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Original Article

Effects of fresh bone marrow mononuclear cell therapy in rat model of retinopathy of prematurity

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ABSTRACT

Introduction: Retinopathy of prematurity (ROP) is a vasoproliferative disease that alters retinal vascular patterns in preterm neonates with immature retinal vasculature. This study was conducted to investigate the effects of cell therapy by bone marrow mononuclear cells (BMMNC) on neurological and vascular damages in a rat model of ROP.

Methods: Ten newborn Wistar rats were divided randomly into the control and the oxygen-induced retinopathy (OIR) groups. Animals in the OIR group were incubated in an oxygen chamber to induce retinopathy. One eye of animals in the OIR group received BMMNC suspension (treated eyes), and the contralateral eye received the same volume of saline injection. Then, all animals underwent funduscopy, angiography, electroretinography, histopathology and immunohistochemical assessments.

Results: Compared to the saline injection group, eyes treated with BMMNC had less vascular tortuosity while veins and arteries had relatively the same caliber, as revealed by fundus examinations. Eyes in the treatment group showed significantly elevated photopic and scotopic B waves amplitude. Neovascularization in the inner retinal layer and apoptosis of neural retina cells in the treatment group was significantly lower compared to untreated eyes. Also, BMMNC transplantation decreased glial cell activation and VEGF expression in ischemic retina.

Conclusions: Our results indicate that intravitreal injection of BMMNC reduces neural and vascular damages and results in recovered retinal function in rat model of ROP. Ease of extraction without in vitro processing, besides the therapeutic effects of BMMNCs, make this source of cells as a new choice of therapy for ROP or other retinal ischemic diseases.

1. Introduction

Retinopathy of prematurity (ROP) is a vasoproliferative disease, which alters retinal vascular patterns in preterm neonates. The condition is widely known as a potent cause of permanent blindness, and according to reports 20,000 neonates become blind or severely visually impaired from the disease, annually [1]. ROP

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pathophysiology consists of two phases: vascular growth arrest due to hyperoxia state and then, neovascularization in hypoxia phase. Absence of physiologic hypoxia due to preterm parturition [2], and subsequent oxygen therapy with high oxygen concentrations induce hyperoxic conditions that suppress normal retinal vascular development, resulting in poor and inadequate vasculature, and subsequent hypoxia [3]. Following the hypoxic conditions and oxidative stress induced inflammation, the retina runs several pathways to preserve and supply oxygen for the hypoxic tissue [4]. Eventually, excessive vascularization and inflammation cause vascular leakage and retinal detachment.

Current ROP treatments include: laser therapy, vitrectomy, anti-vascular endothelial growth factor (VEGF) medications, and direct ablation of abnormal vessels [5]. These conventional treatment options have several limitations including the induction of new vascular abnormalities, long-term systemic disease in neonates treated with anti-VEGF drugs, and disease recurrence [6,7]. A better understanding of the condition's pathophysiology brings about treatment modalities with lower complications and higher success rates. One example would be regenerative medicine approaches using cell therapy, which have demonstrated promising results in treating ROP and other conditions with similar inflammatory and pathophysiologic basis [8]. Stem cells used in animal studies of ROP include; mouse bone marrow derived mesenchymal stem cells, human progenitor cell combination (bone marrow derived and vascular wall derived endothelial colony forming cells), human endothelial progenitor cell, and human peripheral blood stem cells [9–11]. These studies showed that stem cells migrate to avascular regions of the retina and promote vascular repair by inhibiting angiogenesis and making the retina more flexible to oxygen concentration changes mainly by stimulating the hypoxia-inducible factor (HIF)-alpha pathway (Fig. 1). These cells can differentiate into microglial cells that play immunomodulatory roles in ischemia and phagocyte tissue debris and help retinal regeneration [12]. Studies show that stem cell's engraftment to preexisting astrocyte templates promotes retinal vasculogenesis during retinal development. Many cells migrate into the retina's deep layers and develop a vascular network [13].

Bone marrow-derived mononuclear cells (BMMNC) are a heterogeneous population of cells consisting of stem cells (hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells), immune cells (B cells, T cells, monocytes) and several soluble molecules such as cytokines and growth factors [14]. BMMNCs possess anti-inflammatory effects and reduce neurodegeneration defects in ischemic conditions [15]. In preclinical studies, BMMNCs decreased gliosis and also showed regenerative effects in retinal degeneration. In addition, studies on optic nerve crush and mechanical retinal damages in animal models revealed that, cell therapy using whole BMMNCs fraction resulted in neuroprotection and repaired damaged retina by differentiation into retinal neural cells [16–18]. BMMNCs were used in clinical trials on retinitis pigmentosa, Stargardt disease, retinal dystrophy, age related macular degeneration, and diabetic retinopathy. They proved to be effective in terms of improving retinal function and visual accuracy [19–22]. While some researchers have purified and utilized one specific cell line from BMMNCs, others have utilized the whole BMMNC population, with all its cell subtypes in it [23]. Fresh BMMNCs without ex vivo processing reduces the chance of cell contamination and differentiation risk, is cost-beneficial and a time-saving process [24]. We hypothesized that fresh bone marrow mononuclear cells may reduce neural-vascular damage and protect retina from dysfunction during ischemia in rat model of ROP. The aim of this study is to evaluate BMMNC effects on neural and vascular damages in the rat model of ROP to understand whether BMMNC therapy reduces these damages and recovers retinal function.

2. Materials and methods

2.1. Animals

All animal procedures in this study are carried out under the national animal care guidelines and approved by local Ethics committee. 10 new born Wistar rats and their dam, in addition to one male 8 month old GFP rat (as a cell donor) were provided by our institution. Animals were kept in 12-h light/dark cycles and ad libitum access to food and water.

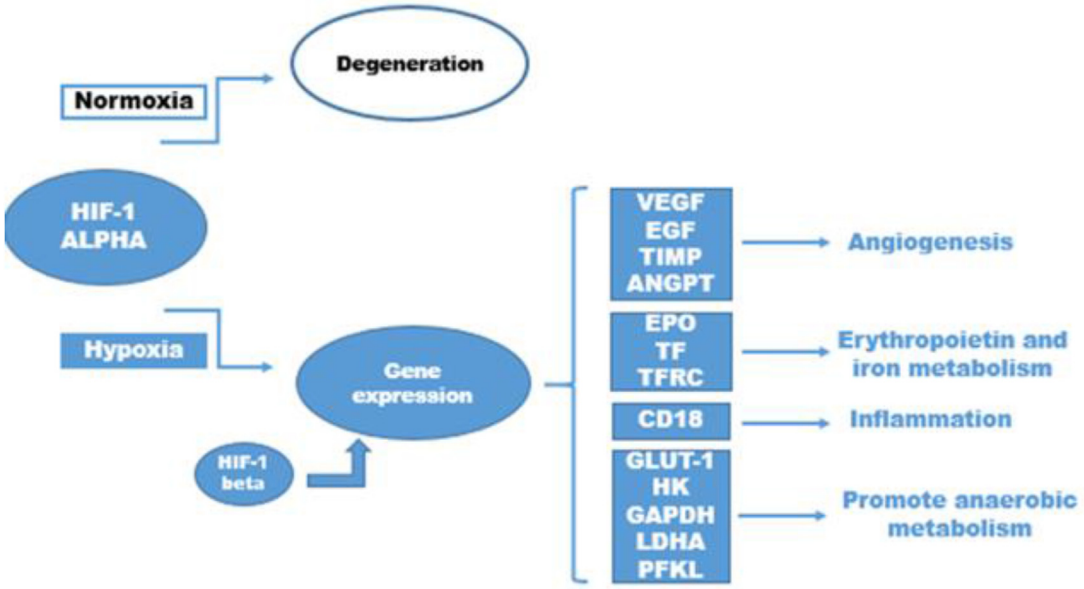

Fig. 1. HIF-1alpha pathway Overview. HIF: Hypoxia-inducible factor. VEGF: Vascular endothelial growth factor. EGF: Epidermal growth factor. TIMP: Tissue inhibitor of metalloproteinase. ANGPT: Angiopoietin. EPO: erythropoietin. TF: Transferrin.

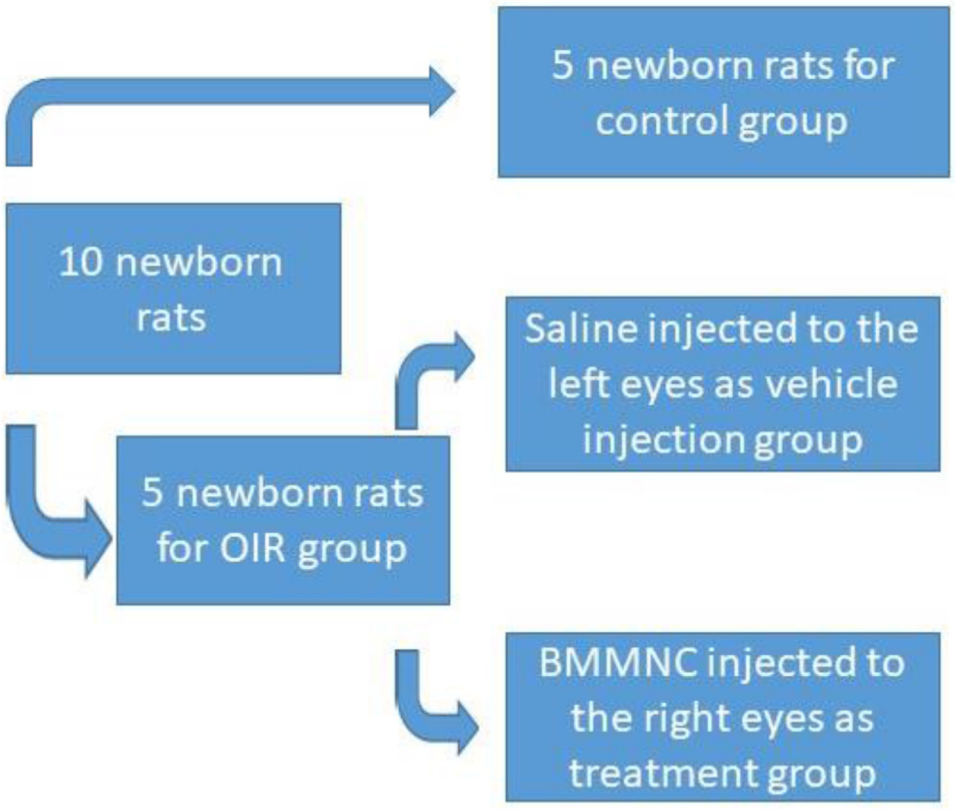

Fig. 2. The experimental design.

2.2. Experimental study design

In this study, 10 newborn Wistar rats were randomly divided into two groups, the control group and the oxygen-induced retinopathy (OIR) group (Fig. 2). Based on previous studies on cell therapy in ROP animal models, the intervention was done at the initiation of phase 2 (hypoxia) of ROP modeling on postnatal day 12 (p12). In animals in the OIR group, one eye received BMMNC suspension (treatment group), and the contralateral eye received the same volume of saline injection (vehicle injection group). On p17, all animals underwent funduscopy and electroretinography. Then, they were euthanized by CO_2 inhalation, and their eyes were harvested for histopathological assessments. Post-natal day 17 was chosen according to Xu et al. study [25].

2.3. Rat model of oxygen induced retinopathy (OIR)

In the current study, we used the conventional protocol of preparing an animal model of ROP described in detail by Smith et al. [26]. As illustrated in Fig. 3, seven-day-old Wistar rats with their nursing dam were incubated for five days in a hand-made isolated chamber with oxygen concentration adjusted to 70–75%. During the incubation period, animals were provided free access to standard food and water, and the oxygen concentration was monitored

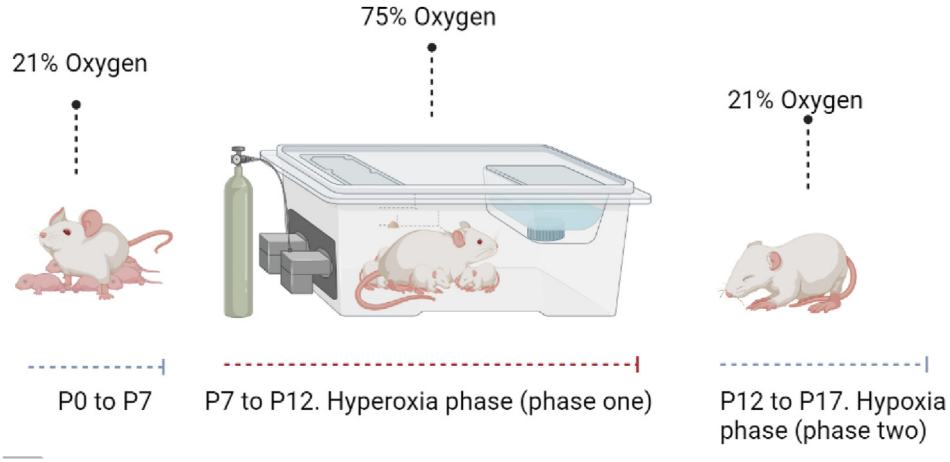

Fig. 3. Oxygen induced retinopathy modeling procedure. Created with BioRender

continuously by using an oxygen indicator (CityCell. the UK). We placed a heated pad under the chamber to maintain the chamber's temperature at 25 °C. On p12, animals were returned to room air (21% O_2) for five days.

2.4. BMMNC extraction and autologous transplantation

Bone marrow was extracted from the dissected humerus, femur, and tibia from a donor GFP rat with cold sterile Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEMF-12) (Gibco, Thermofisher Scientific, MA) and then filtered through 70 μm cell strainer. The suspension was centrifuged at 500*g* for 5 min (Eppendorf, Germany) at room temperature. The supernatant was removed, and cells were resuspended in 5 ml of 1X RBC lysis buffer (Sigma-Aldrich, Germany) and incubated for 3 min at room temperature. The lysis reaction was stopped by adding 20 ml of PBS, and then the solution was centrifuged again and the supernatant was removed. The final solution was achieved by adding 1 ml sterile PBS to the cell pellet. The availability of bone marrow cells was approved by trypan blue staining. Then, one syringe was filled with final suspension [27].

After general anesthesia with an intraperitoneal injection of ketamine/Xylazine (Rotexmedica, Germany) and saline mixture (85/10 mg/kg), a small incision was made on the fissure between the eyelids with a lancet blade (NO 24) to separate them. Ocular surface analgesia was induced by tetracaine 0.5% eye drop. 10 μl of the BMMNC suspended in sterilized PBS, containing 1.2×10^5 cells, was slowly injected intravitreally with a 30-gauge needle to one eye of each neonate rat in the OIR group and equivolume of sterile saline was injected into the contralateral eyes [28].

2.5. Confirmation of injected cells recruitment

In order to confirm BMMNC recruitment in the retinas, immediately after euthanization, eyes were enucleated and cornea, lens and vitreous body were removed. Then, flat mounted retinas were prepared by making four incisions, and they were directly placed onto glass slides. Imaging was performed by an Olympus BX50 microscope and cells with fluorescence reactivity were considered GFP + BMMNCs [29].

2.6. Fundus examination and angiography

We captured fundus photographs from control, ROP and treatment groups and compared retinal vasculature and optic disc properties to evaluate ischemic damage to the eyes. On p17, all animals underwent fundus examination by a slit lamp 78 diopter lens (Volk optical Inc., Germany). Vascular tortuosity, variation in veins and arteries caliber, lack of polygonal vascular pattern, and the hazy appearance of the fundus were considered indicators of retinal vascular damage. Also, optic disc ischemic damage was defined by papilledema (enlarged and pale optical disc) and non-even boundaries of the optic disk [30]. To capture fundus images, we used smartphone fundus photography. The pupil was dilated using 0.5% atropine eye drop. Examiner sat in a chair while holding a slit lamp 78 diopter lens in his hand, wearing a headlight. Proper alignment of the light source, lens, and animal's eye resulted in authentic aerial images recorded using a smartphone camera. Fluorescent angiography was performed using Heidelberg Retina Angiograph - 2 (HRA-2) (Heidelberg, Germany) as described in detail by Huber et al. [31]. Briefly, after appropriate induction of anesthesia by Ketamine/Xylazine combination, pupils were dilated using atropine eye drop and then, 0.15 ml of 10% sodium fluorescein was injected intraperitoneally and animals were placed in front of the camera. HRA 2 device was operated in fluorescence mode and images were captured using the high resolution mode from central retina.

2.7. Electroretinography (ERG)

Full-field ERGs were recorded using the RETIanimal system (Roland Consult, Germany) and a Ganzfeld (Q 450 SC, Roland Consult, Germany). A and B waves amplitude were measured in both dark- and light-adapted eyes. Briefly, on p17, neonatal rats were dark-adapted for 2 h and anesthetized by injection of Ketamine/Xylazine mixture (85/10 mg/kg). The pupils of rats were dilated with 0.5% atropine eye drop, and the body temperature was maintained at 35 °C using an electric heat pad. After sufficient anesthesia of the corneal surface with 0.5% tetracaine eye drop, 12 mm golden electrodes were placed on the cornea, skin, and tail. ERG responses under dark-adapted conditions were evoked by 9 flashes ranging from 0.01 Log cd.s/m^2 to 10 Log cd.s/m^2 in distinct low/high pass filters (low-pass filter was 0.05 Hz and high-pass was 500 Hz). In order to prevent attenuating dark adaptation, flash series were shined from the lowest to the highest intensity. A and B waves were evaluated in scotopic and photopic states, respectively. The waveforms were averaged across each flash series, and the A- and B-wave were taken at 3 Log cd.s/m^2 in photopic conditions. Moreover, A- and B-wave were recorded in scotopic condition with different intensities (−2, 0, 1 Log cd.s/m^2 for B wave and 0, 1 Log cd.s/m^2 for A wave). The A-wave values were calculated as the absolute value of the minimum amplitude following the flash stimulus, while the B-waves amplitudes were calculated from baseline voltage (0 μV) to the peak of the response. Recorded waves were automatically analyzed, and waves amplitudes were measured by RETIanimal software [32].

2.8. Histopathology and immunohistochemistry

After euthanizing the animals, both animals' eyes were enucleated and immediately fixed in 10% neutral-buffered formalin with the fixative solution, which was replaced every two days until the tissues hardened. In addition, each eye was embedded in a paraffin block, and 5 μm sections, with the same distance from optic nerve parallel to its sagittal axis, were prepared and stained with hematoxylin and eosin (H&E). In order to score neovascular nuclei in the internal limiting surface of the retina, Slides were examined qualitatively in 40× magnification under a light microscope (Nikon. Japan) [33]. Endothelial cells or blood vessels with full lumen, apoptosis, and organization of retinal layers were compared qualitatively [34]. After H&E staining, the remaining paraffin sections were used for immunohistochemistry assay to detect activated glial cells by glial fibrillary acidic protein (GFAP) and evaluate vascular endothelial growth factor (VEGF) expression. Immunohistochemistry staining procedure was performed following the manufacturer's instructions. Paraffin sections were deparaffinized and dehydrated, and then the antigens were repaired using a heated citric acid repair liquid (P0083; Beyotime Institute of Biotechnology, Shanghai, China). Endogenous peroxidase activity was blocked by incubating the sections in 3% H2O2 for 20 min; then, the sections were washed and placed in goat serum for approximately 15 min to block non-specific labeling. The sections were incubated at 4 °C overnight with primary mouse monoclonal anti GFAP (1:2000, Sigma-Aldrich, Germany) and polyclonal rabbit anti-VEGF (1:200, Proteintech, USA) antibodies. Finally, the sections were washed, dehydrated, embedded in paraffin, and scanned. ImageJ software was used to quantify GFAP and VEGF expression [35].

2.9. TUNEL assay

TUNEL staining was performed with a kit (Elabscience Biotechnology, China, E-CK-A334). Paraffin sections were dewaxed and hydrated two times in xylene twice followed by one time in ethanol. Slides were washed three times with PBS and were incubated with proteinase K working solution (1 μL proteinase K in 99 μL PBS) in 37 °C for 30 min. The cells nuclei were counterstained with 1 mg/ml DAPI for 30 min at room temperature in the dark. TUNEL-positive cells in retinas were quantified by random field counting. Fluorescence images were scanned with an Olympus BX50 microscope and analyzed with ImageJ software [36].

2.10. Statistics

We used GraphPad Prism version 8.0.0 for statistical analysis and graphing. All values were expressed as the mean ± SEM. Kolmogorov–Smirnov test was used to confirm the normal distribution of data, and differences between groups were calculated using ANOVA test. $P < 0.05$ was considered significant.

3. Results

3.1. Establishment of ROP rat models

Fundus examination in the control group revealed radial straight vascular branches with no tortuosity. Also, veins and arteries were unicaliber (Fig. 5A). However, fundus examination in the ROP group showed abnormal vessel architecture with visible neovascular tufts, distorted distribution and tortuosity, non-polygonal structure, and difference in veins and arteries caliber. In addition, optic disc injury was indicated by papilledema, pale and non-even optic disc boundaries (Fig. 5B).

In ROP group, ERG data indicated significantly weak B waves, with much more difference in lower intensities, in both scotopic ($P \leq 0.05$ in 0 Log cd.s/m^2 and $P \leq 0.01$ in −2 Log cd.s/m^2 intensity) and photopic ($P \leq 0.01$ in +3 Log cd.s/m^2 intensity) conditions, compared to control group (Fig. 6A–C). A significant difference was also seen in photopic A waves between the two groups ($P \leq 0.0001$) (Fig. 6D). However, no significant difference was reported in scotopic A waves amplitudes (Fig. 6E).

Histopathological results in the control group showed clear retina's internal limiting surface with well-organized retinal layers (intact outer plexiform layer and outer nuclear layer). The neovascular nucleus was rarely seen (Fig. 7A). On the other hand, the retina in the ROP group was disorganized in layers and had a non-even internal limiting surface. Besides, extensive hydropic degeneration and apoptosis were seen in the inner nuclear layer (Fig. 7B). It is notable that in the ROP group, the neovascular nuclei count was significantly higher compared to the control group ($P \leq 0.005$) (Fig. 7D). Immunohistochemichal assessments revealed extensive gliosis and significant increase of GFAP positive cells in ROP group ($p < 0.001$) (Fig. 8). Moreover, VEGF expression in retinas of ROP group significantly increased, comparing to control group ($p < 0.05$) (Fig. 9).

According to TUNEL assay, number of apoptotic cells significantly increased in ROP group eyes ($p < 0.0001$) and most of TUNEL positive cells were detected in the outer nuclear layer of retina (Fig. 10).

3.2. Evaluation of BMMNC transplantation effects on ROP affected retina

3.2.1. BMMNC recruitment

To examine whether BMMNC was successfully recruited to the retina, we detected GFP positive cells in fresh flat mounted retinas.

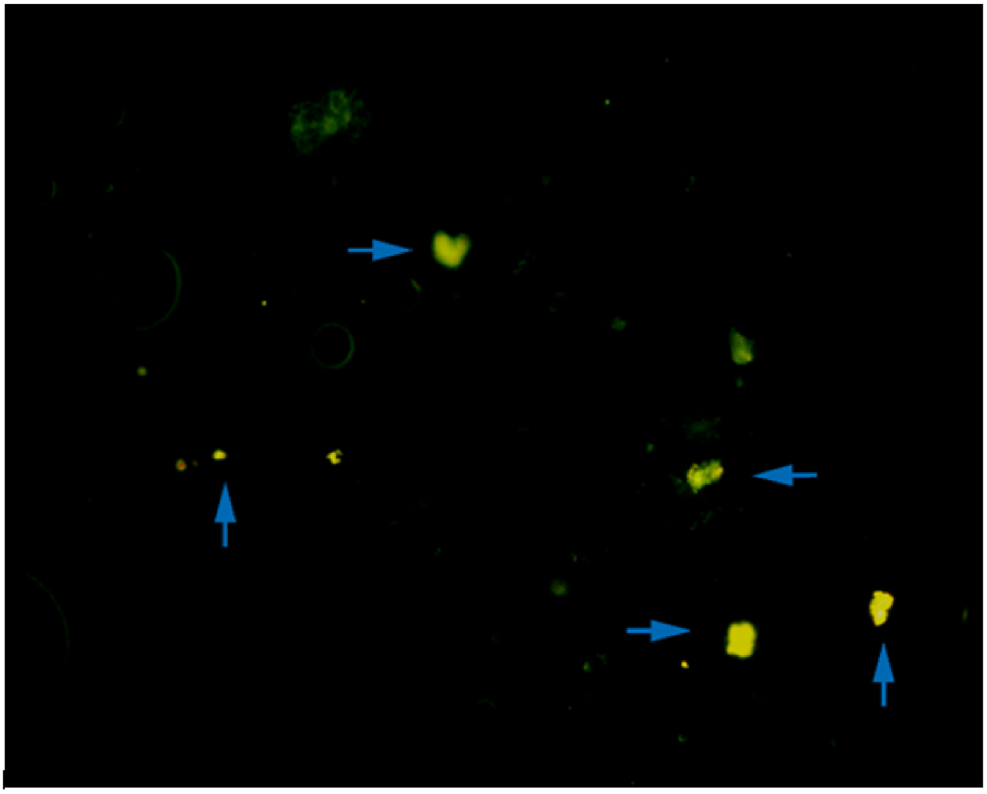

Fig. 4. Recruitment of GFP positive cells (blue flesh) to the inner surface of retina of neonatal rats. Pictures captured from fresh flat mounted retina under fluorescent microscope (× 100).

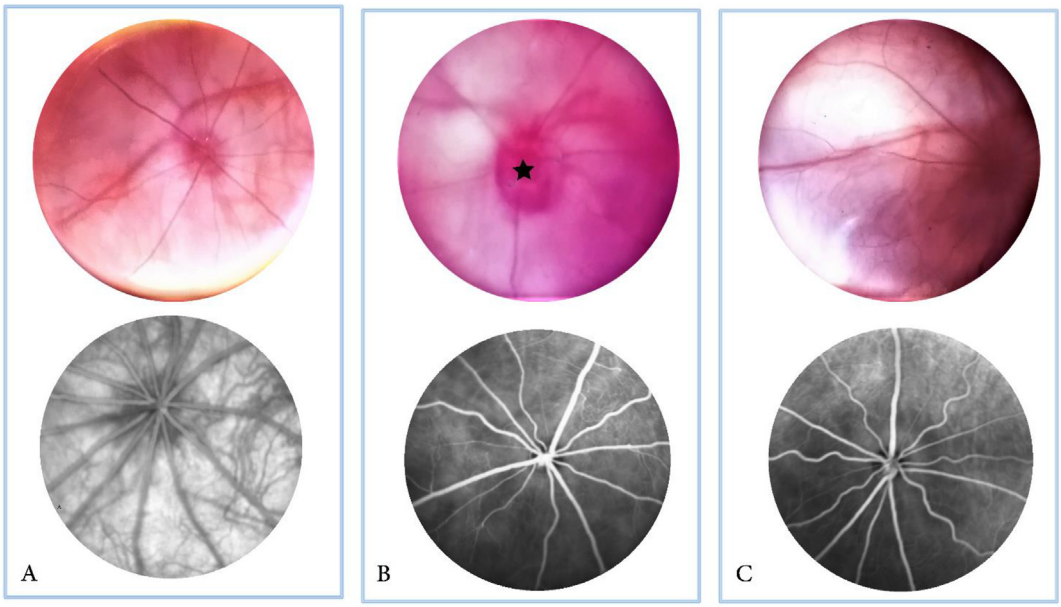

Fig. 5. Fundus photography (up) and fluorescent angiography (down). (A); Control, (B); Vehicle injection, (C); BMMNC treatment. (A): intact retina with straight radial branches without tortuosity and with a polygonal shape. Note the same caliber of veins and arteries and normal appearance of the optic disc with no edema and well even boundaries. (B): ischemic retina with engorged vessels and vascular tortuosity. Veins and arteries do not have the same caliber ad there is no polygonal appearance in vascular structure. Note papilledema (star) with non-even boundaries which indicates optic disc injury in fundus photos. (C): there are more prominent vessels are detectable in the treatment group retina, veins and arteries are relatively unicaliber, vascular tortuosity is detectable but radial branches are relatively straight and make a polygonal shape. In fundus photos, the optic nerve is normal, there is no enlargement and papilledema detected and appearance of the optic nerve is pink with even boundaries.

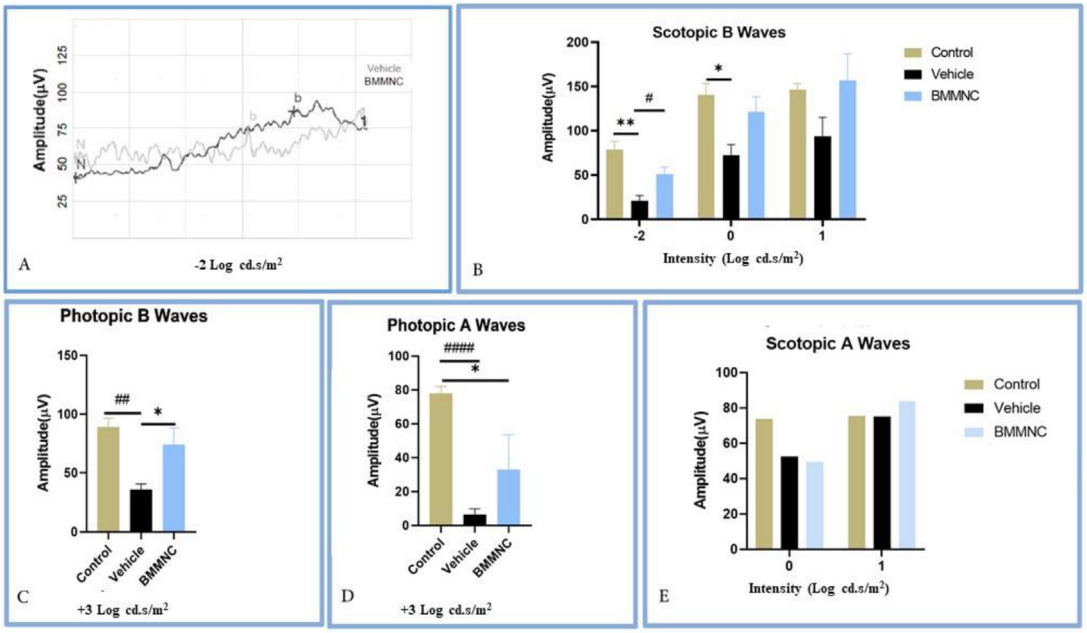

Fig. 6. (A); Simultaneously electroretinography waves of BMMNC treatment and vehicle injection of a rat. Note the high b wave amplitude in BMMNC treatment versus vehicle injection. (B); an average scotopic B wave amplitude in BMMNC, vehicle injection, and control in three ascending intensities. There are significant differences between vehicle injection and BMMNC treatment groups in −2 Log cd.s/m^2 intensity and also between the control group and vehicle injection in 0 Log cd.s/m^2 intensity. There is no significant difference among groups in +1 Log cd.s/m^2 intensity. (C); Average photopic B Wave amplitude in BMMNC and vehicle injection in +3 Log cd.s/m^2 intensity. B wave amplitude has significant difference with control and BMMNC groups. Also, there is a notable difference between vehicle injection and control groups. (D); average photopic B wave amplitude in BMMNC, vehicle injection, and control groups in +3 Log cd.s/m^2 intensity. The significant difference is detectable among BMMNC treatment and vehicle injection groups. Also, there is a notable difference between vehicle injection and control groups. (E); average scotopic A wave amplitude in BMMNC treatment, Vehicle injection, and control groups in 0 and + 1 Log cd.s/m^2 intensity. Data presented. represent mean ± SEM (*, #p < 0.05, ##p < 0.01, ****p ≤ 0.0001).

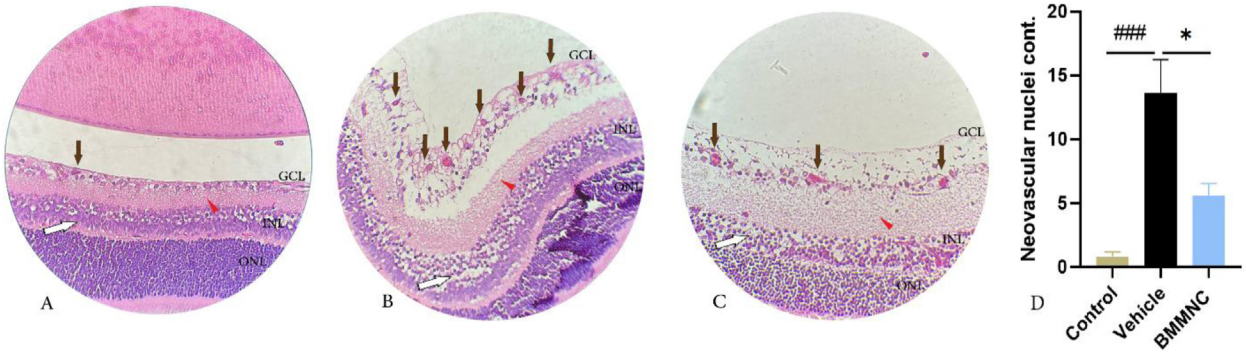

Fig. 7. Histopathology in three groups; (A): Control, (B): Vehicle Injection, (C): BMMNC Treatment. (A): normal vasculature of outer retina (black arrow), intact outer plexiform layer and outer nuclear layer (red and white arrows) note the well-organized layers. (B): numerus neovasculogenesis spots and engorged vessels (black arrows), dissociated inerplexiform layer (red arrow) and extensive hydropic degeneration and apoptosis in innernuclear layer (white arrow), note that disorganization of layers. (C): neovascular spots (black arrows), organized inerplexiform layer (red arrows), hydropic degeneration and apoptosis in innernuclear layers (white arrows), note the organization of layers (H&E × 40). (D): quantification of neovascular nuclei in control, vehicle injection and BMMNC treatment groups. n = 5 retina/group, Data presented. represent mean ± SEM. (*p < 0.05, ###p < 0.005). GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer.

In Fig. 4, GFP positive cells are visible on the inner surface of retina, which confirms BMMNC migration from vitreous to the retina.

3.2.2. Fundus examination and angiography

Fundus examination and angiography in the ROP group showed notable vascular tortuosity, papilledema, noneven boundaries, and non-polygonal engorged veins and arteries with different calibers (Fig. 5B). In comparison to sham group, BMMNC-treated eyes had fewer engorged vessels, the vessels had relatively same calibers, more prominent vessels were noted, and vascular radial branches were relatively straight with polygonal appearance. Also, neovascular tufts were rarely seen in treated eyes, no papilledema was seen in the treatment group, and the appearance of the optic disc was normal (Fig. 5C).

3.2.3. Electroretinography

BMMNC treatment elevated the response to stimuli of the ischemia-affected retinas in rat puppies (Fig. 6A). In the BMMNC treatment group compared to sham, B waves amplitudes significantly increased in −2 Log cd.s/m^2 and +3 Log cd.s/m^2 intensities in

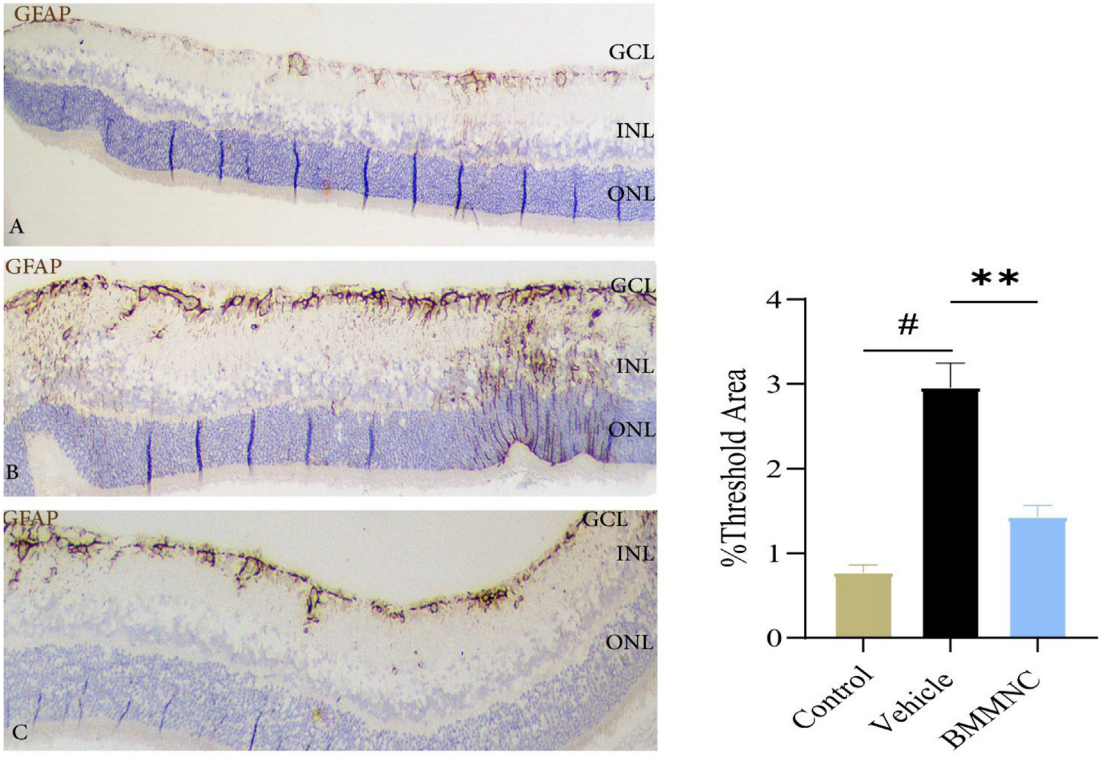

Fig. 8. GFAP immunohistochemistry in the retina sections in three groups; (A): Control, (B): Vehicle Injection, (C): BMMNC Treatment. (A); weak immunoreactivity of GFAP marker and normal distribution of glia cells in inner limiting membrane and GCL in healthy retina. (B); high expression of GFAP marker in all layers of retina indicates infiltration of glia cells from GCL through ONL and extensive gliosis in vehicle injection group. (C); moderate immunoreactivity of GFAP positive cells with limited infiltration to INL indicates low to moderate gliosis in BMMNC treatment retina (× 100). (D): quantification of GFAP expression in control, vehicle injection and BMMNC treatment groups. n = 5 retina/group, Data presented. represent mean ± SEM. (##p < 0.001, **p < 0.001). GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer.

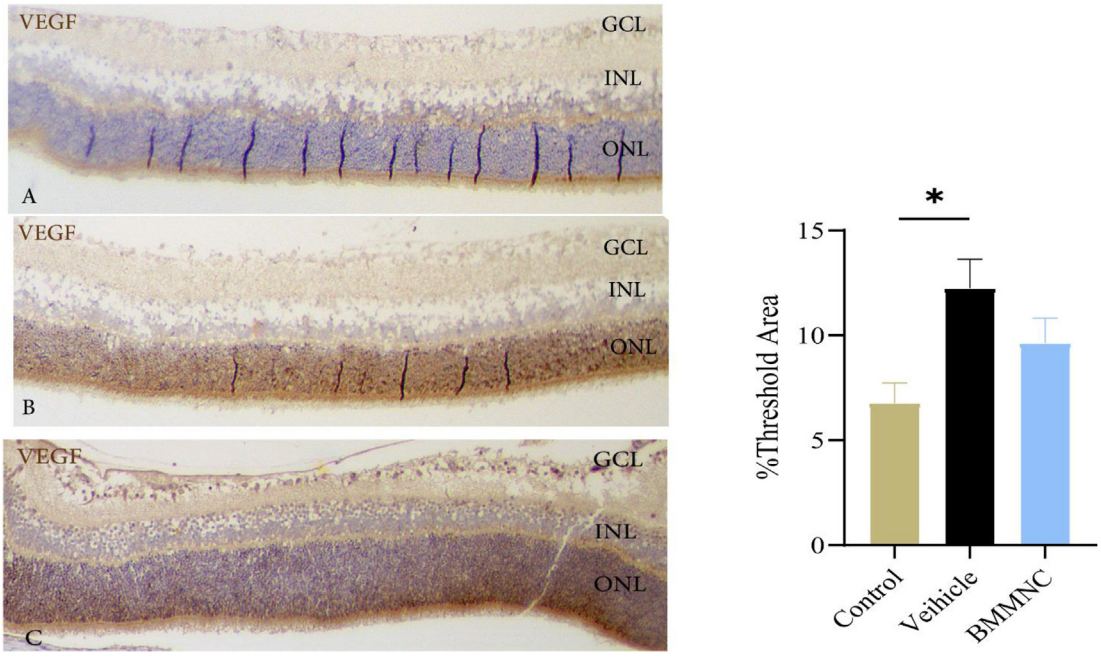

Fig. 9. VEGF immunohistochemistry in the retina sections in three groups; (A): Control, (B): Vehicle Injection, (C): BMMNC Treatment. (A); weak immunoreactivity of VEGF marker indicates low expression of VEGF in healthy retina, (B); high expression of VEGF marker, mostly in ONL, in vehicle injection group, (C); moderate immunoreactivity of VEGF marker in BMMNC treatment (×100). (D): quantification of VEGF expression in control, vehicle injection and BMMNC treatment groups. n = 5 retina/group, Data presented. represent mean ± SEM. (*p < 0.05). GCL: ganglion cell layer, INL: inner nuclear layer, ONL: outer nuclear layer.

scotopic and photopic conditions (P ≤ 0.05) (Fig. 6B, C). A waves amplitudes were reported to be lower in sham compared to BMMNC-treated group, and this difference is more prominent in photopic ERGs. However, these differences are not significant statistically (Fig. 6D, E).

3.2.4. Histopathology and immunohistochemistry

Inner and outer nuclear layers cell apoptosis and hydropic degenerations were relatively lower in BMMNC-treated eyes compared to vehicle-injected group. In the BMMNC treatment group, retinal layers remained organized and were not dissociated, unlike retinal layers in the untreated ROP group (Fig. 7B, C). Also, neovascular nuclei in the internal limiting surface of the treated retinas were significantly lower in number (P ≤ 0.05) (Fig. 4F). Immunohistochemichal results showed that retinas in BMMNC treatment group had significantly lower GFAP immunoreactivity comparing to vehicle injection group (p < 0.05) (Fig. 8). Although the VEGF expression was lower in BMMNC treatment eyes, but this difference was not significant (Fig. 9).

3.2.5. TUNEL assay

TUNEL assay revealed a significant decrease in number of apoptotic cells in the treatment group compared to sham (p < 0.05) (Fig. 10).

4. Discussion

In this study, we examined the hypothesis of whether intravitreal injection of fresh BMMNC can reduce neurological and vascular damages in retinal ischemia in an animal model of ROP. It showed

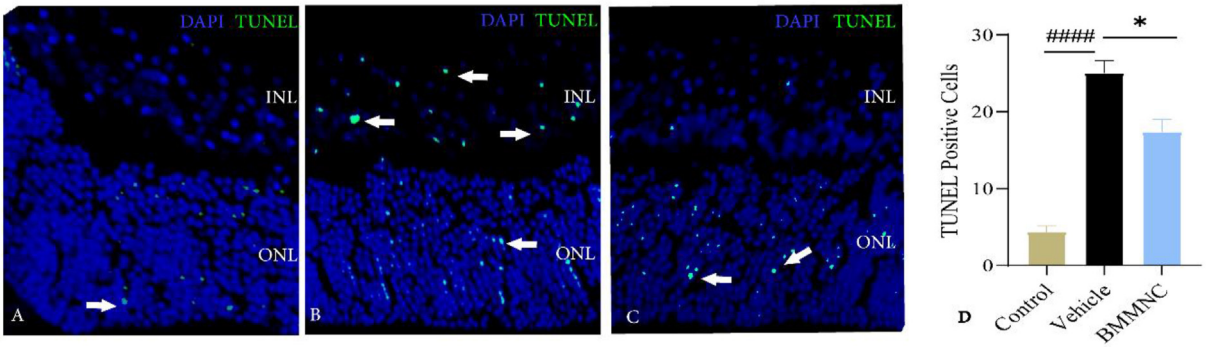

Fig. 10. TUNEL immunoassay on retina section in three groups; (A): Control, (B): Vehicle Injection, (C): BMMNC Treatment. Green stained cells indicate TUNEL positive cells and apoptosis (white flesh). (D): quantification of TUNEL-positive cells in control, vehicle injection and BMMNC treatment groups. n = 5 retina/group. Data presented. represent mean ± SEM. (####p < 0.0001, *p < 0.05). INL: inner nuclear layer, ONL: outer nuclear layer.

that this treatment protects ROP affected retinas from vascular obliteration and significantly suppresses neovascularization. Moreover, BMMNC therapy significantly improves response to visual stimuli in the neural retina, limits excessive gliosis and preserves optic disc from pathological changes, like papilledema. BMMNC transplantation also decreases retinal cell death in animal model of ROP.

HIF 1-alpha gene has established benefits in ROP treatment (Fig. 1). In recent years, scientists have been attracted to different lineages of bone marrow cells, since these cell express high levels of HIF 1-alpha gene. It is presumed that therapeutic effects of BMMNC is associated with high expression of this gene by its cellular composition [37]. Ritter et al. used myeloid progenitor cells, in a murine OIR model, revealing these cells differentiate into microglia and promote vascular repair [12]. In another study, they demonstrated that bone marrow mesenchymal cells reduce apoptosis in a rat model of retinopathy of prematurity [38]. Li Calzi et al. used a combination of human progenitor cells, including bone marrow-derived $CD34^+$ cells and vascular wall–derived endothelial colony-forming cells (ECFCs), to treat the murine OIR model. They reported that intravitreal injection of their cell combination may provide some protection to the neural retina [10].

Several studies have reported beneficial effects of intravitreal administration of whole BMMNC fraction in retinal injuries by downregulating GFAP expression. As demonstrated, intravitreal injection of the entire BMMNC fraction in a rat model of optic nerve crush, decreases gliosis and GFAP expression, which results in axonal outgrowth and increases retinal ganglion cell survival [28]. In other attempts to use whole BMMNC for retinal diseases, two studies reported that intravitreal and subretinal injections of BMMNC, decrease GFAP immunoreactivity and improve photoreceptor survival in models of retinal degeneration [39,40]. These studies suggest that treatment with BMMNC decreases retinal stress, which results in reduced gliosis and GFAP expression. These are in line with another study demonstrating that bone marrow derived $CD34^+$ cells reduce retinal oxidative stress in rat model of ROP by downregulating stress response genes [10]. Our study confirms previous results and shows that application of whole BMMNC fraction, decreases GFAP expression and downregulates inflammatory response in animal model of ROP.

There is a limited number of studies which evaluate VEGF expression in BMMNC-treated animal models of neural damages with different results. For example a study reported that transplantation of bone marrow stromal cells activates the endogenous cell expression of VEGF and VEGFR2 (VEGF receptor 2) in neural ischemic damage while, another study found no significant difference in VEGF levels between BMMNC treatment and other groups in a rat model of optic nerve crush injury [28,41]. In our experiment, BMMNC therapy downregulated VEGF expression, but no significant difference was detected between BMMNC-treated and -untreated groups.

A potential explanation for retinal function recovery after cell therapy with BMMNC is the reservation of rods, cones, and bipolar cells in the retina from cellular death. B waves in ERG originate from these cells. As we reported in histopathological and TUNEL assay results, hydropic degeneration and cell death in the neural retinal layer were reduced in the treatment group, and BMMNC saved the population of retinal cells. We presume that anti apoptotic effects of BMMNC treatment may be due to anti apoptotic properties of its cellular constituents, like bone marrow MSCs which reduce apoptosis of retinal cells in ROP rats by expression of NT-3 (neurotrophin-3) and CNTF (ciliary neurotrophic factor) [38]. Scotopic ERGs show rod-originated signals recovered after the treatment. Photopic ERGs with the saturation of rod photoreceptors revealed that cone-originated B waves also recovered. BMMNC treatment recovers retinal function by saving cone and rod photoreceptors in this disease. Di Pierdomenico et al. studied the effects of BMMNC on retinal degeneration. Although they demonstrated an increase in photoreceptor survival and a decrease in GFAP expression, they found no significant change in their ERG results. This discrepancy between their findings and our results can be explained with the differences in the pathophysiology of the diseases in the used models. In our rat model of ROP, ischemia is the main culprit, and the injection of BMMNCs prevents the injury to retinal neural cells (rods, cones, and bipolar cells). Meanwhile, in their rat model of inherited retinal degeneration, the intrinsic defect resulted in permanent cell death of retinal neural cells, and the injected BMMNCs could not penetrate the inner retinal layers to substitute the lost photoreceptors [39].

Collectively, we assume that neuro- and vaso-protective effects of BMMNC treatment is mainly due to downregulation of excessive gliosis, and prevention of apoptosis of retinal cells. Also, some studies demonstrate role of BMMNC-secreted trophic factors, cytokines and FGF-2 in treatment of different retinal injuries, but evaluation of these factors is beyond the scope of this study. Our recommendation for future studies is to evaluate proinflammatory cytokines, secretion of anti-inflammatory substances, upregulating anti-apoptotic genes and rebalancing stimulators and inhibitors (PEDF, HIF) [25,42]). Furthermore, it is essential to investigate the impact of varying cellular dosages of BMMNC on treatment outcomes to determine the most effective dose for optimal therapeutic effects.

5. Conclusion

The use of whole Bone marrow-derived mononuclear cells fraction can reduce neural-vascular damages and recover retinal function in a rat model of ROP. Not only BMMNC has the therapeutic effects of its cellular constituents, but also it is easy to obtain without the need of time-consuming and expensive procedures for sorting a specific cell line. Moreover, a clinical trial has shown that intravitreal injection of BMMNCs has an acceptable safety profile, and no detectable structural or functional toxicity was reported [43]. This treatment can be considered as a new therapeutic choice for ROP, which requires further investigations.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethical approval and consent to participate

All animal procedures in this study are carried out under the national animal care guidelines and approved by the Ethics committee of the children's medical center, Tehran, Iran.

Authors' contributions

Saman Behboodi Tanourlouee: Methodology, writing the proposal, Study design, Study performance (Animal, Cellular, udusphotography, ERG and Histopathology), writing the manuscript;

Mohammadreza Nasirzadeh: Conceptualization, Study design, Methodology, approval of the final manuscript, supervision; Masoumeh Majidi Zolbin: Methodology, Study performance (Cellular), supervision, approval of the final manuscript; Ashkan Azimzadeh: Study performance (Animal, Cellular), Methodology; Javad Fahanik Babaei: Study performance (ERG), approval of the final manuscript; Masoud Bitaraf: Study performance (Animal, Cellular), writing the manuscript, Editing the manuscript, approval of the final manuscript; Abdol-Mohammad Kajbafzadeh: Conceptualization, approval of the final manuscript, supervision; Shokoufeh Hassani: approval of the final manuscript. Ahmad Masoumi: approval of the final manuscript; Kayvan Mirnia: Conceptualization, Study design, Methodology, approval of the final manuscript, supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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