Special Focus, Neuroophthalmology

Therapeutic potential of human umbilical cord–derived mesenchymal stem cells transplantation in rats with optic nerve injury

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Purpose: There are no effective treatments currently available for optic nerve transection injuries. Stem cell therapy represents a feasible future treatment option. This study investigated the therapeutic potential of human umbilical cord–derived mesenchymal stem cell (hUC-MSC) transplantation in rats with optic nerve injury. **Methods:** Sprague–Dawley (SD) rats were divided into three groups: a no-treatment control group ($n = 6$), balanced salt solution (BSS) treatment group ($n = 6$), and hUC-MSCs treatment group ($n = 6$). Visual functions were assessed by flash visual evoked potential (fVEP) at baseline, Week 3, and Week 6 after optic nerve crush injury. Right eyes were enucleated after 6 weeks for histology. **Results:** The fVEP showed shortened latency delay and increased amplitude in the hUC-MSCs treated group compared with control and BSS groups. Higher cellular density was detected in the hUC-MSC treated group compared with the BSS and control groups. Co-localized expression of STEM 121 and anti-S100B antibody was observed in areas of higher nuclear density, both in the central and peripheral regions. **Conclusion:** Peribulbar transplantation of hUC-MSCs demonstrated cellular integration that can potentially preserve the optic nerve function with a significant shorter latency delay in fVEP and higher nuclear density on histology, and immunohistochemical studies observed cell migration particularly to the peripheral regions of the optic nerve.

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The human optic nerve is made up of the axons of 1.2 million retinal ganglion cells that mediate the electric signals from the retinal photoreceptors on receiving visual stimuli.[1] As with most central nervous system components, the optic nerve is susceptible to degeneration from genetic causes or damage from acquired causes due to the inexistence of spontaneous regeneration in neurons.[2]

Flash visual evoked potential (fVEP) is a noninvasive tool that measures the electrical signal conduction along the visual pathway.[3] The fVEP waveform represents several positive and negative deflections designed as P1, N1, P2, N2, and N3 peaks by Creel *et al*.[4] Any visual pathway abnormality will affect the appearance of the fVEP waveforms.[5]

In rodent models of optic neuritis, latency delay of the N1, P1, and N2 peaks was reported to have a strong correlation with the reduction of myelination of the optic nerve. Similarly, the magnitude of P1 peak, either measured from N1 or N2 peak, was reported to have a strong correlation with the neuronal cell density of the optic nerve.[6]

Human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) have been explored for potential cell-based therapies of various diseases such as ischemic stroke,[7] spinal cord injury,[8] Parkinson disease,[9] cardiovascular diseases,[10] myogenic disease,[11] and cornea-related diseases.[12]

This study aims to utilize an animal model of traumatic optic neuropathy by performing optic nerve crush injury in Sprague–Dawley (SD) rats to examine the therapeutic potential of treatment using hUC-MSC. The outcomes on optic nerve function are evaluated via fVEP and neuronal cell density following immunohistological analysis.

Methods

Animals

SD rats ($n = 18$; weighing between 300 and 350 g; age 10–14 weeks; Laboratory Animal Resource Unit, Kuala Lumpur, Malaysia) were used. All animals were maintained in an air-conditioned

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room with controlled temperature (21 ± 2 ℃), fixed daily 12-hour light/12-hour dark cycles, and in individually ventilated specific pathogen-free cages in an animal laboratory at the Tissue Engineering Centre, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia. The study was approved by the institutional Animal Ethics Committee (AEC; Approval number: FP/OFTAL/2012/MAE-LYNN/20-SEPT/462/JAN2013-DEC2014). All procedures involving animals were conducted in accordance with the guidelines drawn by the institutional AEC and conformed to the ARVO (The Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

The animals were allocated to three groups ($n = 6$ per group). Two control groups, namely, the negative control (Group A) and the sham control (Group B) were employed with the former receiving no treatment after the optic nerve injury and the latter receiving an injection of Hank's balanced saline solution (HBSS) into the peribulbar space of the right eyes. The treatment group (Group C) consisted of rats that received treatment injections of a suspension of hUC-MSCs cells into the peribulbar space of the right eyes.

Each animal was anesthetized with 0.1 mL/kg bodyweight of intramuscular injection of anesthesia regime containing 12.5 mg of tiletamine hydrochloride (Zoletil-50, Virbac Lab, France), 12.5 mL of xylazine hydrochloride (Xylazil-20, Tryo Lab, Australia), and 2.5 mL of ketamine hydrochloride (Bioketan, Vetoquinol Biowet, Poland). The fVEP was measured before optic nerve crush, 3 weeks after treatment, and 6 weeks after treatment. After 6 weeks, the rats were sacrificed for histological analysis [Fig. 1].

The rats were excluded when any complications that could interfere with vision were present. The complications included hemorrhage, endophthalmitis, or media opacities such as cataract. Rats unfit to undergo anesthesia, those with a gross physical abnormality, those with only one functional eye, those having flat or unrecordable optic nerve function on fVEP, or those that demonstrated histological evidence of postmortem degradation were also excluded.

Stem cell preparation

The hUC-MSC used were obtained from a local stem cell bank (CryocordTM, Malaysia), which prepares the stem cells in a good manufacturing practice–accredited laboratory. The cells were subjected to evaluation according to the International Society for Cellular Therapy criteria for mesenchymal stem cell. Additionally, they were also tested for differentiation capacity into neurons. Isolation of the mesenchymal stem cells has been described previously.[13] The method of obtaining and preparing the stem cells has also been described in detail by Leow *et al*.[14] in their 2015 article.

The cells were cultured in a medium (low glucose Dulbecco's modified Eagle's medium; 10% human serum; 100 U/mL penicillin; 100 µg/mL streptomycin; 0.25 µg/mL amphotericin; Gibco, USA). The cells were expanded until reaching an appropriate density at passages 4 to 5, and used throughout the experiment.

fVEP

Baseline visual function was evaluated by fVEP before crushing the optic nerve. Following the optic nerve crushing, fVEP was measured again at Week 3 and Week 6 after hUC-MSCs transplantation. The fVEP recording was done under full anesthesia.

ROLAND RETI-port (Roland Consult, Brandenburg, Germany) visual electrophysiology system with platinum needle electrodes was used. The recording needle electrode was placed at the rat's occipital tuberosity, and the reference needle electrode was inserted at the central frontal region, as shown in Fig. 2a. The ground needle electrode was placed at the animal's ear.

Full visual field white flash stimulation was applied, with a flash intensity of 3.93 cd/m^2 without background light and with a stimulation frequency of 2 Hz, a band pass width of 1 to 100 Hz, and at 20,000 × magnification. The time for each sampling was 25 ms, and the waveform was superimposed 50 times. The VEP for every rat was recorded three times at an interval of 10 minutes. Data were collected for latency at N1, P1, N2, and amplitude of N1-P1 and P1-N2 as depicted in Fig. 2b.

Optic nerve crush

Following measurement of baseline fVEP, the right eye optic nerve was crushed while the contralateral eye acted as a control. All operations were performed under full anesthesia. Prior to the procedure, 0.5% proparacaine hydrochloride (Alcon Laboratories, Fort Worth, USA) was applied to the eyes for topical anesthesia, and 5% povidone (Alcon Laboratories, Inc.) was used as an antiseptic to prevent infection.

The rats were placed in the lateral decubitus position during the procedure. A small incision was made under a binocular microscope with spring scissors over the temporal conjunctiva. Care was taken on the depth of incision to avoid cutting into the underlying musculature (lateral rectus, inferior rectus, and inferior oblique muscles). With microforceps, the edge of the conjunctiva next to the globe was grasped and retracted, rotating the globe nasally.

The exposed posterior aspect of the globe allows optic nerve visualization. Retrobulbar tissues were further retracted to expose the optic nerve. The optic nerve was clamped for 7 seconds using an atraumatic vascular clip (60 g microvascular clip, World Precision Instruments, FL, USA) 2.0 mm behind the eyeball to cause optic nerve injury. The vascular clip applied a constant and consistent force on the optic nerve. The clip was then released and removed after 7 seconds, thus allowing the eye to rotate back into place.

Maxitrol™ (neomycin and polymyxin B sulfates and dexamethasone, Novartis, Switzerland) eye ointment was used after the operation to avoid infection. The rats were then placed on a warm pad and monitored until they had fully recovered from anesthesia. Subsequently, rats were monitored and given Maxitrol™ eye drops every 6 hours, Maxitrol™ ointment at night, and analgesics (oral ibuprofen continuously in water, 15 mg/kg/day) for 1 week. The rats were monitored after the procedures for possible complications, including infection and bleeding.

Stem cell injection

After the right eye optic nerve crush was performed, treatment injections of hUC-MSC cells suspension into the peribulbar space of the right eyes were administered (1×10^7 cells, 100 µL per eye). The injection was performed using an insulin syringe (30G) through the inferotemporal quadrant, passing

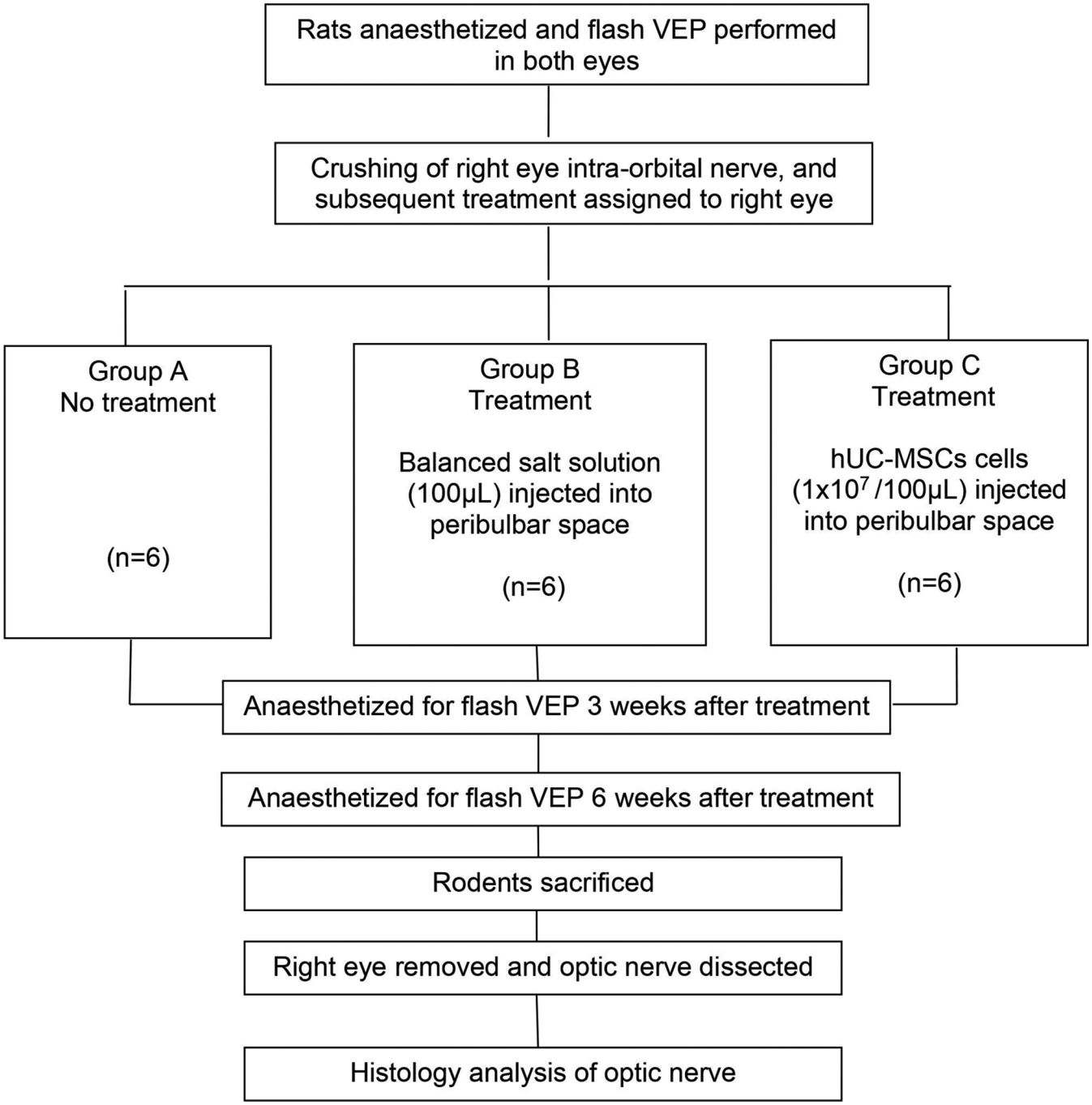

Figure 1: Flow chart of the study methodology

through the conjunctiva, between the lateral third and the medial two thirds of the inferior orbital edge. It was positioned almost parallel to the orbital floor and advanced to 8 to 10 mm in depth. Cyclosporine-A (Bioshop, Canada) was administered through drinking water (210 mg/L) resulting in a blood concentration of 250-300 µg/L, which was given 2 days before cell injection. This immunosuppressive agent that reduces rejection of the transplanted cells has to be given ahead of the transplant, because it does not work immediately and takes some time to work.

Animals with complications from the peribulbar injection such as hemorrhage, endophthalmitis, and cataract were excluded from the study. The hUC-MSCs were obtained from the same supplier in the same batch, and all peribulbar injections were carried out by the same researcher to minimize variability.

Histological analysis of the optic nerve

The rats were sacrificed after fVEP recording under anesthesia at Week 6 using a lethal dose of sodium pentobarbital (100 mg/kg bodyweight) given intraperitoneally. The eyes were enucleated with complete dissection of the optic nerve. The eyes were then immersed in 2% paraformaldehyde for 1 hour, infiltrated with sucrose. The eyeballs with about 5 to 7 mm of the optic nerve were harvested and embedded in a paraffin

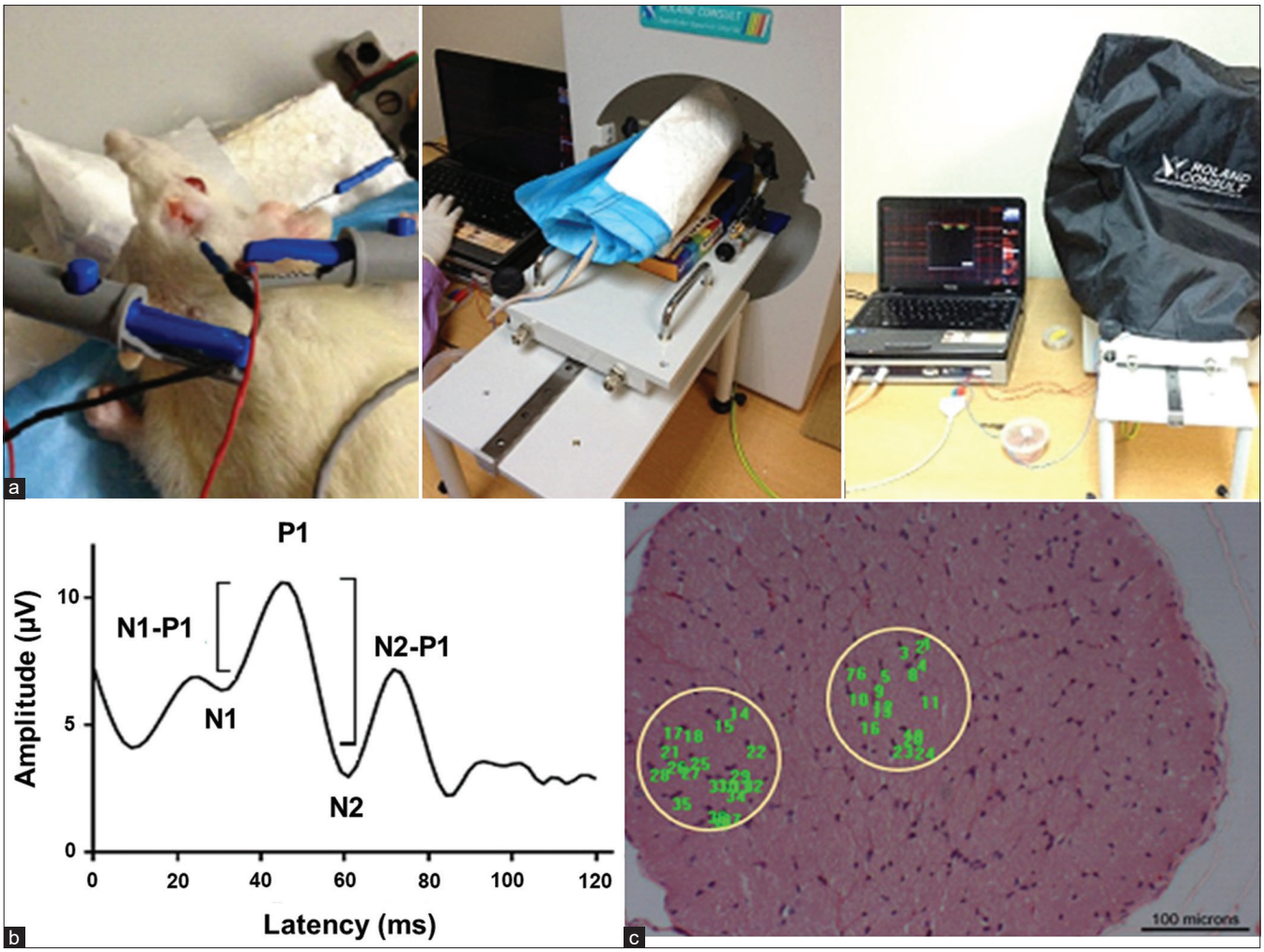

Figure 2: Methodology of the study.(a) fVEP setting: Electrodes (black wire, the recording electrode; red wire, the reference electrode and ground electrode placed at ear) placed with clip for stabilization; rat was covered with warm pad into the VEP machine; output was recorded through a laptop connected to the VEP machine. (b) Representative VEP tracing from a rat in stem cell treatment Group C at Week 0; parameters labeled as P1, N1, N2, N1-P1, and N2-P1. (c) Histology assessment method of cellular density

block for histology studies. Coronal sections were done with a microtome machine. The optic nerve was cut 2 mm away from the globe for a length of 1 mm. This portion of the optic nerve was then cut into multiple cross-sectional slices with a thickness of 3.0 μm before tissues were stained with hematoxylin and eosin (HE) for histological analysis.

Digital images were obtained at 10 × magnification using a light microscope (Olympus BX40; Olympus Optical Co. Ltd., Tokyo, Japan), and the histological examination of axonal density was done using software Q Capture Pro Version 5.1. Axonal density was calculated from the central region and the peripheral region of the optic nerve as shown in Fig. 2c.

Axons reviewed as nonviable had visibly long swollen axons, shrunken axons or axons with layer splitting of myelin sheaths, and fibrotic axons. Two different examiners counted the number of viable axons and compared it between the groups.

Immunohistological staining

A 4 μm thick tissue was fixed in 4% paraformaldehyde before being immersed in a preheated target retrieval solution and blocked with 10% goat serum to prevent nonspecific binding sites. Then the tissues were stained with primary mouse monoclonal antibody STEM121 (U.S. Stem Cell Inc., Florida, USA) specific for a human cytoplasmic marker to highlight the hUC-MSCs, and anti-S100 beta antibody (BD Biosciences, NJ, USA), which was a glial tissue-specific protein only expressed by a subtype of mature astrocytes. STEM121 specifically stains human protein, whereas anti-S100B antibody stains both human and rat proteins.

Primary antibody was detected by Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 488 (Life Technologies, USA) for anti-S100B antibody and Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 594 (Life Technologies) for STEM121. 4,6-Diamidino-2-phenylindole was used for nuclear staining. The sections were examined using fluorescence microscopy (Nikon, Japan).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 19.0; IBM, Chicago, IL, USA) presented as the median ± interquartile range (IQR). Kruskal–Wallis and

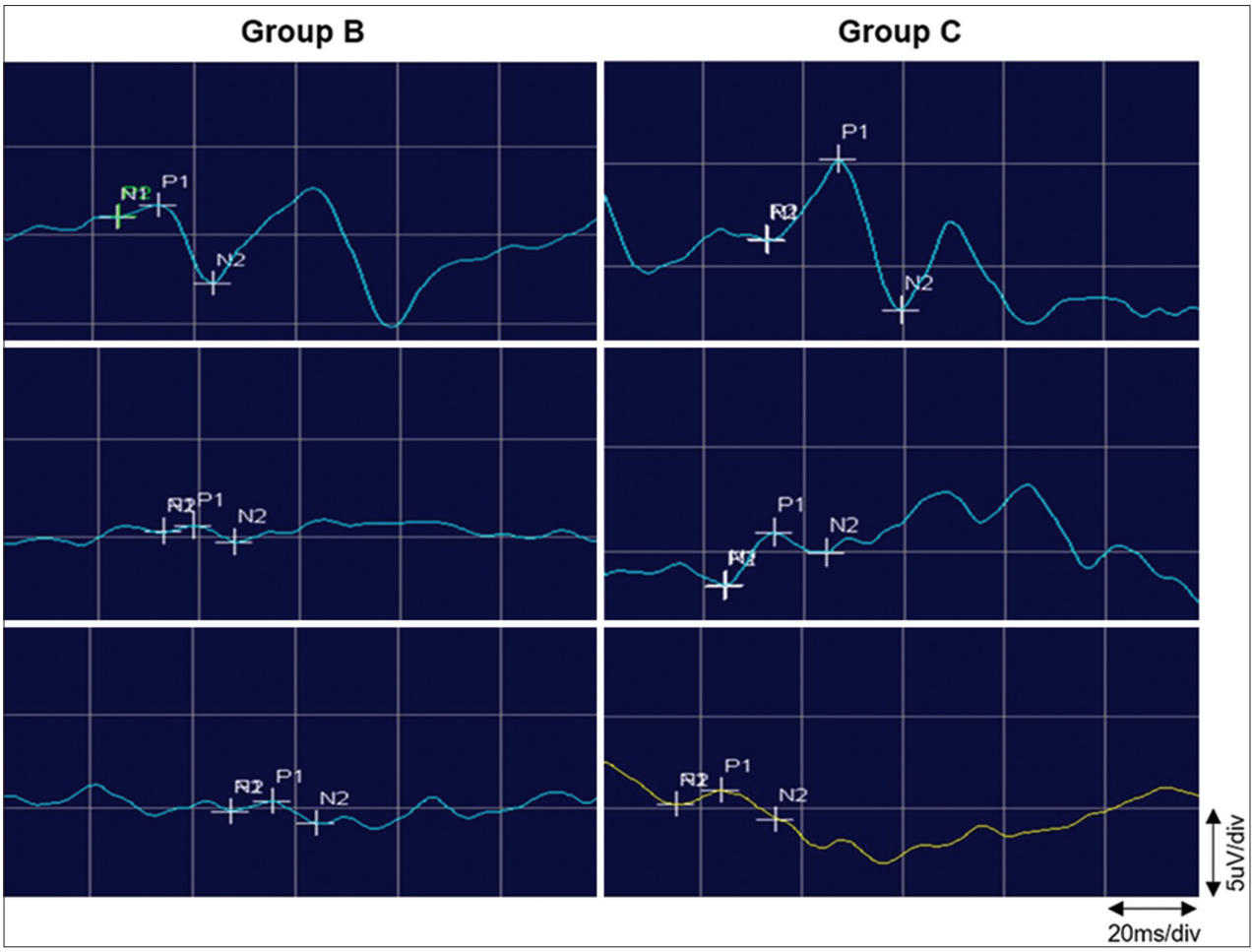

Figure 3: Example of VEPs representative in Group B and Group C rats

Mann–Whitney *U* test were used to compare the outcome between independent groups. A *P* value <0.05 indicated a statistically significant difference.

Results

Following optic nerve crush, all the rats from all groups survived without any presentation of infection or tumor growth. However, one rat from the sham control group and four rats from the stem cell treatment group were excluded from the analysis following the exclusion criteria outlined. Two rats were excluded from the stem cell treatment group due to the presentation of hemorrhage during the optic nerve crush procedure. One rat from the sham control group and two rats from the stem cell treatment group were excluded from the analysis for having histological evidence indicative of postmortem degradation. This includes extensive tissue swelling, fibrotic axons, and shrunken axons with layer splitting of myelin sheaths, which made them unsuitable for data analysis.

Fig. 3 shows a representative fVEP trace obtained from rats in the sham control group and the stem cell treatment group before the optic nerve crush, as well as 3 and 6 weeks after treatment. The fVEP traces from the negative control group were excluded from the analysis following a noticeable difference between its baseline fVEP parameter values compared with

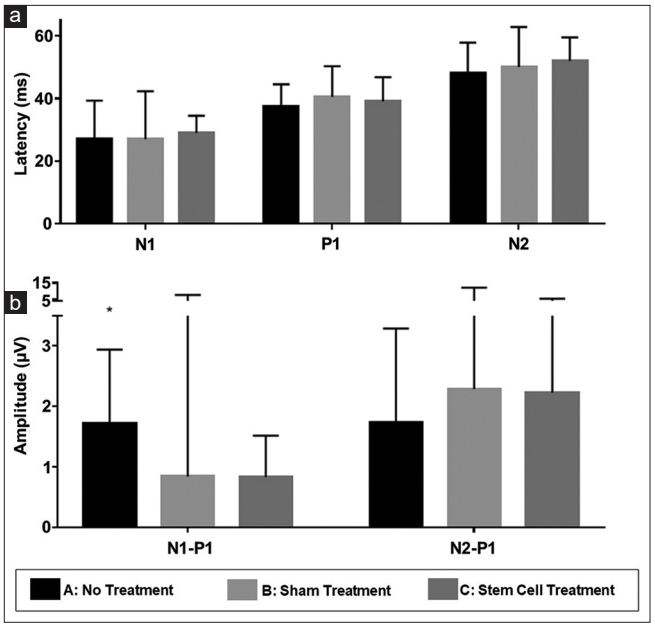

Figure 4: Comparison of baseline parameters of (a) latency of N1, P1, and N2; (b) amplitude N1-P1 and N2-P1 between no treatment control, sham treatment control, and stem cell groups. Kruskal–Wallis test revealed a significantly higher N1-P1 amplitude in no treatment group compared with the other two groups (*$P < 0.05$)

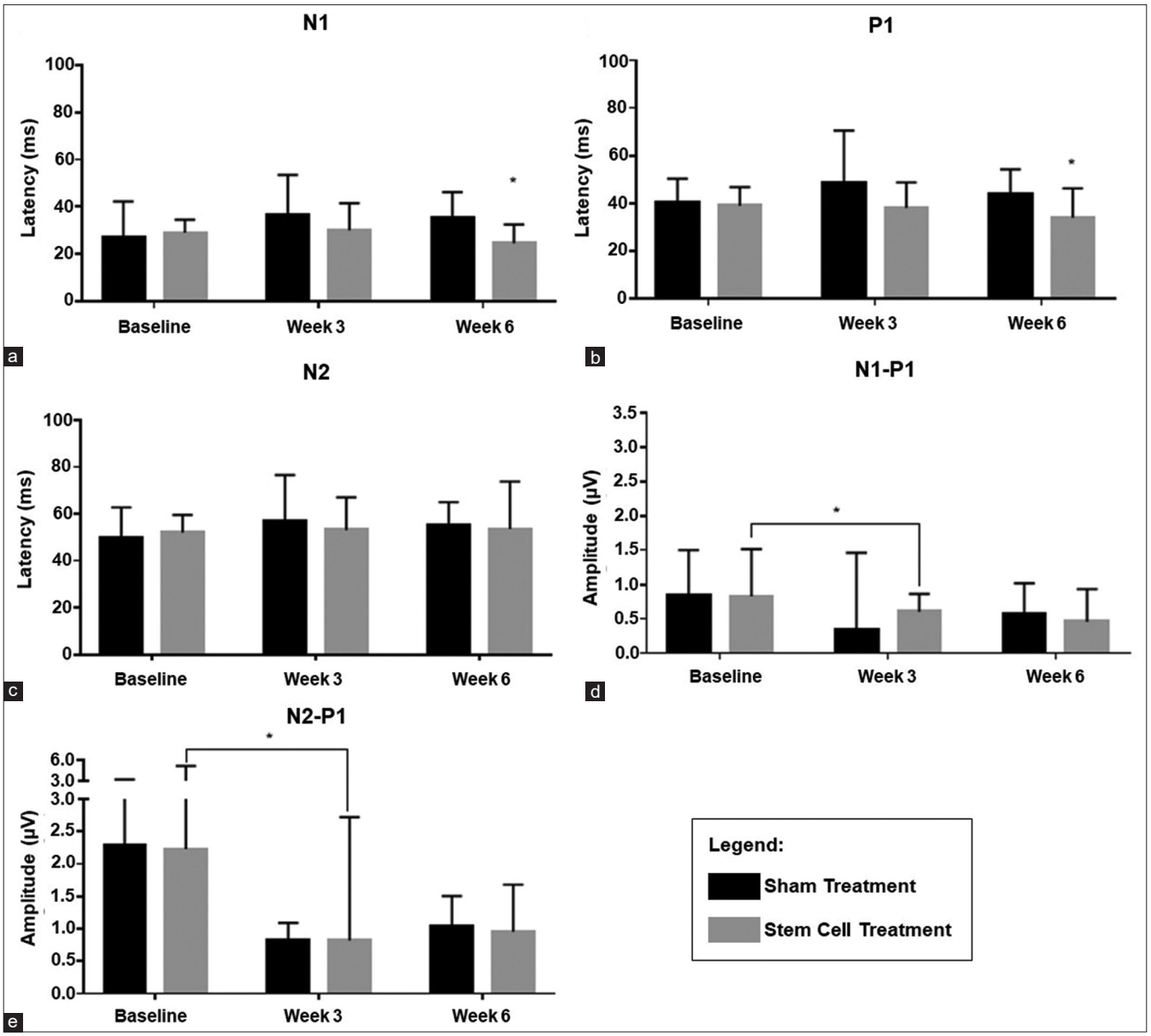

Figure 5: Comparison of latency parameters (a) N1, (b) P1, (c) N2, and amplitude parameters (d) N1-P1 and (e) N2-P1 between sham treatment control and stem cell groups at baseline, Week 3, and Week 6 posttreatment. Kruskal–Wallis test revealed significant differences in parameters of N1 at Week 6, P1 at Week 6 between the two groups, as well as N1-P1 and N2-P1 amplitude in stem cell group between Week 3 and baseline (*$P < 0.05$)

the rest of the experimental groups [Fig. 4]. In particular, the negative control group showed significantly higher N1-P1 amplitude compared with the other two groups ($P = 0.039$).

When latency values were plotted, statistically significant differences were noted in the latency parameters of N1 ($P = 0.015$) and P1 ($P = 0.041$) when comparing between the two groups at 6 weeks after treatment [Fig. 5a-c]. However, none of the differences in the latency values for N2 latency at 3 weeks after treatment or for all latency values at 6 weeks after treatment were statistically significant between the two groups.

In terms of amplitude, there was a significant deterioration of N1-P1 values in the stem cell treatment group ($P = 0.0036$) from baseline compared with after 3 weeks of treatment [Fig. 5d]. Alternatively, significant deterioration of N2-P1 amplitude values was observed in the sham control group ($P = 0.0036$) from baseline to 3 weeks after treatment [Fig. 5e].

Histological outcomes

Histopathology of optic nerve crush areas was observed with H and E. The specimens were found to be highly cellular on H and E staining. In the BSS treated group, as shown in Fig. 6a and b, there were more circumscribed areas of fibrotic axons and shrunken axons with splitting in the layers of myelin sheaths. These were areas of vacuolation and optic nerve edema with swollen axons. However, in the group treated with stem cells as shown in Fig. 6c and d, there was an increase in the cellular density within the injured axonal tissue, and at areas surrounding the injured optic nerve.

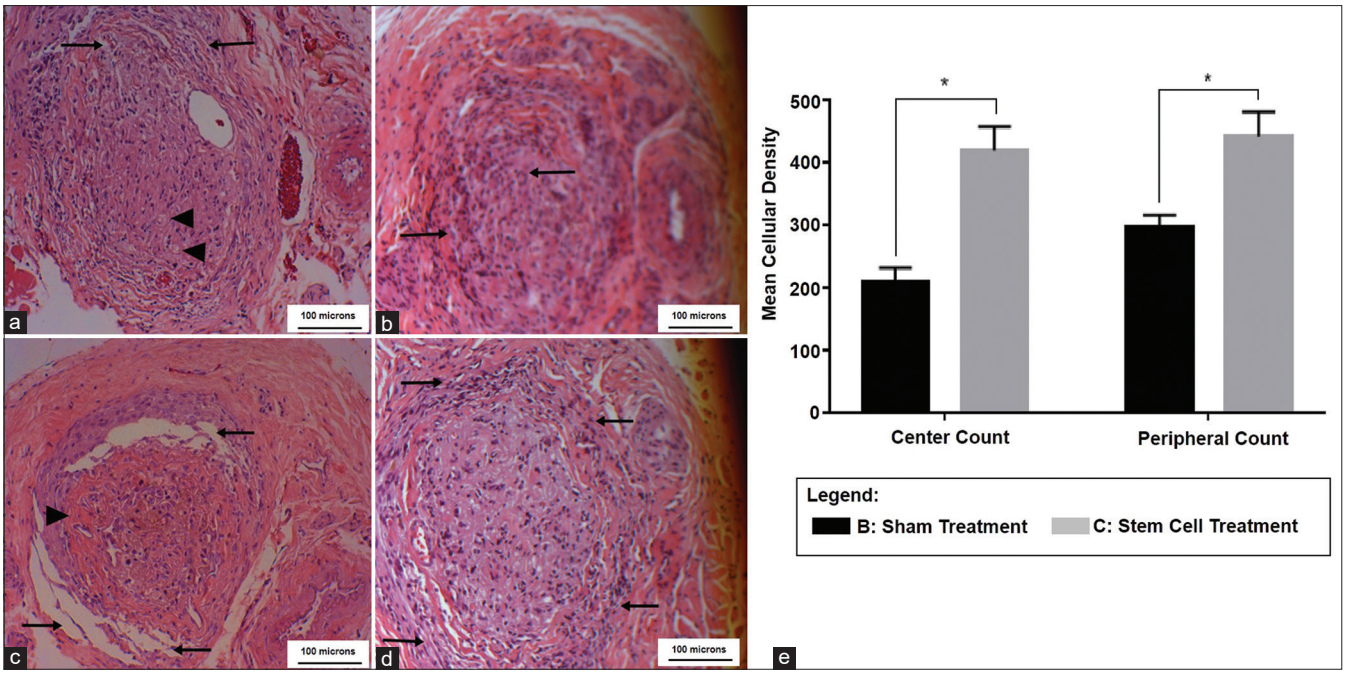

Figure 6: Cross-sectional histological images of optic nerve at 6 weeks after optic nerve crush injury (hematoxylin–eosin stain) of sham treatment group (a and b) and stem cell treatment group (c and d). (a) Circumscribed area of fibrotic axons (arrows). Areas of optic nerve swollen with vacuolation (arrowheads). (b) Shrunken axons with layer splitting of myelin sheath (arrows). Areas of optic nerve edema with swollen axons (arrowhead). (c) Narrow nerve fiber bundles with increased cellular density in the interaxonal tissue. (d) Increased cellular density surrounding optic nerve. (e) Central and peripheral areas cellular count in BSS treatment Group B and stem cells treatment Group C

Cellular density counts were lower in the BSS treatment Group B compared with the stem cell treatment Group C, with the representative images of each group with center and peripheral counts shown in Fig. 6e. The mean cellular density counts were higher at 418.6 cells at the center area and 440.6 cells at the peripheral area following optic nerve injury treatment in the stem cell treatment group. In comparison, the BSS treatment Group B had cellular density counts of 209.3 cells at the center area and 295.2 cells at the peripheral area. There is a statistically significant difference between BSS treatment group and stem cell treatment group in terms of center and peripheral mean cellular density counts ($P = 0.001$ and $P = 0.038$, respectively).

The fluorescence microscopy images shown in Fig. 7a depict the localization of hUC-MSCs at Week 6 within the injured optic nerve. The hUC-MSCs appear to have survived and migrated to the peripheral and central regions of the injured optic nerve. Higher hUC-MSC density was seen at the peripheral regions of optic nerve injury compared with the central region.

In terms of neural differentiated cells, anti-S100B stained cells were found to prominently reside in the peripheral regions of the optic nerve injury [Fig. 7b]. This suggested there had been a differentiation of hUC-MSCs into glial tissues and mature astrocytes, as indicated by the peripheral region having a higher density of hUC-MSCs compared with the central region.

Discussion

From this study, it was observed that the hUC-MSCs showed potential as a therapy for optic nerve injury in a rat model. This study has demonstrated significant functional improvement following hUC-MSC transplant on fVEP, in some parameters in latency, although the same was not observed in the amplitude of the response. Furthermore, hUC-MSCs showed better histological evidence of cell recovery than saline.

The effects observed could be attributed to the potential anti-inflammatory effects of hUC-MSC transplantation, whereby inflammatory responses that occur when there is an injury are being controlled. Hence, the reduction in inflammation that results aids in the repair process and further triggers cell repair and optic nerve regeneration.[6] Furthermore, the absence of significant reduction of fVEP parameters suggests that there is potential maintenance of visual function with stem cell treatment. However, further studies are warranted as longer follow-up, increased frequency of stem cell transplantation, and increased quantity of stem cells may optimize stem cell function in optic nerve regeneration.

Although the peribulbar route of administration chosen in this study has the advantage of direct implantation to the site of injury where the injured neurons are targeted and systemic spread was minimized, the dosage for injection of hUC-MSCs could still be inadequate. A higher dose could be needed as peribulbar transplantation of stem cells may have caused a distribution of the cells throughout the peribulbar space leading to a lower concentration in the subretinal space. Hence, repeated injections with transplantation of stem cells with longer follow-up duration could be done in the future studies to look for the optimal dosage needed for functional efficacy. Alternatively, future studies may consider a different site for stem cell delivery such as injection of stem cells directly into the optic nerve.

Second, the minimal changes in visual function observed could be due to fVEP in the study being not sensitive enough to detect the changes, and hence revealing inconsistent results. An

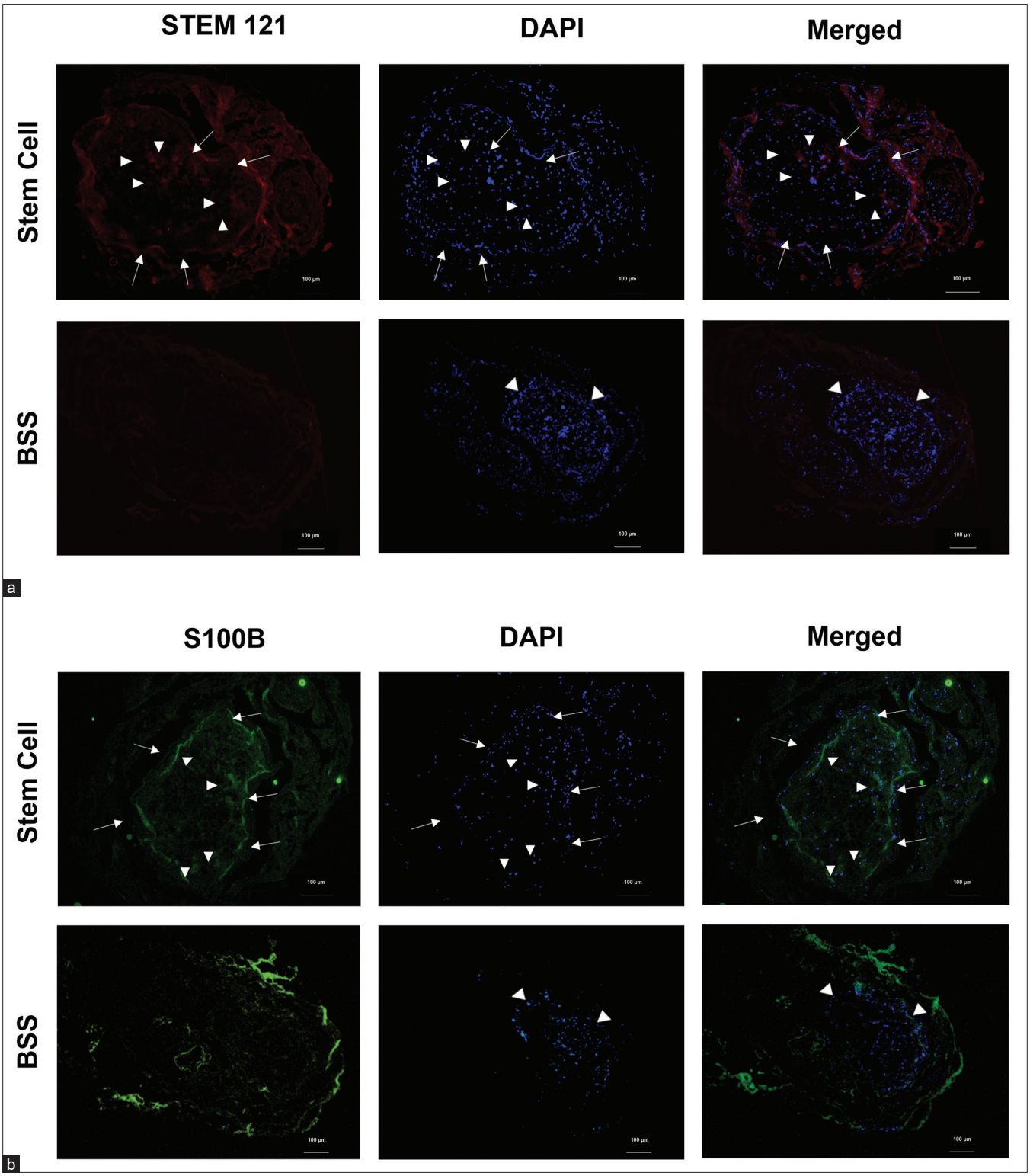

Figure 7: Fluorescence microscopy of (a) STEM121 at Week 6 in Group B and C. Scale bar represents 100 μm. STEM121 antibody marker (red) for mesenchymal stem cells and counterstained with DAPI (blue) to label the nucleus. Merged immunofluorescence image revealed co-localization of nuclei-positive cells, DAPI with STEM121. Arrows (peripheral) and arrowheads (center) indicate double-labeled cell areas. There was absence of STEM121 antibody expression in Group B. Labeled nuclei were seen to be more centrally located with some not expressing STEM121. (b) S100B at Week 6 in Groups B and C. Scale bar represents 100 μm. S100B antibody marker (green), which was glial specific, was used to stain mature astrocytes and counterstained with DAPI (blue) to label the nucleus. Merged immunofluorescence image revealed co-localization of nuclei-positive cells, DAPI with S100B antibody. Arrows (peripheral) and arrowheads (center) indicate double-labeled cell areas. Anti-S100B antibody expressed at the peripheral region. Labeled nuclei were seen to be more centrally located with some not expressing S100B

fVEP performed through mini-Ganzfeld and screw electrodes could give better and more persistent results as shown by You *et al*.[6] Besides, other electrophysiological tests associated with macula function such as pattern electroretinogram or multifocal electroretinogram that allow improved interpretation of an abnormal VEP could be done in the future studies. This would have allowed us to evaluate all the levels of electrophysiological response to determine the point at which pathology interfered with the signal. Unfortunately, due to cost and equipment issues, they could not be performed in this study.

Despite these limitations, histological analysis was able to demonstrate signs of early recovery. Still, the data were too small for statistical calculation. Stem cell treatment showed higher cellular density in the surrounding the optic nerve and intra-axonal regions, and improved optic nerve fibrosis, edema with vacuolation, and shrunken axons with layer splitting of the myelin sheath that were observed with HBSS treatment.

Detection of higher cellular density human STEM121 at the peripheral as well as central areas of optic nerve suggests that hUC-MSCs have the potential to differentiate into axon-like cells *in vivo*. Similarly, anti-S100B stained glial tissues and mature astrocytes were also found at higher cellular density in the peripheral and central regions. These findings further suggest that the hUC-MSCs have the potential to differentiate into axonal-like cells *in vivo*. Other studies have reported successful *in vitro* induction of hUC-MSCs into neurons and glia,[15] and retinal progenitor cells.[16] This showed the potential of hUC-MSCs to differentiate into different cell lineages including cells that constitute optic nerve.

Although this pilot study has many limitations, the potential of hUC-MSC as a cell therapy for optic nerve injury warrants further studies with a longer duration of follow-up that can demonstrate neuronal synapses with an improvement of visual function.

Conclusion

In conclusion, peribulbar transplantation of hUC-MSCs demonstrated cellular integration that can potentially preserve the optic nerve function as shown by the significantly shorter latency delay in fVEP, histological changes of higher nuclear density, and immunohistochemistry with STEM121 and anti-S100B antibody studies observed in areas of higher nuclear density, both in the central and peripheral regions of the optic nerve.

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Conflicts of interest

There are no conflicts of interest.

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