Original Article

Mesenchymal stem cells ameliorate intra-amniotic inflammation-related neonatal complications in rats

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Objective: **The aim of this study was to establish a novel rat model of neonatal complications secondary to intra-amniotic infection/inflammation in order to investigate the therapeutic efficacy of rat umbilical cord-derived mesenchymal stem cells (rUCMSCs).**

Methods: **On gestational age day (GAD) 16, approximately 0.2 μg of lipopolysaccharide (LPS) was directly injected into the amniotic cavity of a pregnant rat. Placental inflammation on GAD 20 was histologically evaluated and the cytokine (*Il-1β, Tnfa, Mcp-1, Il-6, Cxcl-1*) and prostaglandin synthesis enzyme (*Cox-1, Cox-2*) expression patterns were analyzed by quantitative real-time polymerase chain reaction. Neonatal lung and brain injuries on postnatal day (PND) 14 were assessed histologically. rUCMSCs were injected intravenously into pups to investigate their therapeutic efficacy.**

Results: **LPS significantly decreased alive-birth rates. Significant increases in inflammatory cell infiltration and up-regulation of *Mcp-1* and *Cox-2* expression were observed in the placenta. In the neonates, the areas staining positive for myelin basic protein in the brain and radial alveolar counts in the lungs were significantly reduced in the LPS group compared with the control group. rUCMSCs improved myelination and alveolarization.**

Conclusion: **Intrauterine injection of LPS causes placental inflammation along with neonatal brain and lung injuries in neonatal rats. Postnatal administration of rUCMSCs alleviates these neonatal complications.**

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Key words chorioamnionitis, periventricular leukomalacia, bronchopulmonary dysplasia, preterm birth, fetal inflammatory response syndrome

Introduction

Advances in modern neonatal intensive care have resulted in decreased perinatal mortality, although high morbidity rates due to periventricular leukomalacia (PVL) and bronchopulmonary dysplasia (BPD) are still observed[1, 2]. PVL, which is also referred to as immature cerebral white matter injury, leads to localized necrosis or a decrease in myelination[3, 4]. It causes cerebral palsy and cognitive/behavioral deficits in neonates[5]. Although ischemia/reperfusion injury is considered to be either the only or at lease most common cause of PVL, intra-amniotic infection/inflammation (diagnosed pathologically as chorioamnionitis) also plays an additional role in its etiology[6, 7]. Intra-amniotic infection/inflammation has been reported to be involved in BPD[8, 9]. BPD interferes with normal lung development processes, such as alveolarization and septation[10]. Although the normal signals required for alveolarization are not clearly understood, transgenic mice overexpressing cytokines in the lung such as tumor necrosis factor (TNF) α[11], transforming growth factor (TGF) α[12] and interleukin (IL)-6[13] exhibit problems with alveolarization. However, the etiology of neonatal complications is an intricate process, involving immaturity, fetal distress and postnatal intervention, and exposure of the fetus to intra-amniotic infection/inflammation is an important factor that may greatly impinge upon the general condition of the neonate.

When intra-amniotic infection/inflammation is suspected in pregnant women, physicians have no choice but to deliver the baby prematurely before the inflammation spreads and harms the fetus, regardless of the clinical benefit that might result from prolonging the pregnancy. Physicians often encounter difficulties in diagnosing intra-amniotic infection/inflammation when the maternal inflammatory response is minor or subclinical. This leaves the fetus exposed to a chronic inflammatory response until delivery, and complications such as PVL or BPD only become apparent postnatally. Currently, no fundamental therapy for PVL or BPD is available.

Postnatal hypoxic-ischemic animal models have often been used as models of PVL. Unilateral carotid artery ligation followed by the exposure of immature animals to hypoxia for a few hours is one such cerebral hypoxia model[14, 15]. Although various inflammation models in which brain injury was induced by the administration of Escherichia coli or lipopolysaccharide (LPS) in the antenatal or postnatal period were also used to study PVL[16], only a few animal models utilizing the neonatal condition after exposure to chronic intra-amniotic infection/inflammation exist.

Clinically, brain injury commences and then progresses as a consequence of intrauterine infection during the fetal period, with ongoing manifestations during the postnatal period. Additionally, Rees and Inder reported that fetal brain injury is more severe in the presence of maternal inflammation and infection[17]. Hence, to investigate the etiology and to develop a novel therapy as well as prophylaxis for these complications, we established an animal model in which the fetuses are exposed to extended inflammation and investigated the therapeutic efficacy of mesenchymal stem cells (MSCs), which have been reported to be effective in hypoxic-ischemic neonatal rodent models[14, 18].

MSCs are multi-potent cells with a self-renewal capacity as well as osteogenic, adipogenic, and chondrogenic differentiation *in vitro*[19]. Their anti-inflammatory, anti-apoptotic, angiogenic and immunomodulatory potentials have been reported[20]. These cells can be isolated from almost any adult tissues, including the bone marrow, adipose tissue and teeth. Since the umbilical cord is generally considered “medical waste”, MSCs can be abundantly and non-invasively isolated from the umbilical cord (UCMSCs)[21].

In this study, we successfully generated a rat intra-amniotic infection/inflammation model by the administration of a relatively low-dose of LPS into the amniotic cavity. We also demonstrated that intravenous injection of rat UCMSCs (rUCMSCs) into these neonatal rats attenuated the reduction of myelination and the deficiency in alveolarization.

Materials and methods

1)Animals

This study was approved by the Committee for Animal Experiments of Tokyo Medical and Dental University (#0130144A). Pregnant Sprague-Dawley (SD) rats were purchased from the Sankyo Labo Service (Tokyo, Japan) on gestational age day (GAD) 14. The gestational period of this animal was 22.5 days. The rats were kept in clean cages with free access to food and water.

2)Intra-amniotic infection model and tissue collection

To develop a rat intra-amniotic infection/inflammation model, we referred to previous studies on preterm births and neonatal complications[15, 22, 23]. On GAD 16, laparotomy under inhalation anesthesia (2% isoflurane in air) was

performed on the pregnant rats. The uterus was gently withdrawn from the peritoneal cavity and the number of live fetuses was counted. LPS (0.2μg, E. coli 055:B55; Sigma-Aldrich, St. Louis, MO, USA) in 20 μL saline or saline alone was injected into the individual gestational sacs of the experimental and control group animals, respectively. Then, the uterus was returned to the peritoneal cavity and the abdominal incision was closed. On GAD 20, some of the rats were anesthetized and their placentas were harvested in order to histologically analyze their inflammatory reaction. In separate experiments, other rats delivered spontaneously 6 days after LPS administration. The number of live neonates was counted one day after delivery. The alive-birth rate was calculated as follows:

Alive-birth rate = number of live pups on postnatal day (PND) 1/number of live fetuses on GAD 16.

The pups in the experimental group received 20 μL of α minimum essential medium: αMEM) intravenously for the subsequent therapeutic experiment.

3)Tissue preparation

On GAD 20, the rats that were previously injected with LPS or saline were once again anesthetized and laparotomy was performed to collect their placentas. The placentas were separated into two parts. The part that included the umbilical cord insertion site was fixed with 4% paraformaldehyde (PFA), while the other was placed in RNAlater® (Life Technologies Japan, Tokyo, Japan) for a week and stored at 80°C for further experiments. On PND 14, the pups that were born from the experimental and control group animals were anesthetized with Nembutal and perfusion fixation with 4% PFA was performed to harvest the neonatal brains and lungs. The brains and lungs were collected and immersed in 4% PFA at 4°C for several days.

4)Immunohistochemistry

The placentas, brains and lungs were embedded in paraffin blocks, and serial coronal sections of 5 μm thickness were obtained. Brain sections containing the choroid plexus and placental sections containing the umbilical cord insertion site were chosen from each individual series. After deparaffinization and rehydration, the sections were boiled in a microwave in 10 mM sodium citrate for 15 min for antigen retrieval. The sections were incubated in a solution of 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, followed by blocking with 2% horse serum solution. The brain sections were then incubated overnight with an anti-myelin basic protein (MBP) monoclonal antibody (1:500, mouse monoclonal antibody, BioLegend, San Diego, CA, USA), while the placental sections were incubated overnight with an anti-myeloperoxidase (MPO) polyclonal antibody (1:1, rabbit polyclonal antibody, Thermo Scientific, Rockford, IL, USA) in a humid chamber at 4°C, and then incubated with a biotin-conjugated IgG secondary antibody (1:200) for 30 min at room temperature. An avidin-biotinylated peroxidase complex detection system was used to enhance sensitivity. Color development of the sections was performed by using 3,3'-diaminobenzidine (DAB) and nuclear staining was performed with methyl green.

5)Placental immune cell counts

Placental sections containing the umbilical cord insertion site were stained with hematoxylin and eosin (H&E). Five random areas in the labyrinth zone in each placenta were captured using a photomicrograph (x400 magnification, Leica, Wetzlar, Germany). The number of segmented leukocytes in the maternal blood space was blindly counted. Infiltrated neutrophils were quantified by counting MPO-positive cells.

6)RNA extraction and real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA from the placentas was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The purity and concentration of the extracted RNA were measured by spectrophotometry (Gene Quant pro, GE Healthcare Japan, Tokyo, Japan). One microgram of total RNA was reverse-transcribed using a first strand cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. RT-PCR was performed using SYBR Green PCR Master Mix (Roche) and gene specific primers with a Light Cycler 480 II (Roche). Each experiment was performed in triplicate. The primers used for RT-PCR were as follows: *Il-1β* forward, 5'-CACCT CTCAAGCAGAGCACAG-3'; *Il-1β* reverse, 5'-GGGTTCCA TGGTGAAGTCAAC-3'; *Tnfa* forward, 5'-ATGTGGAACTG GCAGAGGAG-3'; *Tnfa* reverse, 5'-ACGAGCAGGAATGAG AAGAGG-3'; *Mcp-1* forward, 5'-AGCCAGATGCAGTTAAT GCCC-3'; *Mcp-1* reverse, 5'-ACACCTGCTGCTGGTGATT CTC-3'; *Il-6* forward, 5'- AGTTGCCTTCTTGGGACTGA-3'; *Il-6* reverse, 5'-CAGAATTGCCATTGCACAAC-3'; *Cox-1* forward, 5'-GCCTCGACCACTACCAATGT-3'; *Cox-1* reverse, 5'-AGGTGGCATTCACAAACTCC-3'; *Cox-2* forward, 5'-T

CAAGACAGATCAGAAGCGA-3'; *Cox-2* reverse, 5'-TACC TGAGTGTCTTTGATTG-3'; *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) forward, 5'-ACCACAGTCCATGC CATCAC-3'; and *Gapdh* reverse, 5'-TCCACCACCCTGTT GCTGTA-3'. Melting curves were assessed to evaluate the PCR specificity. For quantification, standard curves were obtained using serial dilutions of the appropriate sample. mRNA expression was normalized to that of Gapdh mRNA used as an internal control. The data are presented as the relative mRNA levels.

7)Assessment of white matter injury in the neonatal rats

In neonatal brain sections stained with MBP, the periventricular region was captured using a photomicrograph (Leica, x40 magnification) operated by an investigator who was blinded to the groups. Determination of the MBP-positive areas was performed by computer-assisted planimetry (ImageJ software, version 1.27z, National Institute of Health, Bethesda, MD, USA).

8)Assessment of pulmonary alveolar maturation in neonatal rats

The neonatal lung sections were stained with H&E. Neonatal lung images were captured under microscopy (Biozero; Keyence Co., Osaka, Japan, x32 magnification) and lung maturation was assessed using the radial alveolar count method (RAC)[24, 25]. Briefly, the number of alveoli on a radial line from the pleura to the nearest terminal bronchiole was counted in five random areas in each section.

9)Isolation and administration of rUCMSCs

rUCMSCs were isolated by using the tissue explant method[21] from the umbilical cords collected from a pregnant rat (dam) by cesarean section on GAD 20. Pups born from intra-amniotic infection/inflammation model dams were injected with approximately 100,000 cells in 20 μL αMEM (LPS+MSC group) or 20 μL αMEM alone (LPS group) intravenously on postnatal day 1 and 7. Tissue collection and assessment of periventricular white matter damage was performed as described above.

10)Statistical analysis

Statistical analysis was conducted using the JMP software program (SAS Institute Inc., NC, USA). Differences between the control and LPS group were assessed using Student's *t*-test. Evaluation of the control, LPS, and LPS+MSC group was performed by analysis of variance and Turkey-Kramer tests. *P* values <0.05 were considered statistically significant.

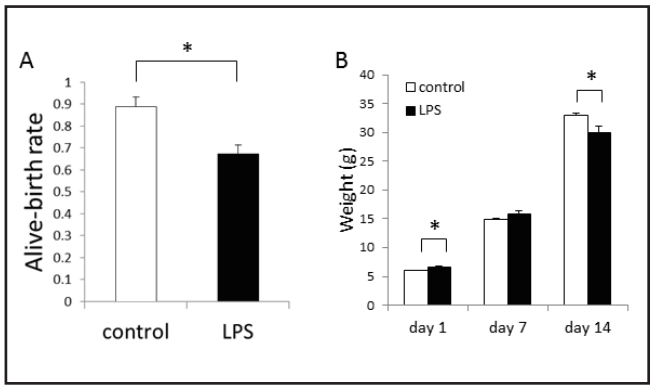

Fig. 1 Pregnancy outcome of this intra-amniotic infection/inflammation model in rat

(A)Alive-birth rate (control group n=5, LPS group n=12).
(B) Weight of the pups (day 1 LPS n=23, control n=18. Day 7 LPS n=15, control n=17. Day 14 LPS n=7, control n=12).
Mean±S.E.M, Student's *t*-test, ＊ $p<0.05$

Results

On GAD 16, LPS or saline was injected into individual gestational sacs. Preterm delivery was not observed. The alive-birth rate was significantly lower in LPS-injected animals (LPS group) compared with saline-injected animals (control group) (67±4% vs. 89±4% respectively, means±S.E.M. $p=0.006$) (e.g., Fig. 1A). The weight of the pups was significantly higher in the LPS group on PND 1 and higher in the control group on PND 14 (e.g., Fig. 1B).

First, placental inflammation was examined histologically. A significant increase in inflammatory cell infiltration was observed in the placenta of the LPS-administered animals (LPS group: 3.83±0.28/high power field (hpf) vs. control group 2.38±0.28/hpf, means±S.E.M. $p=0.002$) (e.g., Fig. 2 A-C). The interstitium of the placental labyrinth zone tended to be thickened in the LPS group. MPO-immunostaining revealed greater MPO-positive cell infiltration in the placenta of the LPS group of animals when compared to the control animals (e.g., Fig. 2 D-F).

Next, we investigated the expression of inflammatory cytokines and enzymes in the placenta. A significant up-regulation of *Mcp-1* (5.4±1.7-fold change, means±S.E.M. $p=0.006$) and *Cox-2* (8.0±2.1-fold change, means±S.E.M. $p=0.017$) expression was observed in the LPS group 4 days after administration (e.g. Fig. 2G). On the other hand,

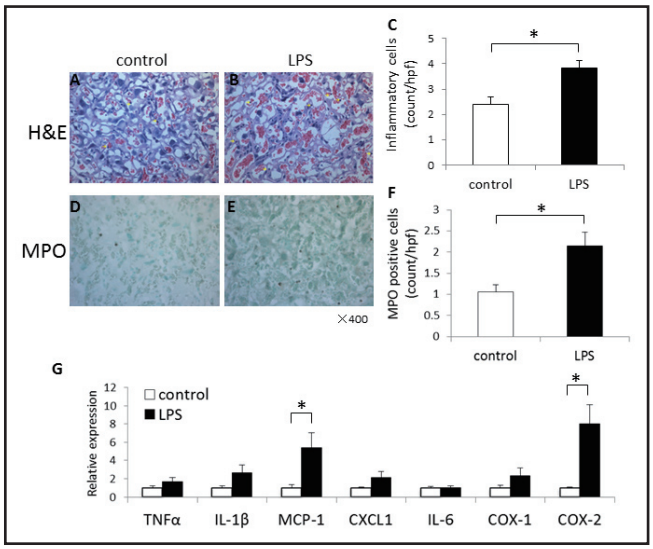

Fig. 2 Intra-amniotic injection of LPS caused chronic inflammation in placenta

(A)H&E staining of the placenta (labyrinth zone) from the LPS group animals and (B)from the control group animals.
(C)Number of infiltrated inflammatory cells (control group n=12, LPS group n=12).
(D)MPO (neutrophil marker) staining of the placenta (labyrinth zone) from LPS group animals and (E) from control group animals.
(F)Number of infiltrated MPO-positive cells (control group n=12, LPS group n=12).
(G)Inflammatory cytokine expression in the placenta (control group n=5-8, LPS group n=7-9).
Mean±SEM, Student's *t*-test, * $p<0.05$

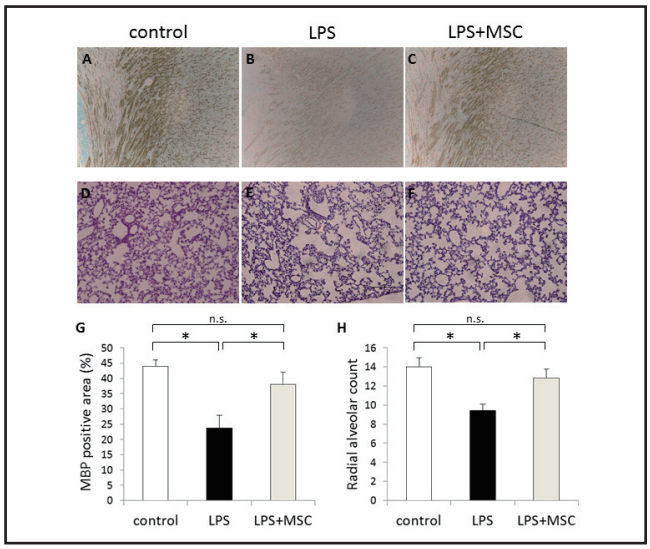

Fig. 3 The administration of rUCMSCs to neonates ameliorates both brain and lung injuries caused by intra-amniotic injection of LPS

(A)MBP immunostaining of the periventricular region in neonatal rat brains in the control group, (B)LPS group, and (C) LPS+MSC group.
(D)H&E staining of the neonatal lung in the control group, (E)LPS group, and (F) LPS+MSC group.
(G)Percentage of MBP positive area (control group n=7, LPS group n=6, LPS+MSC group n=6).
(H)Radial alveolar count (control group n=3, LPS group n=5, LPS+MSC group n=5).
Mean±SEM, Analysis of variance and Turkey-Kramer tests, *$p<0.05$

the expression of acute inflammatory cytokines such as *Il-1β, Il-6*, and Tnfα tended to be up-regulated, although not significantly, in the LPS group.

We then investigated the neonatal pathology and tested the therapeutic potential of rUCMSCs in this model. In the neonatal rat brains, the percentage of MBP-positive areas was significantly lower in the LPS group (LPS group: 23.6 ±4.25% vs. control group: 44.5±5.67%, means±S.E.M. $p<0.01$), suggesting a delay or impairment of myelination (e.g., Fig. 3A,B,G). At 7 days after the administration of rUCMSCs, the MBP-positive area significantly increased in the periventricular region of control rats as compared to that in the LPS group (e.g., Fig. 3 B,C,G). In the neonatal lungs, a significant reduction of RAC was observed in the LPS group (LPS group: 14.0±0.68/line vs. control group: 9.40±0.95/line, means±S.E.M. $p<0.05$) (e.g., Fig. 3D,E,H). A thicker interstitium was observed in the LPS group, which is a pathognomonic feature of chronic lung disease. In the LPS+MSC group, RAC was significantly increased (e.g., Fig. 3E,F,H). These results suggest that rUCMSCs ameliorate both myelination and alveolarization.

Discussion

We demonstrated that LPS administration into the amniotic cavity of pregnant rats induces placental inflammation with signs of neonatal brain and lung injury. Furthermore, the therapeutic effects of rUCMSCs against these injuries were clearly shown in this model.

The etiology of neonatal complications is multifactorial, including immaturity, intra-amniotic infection/inflammation, hypoxia/ischemia and postnatal intervention effects, and individual contribution of the various factors to disease progression remains unclear. PVL occasionally appears without any hypoxic/ischemic episode in neonates in cases that present with chorioamnionitis. We successfully generated neonatal brain and lung injuries that mimic PVL and BPD by the administration of a relatively low dose of LPS into the amniotic cavity.

We found that inflammatory cell infiltration and the expression of the protein markers for chronic inflammation significantly increased in the rat placenta on GAD 20. Furthermore, a decrease in myelination and alveolarization was found in the rats on PND 14.

In this study, we used LPS, which is wall component of gram negative bacteria. Several reports have demonstrated that intra-amniotic infection with Escherichia coli (E. coli) leads to preterm-stillbirth in animal models[26-28], while the infusion of LPS has been reported to cause both preterm-stillbirth[29] and neonatal injuries without preterm birth[30], implying the involvement of different underlying mechanisms. In this study we utilized LPS infusion to cause intra-amniotic infection/inflammation with fetal injuries in order to evaluate the brain and lung injuries as well as whether MSCs exert any effect on them. Since the cause of intrauterine infection is typically a polymicrobial infection such as Gardnerella vaginalis, bacteroides, Group B streptococcus and Escherichia coli as well as genital mycoplasmas such as Ureaplasma urealyticum and Mycoplasma hominis, further studies are needed to determine whether MSCs also exert a therapeutic in their individual cases.

LPS injected into amniotic cavity triggers a maternal immune response that leads to an increase the number of infiltrated immune cells and the expression of cytokines in the placenta[31], indirectly stimulating the fetal immune response via maternal-fetal blood circulation. Also, LPS injected into the amniotic cavity can directly trigger the fetal immune response by fetus aspiration of amniotic fluid. Fetal systemic inflammation injures the fetal brain, lung and other organs. In terms of white matter injury, it has been reported that activated microglia play a key role in injuring cells such as premyelinating oligodendrocytes and neurons via the secretion of proinflammatory cytokines and reactive oxygen species[32-34]. The clinical relation between BPD and intra-amniotic infection/inflammation has been widely accepted[35, 36]. A few reports have demonstrated the causative mechanism of intra-amniotic infection/inflammation (induced) induces BPD. Benjamin et al. showed that LPS exposure resulted in a decreased expression of fibroblast growth factor-10, a critical protein in bronchial and bronchiolar development[37]. Miller et al. found that prenatal LPS exposure increases the production of angiogenic CC chemokines, such as macrophage inflammatory protein-1α and monocyte chemoattractant protein-1, in the fetal lung, speculating that an inflammation-mediated surge in angiogenesis leads to the formation of aberrant alveolar capillaries in the lungs[38]. Further studies are required to investigate whether these mechanisms are involved in the model reported here.

As both the blood circulation and amniotic/alveolar fluid pathways might be involved in the extension of inflammation to the fetus, the dose of LPS used, which was relatively lower than those used in previous reports[15, 23], was enough to cause the brain and lung injuries in this model.

We showed the therapeutic effect of rUCMSCs on these brain and lung injuries. Therapeutic effects of MSCs have previously been reported for ischemia/hypoxia encephalopathy and hyperoxia-induced lung injury[39, 40]. However, the effect of MSCs on PVL or BPD caused by intra-amniotic infection/inflammation is not wells understood. Surprisingly, the transvenous administration of rUCMSCs on PND 1 and 7 (approximately one or two weeks after LPS exposure) improved periventricular myelination and alveolarization 2 weeks after birth. This result suggests that intra-amniotic infection/inflammation causes consecutive disorders in the brain and lung and worsens the symptoms without intervention. In addition, these results may expand the possibility of treatment for PVL and BPD to the period after birth.

Recently, Lei et al. reported that maternal administration of adipocyte-derived MSCs 15 hours prior to inducing injuries with LPS improved the preterm birth rate, neonatal encephalopathy and dyskinesia in a mouse model[41]. This study is clinically significant since the prolongation of the pregnancy period and the reduction of neonatal complications were achieved at the same time. In our study, we demonstrated the possibility of postnatal treatment for fetuses exposed to chronic inflammation. In future studies, we will examine the effects of maternal administration of rUCMSCs in this rat intra-amniotic infection/inflammation model.

In this study, we did not examine the mechanism underlying the effect of rUCMSCs treatment. Previous studies have reported anti-inflammation, anti-apoptosis, and angiogenesis potential of MSCs[20] might contribute to the improvement of symptoms following their administration, but further investigation is still ongoing.

The animal model established in this study should prove useful in the investigation of pathological conditions induced by an exposure to chronic inflammation due to the prolongation of pregnancy or other causes, as well as in the development of novel strategies for therapy or prophylaxis of neonatal complications.

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Conflict of interests

The authors have no conflict of interest to disclose.

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