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ORIGINAL ARTICLE

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Progesterone-mediated angiogenic activity of endothelial progenitor cell and angiogenesis in traumatic brain injury rats were antagonized by progesterone receptor antagonist

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Abstract

Objectives: Progesterone (P4) has the potential therapeutic effects for traumatic brain injury (TBI) whose recovery depended on the enhanced angiogenesis. Endothelial progenitor cell (EPC) plays an essential role in vascular biology. We previously demonstrated that P4 administration improved circulating EPC level and neurological recovery of rat with TBI. Here, we hypothesized that P4 augmented angiogenic potential of EPC and the angiogenesis-related neurorestoration after TBI through classical progesterone receptor (PR).

Materials and methods: EPC derived from rats were stimulated with graded concentrations (0, 10^{-10}, 10^{-9}, 5×10^{-9}, 10^{-8}, 10^{-7} mol/L) of P4 or 10^{-6} mol/L ulipristal acetate (UPA, a PR antagonist). Male rats were subjected to cortical impact injury and treated with (i) DMSO (dimethyl sulfoxide), (ii) P4 and (iii) P4 and UPA.

Results: It showed that P4 improved the angiogenic potential of EPC, including tube formation, adhesion, migration and vascular endothelial growth factor secretion, in a dose-dependent fashion with the maximal effect achieved at 10^{-9} mol/L P4. High concentration (10^{-7} mol/L) of P4 impaired the angiogenic potential of EPC. Notably, 10^{-6} mol/L UPA antagonized the stimulatory effects of 10^{-9} mol/L P4. After administrating P4, a significant improvement of neurological function and the restoration of the leaked blood-brain barrier were observed as well as a reduction of the brain water content. Both vessel density and expression of occludin of vessels were increased. When UPA was administered with P4, the neural restoration and angiogenesis were all reversed. Western blot showed that 10^{-9} mol/L P4 increased the content of PRA and PRB of EPC, while 10^{-7} mol/L P4 reduced the content of both PR isoforms, but there was no change found in the TBI rats.

Conclusions: It may suggest that P4-mediated angiogenic activity of EPC and angiogenesis in TBI rats were antagonized by PR antagonist.

Peng Yu, Shengjie Li and Zhifei Zhang contribute to the paper equally.

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1 | INTRODUCTION

Progesterone (P4), an endogenous sex hormone and neurosteroid, is naturally synthesized in ovary, placenta and brain.[1] Beyond the known effect on regulation of reproductive organs, P4 has potent protective properties in vascular system, which is independent of oestrogen.[2,3] In models of central nervous system diseases,[4-6] P4 exerts neuroprotective effects, which are related to the repair of blood-brain barrier (BBB).

Endothelial progenitor cell (EPC) plays a significant role in maintenance of vascular homeostasis, characterized by homing to injured sites, adhesion, migration, proliferation, secretion and differentiation of EPC to replenish damaged endothelium.[7,8] Notably, poor microenvironments can impair circulating levels, migration and proliferation ability of EPC and hinder EPC from becoming a promising intervention for vascular diseases.[9,10] Available evidence suggests that P4 augments proliferative activity of EPC derived from the peripheral blood of female subjects.[11] However, whether P4 affects angiogenic activity of EPC is still unknown and the underlying mechanism needs to be elucidated.

Traumatic brain injury (TBI) is still one of the major diseases which lead to death and disability and its recovery depend on the enhanced angiogenesis around the injured brain tissue. P4 could pass through BBB easily because of its lipid solubility. In addition, P4 has been proved to reduce brain oedema, stabilize the disrupted BBB, attenuate the apoptosis, modulate the inflammation cytokines, evacuate the toxic free radicals, stop the Ca^{2+} influx, enhance angiogenesis and promote the recovery of the neural function in TBI.[4,5,12-18] Moreover, our previous study demonstrated that P4 increased levels of circulating EPC and reinforced neurovascular repair following TBI in rats[19] which indicates that the EPC play an essential role in the neurovascular repair after TBI.

During the new vessel formation, angiogenesis includes multifarious process.[20] There are growing evidences that suggest that P4 and progesterone receptor (PR) play an important role in different kinds of tissue angiogenesis, including endometrium and tumour.[21-23] Vascular endothelial growth factor (VEGF) is the key growth factor for neovascularization, vascular remodelling and angiogenesis.[24-26] The level of VEGF mRNAs was increased significantly by P4 in the bovine granulosa cells.[27] Okada confirms that in human endometrial stromal cells, high level of P4 (100 nmol/L) could decrease CXCL12 and stradiol-induced VEGF expression.[28] We hypothesized that P4 may exert the EPC angiogenic activity by mediating the level of VEGF and PR.

Ulipristal acetate (UPA) is usually defined as a selective PR modulators.[29,30] UPA has been used as a contraceptive pill, and its safety and efficacy in treating uterine fibroids was successfully demonstrated.[29,31] UPA has also been introduced as P4 antagonist in several studies.[31-33]

2 | MATERIALS AND METHODS

2.1 | Cell culture and characterization

EPC cultivation and identification were described in our previous paper.[34] To exclude the effects of cytokines, we used the EBM2 (endothelial cell basal medium) without cytokines in all the tests.

2.2 | Tube formation assay

Briefly, 50 μL/well of chilled Matrigel (BD Biosciences, Bedford, MA, USA) was laid into ice-cold 96-well plates.[35,36] About 100 μL EPC (1×10^4 cells) in EBM-2 with P4 (Sigma-Aldrich, St. Louis, MO, USA 1568007. 0, 10^{-10}, 10^{-9}, 10^{-8} 5×10^{-9} or 10^{-7} mol/L,) or VEH (vehicle, 0.016% alcohol) was seeded in each well with solidified Matrigel and incubated for 3, 6, 12 and 24 hour. Microscopic images were captured at corresponding time points. To evaluate the effect of UPA on P4 action, cells were coincubated with 10^{-6} mol/L UPA and P4 at optimal stimulating conditions of P4 (10^{-9} mol/L). Tube formation was defined as a structure exhibiting a length four times its width.[37] Total tube numbers and tube length were calculated in five random fields of well (10×10 objective lens), six wells/group, using Wimasis WimTube software (Wimasis GmbH, Munich, Germany).

2.3 | Adhesion assay

After 6 hours of treatment by VEH or indicated concentration of P4, EPC was gently detached and collected in EBM-2. Identical cell numbers were inoculated onto fibronectin-coated plates and allowed to adhere for 30 minutes in a 37°C incubator. The non-adherent cells were then vigorously washed out and adherent cells were counted manually per field (five random fields/well, six wells/group) by an independent blinded observer. To evaluate the effect of UPA on P4 action, cells were coincubated with 10^{-6} mol/L UPA and P4 at optimal stimulating conditions of P4 (10^{-9} mol/L).

2.4 | Transwell migration assay

After 6 hours of incubation in VEH or indicated concentration of P4, EPC was harvested, resuspended (1×10^6 cells/mL) and placed in upper chamber of 24-well transwell plates (3422, Corning, NY, USA). After incubation at 37°C for 8 hours, non-migrated cells in the upper chamber were removed and cells migrating forwards low chamber were stained with crystal violet (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The stained cells were photographed and counted (five random fields/well, six wells/group) by a blinded observer. To evaluate the effect of UPA on P4 action, cells were coincubated with 10^{-6} mol/L UPA and P4 at optimal stimulating conditions of P4 (10^{-9} mol/L).

2.5 | Induction of necrosis and apoptosis

To clarify if P4 could influence the apoptosis and necrosis of EPC, annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were employed. Treated (6 hours) cells were harvested by trypsin and labelled by annexin V-FITC and PI (BD Biosciences), and then EPCs (50 000) were subjected to FACScan laser flow cytometer (FACSCalibur™; BD Biosciences).[38]

2.6 | Animals and TBI model

Male Wistar rats (250-300 g) were obtained from Military Medical Academy of China. The animals were randomly divided into three

groups: (i) TBI group: receiving the equal volume of vehicle (DMSO) of P4 fluid by intraperitoneal injection; (ii) P4 group, receiving P4 (16 mg/kg; Sigma) dissolved in DMSO by intraperitoneal injection; and (iii) P4+UPA group, receiving both P4 and UPA (3 mg/kg; Selleck Chem, Houston, TX, USA) by intraperitoneal injection.

For the controlled cortical impact model, rats were anaesthetized with chloride hydrate (3.0 mL/kg, intraperitoneal injection) and immobilized in a Kopf stereotaxic frame. A craniotomy (6 mm in diameter; Bregma −2.8, 2.5 mm lateral) was performed using a Michele trephine to expose the motor cortex. An electronically controlled cortical impact device (5 mm tip diameter; Precision Systems and Instrumentation LLC, Fairfax, VA, USA) was used to administer a 1.5 mm cortical deformation (speed 3.5 mm/s; 400 ms dwell time).[39]

2.7 | Modified Neurological Severity Score test

The modified Neurological Severity Score (mNSS) test was used to evaluate the neurological function of experimental rats. The test was performed in all rats before injury and at 1, 7 and 14 days after TBI. The mNSS could reflect the motor, sensory and reflex functional. Neurological function was graded on a scale of 0-18. The higher the score, the more severe the injury is. The mNSS test was performed by an investigator who was blinded to the experiment.

2.8 | Determination the brain water content

A dry-wet method was used to determine the brain water content on the third day after TBI. Rats were sacrificed to remove the brain and weighted (wet weight). Brain samples were incubated at 60°C for 72 hours and then reweighted (dry weight). The brain water content was then expressed as a percentage of wet weight using an equation published previously: 100 percent × ([wet weight−dry weight]/wet weight).[40]

2.9 | Evans blue dye extravasations

Briefly, Evans blue (EB, 2% in PBS, 4 mL/kg), as BBB permeability tracer, was injected into the tail vein of the rats on third day after TBI. One hour after EB injection, the rat was perfused by 200 mL heparinized saline under deep anaesthesia. The brain tissue was then obtained, weighted and homogenized in formamide (1 mL) and incubated at 60°C overnight and then centrifuged (30 minute at 22 000 x g) to get the supernatant. Using a spectrophotometer (UV/VIS, Spectrometer, UK) to measure the optical density of the supernatant at 625 nm. The amount of extravasated EB dye (μg/g wet weight) was quantified according to a linear standard curve.

2.10 | Measurement of vessel density and occludin

On the third day after therapy, rats were decapitated under anaesthesia and perfused with ice-cold, heparinized PBS followed by 4% paraformaldehyde. Brain tissue was removed and immediately placed in buffered formaldehyde. A series of 6 μm sections was cut from the standard paraffin block (through the lesioned area). Antibodies against von Willebrand factor (vWF, an endothelial cell marker, 1:50; GeneTex, Irvine, CA, USA) and occludin (a tight junction and BBB protein marker, 1:100; Abcam, Cambridge Cambridgeshire, UK) were employed. For measurement of vascular density, each vWF immunostained section was digitized using a 20× objective *via* the MCID computer imaging analysis system. The number of vessels was counted throughout each field of view. The total number of vessels was divided by the total tissue area to determine vascular density. For quantification, occludin-immunoreactive-positive cells were measured in total 20 enlarged positive blood vessels. The data are presented as percentage of positive cell number of vessels around the injury.

2.11 | Western blot assay

Briefly, equal protein aliquots were electrophoresed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and then blocked in 5% fat-free milk for 2 hours at room temperature. Blocked blots were incubated with specific antibodies against PR (1:1000; Santa Cruz Biotechnology) and GAPDH (an internal control, 1:5000; Solarbio, Beijing, China) overnight at 4°C. The blots were then immunostained for 2 hours at room temperature with corresponding horseradish peroxidase-conjugated secondary antibodies (1:1500; Santa Cruz Biotechnology) and incubated with the SuperSignal West Pico Chemiluminent Substrate (Pierce, Rockford, IL, USA). Protein bands were detected using a G: BOX F3 imaging system (Syngene, Cambridge, UK).

2.12 | VEGF secretion assay

After 6 hours of treatment by VEH or indicated concentration of P4, EPCs were cultured (1×10^6 cells/well) in growth factor-free medium EBM-2 for 24 hours. VEGF level in the supernatant (six wells/group) was measured by ELISA (Quantikine; R&D Systems, Minneapolis, MN, USA) and normalized to 1×10^6 cells. To evaluate the effect of UPA on P4 action, cells were coincubated with 10^{-6} mol/L UPA and P4 at optimal stimulating conditions of P4 (10^{-9} mol/L).

2.13 | Statistical analysis

All data were presented as mean ± SD of at least three independent experiments. Statistical analysis of the differences between the two groups was performed using the Student's *t*-test. Multiple comparisons were performed by ANOVA followed by Bonferroni correction using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). $P<.05$ was considered statistically significant.

3 | RESULTS

3.1 | Concentration- and time-dependent effects of P4 on EPC tube formation

We examined the effect of P4 on the tube formation activity of EPC. Interestingly, the quantification of tube numbers (Figure 1J)

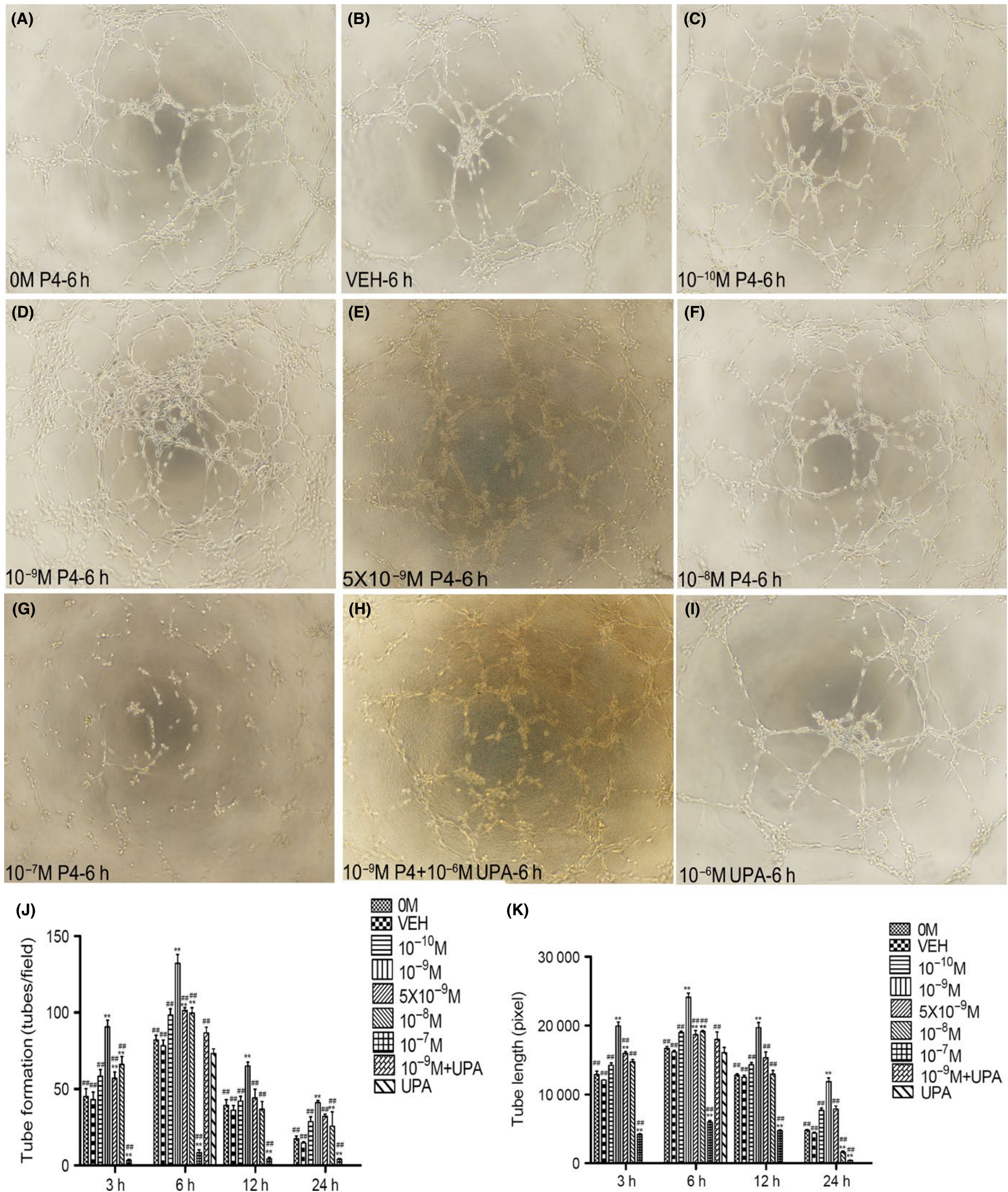

FIGURE 1 Concentration- and time-dependent effects of P4 on endothelial progenitor cell (EPC) tube formation. (A-I) Representative micrographs showing tube formation of EPC after 6 h of treatment by P4 (10×10 objective lens). (J, K) The concentration- and time-dependent effects of P4 on tube formation capability of EPC. Data are presented as mean±SD (n=6). $^{**}P<.05$ vs untreated group; $^{\#\#}P<.05$ vs 10^{-9} mol/L group

and length (Figure 1K) revealed that tube formation capability of EPC-treated by low concentration of P4 (10^{-10}, 10^{-9}, 5×10^{-9} and 10^{-8}mol/L) increased in a time-dependent fashion, achieving the best state after 6 hours of stimulation. Figures 1A-H were the representative pictures of tube structures formation of EPC 6 hours after stimulation by P4. At the peak time (6 hours), cells exhibited

the highest sensitivity to 10^{-9} mol/L P4 compared with 10^{-10} mol/L, 5×10^{-9} and 10^{-8} mol/L. However, 10^{-6} mol/L UPA blocked the tube formation capacity of EPC stimulated by 10^{-9} mol/L P4. High concentration (10^{-7} mol/L) of P4 significantly inhibited tube formation capacity of EPC at all incubation time points.

Based on the temporal kinetics of the concentration-dependent effects of P4 on EPC viability and tube formation capability and the fact that EPC-mediated tube formation is the main manifestation of vascular repair capacity of EPC, 6 hours of stimulation time was chosen for subsequent experiments.

3.2 | Effect of P4 on EPC adhesion

The adhesion of EPC to endothelial cells initiates the first step of vascular repair process.[7,41] The adhesion of EPC-treated by low concentrations of P4 (10^{-10}, 10^{-9}, 5×10^{-9} and 10^{-8} mol/L) exceeded that of untreated EPC (Figure 2A-I) with the maximal effect achieved at 10^{-9} mol/L. However, 10^{-6} mol/L UPA inhibited 10^{-9} mol/L P4-mediated EPC adhesion improvement. High concentration P4 impaired the adhesion capability.

3.3 | Effect of P4 on EPC migration

EPC migration is a crucial step in vascular remodelling following adhesion process.[7,41] As shown in Figure 3A-I, 6 hours of P4 stimulation caused a dose-dependent improvement of migration potential of EPC and the optimal concentration is 10^{-9} mol/L. However, 10^{-6} mol/L UPA inhibited 10^{-9} mol/L P4-mediated EPC migration improvement. Increasing concentration (10^{-7} mol/L) of P4 did not further augment migrated cells proportion but caused a reduced migration level.

3.4 | Effect of P4 on EPC necrosis and apoptosis

It was shown that low concentrations of P4 (10^{-9} mol/L) decreased the later apoptosis after 6 hours P4 stimulation. There was no difference of necrosis and apoptosis in other concentrations of P4 groups as compared with control. 10^{-6} mol/L UPA inhibited 10^{-9} mol/L P4-mediated EPC later apoptosis improvement (Figure 4).

3.5 | P4 promote neural functional recovery after TBI

The rats were subjected to mNSS and the rats treated with P4 significantly improved their neural function when compared either with the control or the P4+UPA, which indicated that the inhibitory PR attenuated the therapeutic effect of P4 (Figure 5A).

3.6 | P4 reduced the leakage of Evans blue disruption of BBB and brain water content following TBI

EB leakage was observed after TBI. It was shown that P4-treated rats reduce the EB extravasation when compared with control TBI group. When P4 treatment was mixed with UPA, the P4-induced reduction of the EB extravasation was reversed significantly (Figure 5B,C). These data indicated that a significant decrease in the permeability of the BBB was induced by the P4, which could be attenuated through blocking PR.

To examine the effects of P4 on oedema formation after TBI, we evaluated the brain water content of each groups. In accord with the EB dye extravasation, there was a significant decrease of brain water content in P4-treated group compared with the control. The UPA combined with P4 treatment then increased the water content in the brain tissue when compared with the P4-treated group (Figure 5D).

3.7 | Progesterone increases the density of brain vessels and occludin around the damage area

To assess the density of angiogenesis after TBI in each group at day 3, we employed vWF immunostaining. P4-treated group exhibited a significant increase in vascular density compared with the control. When P4 combined with UPA, the vascular density in the brain tissue of the TBI rats was significantly less than the P4-treated group (Figure 6A-D). Occludin expression was consistent with P4-mediated brain vessels increase. The low level of the occludin indicates the broken of BBB, while the high level of occludin indicates the breakage of BBB is repaired. P4 significantly increases the occludin expression and the UPA attenuated P4-induced high occludin expression (Figure 6E-H).

3.8 | Effect of P4 on PR expression

Western blot showed that two bands (80 and 120 kDa) corresponding to PRA and PRB were detected and PRA content was higher than that of PRB in EPCs without P4 treatment. 10^{-9} and 5×10^{-9} mol/L P4 increased the content of PRA and PRB after 6 hours of treatment. In contrast, 10^{-7} mol/L P4 reduced the content of both PR isoforms after 6 hours of treatment (Figure 7A,B).

On the other hand, we found that there was no expression change of PRA and PRB in the damaged brain after treating TBI mice with P4 or UPA (Figure 7D-F).

3.9 | Effect of P4 on VEGF secretion

VEGF is a powerful mediator of the revascularization.[42,43] As illustrated in Figure 7C, P4 dose-dependently increased VEGF level in the supernatant with peak value observed in the presence of 10^{-9} mol/L P4. Notably, 10^{-6} mol/L UPA blocked the stimulatory effect of 10^{-9} mol/L P4. 10^{-7} mol/L P4 impaired VEGF secretion potential of EPC.

4 | DISCUSSION

Some of clinical trails showed that P4 could improved outcome of TBI patients, the effectiveness of the treatment was still controversial.[44-47] The aim of the study was finding out what roles may P4 and

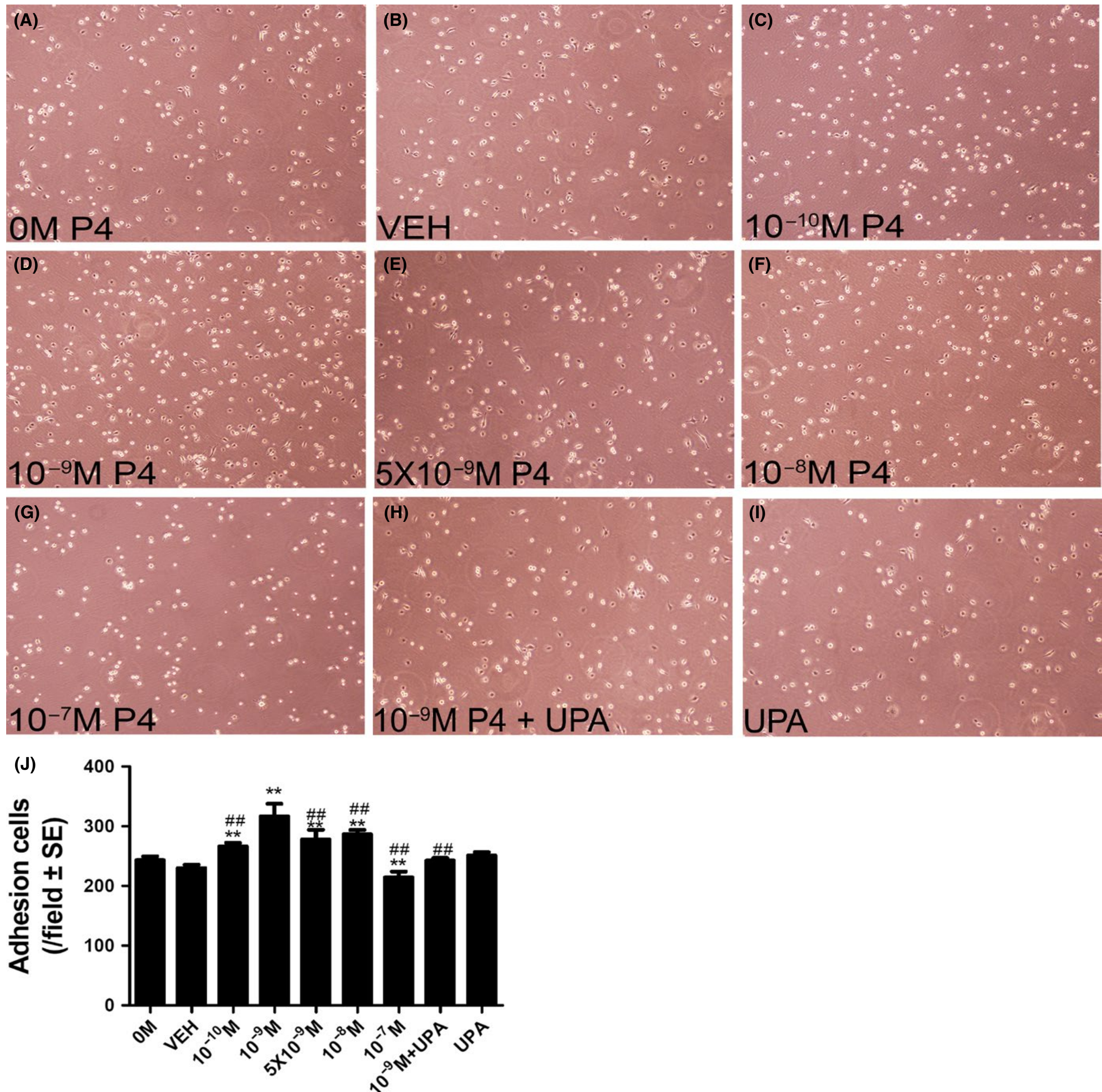

FIGURE 2 Effect of P4 on EPC adhesion (A-I) EPC adhesion in response to P4 treatment (10×10 objective lens). (J) Quantification of EPC adhesion. P4 induced a dose-dependent increase of EPC adhesion. Data are presented as mean±SD (n=6). *****P***<.05 vs untreated group; $^{\#\#}$***P***<.05 vs 10^{-9} mol/L group

PR play in mediating the angiogenic activity of EPC and angiogenesis after TBI rats.

The multi-faceted effect of P4 was also reflected in studies on mature endothelial cell or EPC. Hsu et al. demonstrated that P4 (5×10^{-9}–5×10^{-7} mol/L) inhibited proliferation of human umbilical venous endothelial cells (HUVECs) through a p53-mediated pathway.[48,49] Lee et al. found that P4 (5×10^{-9}–5×10^{-7} mol/L) inhibited migration of HUVECs in a dose-dependent manner.[50] On the contrary, yuko et al. found that incubation with P4 increased proliferation of EPC.[11] It is similar in Kempisty's study that they found that low dose of P4 rose the proliferation of luminal epithelial cells, but the high dose of P4 decrease the proliferation.[51] Our group confirmed that low concentration of P4 improved the viability of EPC through the CXCL12/CXCR4/PI3K/Akt signalling pathway.[34] It is essential for EPC to have these potential tube formation capacities in vascular repair and angiogenesis. Thus, in order to confirm what role of progesterone is played in mediating angiogenic activity of EPC, EPC was stimulated with graded concentrations (0, 10^{-10}, 10^{-9}, 5×10^{-9}, 10^{-8}, 10^{-7} mol/L) of P4 and tube formation, adhesion and migration tests were employed. Our data showed that low

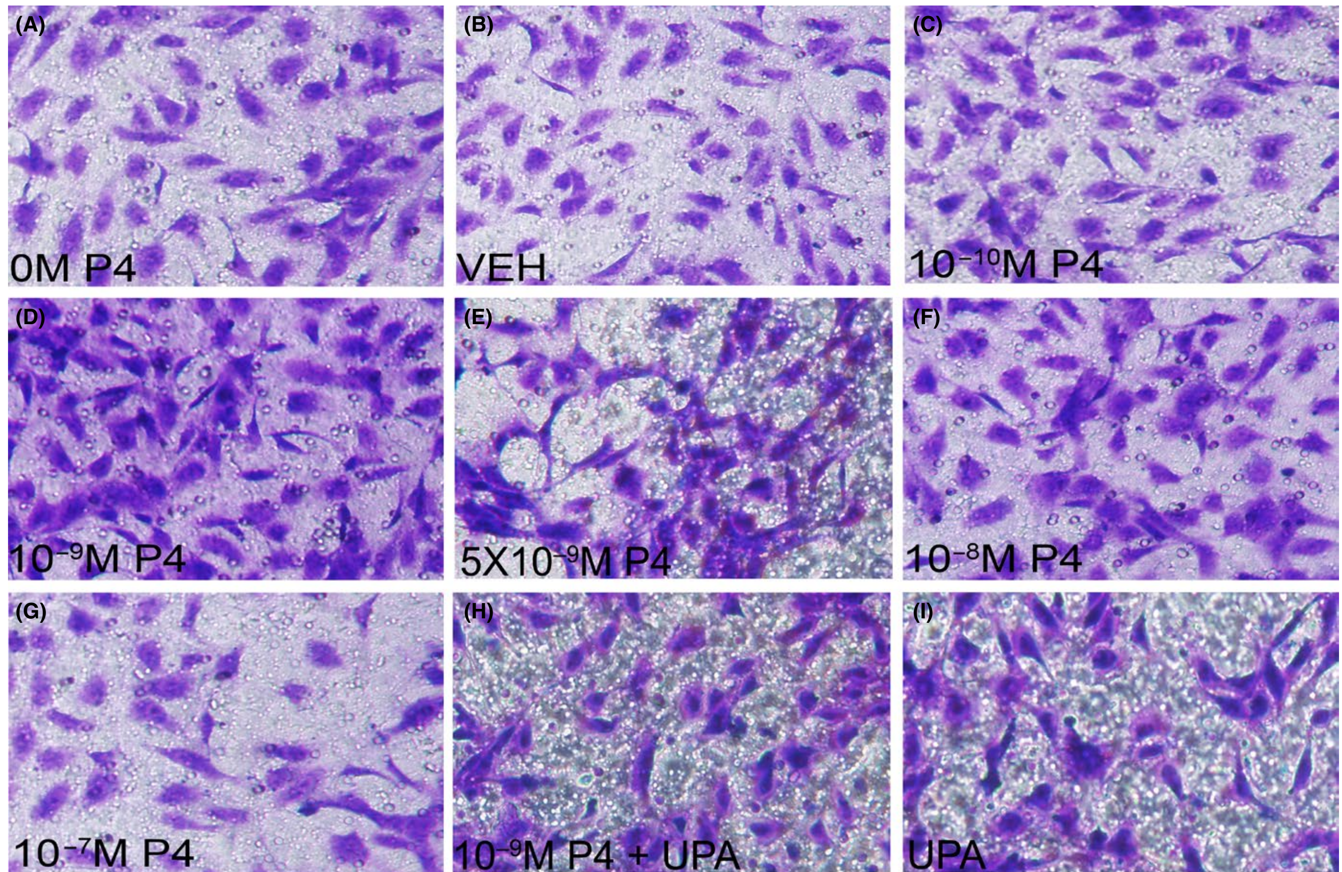

FIGURE 3 Effect of P4 on EPC migration (A-I) EPC migration in response to P4 treatment (10×20 objective lens). (J) Quantification of EPC migration. EPC exhibited a dose-dependent migratory response towards P4. Data are presented as mean±SD (n=6). $^{**}P$<.05 vs untreated group; $^{\#\#}P$<.05 vs 10^{-9} mol/L group

concentration of P4 exhibited stimulatory effect on tube formation, adhesion and migration of EPC which could enhance angiogenic activity of EPC, and the stimulatory effect was achieved within a short term (no more than 24 hours). On the other hand, high concentration (10^{-7} mol/L P4) of P4 impaired angiogenic activity of EPC. Meanwhile, low concentrations of P4 (10^{-9} mol/L) decreased the later apoptosis after 6 hours P4 stimulation. There was no difference of necrosis and apoptosis in other concentrations of P4 groups as compared with control. Coincubation with 10^{-6} mol/L UPA inhibited 10^{-9} mol/L P4-mediated decreasing the later apoptosis of EPC. The data may indicate that P4 has the concentration- and time-dependent effects on angiogenic activity of EPC and do not lead

(A) 0M
Q1 0.3% Q2 1.5% Q3 94.1% Q4 4.1%
(B) VEH
Q1 0.4% Q2 1.2% Q3 94.9% Q4 3.5%
(C) 10−10M
Q1 0.4% Q2 0.8% Q3 95.0% Q4 3.8%
(D) 10−9M
Q1 0.4% Q2 0.4% Q3 96.1% Q4 3.0%
(E) 5X10−9M
Q1 0.7% Q2 0.7% Q3 94.2% Q4 4.4%
(F) 10−8M
Q1 0.4% Q2 0.7% Q3 94.4% Q4 4.5%
(G) 10−7M
Q1 0.5% Q2 0.9% Q3 94.6% Q4 4.0%
(H) 10−9M+UPA
Q1 0.5% Q2 1.1% Q3 94.4% Q4 4.0%
(I) UPA
Q1 0.3% Q2 1.1% Q3 94.0% Q4 4.5%
PI-A
Annexin V-FITC-A

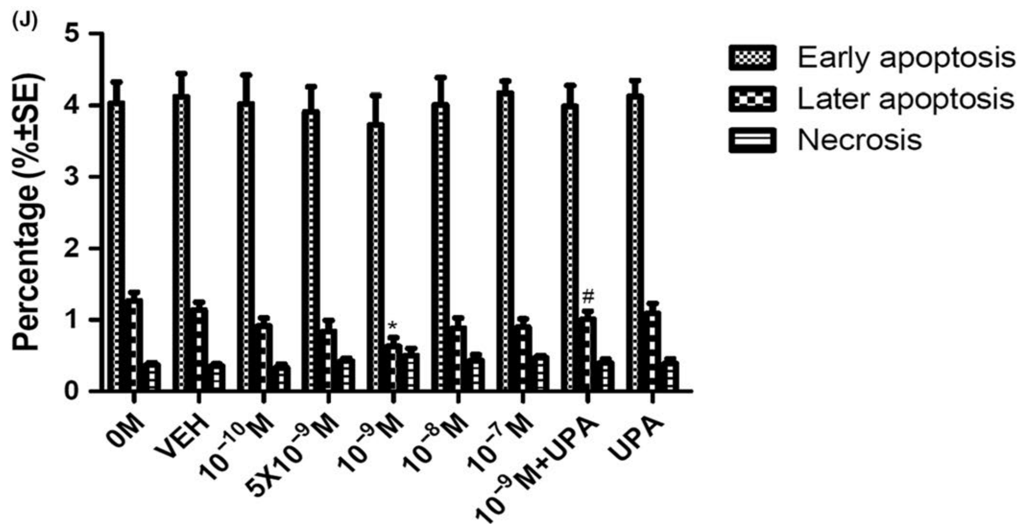
(J)
Percentage (%±SE)
0
1
2
3
4
5
Early apoptosis
Later apoptosis
Necrosis
*
#
0M
VEH
10−10M
5X10−9M
10−9M
10−8M
10−7M
10−9M+UPA
UPA

FIGURE 4 Effect of P4 on EPC necrosis and apoptosis. The viable cells were in lower left quadrant (annexin V-FITC and PI negative). Early apoptosis cells were in lower right quadrant (annexin V-FITC positive but PI negative). Late apototic cells were in upper right quadrant (annexin V-FITC positive and PI positive). Necrotic cells were in upper left quadrant (annexin V-FITC negative and PI positive). Low concentrations of P4 (10^{-9} mol/L) decreased the later apoptosis after 6 h of P4 stimulation. There was no difference of necrosis and apoptosis in other concentrations of P4 groups as compared with control. Coincubation with 10^{-6} mol/L UPA inhibited 10^{-9} mol/L P4-mediated decreasing the later apoptosis of EPC. **P*<.05 P4 (10^{-9} mol/L) vs P4 (0M), $^{\#}$*P*<.05 P4 (10^{-9} mol/L) vs P4 (10^{-9} mol/L) +UPA

to necrosis and apoptosis, which may be involved in the improved angiogenic activity following TBI.

Growing evidences suggest that angiogenesis and neurogenesis after TBI play a vital part in brain functional recovery. The enhanced angiogenesis will provide a better microenviroment to increase oxygen and blood supplements, decrease the brain tissue oedema, eliminate toxic chemical molecules (free radicals and inflammation cytokines) and reduce apoptosis. Recent finding showed that statins could improve the functional outcome after stroke and meanwhile increase angiogenesis and neurogenesis in aged animals.[52,53] Most of the neurorestorative agents contribute to regulating angiogenesis and neurogenesis and leading to improved functional recovery after TBI and stroke.[54-56] Previously, we found that P4 administration following brain injury in aged male rats improved circulating EPC level, vascular repair and neurological recovery.[19] We also confirmed that human EPC homed to injured brain which promoted angiogenesis in TBI mouse after EPC intravenously infusion.[57] Thus, we hypothesized that P4 enhance angiogenesis by promoting the angiogenic activity of EPC after TBI. In this manuscript, we already confirmed that P4 could enhance the angiogenic activity of EPC which played an essential role in the replenishing damaged endothelium. In the in vivo study, we found that TBI rats are also benefited from P4 application. In addition to the improvement in neural behavior, the reduction of the BBB permeability and brain water content as well as the increasing expression of occuludin and vessel density were also revealed in the TBI rats after P4 treatment. Thus, according to our previous study, it may indicate that P4 could induce angiogenesis after TBI through enhancing the angiogenic activity of EPC.

However, the mechanism of P4-induced stimulatory effects of EPC and angiogenesis after TBI are unknown. The P4-mediated responses are regulated through PR, including classic PRA and PRB and several transmembrane PR. Lee et al. found that P4-induced migratory inhibition of HUVECs was blocked by PR agonist-antagonist.[50] In endometriotic cells, dienogest which is a PR agonist could attenuate neuroangiogenesis and inflammatory factors through PR isoforms A and B.[58] It also has been documented that P4 plays an important role in angiogenesis through the PR.[59] But growing evidences suggested

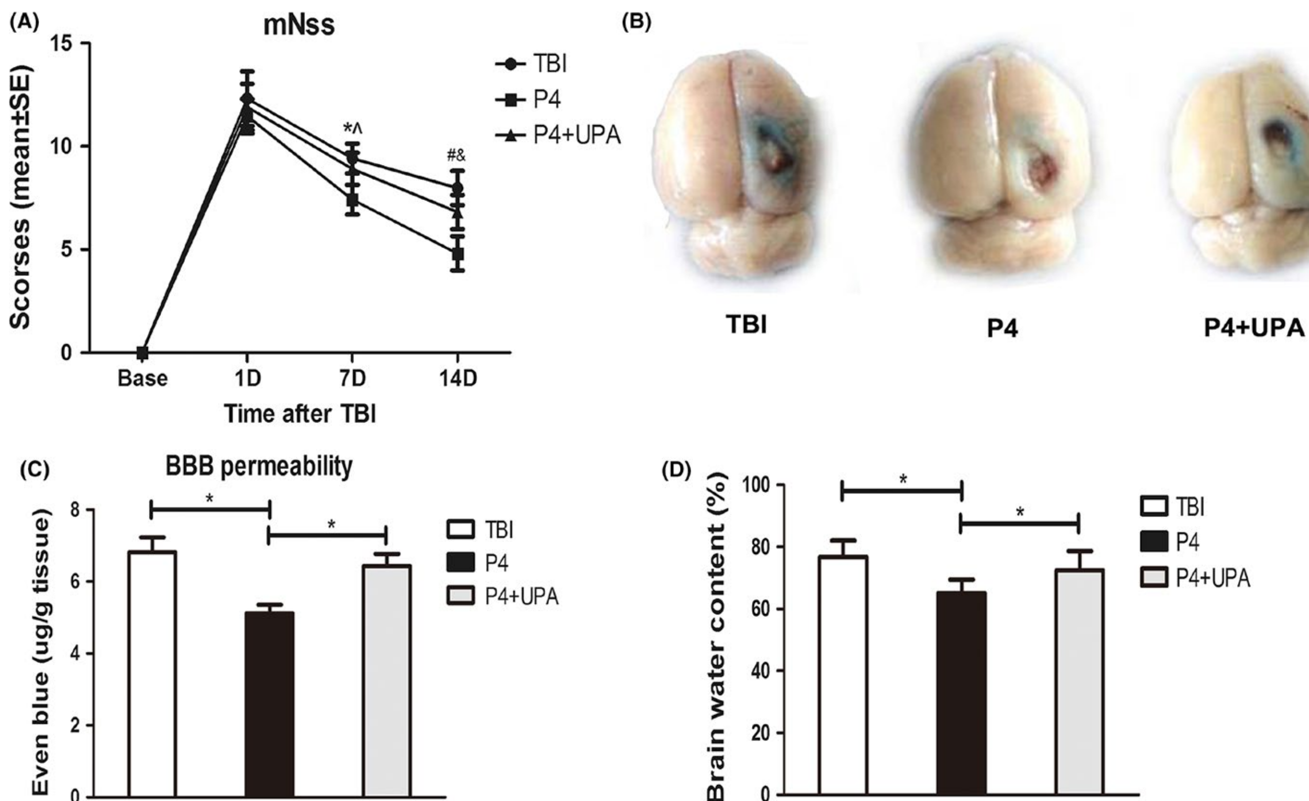

FIGURE 5 (A) Modified Neurological Severity Score (mNSS) test. P4 treatment improves functional outcome after TBI which could be attenuated by UPA in rats. **P*<.05 P4 vs TBI at day 7, ^*P*<.05 P4 vs P4+UPA at day 7, $^{\#}$*P*<.05 P4 vs P4+UPA at day 14, and *P*<.05 P4 vs TBI at day 14. (B,C) P4 treatment reduced the leakage of Evans blue after TBI which was reversed by UPA. **P*<.05 P4 vs TBI and P4+UPA. (D) P4 reduced the brain water content compared with TBI group which could be attenuated by UPA in rats. **P*<.05 P4 vs TBI and P4+UPA

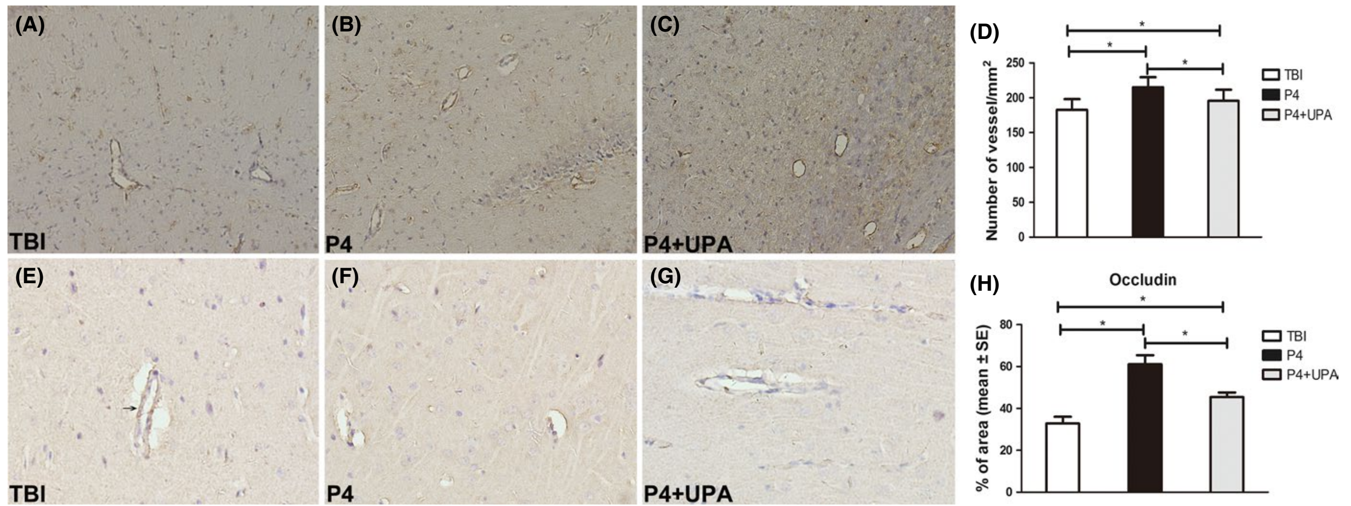

FIGURE 6 P4 induced angiogenesis. (A-D) The density of brain vessels around the damage area was increased by P4 treatment after TBI, which could be reversed by UPA.*P<.05. (E-H) The expression of occludin around the vessels was increased by P4 treatment after TBI, which could be reversed by UPA. *P<.05

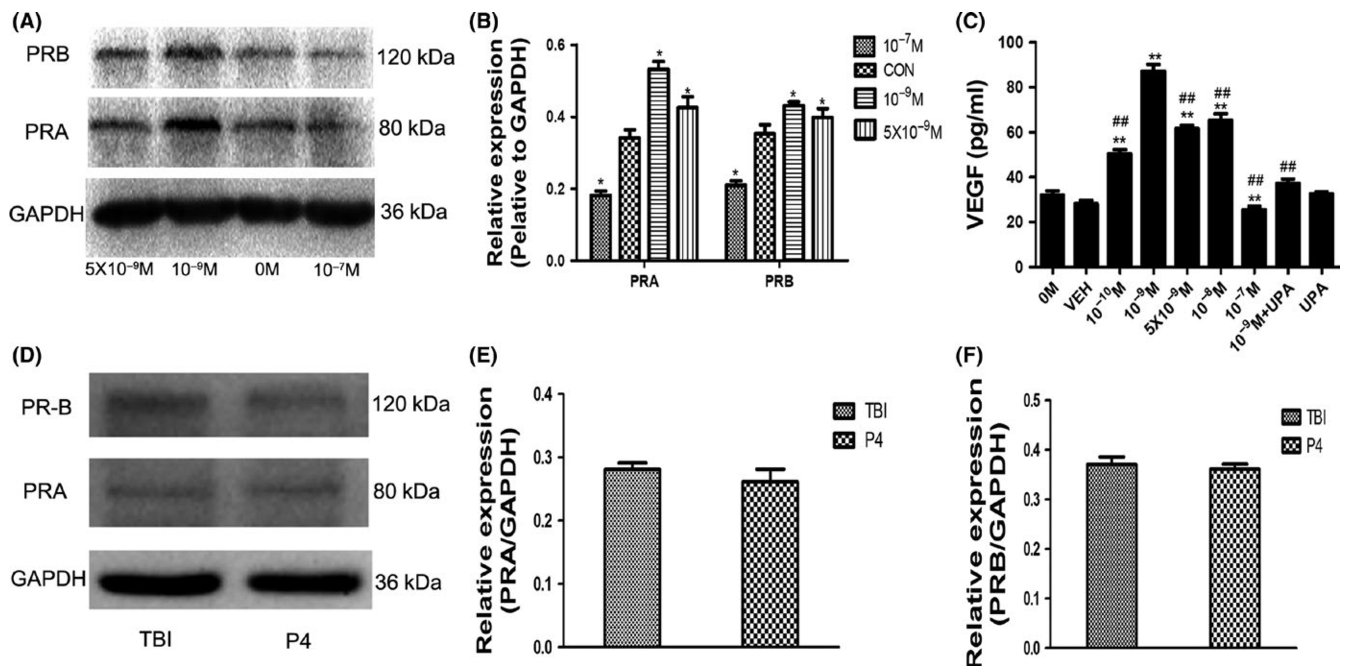

FIGURE 7 Effect of P4 on PR expression. (A, B) Western blot detected that both PRA and PRB isoforms were expressed in EPC and the content of PRA is higher. The expression of PRA and PRB was upregulated by 10^{-9} mol/L and 5×10^{-9} mol/L P4 but downregulated by 10^{-7} mol/L P4. GAPDH was used as a loading control. (C) VEGF secretion of EPC in response to P4 treatment and UPA. P4 stimulated the VEGF secretion dose-dependently and the optimal stimulating concentration was 10^{-9} mol/L. **P<.05 vs untreated group; ##P<.05 vs 10^{-9} mol/L group. (D-F) There is no different of PRA and PRB expression after P4 treatment in brain of TBI rat. GAPDH was used as a loading control

that classic PR may not mediate the therapeutic effects through progesterone classic signalling in the brain.[60,61] P4 was converted to several metabolites in vivo, including allopregnanolone (ALLO) and 5alpha-dihydroprogesterone. The ALLO could act through γ-amino butyric acid type A (GABAA) receptors rather than PR. The P4 effects could be abolished by administration of finasteride which was utilized to prevent metabolism from P4 to ALLO.[62] EPC takes an essential part in angiogenesis which involves endothelial cell migration and proliferation especially after neurovascular injury.[63,64] In this study, we found that 10^{-9} mol/L P4 exhibited optimal stimulatory effect on tube formation, adhesion and migration which could be abolished by UPA. In vivo, the P4-induced therapeutic effect and angiogenesis could also be inhibited by UPA. It was suggested that progesterone may induce angiogenic activity through progesterone classical signalling pathway. Hence, we hypothesized that P4 may exert the therapeutic effects by regulating the EPC rather than neuron through classic PR after TBI.

It was reported that there are different functions and activities between PRA and PRB isoforms. PRA played as a transcriptional inhibitor role of PRB when both of them present in a same cell.[65] Moreover, P4 fail to induce the inhibition of oestrogen-mediated proliferation of epithelial cell when selective ablation of PRA rather than PRB.[66] PRB play an essential role in the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway, cell proliferation and apoptosis and alteration of adhesion molecules in human EC cell lines.[67-70] In our study, we found that PR distribution was detected in EPC and its expression was upregulated by 10^{-9} mol/L P4 but down-regulated by 10^{-7} mol/L P4, which is consistent with the finding that PR content in reproductive and non-reproductive tissues fluctuated in response to concentration change of sex hormones.[3] Thus, we hypothesize that low concentration of P4 induce EPC angiogenic activity through PRB and simultaneously increase the PRA level which as a result inhibit the P4-mediated EPC angiogenic activity for more than 12 hours. High concentration of P4 decreased the concentration of PRB which may lead to the opposite results. In addition, PR-mediated responses are based on the coordinated activation of individual PR isoforms which results in differential responses.[65] The expression of the PRA and PRB was not changed after administration of P4 in TBI rat brains. It may indicate that P4 improve neural recovery by mediating EPC angiogenesis function through PR rather than other cells in brain after TBI.

VEGF was a key role of P4-mediated angiogenesis.[21] We found that dose-dependently increased VEGF level in the supernatant with peak value was observed in the presence of 10^{-9} mol/L P4 and 10^{-7} mol/L P4 impaired VEGF secretion potential of EPC, which indicated that P4 may promoted EPC angiogenic activity through increasing the VEGF. Notably, 10^{-6} mol/L UPA blocked the stimulatory effect of 10^{-9} mol/L P4 which suggested that PR may take an important part in VEGF secretion of EPC which may involve in the angiogenesis after TBI. Above all, the classical P4 signalling pathway appears to be involved in P4-induced EPC angiogenic activity, which may take responsibility for P4-mediated angiogenesis after TBI. The limitation of the study was failed to conform the functions of PRA and PRB respectively which was needed to be further investigated.

5 | CONCLUSION

Progesterone-mediated angiogenic activity of EPC and angiogenesis in TBI rats were antagonized by progesterone receptor antagonist.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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