Benefits of progesterone on brain immaturity and white matter injury induced by chronic hypoxia in neonatal rats

Gang Liu, MD,[a] Yichen Yan, MD,[a] Bowen Shi, MD,[a] Junrong Huang, MD,[b] Hongwei Mu, MD,[a] Cong Li, MD,[a] Huiwen Chen, MD, PhD,[a] and Zhongqun Zhu, MD, PhD[a]

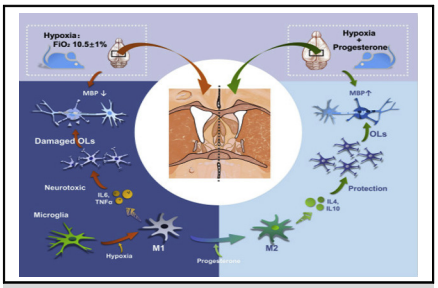
ABSTRACT

Objectives: This study aims to evaluate the protective effects of progesterone on white matter injury and brain immaturity in neonatal rats with chronic hypoxia.

Methods: Three-day old Sprague–Dawley rats were randomly divided into 3 groups: (1) control (n = 48), rats were exposed to normoxia (fraction of inspired oxygen: 21% ± 0%); (2) chronic hypoxia (n = 48), rats were exposed to hypoxia (fraction of inspired oxygen: 10.5% ± 1.0%); and (3) progesterone (n = 48), rats were exposed to hypoxia and administrated with progesterone (8 mg/kg/d). Hematoxylin–eosin staining, immunohistochemistry, real-time quantitative polymerase chain reaction, and Western blot analyses were compared on postnatal day 14 in different groups. Motor skill and coordination abilities of rats were assessed via rotation experiments.

Results: Increased brain weights ($P < .05$), narrowed ventricular sizes ($P < .01$), and rotarod experiment scores ($P < .01$) were better in the progesterone group than in the chronic hypoxia group. The number of mature oligodendrocytes and myelin basic protein expression increased in the progesterone group compared with the chronic hypoxia group ($P < .01$). The polarization of M1 microglia cells in the corpus callosum of chronic hypoxia-induced hypomyelination rats was significantly increased, whereas there were fewer M2 microglia cells. Conversely, progesterone therapy had an opposite effect and caused an increase in M2 microglia polarization versus a reduction in M1 microglia cells.

Conclusions: Progesterone could prevent white matter injury and improve brain maturation in a neonatal hypoxic rat model; this may be associated with inducing a switch from M1 to M2 in microglia. (J Thorac Cardiovasc Surg 2020;160:e55-66)



Benefits of progesterone on brain immaturity and WM injury caused by chronic hypoxia.

CENTRAL MESSAGE

Chronic hypoxia led to brain immaturity and WM injury by inducing microglial activation. The benefits of progesterone to the brain were achieved by switching the microglial activation state from M1 to M2.

PERSPECTIVE

Fetal brain immaturity and WM injury may predispose to neurodevelopmental deficits after neonatal cardiac surgery. Progesterone is beneficial to brain development and WM protection in a neonatal chronic hypoxic rat model equivalent to the third trimester in humans. Progesterone supplement before birth is expected to improve long-term neurologic outcomes in cyanotic CHDs after heart surgery.

See Commentaries on pages e67 and e69.

Neurodevelopmental deficits are common in children with cyanotic congenital heart defect (CHD) who underwent surgery in early infanthood.[1,2] Previous investigations have focused on the conduct of surgery and management of cardiopulmonary bypass to improve neurodevelopmental outcomes. However, no significant gains have been made despite advances in surgical techniques and perfusion strategies.[3]

From the [a]Department of Cardiothoracic Surgery, Heart Center, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, China; and [b]Department of Cardiothoracic Surgery, Shenzhen Children's Hospital, Shenzhen, China.

Funding for this study was supported by the Shanghai Municipal Health Commission (20194Y0301, 2017BR049), Research Project of Pudong New Area Health and Family Planning Commission (PW2017D-8), "GaofengGaoyuan" Plan by Shanghai Jiaotong University (20172027), and Shanghai Science and Technology Commission (19411964900, ZH2018ZDA24).

G.L., Y.Y., and B.S. contributed equally to this work.

Received for publication Nov 24, 2019; revisions received March 15, 2020; accepted for publication March 18, 2020; available ahead of print April 4, 2020.

Address for reprints: Zhongqun Zhu, MD, PhD, Department of Cardiothoracic Surgery, Heart Center, Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, 1678 Dongfang Rd, Shanghai, China (E-mail: zzqheart@aliyun.com).

0022-5223/$36.00

https://doi.org/10.1016/j.jtcvs.2020.03.057

Abbreviations and Acronyms

Arg1 = arginase 1
CHD = congenital heart defect
Ig = immunoglobulin
IL = interleukin
MBP = myelin basic protein
OL = oligodendrocyte
TNF-α = tumor necrosis factor alpha
WM = white matter

Recent prenatal magnetic resonance imaging studies show a progressive decline in white matter (WM) volume and WM injury, as well as smaller total brain volumes and an abnormal brain metabolism in hypoxic CHD fetuses.[4,5] Postnatal magnetic resonance imaging studies also showed that WM injury is evident in as much as 13% to 42% of infants before cardiac surgery[6-8] and that brain maturation is delayed by approximately 1 month in neonates with cyanotic severe/complex CHD relative to normative controls at birth.[9] Intrauterine or postnatal WM injury and brain immaturity can increase the vulnerability of cerebral ischemia in subsequent CHD surgery and significantly increase the occurrence of new and worsened WM injury on postoperative brain magnetic resonance imaging.[7,9] To prevent preoperative brain development delay and WM injury, and to improve the cerebral ischemia and hypoxia tolerance for these vulnerable infants, some novel neuroprotective strategies may need be initiated before birth or immediately after birth, not only at the time of cardiac surgery.[10,11]

Chronic hypoxia in utero is considered to be a main cause leading to fetal brain immaturity and WM injury in cyanotic CHD.[4,5,7-10] Progesterone is a sex hormone that has been proven to play an important role in fetal brain development, neuroprotection, and anti-inflammation[12]; however, the cellular mechanisms are not fully clear. Microglia, the resident innate immune cells in the brain, are activated in response to brain injuries. Activated microglia adopt different phenotypes, generally categorized as proinflammatory phenotype M1 and the anti-inflammatory phenotype M2. The microglial polarization and function can be regulated.[13]

In this study, a neonatal rat model of chronic hypoxia during development that is the equivalent time period to the third trimester in humans was established to produce the brain immaturity and diffuse WM injury seen in a fetus with cyanotic CHD.[14] More important, the aim of the present study is to determine the protective effects of progesterone on these brain injuries and whether the roles of progesterone are related to the regulation of microglia polarization.

MATERIALS AND METHODS

Animals

Neonatal (postnatal day 3) Sprague–Dawley rat pups of both sexes were exposed to hypoxia from days 3 to 14; these rats were used as a model to investigate WM injury and brain immaturity because they have a maturation level similar to the human fetus at the third trimester and newborn.[14,15] All animal experiments were approved by the Ethics Committee for Laboratory Animals, Shanghai Institute of Materia Medical, Chinese Academy of Sciences (Shanghai, China).

Hypoxia Experiment and Progesterone Treatment

Three-day old Sprague–Dawley rats (n = 144) were randomly divided into 3 groups: (1) control (n = 48), rats were exposed to normoxia (fraction of inspired oxygen 21% ± 0%); (2) chronic hypoxia (n = 48), rats were exposed to hypoxia (fraction of inspired oxygen 10.5% ± 1.0%)[14,16]; and (3) progesterone (n = 48), rats were exposed to hypoxia and administered progesterone 8 mg/kg/d (Sigma-Aldrich, St Louis, Mo). Rats in the chronic hypoxia and progesterone groups (n = 96) were placed with the dams (n = 8) in a plexiglass chamber in which ambient oxygen levels (fraction of inspired oxygen 10.5% ± 1%) were continuously monitored and controlled.[16] The temperature and humidity were also monitored; each of these parameters inside the chamber was similar to the normoxic condition (normoxia). All rats were raised with dams from days 3 to 14 (n = 144), with a 12 hour:12 hour/light:dark cycle at 20°C.

Histopathology

Postnatal day 14 rats were deeply anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.1 mL) and transcardially perfused with saline, and then 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 24 hours at 4°C, and cryoprotected for at least 48 hours in phosphate-buffered saline containing 30% sucrose. All brains were stored at −80°C until further processing. Brains were embedded and coronally sectioned at 5 μm per slice. After deparaffinization, hematoxylin–eosin staining was performed in the corpus callosum and lateral ventricle areas. Images were captured on a confocal microscope (Olympus Fluoview 300 Confocal Microscope; Tokyo, Japan); an image analysis system (Image J; National Institutes of Health, Bethesda, Md) was used to measure the areas of the left and right ventricles as well as the whole brain. The ratio between the total area of both ventricles and that of the whole brain was calculated as the ventricle size index.[17,18]

Immunohistochemistry

For immunofluorescence staining, brains of each group were sectioned into 20-μm slices and permeabilized with 0.5% Triton X-100 for 1 hour at room temperature, then blocked for 1 hour using 1% bovine serum albumin and 10% goat serum in phosphate-buffered saline. Afterward, sections were incubated in the following antibodies: anti-CC1 (1:500, Cell Signaling Technology, Danvers, Mass) for oligodendrocytes (OLs), anti-CD68 (Sigma, St Louis, Mo) for microglia, anti-ionized calcium-binding adapter molecule 1 (1:500, Sigma) for M1, anti-arginase 1 (Arg1; 1:500, Sigma) for M2, and anti–myelin basic protein (MBP; 1:400, Biolegend, San Diego, Calif); then, sections were diluted in blocking buffer at 4°C overnight. Next, sections were washed 3 times with phosphate-buffered saline, then incubated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (Ig)G/IgM (1:400; Invitrogen, Carlsbad, Calif), Alexa Fluor

555-conjugated goat anti-rabbit IgG (1:400; Invitrogen), and 4′,6-diamidino-2-phenylindole (1:1000; Invitrogen) for 1 hour at room temperature. The positive cells were counted under fluorescence microscopy. To assess the effects of chronic hypoxia and progesterone on microglial morphology, we counted microglia and classified Iba-1–labeled cells into 4 morphological types based on their cell shape and configuration of their cytoplasmic processes: round microglia, microglia with stout processes; microglia with thick, long processes; and microglia with thinner, more ramified processes.[19] Microglial subtypes counts were compared as percentages of the total number of classified microglia in per high-power fields. Three randomly chosen high-power fields in the corpus callosum were selected for assessment from each section. The positive cells were quantified by Image-Pro Plus (Media Cybernetics Inc, Rockville, Md), and cell counting was performed on 3 sections per animal and 16 animals per group.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from brain tissue using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and then complementary deoxyribonucleic acid was synthesized with the high-capacity complementary deoxyribonucleic acid reverse transcription kit (Applied Biosystems, Waltham, Mass). Quantitative real-time polymerase chain reaction was performed with the Step One Real Time PCR system (Applied Biosystems) and Real Q Plus 2× Master Mix Green (Ampliqon, Odense, Denmark) using the corresponding primers: interleukin (IL)-6, forward (F): TCCTCGACGG CATCTCA and reverse (R): TTTCACCAGGCAAGTCTCCT; tumor necrosis factor-alpha (TNF-α), F: ATGGCCTCCCTCTCATCAGT and R: TGGTTTGCTACGACGTGGG; Arg1, F: TCACCTGAGCTTTGATG TCG and R: CTGAAAGGAGCCCTGTCTTG; IL-10, F: CAAGCTGA GAACCAAGACCC and R: AAGATGTCAAACTCACTCATGGC; and glyceraldehyde 3-phosphate dehydrogenase, F: GTGTTTCCTCGTC CCGTAGA and R: AATCTCCACTTTGCCACTGC. The specificity of polymerase chain reaction products was confirmed by a melting curve analysis (data not shown). All reactions were performed for 40 cycles using the following temperature profiles: 98°C for 30 seconds (initial denaturation), 55°C for 30 seconds (annealing), and 72°C for 3 seconds (extension). Messenger RNA expression levels were then reported relative to the reference gene glyceraldehyde 3-phosphate dehydrogenase.

Western Blot

Brain tissues stored at −80°C (50 mg corpus callosum per brain) were minced and homogenized in ice-cold radioimmunoprecipitation assay lysis buffer, followed by centrifugation at 15,000 RCF for 15 minutes at 4°C to remove debris. Based on the concentrations measured by a bicinchoninic acid assay, equal amounts of MBP and β-actin per line were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation, and then transferred onto a polyvinylidene difluoride membrane. Target blots were blocked with 5% nonfat milk for 1 hour at room temperature and incubated overnight at 4°C using specific antibodies against MBP (1:1000, Biolegend), and β-actin (1:10,000, Invitrogen). The bands were then incubated for 1 hour at room temperature with the corresponding secondary antibodies after washing with TBST 3 times. Enhanced chemiluminescence detection reagents and a gel imaging system (Tanon Science & Technology Co, Ltd, Shanghai, China) were used to visualize the protein sheets. The sheets were analyzed with Image J software (National Institutes of Health).

Rotarod Experiment

Motor skills and coordination function were evaluated using a rotarod experiment (Columbus Instruments, Columbus, Ohio) at postnatal day 30 (n = 16 per group). Each session consisted of 3 trials on the elevated accelerating rotarod, beginning at 5 rpm/min and progressing to 20 rpm/min in 30 seconds in a trial, to measure the time on the rotarod for each rat; data for the trials were averaged (Video 1).

VIDEO 1. Rotarod experiment. Control (left), chronic hypoxia (middle), and progesterone (right). Total rotarod time is 300 seconds. Video available at: https://www.jtcvs.org/article/S0022-5223(20)30735-2/fulltext.

Statistical Analysis

Data were expressed as the mean ± the standard deviation and assessed using 1-way analysis of variance, followed by a Bonferroni post hoc test using InStat (GraphPad Software Inc, La Jolla, Calif).

RESULTS

Chronic Hypoxia Causes Brain Development Abnormalities

In neonatal Sprague–Dawley rats reared in chronic hypoxia (10.5% ± 1% oxygen) from postnatal day 3 to 14 compared with control rats (Figure 1), ventricular size enlargement ($P < .01$) and WM loss are visible in brain sections of chronic hypoxia rats in the hematoxylin–eosin staining results (Figure 2, *A* and *B*; chronic hypoxia vs control); also, brain weights were lower compared with those reared in room air ($P < .01$) (Figure 2, *C*; chronic hypoxia vs control). From the rotarod experiment results, postnatal day 30 rats in the chronic hypoxia group performed poorly compared with those in the control group (Figure 3; chronic hypoxia vs control, $P < .01$). All these results show hypoxia may lead to brain development abnormalities.

Chronic Hypoxia Reduces the Number of Mature Oligodendrocytes and Myelin Basic Protein Expression

A primary constituent of WM is MBP, a lipid-rich membrane synthesized by specialized OL cells. MBP encases and insulates axons via axonal myelination. OL progenitor cells migrate to WM for differentiation from OL progenitor cells to pre-OLs (O4 +) to immature OLs (O1 +) to mature OLs that can synthesize MBP.[20] Because hypoxia caused by complex CHD affects WM, the effects of hypoxia were examined regarding OL development and MBP expression in our animal model. On the basis of the immunohistochemistry OL results, the number of OLs was lower in chronic hypoxia brains compared with the control group ($P < .01$) (Figure 4; chronic hypoxia vs control). Decreased MBP expression was also evident in the

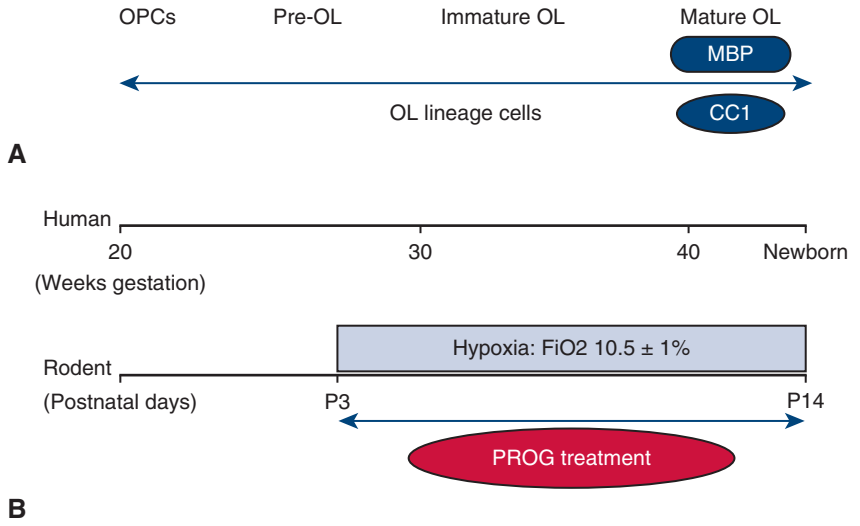


FIGURE 1. A, Developmental stages of OL lineage cells. B, Study design and equivalent time period for WM development between rats and humans. *OPC,* Oligodendrocyte progenitor cell; *OL,* oligodendrocyte; *MBP,* myelin basic protein; *CC1,* mature oligodendrocyte maker; *FiO2,* fractional inspired oxygen concentration; *PROG,* progesterone.

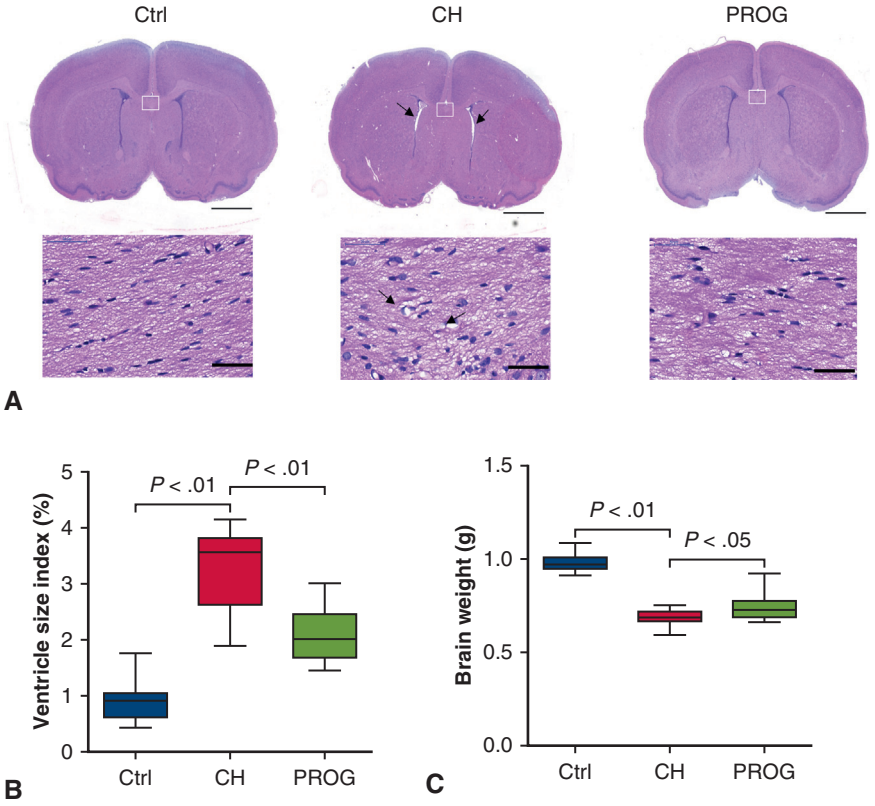


FIGURE 2. A and B, Histopathologic results: hypoxia-induced WM disordered (A, chronic hypoxia vs control) and enlarged ventricle size (B, chronic hypoxia vs control, $P < .01$); progesterone treatment repaired WM (A, progesterone vs chronic hypoxia) and blocked ventricular dilation in rat brains after chronic hypoxia exposure (B, progesterone vs chronic hypoxia, $P < .01$). Data are expressed as box-and-whisker plots. B, 5 μm/section, focus on the WM region (corpus callosum). n = 16 animals/group, 3 sections/animal scale bars = 2000 μm, 50 μm. C, Brain weight: Part of the rat brains from each group were weighed at postnatal day 14. Brain weights of rats in chronic hypoxia were lighter than those in controls, $P < .01$; brain weights of rats in the progesterone group were heavier than those in the chronic hypoxia group, $P < .05$. n = 16 animals/group. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *Ctrl,* Control; *CH,* chronic hypoxia; *PROG,* progesterone.

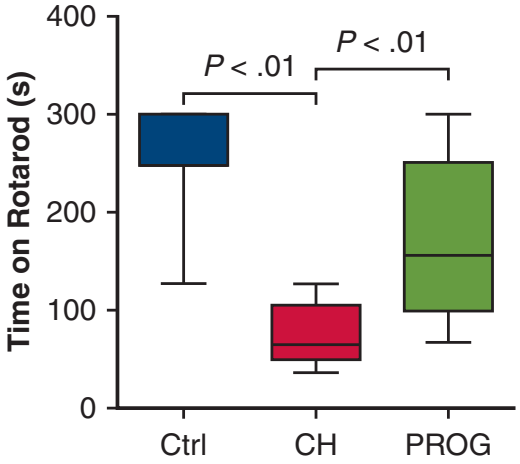


FIGURE 3. Effect of progesterone on movement and coordination. Some of the rats were raised until postnatal day 30 in normoxia for neurodevelopmental outcome analysis, rotarod test used to measure movement, and coordination of rats. A significant reduction of latency to fall indicating worse coordination was observed in chronic hypoxia groups compared with control (chronic hypoxia vs control, $P < .01$). Progesterone treatment increased time on rotarod of rats (progesterone vs chronic hypoxia, $P < .01$). N = 16 animals/group. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *Ctrl,* Control; *CH,* chronic hypoxia; *PROG,* progesterone.

immunohistochemistry and Western blot results in the chronic hypoxia group rats ($P < .01$) (Figure 4; chronic hypoxia vs control). These results indicate that hypoxia leads to WM injury.

Progesterone Improves Brain Maturity of Rats That Had Hypoxia

To determine whether progesterone may improve brain maturity and prevent WM injury, rats exposed to hypoxia were administered progesterone (8 mg/kg/d) from postnatal day 3 to 14. Brain weights were increased compared with those in the chronic hypoxia group (Figure 2, *C*; progesterone vs chronic hypoxia, $P < .05$). Hematoxylin–eosin staining analysis of the brains of progesterone group rats demonstrated ventricular narrowing and more WM (Figure 2, *A* and *B*; progesterone vs chronic hypoxia, $P < .01$). The rotarod experiment results show that postnatal day 30 progesterone group rats perform better than those in the chronic hypoxia group (Figure 3; progesterone vs chronic hypoxia, $P < .01$). These results indicate progesterone may improve the brain maturity of rats that had hypoxia.

Progesterone Promotes the Number of Mature Oligodendrocytes and Myelin Basic Protein Expression After Hypoxia

The CC1-positive immunohistochemistry results demonstrate that the number of mature OLs increased in the progesterone group compared with the chronic hypoxia group (Figure 3; progesterone vs chronic hypoxia, $P < .01$). Increased MBP expression is also evident in the Western blot results of progesterone group rats (Figure 5; progesterone vs chronic hypoxia, $P < .01$). These results signify progesterone may promote the number of OLs and MBP expression after hypoxia.

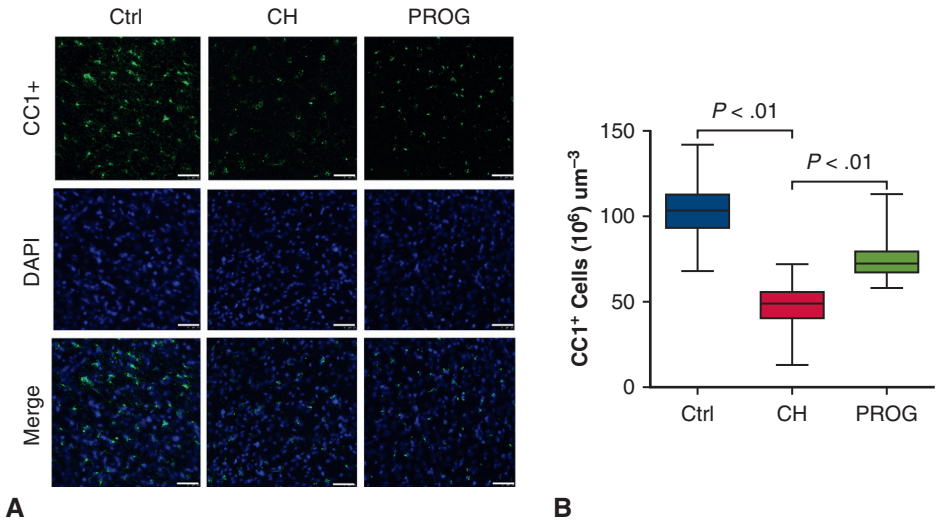


FIGURE 4. Effect of progesterone on the number of CC1 + OLs in WM. A, The immunohistochemistry OLs result. B, The cell counts of OLs. The results showed the number of OLs in WM of chronic hypoxia groups less than those of control groups (chronic hypoxia vs control, $P < .01$). Progesterone treatment increased the number of OLs (progesterone vs chronic hypoxia, $P < .01$). 4′,6-diamidino-2-phenylindole: blue OLs: green, Scale bar 25 μm. N = 16 animals per group. Three sections/animal. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *Ctrl,* Control; *CH,* chronic hypoxia; *PROG,* progesterone; *CC1,* mature oligodendrocyte maker; *DAPI*, 4′,6-diamidino-2-phenylindole.

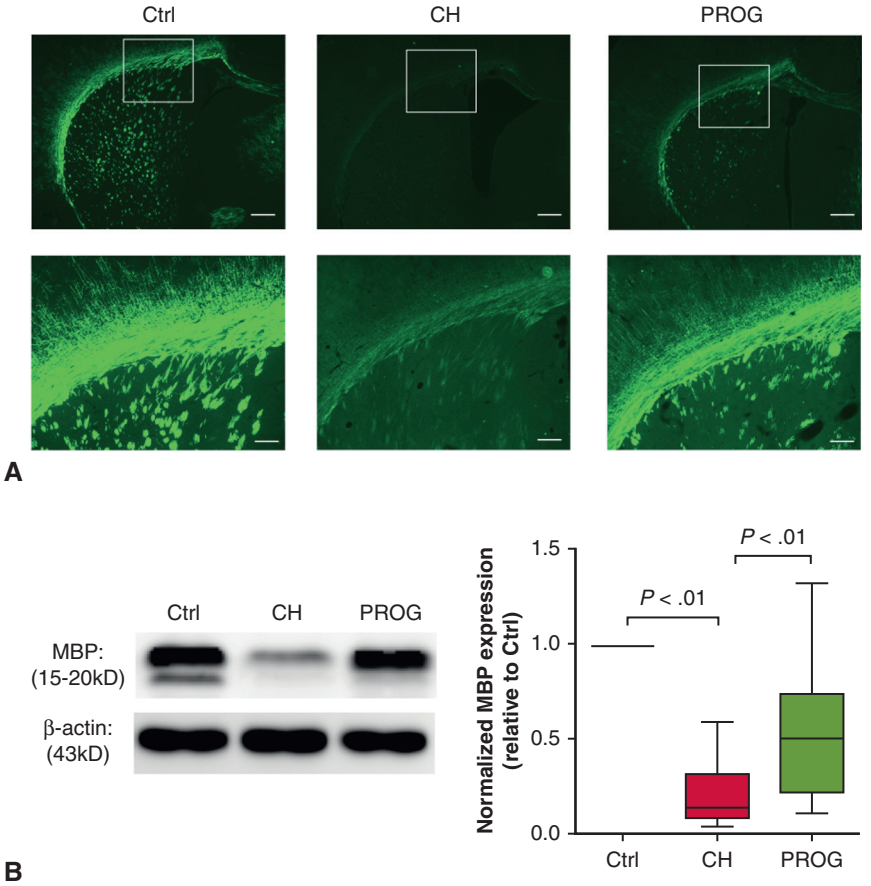


FIGURE 5. A, Effect of progesterone on expression of MBP. B, Western blot for quantitative analysis of expression of MBP. Expression of MBP were control = 1. The result showed less MBP in WM of rats exposed to hypoxia (chronic hypoxia vs control, $P < .01$); progesterone treatment increased expression of MBP (progesterone vs chronic hypoxia, $P < .01$). Scale bars 500 μm, 200 μm. N = 16 animals per group. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *Ctrl,* Control; *CH,* chronic hypoxia; *PROG,* progesterone; *MBP,* myelin basic protein.

Progesterone Induces an M1 to M2 Switch in Microglia

To explore the mechanism of progesterone in promoting brain development, increasing OL numbers, and fostering MBP expression, progesterone administration was evaluated regarding whether it can induce M1-M2 phenotype switching. Microglia polarization was first analyzed at the transcriptional level by quantitative real-time polymerase chain reaction at postnatal day 14 using M1- (IL-6, TNF-α) and M2-assosiated (Arg1, IL-10) markers (Figure 6). Chronic hypoxia rats show significantly higher expression levels of M1 microglia marker genes compared with control rats (Figure 6, *A* and *B*; chronic hypoxia vs control, $P < .01$). However, progesterone rats had a significant reduction in M1 marker expression levels compared with chronic hypoxia rats (Figure 6, *A* and *B*; progesterone vs chronic hypoxia, $P < .01$), emphasizing the anti-inflammatory role of progesterone in demyelination by suppressing M1 microglia polarization. Conversely, the expression of M2-related markers was reduced in chronic hypoxia rats compared with control rats. However, progesterone rats had significantly increased levels compared with chronic hypoxia rats (Figure 6, *C* and *D*). These results clearly indicate that progesterone promotes M2 microglia polarization and attenuates the M1 phenotype.

Microglia polarization was then studied at the translational level in the corpus callosum using immunofluorescence for Iba-1 and Arg1 (Figure 7). Consistent with the transcriptional studies, quantification of immunofluorescent images demonstrated that inducing demyelination by chronic hypoxia caused a significant increase in the number of Iba-1 positive cells ($P < .01$) compared with controls (Figure 7, *A*). However, data show that progesterone therapy resulted in a significant decrease in the number of Iba-1–positive cells ($P < .01$) compared with chronic hypoxia rats (Figure 7, *A* and *B*). In contrast, the expression of Iba-1–positive cells significantly decreased in progesterone rats in comparison with the chronic hypoxia group, whereas the expression of the M2

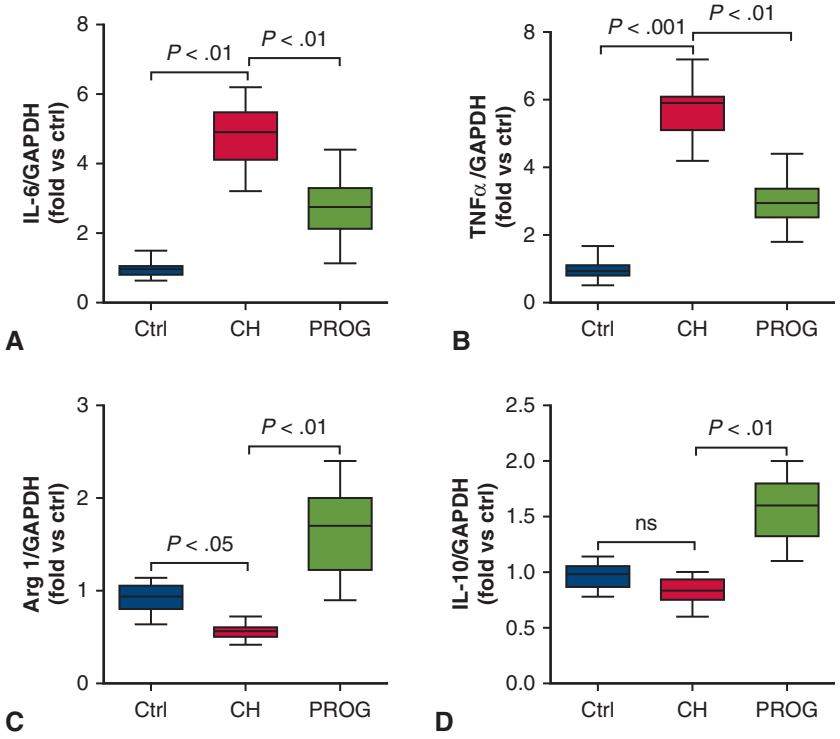


FIGURE 6. Effect of progesterone on M1 and M2 microglia–associated markers. Demyelination was induced by chronic hypoxia exposure to the rats that were then treated with progesterone. Brain samples were isolated at the end of study, at day 14. A and B, Quantitative real-time polymerase chain reaction using specific primers for the M1-microglia associated markers IL-6 and TNF-α. C and D, Quantitative real-time polymerase chain reaction analysis for the M2-microglia associated markers Arg1 and IL-10. Results indicated the upregulation of IL-6 and TNF-α messenger RNA levels in chronic hypoxia rats, which were significantly decreased by administration of progesterone. Meanwhile, results showed the downregulation of Arg1 and IL-10 messenger RNA levels in chronic hypoxia rats, which were significantly increased by administration of progesterone. GAPDH was used as an internal control for normalization. N = 16 animals per group. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *IL,* interleukin; *Ctrl,* control; *CH,* chronic hypoxia; *PROG,* progesterone; *TNF-α,* tumor necrosis factor alpha.

marker Arg1 showed the opposite trend and was significantly increased in progesterone rats compared with the chronic hypoxia group (Figure 7, *C* and *D*).

Progesterone Influences the Morphology of Microglia in Corpus Callosum

The distribution of microglial subtypes among groups in corpus callosum is shown in Figure 8. In the control group, the majority of microglia were mature and ramified (74.3% ± 3.4%, $P < .01$. In the chronic hypoxic group, microglia were largely in an activated or amoeboid state (chronic hypoxia vs control: 68.1% ± 5.3% vs 15.6% ± 2.9%, $P < .01$). In contrast, microglia with thick, long processes predominate in the progesterone treatment group (progesterone vs chronic hypoxia: 56.7% ± 6.1% vs 11.6% ± 2.8%, $P < .01$, which reflected activated or relatively immature state).

Taken together, these results show that progesterone may play a neuroprotective role via inducing an M1 to M2 switch and resulting in morphological changes in microglia.

DISCUSSION

In this study, we found that chronic hypoxia could lead to brain developmental abnormalities such as brain morphologic changes, brain weight loss, OL maturation disorder, and hypomyelination. These microstructural and cellular alterations were similar to preoperative pathological changes in neonates with cyanotic CHD.[7-9] Moreover, we found that chronic hypoxia induced the microglial activation that produced proinflammatory cytokines, while progesterone could regulate microglial M1/M2 polarization and promote microglia phenotype switch from M1 to M2 that released anti-inflammatory cytokines (Figure 9).

Chronic hypoxia in utero was considered to be an important risk factor for fetal brain immaturity and WM injury.[6,9,11,21] Abnormal fetal circulation caused by heart structural lesions led to diminished cerebral blood flow or decreased oxygen supply to the brain.[9,11,21] Hypoxia could directly cause immature OL injury, which were particularly sensitive to hypoxia during the premyelinating stage of development and led to the decrease of mature OL numbers,

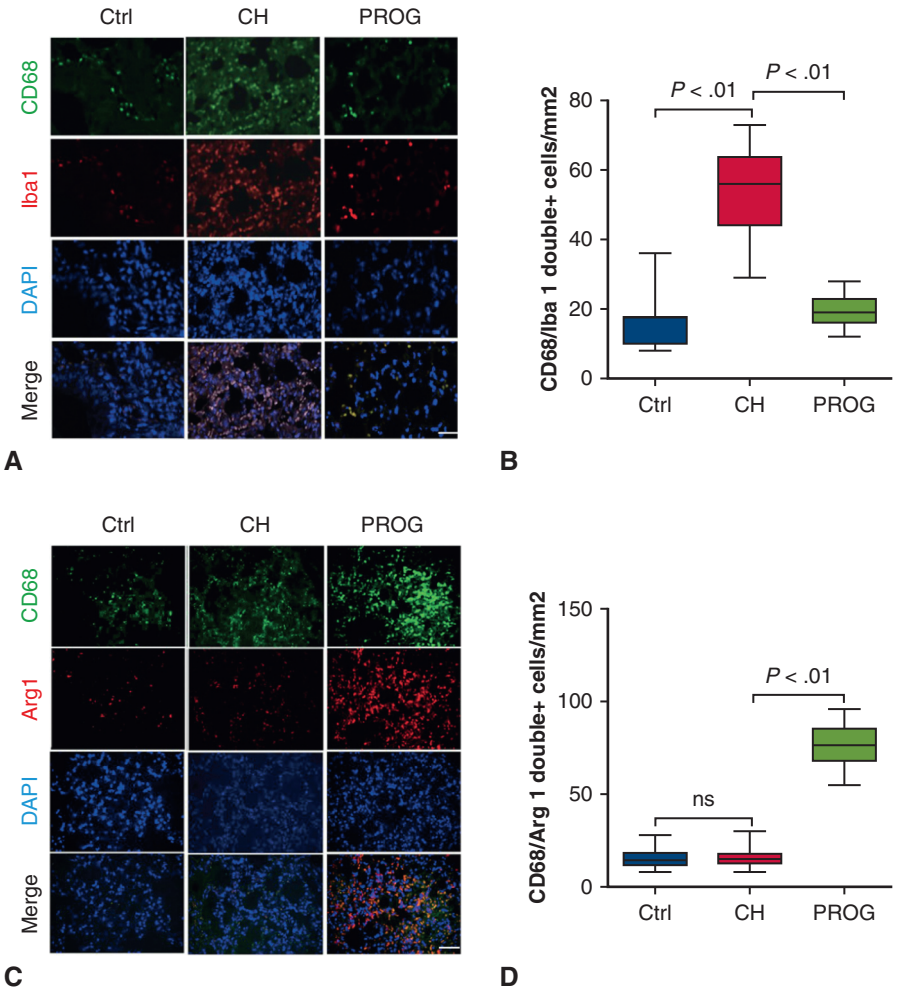


FIGURE 7. Immunohistochemical study of M1 and M2 microglia–associated markers. A and B, Representative captures in corpus callosum sections of rats' brain (nuclei, *blue*), total microglia (CD68, *green*), M1-microglia phenotype (Iba-1; ionized calcium binding adaptor molecule 1, *red*), and M2-microglia phenotype (Arg1, *red*). C and D, Quantitative analysis for the number of CD68+, Iba-1+, and Iba-1+/CD68+ cells, as M1-phenotype markers, and for the number of CD68+, Arg1+, and Arg1+/CD68+ cells, as M2-phenotype markers, obtained from immunofluorescence captures. Scale bar 25 μm. N = 16 animals/group. Three sections per animal. Data are expressed as box-and-whisker plots. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of non-outliers. *Ctrl,* Control; *CH,* chronic hypoxia; *PROG,* progesterone; *DAPI*, 4′,6-diamidino-2-phenylindole.

finally resulting in axonal hypomyelination and diffuse WM injury in CHD.[22] Agematsu and colleagues[23] confirmed that chronic hypoxia diminished the neuroprotective function of WM astrocytes and increased postoperative WM injury in the immature brain. In our previous study, microglial activation played a key role in pre-OL injury in the ex vivo neonatal rat brain slice perfusion and oxygen glucose deprivation model.[24] In this study, we also found that chronic hypoxia could induce microglial activation and initiate the inflammation process, and aggravates and exaggerates WM injury (Figures 2-5).

Microglial cells are the resident macrophages in the central nervous system. During development, microglia possess immature, round/amoeboid morphology initially and gradually progress a series of middle morphologic forms with stout processes, thick, long processes, and eventually differentiate into fully mature, thin, ramified microglia.[25] These cells have a specific ramified morphological phenotype termed "resting microglia" in the normal brain.[26] Immunocompetent macrophages act as neuropathology sensors to detect and respond swiftly to subtle changes in the brain tissues in various pathological conditions. Upon any detection of signs for brain lesions or nervous system dysfunction, microglial cells undergo a complex, multistage activation process that converts them into an amoeboid form phenotype termed "activated microglia."[26] The classic activated phenotype, M1, releases proinflammatory molecules, such IL-6 and TNF-α, and has been associated with neurotoxic activity. Conversely, the alternative activated phenotype, M2, is characterized by anti-inflammatory

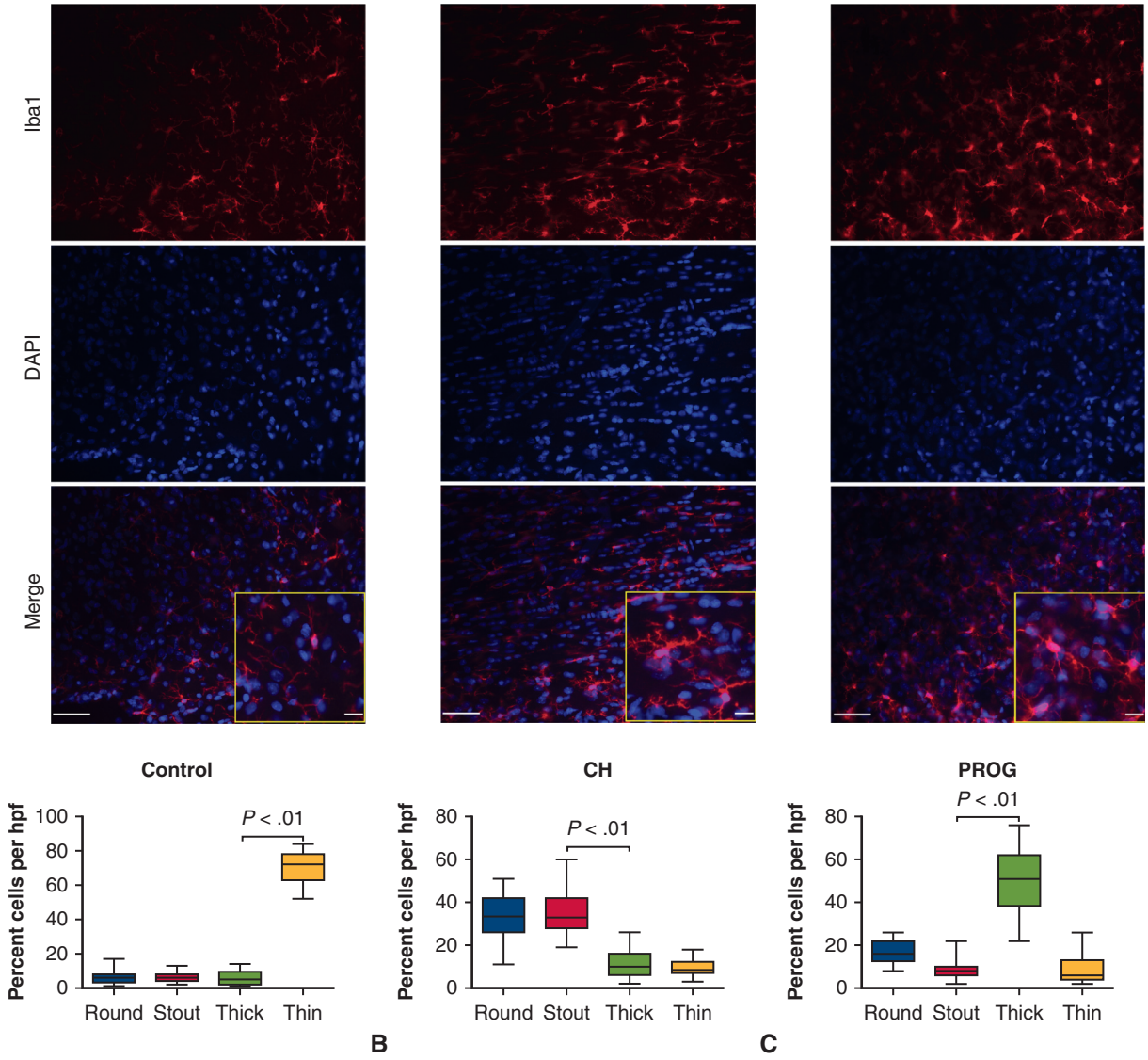


FIGURE 8. Microglia were classified into 4 primary microglial morphological states on postnatal day 14 according to cell shape and configuration of cytoplasmic processes. A, Control group: The thin microglial phenotype predominates in control rats. B, Chronic hypoxia group: The stout and round microglial phenotype predominates in chronic hypoxia rats. C, Progesterone group: The thick microglial phenotype predominates in progesterone treatment rats. Scale bar main panels, 25 μm; inset panels 12.5 μm. N = 16 animals/group. Three sections/animal. *Box-and-whisker plots* below each panel show the distribution of microglial phenotypes in per high-powered fields. The *upper* and *lower borders* of the *box* represent the upper and lower quartiles. The *middle horizontal line* represents the median. The *upper* and *lower whiskers* represent the maximum and minimum values of nonoutliers. *DAPI*, 4′,6-diamidino-2-phenylindole; *CH,* chronic hypoxia; *PROG,* progesterone.

properties that promote tissue remodeling and repair by releasing IL-4 and 10, and trophic factors such as insulin-like growth factor-1 and transforming growth factor-β.[27] Moreover, switching the polarization states of microglia through inhibiting the M1 phenotype and promoting the M2 phenotype could alleviate cerebral damage and improve neurologic function recovery.[28]

As a natural sex steroid, progesterone not only contributes to pregnancy maintenance and menstrual cycle regulation but also plays a crucial role in fetal brain development and neuroprotection.[29] Progesterone plays an active role in oligodendrogenesis, axonal myelination, MBP expression, and behavioral scores. However, the cellular mechanisms of its anti-inflammatory and neuroprotective effects[30] are unclear. Progesterone had been reported to attenuate M1 microglial polarization[31] and regulate the M1 to M2 phenotype switch.[32] A similar phenomenon was observed in this study in which progesterone therapy induced M2 microglia polarization versus a reduction in M1 microglia cells with increasing expression of anti-inflammatory cytokine,

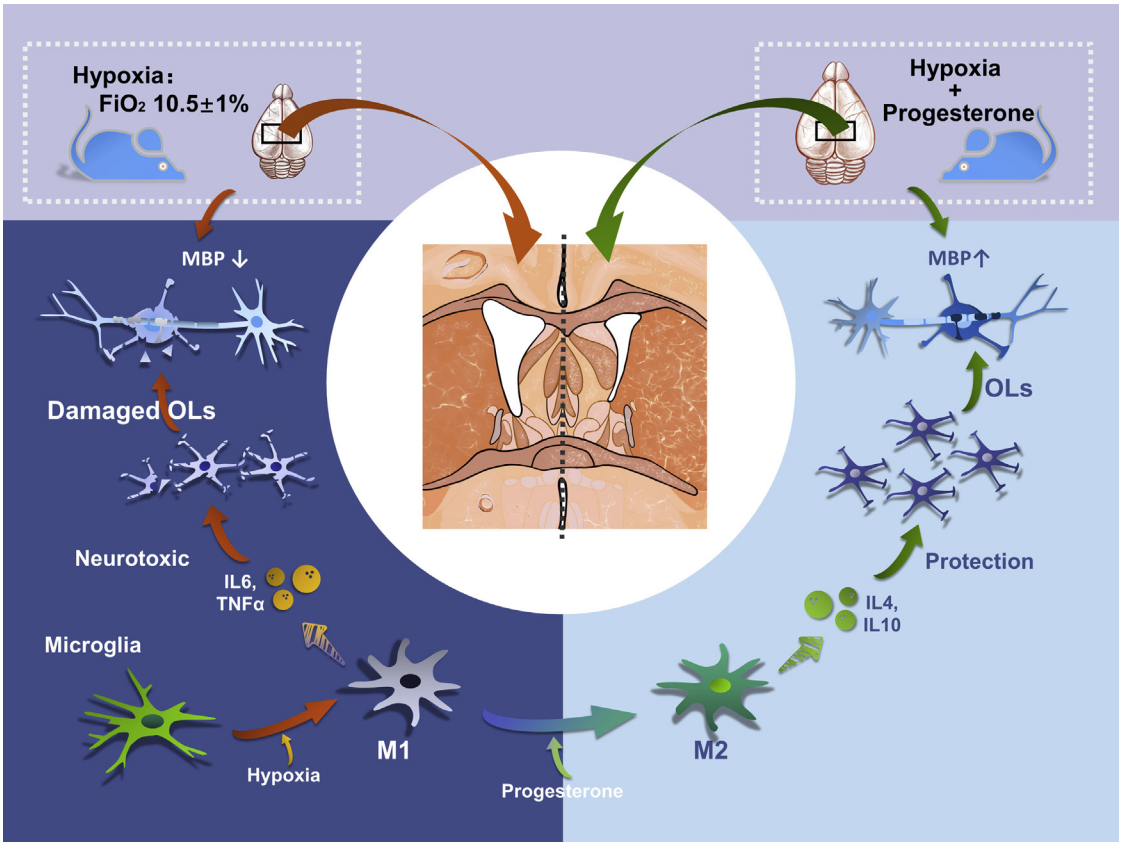


FIGURE 9. Benefits of progesterone on brain immaturity and WM injury caused by chronic hypoxia. *FiO2,* Fractional inspired oxygen concentration; *MBP,* myelin basic protein; *OL,* oligodendrocyte cell; *TNF-α,* tumor necrosis factor alpha.

accompanied by an improvement in neural behavior (Figures 3, 6, and 7). Microglial cells could express subsets of classic and nonclassic progesterone receptors in a highly dynamic way, which signified that microglia are extremely susceptible to functional modulation by progesterone and the potency of sex hormones to switch microglia from a proinflammatory M1 to neuroprotective M2 phenotype.[33]

Lawrence and colleagues[34] reported that prenatal hypoxemia altered microglial morphology with an increase in the number of round or amoeboid subtype of microglial cells, but without an increase in apoptosis or inflammatory lesion and gliosis or necrosis using an ex utero fetal sheep hypoxic perfusion model. In contrast, we developed a neonatal rat ambient hypoxic model and found chronic hypoxia could result in a round or stout subtype of microglial cells with proinflammatory cytokine expression (TNF-α, IL-6), known to be M1 functional phenotype, whereas progesterone could lead to an increase in a number of thick, ramified cytoplasmic process subtypes with an increase in anti-inflammatory cytokines (IL-10) and Arg1+ marker expression known to be an M2 functional phenotype (Figures 6 and 7). Our data showed that the morphological changes might correlate with the functional phenotype (M1/M2) switch. The differences in results between the 2 studies may be caused by inconsistent experimental conditions, such as animal selection and hypoxic modes. Moreover, we hypothesized, in part, that microglia may be primed rather than activated classically by prenatal hypoxia in Lawrence and colleagues study[34]; then there is no inflammation or any other pathologic lesions, although the morphology of glial cells is similar to that of "activated" cells.[25] However, the primed microglia when subjected to secondary insults may overproduce cytokines within the brain compared with cells that were not previously primed.[25] In a word, there is a potential benefit of changing the microglial cell phenotype with supplementation of progesterone during pregnancy to enhance brain maturation and reduce WM injury in fetus with cyanotic CHD, which could reduce the brain vulnerability to the secondary ischemic/hypoxic or inflammatory injury from cardiopulmonary bypass during neonatal cardiac surgery.

Another important cause eliciting brain hypoxia in a CHD fetus is placental dysfunction.[35,36] Goff and colleagues[35] demonstrated placental abnormalities in fetal CHD, including placental weights and vascularity. Sun and colleagues[36] used fetal magnetic resonance imaging to measure cerebral blood flow and cerebral oxygenation in fetuses with and without serious CHD, and found

reduction in umbilical vein oxygen content, 10% reduction in the oxygen saturations of ascending aortic blood, and 15% reduction in cerebral oxygen delivery in fetuses with CHD, which were associated with a 13% reduction in fetal brain volume. These results suggested that placental pathology may be an important contributor to the brain changes observed in the newborn with CHD. As is well known, normal uterine and placental vasculature development is potentially dependent on progesterone concentrations. More important, progesterone acts as a modulator of uterine vessels, decreases the resistance of spiral uterine arteries, and contributes to placental vasculogenesis and angiogenesis.[37] These processes are involved in controlling a trophoblast invasion and remodeling uterine arteries to ensure an adequate blood supply to the uterine-placental region and transporting oxygen and nutrients to a growing fetus.[37] Although the protective effects of progesterone on placental function were not demonstrated in the present study, progesterone supplementation during pregnancy may be helpful for preventing placental dysfunction and brain development delay in a fetus with CHD.[38]

Study Limitations

Although the animal model used in this study replicated WM injury and delayed brain development, this model is still not completely representative of brain hypoxic injury caused by hemodynamic abnormalities in CHD. Second, progesterone could reduce WM injury and brain immaturity secondary to a hypoxic injury. Whether this protective effect can improve the vulnerability of an immature brain to secondary ischemic/hypoxic damage or inflammation in subsequent operations needs to be verified in future experiments. Third, although the morphologic and phenotypic diversity presented among microglia in development, hypoxic injury, and response to progesterone in the present study, their transcriptional profile and epigenetic diversity are not well understood. The microglial transcriptome and epigenetic changes at single cell resolution will be helpful for characterizing and demystifying microglia in various physiologic and pathologic conditions at a molecular level in the future.[39,40] This study could not reveal the regulatory mechanisms of progesterone for the microglial phenotype switch and failed to demonstrate the effects of progesterone on placental function because of the limitations of this animal model. More in vivo studies are needed to determine the clinical application of progesterone.

CONCLUSIONS

Preoperative WM injury and brain immaturity are 2 high risks for long-term neurodevelopmental abnormality after cardiac surgery. WM injury and delayed brain development were clearly mimicked in a neonatal hypoxic rat model. Progesterone could prevent WM injury and improve brain maturation in this model; these benefits may be associated with inducing an M1 to M2 switch among microglia. Thus, intrauterine or preoperative progesterone administration may reduce long-term neurologic defects after cardiac surgery in neonates with cyanotic CHD.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

The authors thank Linyin Feng and colleagues, from the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medical, and Chinese Academy of Sciences, for providing laboratory and technical instructions.

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Key Words: congenital heart defects, microglia, neuroprotection, progesterone, white matter injury