SR, Wang P & Cheng CY 2016 Effects of prenatal maternal mental distress on birth outcomes. *Women and Birth* **29** 376–380. (https://doi.org/10.1016/j.wombi.2016.03.004)

Löf M, Hilakivi-Clarke L, Sandin S & Weiderpass E 2008 Effects of pre-pregnancy physical activity and maternal BMI on gestational weight gain and birth weight. *Acta Obstetricia et Gynecologica Scandinavica* **87** 524–530. (https://doi.org/10.1080/00016340802012288)

Meher S & Duley L 2006 Exercise or other physical activity for preventing pre-eclampsia and its complications. *Cochrane Database of Systematic Reviews* CD005942. (https://doi.org/10.1002/14651858.CD005942)

Mendes-Lima T, Kirsten TB, Rodrigues PS, Sampaio ACS, Felício LF, Rocha PRDA, Reis-Silva TM, Bondan EF, Martins MFM, Queiroz-Hazarbassanov N *et al.* 2020 Prenatal LPS induces sickness behaviour and decreases maternal and predatory behaviours after an LPS challenge. *International Journal of Neuroscience* Epub. (https://doi.org/10.1080/00207454.2019.1706505)

Mlynarik M, Johansson BB & Jezova D 2004 Enriched environment influences adrenocortical response to immune challenge and glutamate receptor gene expression in rat hippocampus. *Annals of the New York Academy of Sciences* **1018** 273–280. (https://doi.org/10.1196/annals.1296.032)

Mohn CE, Fernandez-Solari J, De Laurentiis A, Bornstein SR, Ehrhart-Bornstein M & Rettori V 2011 Adrenal gland responses to lipopolysaccharide after stress and ethanol administration in male rats. *Stress* **14** 216–226. (https://doi.org/10.3109/10253890.2010.532254)

Nallasamy S, Akins M, Tetreault B, Luby-Phelps K & Mahendroo M 2018 Distinct reorganization of collagen architecture in lipopolysaccharide-mediated premature cervical remodeling. *Biology of Reproduction* **98** 63–74. (https://doi.org/10.1093/biolre/iox155)

Narendran S, Nagarathna R, Narendran V, Gunasheela S & Nagendra HRR 2005 Efficacy of yoga on pregnancy outcome. *Journal of Alternative and Complementary Medicine* **11** 237–244. (https://doi.org/10.1089/acm.2005.11.237)

Nold C, Stone J, Graham M, Trinh J, Blanchette A & Jensen T 2018 Is nitric oxide an essential mediator in cervical inflammation and preterm birth? *Journal of Maternal-Fetal and Neonatal Medicine* **31** 1735–1741. (https://doi.org/10.1080/14767058.2017.1326898)

Otaki M, Hirano T, Yamaguchi Y, Kaida K, Koshika S, Nagata K, Nishimura M, Kakinuma S, Shimada Y & Kobayashi Y 2018 Changes in the function and phenotype of resident peritoneal macrophages after housing in an enriched environment. *International Immunopharmacology* **65** 44–49. (https://doi.org/10.1016/j.intimp.2018.09.037)

Patten AR, Yau SY, Fontaine CJ, Meconi A, Wortman RC & Christie BR 2015 The benefits of exercise on structural and functional plasticity in the rodent hippocampus of different disease models. *Brain Plasticity* **1** 97–127. (https://doi.org/10.3233/BPL-150016)

Pujol Lopez Y, Steinbusch HWM, Rutten B, Kenis G, Van Den Hove DL & Myint AM 2015 Effects of subcutaneous LPS injection on gestational length and intrauterine and neonatal mortality in mice. *Neuroimmunomodulation* **22** 274–278. (https://doi.org/10.1159/000368554)

Pustovrh MC, Jawerbaum A, Capobianco E, White V, López-Costa JJ & González E 2005 Increased matrix metalloproteinases 2 and 9 in placenta of diabetic rats at midgestation. *Placenta* **26** 339–348. (https://doi.org/10.1016/j.placenta.2004.06.011)

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access

Rienzo JA, Guzman AW & Casanoves F 2002 A multiple-comparisons method based on the distribution of the root node distance of a binary tree. *Journal of Agricultural, Biological, and Environmental Statistics* **7** 129–142. (https://doi.org/10.1198/10857110260141193)

Rinaldi SF, Catalano RD, Wade J, Rossi AG & Norman JE 2014 Decidual neutrophil infiltration is not required for preterm birth in a mouse model of infection-induced preterm labor. *Journal of Immunology* **192** 2315–2325. (https://doi.org/10.4049/jimmunol.1302891)

Romero R, Mazor M, Munoz H, Gomez R, Galasso M & Sherer DM 1994 The preterm labor syndrome. *Annals of the New York Academy of Sciences* **734** 414–429. (https://doi.org/10.1111/j.1749-6632.1994.tb21771.x)

Romero R, Espinoza J, Gonçalves LF, Kusanovic JP, Friel LA & Nien JK 2006*a* Inflammation in preterm and term labour and delivery. *Seminars in Fetal and Neonatal Medicine* **11** 317–326. (https://doi.org/10.1016/j.siny.2006.05.001)

Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, Chaiworapongsa T & Mazor M 2006*b* The preterm parturition syndrome. *BJOG* **113** (Supplement 3) 17–42. (https://doi.org/10.1111/j.1471-0528.2006.01120.x)

Romero R, Dey SK & Fisher SJ 2014 Preterm labor: one syndrome, many causes. *Science* **345** 760–765. (https://doi.org/10.1126/science.1251816)

Sale A, Berardi N & Maffei L 2014 Environment and brain plasticity: towards an endogenous pharmacotherapy. *Physiological Reviews* **94** 189–234. (https://doi.org/10.1152/physrev.00036.2012)

Sandall J, Soltani H, Gates S, Shennan A & Devane D 2016 Midwife-led continuity models versus other models of care for childbearing women. *Cochrane Database of Systematic Reviews* **4** CD004667. (https://doi.org/10.1002/14651858.CD004667.pub5)

Shi Z, Vasquez-Vivar J, Luo K, Yan Y, Northington F, Mehrmohammadi M & Tan S 2018 Ascending lipopolysaccharide-induced intrauterine inflammation in near-term rabbits leading to newborn neurobehavioral deficits. *Developmental Neuroscience* **40** 534–546. (https://doi.org/10.1159/000499960)

Sorensen TK, Williams MA, Lee I-M, Dashow EE, Thompson ML & Luthy DA 2003 Recreational physical activity during pregnancy and risk of preeclampsia. *Hypertension* **41** 1273–1280. (https://doi.org/10.1161/01.HYP.0000072270.82815.91)

Stuebe AM, Oken E & Gillman MW 2009 Associations of diet and physical activity during pregnancy with risk for excessive gestational weight gain. *American Journal of Obstetrics and Gynecology* **201** 58.e1–58.e8. (https://doi.org/10.1016/j.ajog.2009.02.025)

Timmons BC, Reese J, Socrate S, Ehinger N, Paria BC, Milne GL, Akins ML, Auchus RJ, McIntire D, House M *et al.* 2014 Prostaglandins are essential for cervical ripening in LPS-mediated preterm birth but not term or antiprogestin-driven preterm ripening. *Endocrinology* **155** 287–298. (https://doi.org/10.1210/en.2013-1304)

Törnblom SA, Maul H, Klimaviciute A, Garfield RE, Byström B, Malmström A & Ekman-Ordeberg G 2005 mRNA expression and localization of bNOS, eNOS and iNOS in human cervix at preterm and term labour. *Reproductive Biology and Endocrinology* **3** 33. (https://doi.org/10.1186/1477-7827-3-33)

Vakharia K & Hinson JP 2005 Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism. *Endocrinology* **146** 1398–1402. (https://doi.org/10.1210/en.2004-0882)

Vollrath ME, Sengpiel V, Landolt MA, Jacobsson B & Latal B 2016 Is maternal trait anxiety a risk factor for late preterm and early term deliveries? *BMC Pregnancy and Childbirth* **16** 286. (https://doi.org/10.1186/s12884-016-1070-1)

Vrekoussis T, Kalantaridou SN, Mastorakos G, Zoumakis E, Makrigiannakis A, Syrrou M, Lavasidis LG, Relakis K & Chrousos GP 2010 The role of stress in female reproduction and pregnancy: an update. *Annals of the New York Academy of Sciences* **1205** 69–75. (https://doi.org/10.1111/j.1749-6632.2010.05686.x)

Wadhwa PD, Entringer S, Buss C & Lu MC 2011 The contribution of maternal stress to preterm birth: issues and considerations. *Clinics in Perinatology* **38** 351–384. (https://doi.org/10.1016/j.clp.2011.06.007)

Wang L, Luo P, Zhang F, Zhang Y, Wang X, Chang F, Zhang Y, Tang H & Xia Z 2017 Toll-like receptor 4 protects against stress-induced ulcers via regulation of glucocorticoid production in mice. *Stress* **20** 2–9. (https://doi.org/10.1080/10253890.2016.1224843)

Wen J, Xun P, Chen C, Quan M, Wang R, Liu Y & He K 2017 Non-occupational physical activity during pregnancy and the risk of preterm birth: a meta-analysis of observational and interventional studies. *Scientific Reports* **7** 44842. (https://doi.org/10.1038/srep44842)

Wi S, Lee JW, Kim M, Park CH & Cho SR 2018 An enriched environment ameliorates oxidative stress and olfactory dysfunction in Parkinson's disease with α-synucleinopathy. *Cell Transplantation* **27** 831–839. (https://doi.org/10.1177/0963689717742662)

Williamson LL, Chao A & Bilbo SD 2012 Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus. *Brain, Behavior, and Immunity* **26** 500–510. (https://doi.org/10.1016/j.bbi.2012.01.003)

Received 29 November 2019
First decision 27 December 2019
Revised manuscript received 23 January 2020
Accepted 30 January 2020

Downloaded from Bioscientifica.com at 12/14/2021 02:57:46PM
via free access