Multifocalelectroretinography Result before and after Peribulbar Injection of Allogeneic Umbilical Cord – Mesenchymal Stem Cell Secretome for Late-Stage Retinitis Pigmentosa

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Abstract

Background: Retinitis Pigmentosa (RP) is a rare retinal genetic disease without available treatment to date. Previous studies show growth factor and regenerative ability of secretome from cultured allogeneic umbilical chord mesenchymal stem cells (UC-MSC). This studyaimed to report multifocal electroretinography of retinal photoreceptor responsefrom RP patients, before and after injection of secretome from allogeneicUC-MSC.

Patients and Methods: Four subjects with severely damage retina (visual field defect of 25 %- 50% in initial Humphrey perimetry examination) were recruited and givenperibulbar injection of secretome from allogeneic cultured UC-MSC. Visual acuity, visual field examination, multifocal electroretinogram of retinal photoreceptor examination were observed before and periodically after injection until six months period

Results: Overall, we observed subtle changes in N1 and P1 amplitude and implicit time only in ring 1 at 1, 3 and 6 month post secretome injection.

Conclusion: Peribulbar injection of allogeneic UC-MSC secretome had very miminal influence on photoreceptor activity in late-stage RP patients.

Keyword*: Retinitis Pigmentosa, Secretome, Umbilical Chord, Allogeneic,Electroretinogram.*

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Introduction

Retinitis Pigmentosa (RP) is a retinal disease characterized by progressive peripheral retinal photoreceptors damagecausing blindness mostly in productive age. Mutation of genes, disruption of oxygen supply, and chronic inflammation of the Retinal Pigment Epithelium (RPE) are considered to be etiology of RP[1,2].

A study by Yoshida et al [3] found a significant level of inflammatory cytokines in vitreous humor and aqueous humor of RP patients. This inflammation was speculated to contribute to RPE damage and led to photoreceptor death[3]. However to date,despite extensive understanding about the pathophysiology of RP, there has been no definitive therapy for the disease[1,3]

Mesenchymal stem cells (MSC) have an ability to modulate immune system and suppress inflammatory cytokine activity.Previous evidence suggested that stem cells are also able to control immune response and produce microRNA (miRNA), Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Ciliary Body Neurotrophic Factor (CBNF) and other neurotrophic factors to revitalize the residual bipolar and photoreceptor cells in retina of RP patients. In support of this, more recent study using bone marrow autologous mesenchymal stem cell with intravitreal injection technique showed a temporary visual acuity improvement in a small group of RP patients [2,4], which opens a potential venue for RP therapy using stem cells.

Cultured umbilical cord-mesenchymal stem cells (UC-MSC) releases growth factors and genetic materials in its secretome which has a regenerative capability. Exosome found in the secretome, contains miRNA – a non-coding RNA, which contributes to epigenetic control. Several studies have suggested that miRNA may influences the epigenetic of cells to either silence or activate the genes[5].Taken together, we speculated that secretome taken from UC-MSCcontribute to influence mutated genes through modification of transcription, acetylation and methylation of histone which hopefully can improve metabolism as well as function of photoreceptors[5,6]. Nevertheless, none of previous studies have used UC-MSC in patients with RP.

We performed a preliminary study to observe the effect of UC-MSC secretome on patients with RP. In this case series, we investigated the changes in electrophysiology, as a proxy of sub-clinical photoreceptor activity, of peribulbar injection of secretome from UC-MSC for patients with RP. The rationale of this study are two folds: first, peribulbar has extensive capillary blood vessels, therefore is a good entrance to ocular's blood circulation[7]. Second, secretome is rich of growth factors and genetic materials which potentially incite regenerative process and epigenetic control to improve photoreceptor function in patients with RP and slow the progression of RP [8-10].

Material and Methods

Study Design and Patient Selection

We performed an interventional case series of 4 patients with confirmed RP based on their clinical history, symptoms, dilated fundus examinations, Humphrey visual field analyzer (HFA) and electroretinography (ERG) examination.The Study conducted in June 2018 – June 2019 at Dr. Sardjito General Hospital, Yogyakarta, Indonesia. Patients were selected from our existing RP registry. We only included patients with visual acuity better then 20/100 or cone receptor ERG amplitude more then 0,68 μV or with visual field wider then 10 º. This condition is better then floor effect which is the final stage of RP.We speculated that there will be photoreceptors still functioning in the retina. Written consent was obtained from each participant. This study was conducted in accordance to Declaration of Helsinki (1964). This study was registered in www.clinicaltrial.gov with identifier NCT04315025 and ethical approval was obtained from Medical and Health Research Ethics Committee, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada (KE/ FK/ 0616 / EC/2018).

The study was conducted at Public Hospital Dr. Sardjito, Yogyakarta. The stem cells isolation was performed at Prodia Stem Cell Indonesia (ProSTEM) Laboratory, Jakarta.

Stem Cell Isolation and Secretome Production

Isolation and Culture of Umbilical Cord Mesenchymal Stem Cell

Fresh Umbilical Cord (UC) tissue sample was obtained, minced into less then 5 cm pieces then stored into transport medium containing PBS with 1% antibiotic-antimycotic. To obtain a pellet of cells, initially the minced UC tissue was digested with sterile solution of 0.3% type I and/or II collagenase in 50 mL conical centrifuge tube. We subsequently added growth medium of approximately twice volume of collagenase solution and centrifuged at 500G for 5 mins. The tissue was then stored at 37°C for 1 hour. The supernatant

was removed, and remaining cells were resuspended in culture medium. The viability of cells can be assessed by using Trypan blue exclusion Culture cells T75 culture dishes in 10 ml of complete MSC medium at a density of 25 × 106 cells/ml. The plate of cells was incubated at 37 °C with 5% CO2 mixture in a moistened chamber until 3 hours. After removing the non-adherent cells that accumulate on the surface of the dish, then we left the remaining cells for 8 additional hours incubation for culture. Next, we replaced the medium with 10 ml of fresh complete medium. This procedure was frequently performed for every 8 hours until 72 hours from initial culture. Afterwards Cells were frozen in MSC growth media plus 10% DMSO at a density of 2x106 cells/vial [11,12].(Figure 1)

Collection of Conditioned Medium

We collected MSC at 4–5th passages and they were seeded with a density of 3 × 103 cells/cm^2 in Corning ® uncoated culture plastic. Cells were cultured to 70–80% density in 100 mm culture dishes. MSC bathed thoroughly 3 times using 10 mL of HBSS without Ca2+ and Mg2+, and recultured again with low glucose MSC DMEM (DMEM-LG, HyClone). Cells were cultured in a chamber moistened with 5% CO2 at 37°C for different time periods. Conditioned media samples containing secretome were collected and conitinue with centrifugation at 3000 rpm for 10 min at 4 ◦C to remove debris, then frozen in aliquots at −70ºC [11,12].

Marker Detection

MSCs population was successfully cultured with positive stained for CD 73, CD 90 and CD105. The secretome was then collected from the culture [12,13]. (Figure 1)

Clinical Examinations and Secretome Injection

Clinical evaluations were performed at pre-injection, 1, 3, and 6 month post-injection. Each evaluation included general eye examination, dilated fundus examination by a retinal specialist, visual field examination using Zeiss Humprey Field Analyzer 3 (Carl Zeiss Meditec AG, Jena, Germany), multifocal ERG (mf-ERG) examination using Metrovision MonPackOne (Metrovision, Perenchies, France), fundus photography and optical coherence tomography (OCT) using Zeiss Cirrus HD-OCT (Carl Zeiss Meditec AG, Jena, Germany). Best corrected visual acuity (BCVA) was measured with Snellen chart and converted to LogMar visual acuity value.

Electroretinography Examination

Mf-ERG examination was performed by a single trained operatorwith Metrovision® device. The examinationwas done alternately on each eye. Non-examined eye was patched with black, non-transparent eyepatch.We followed a standardized mfERG examination protocol by the International Society for Clinical Electrophysiology of Vision [14]. Brielfly, a centre forehead AgCl ground electrode, an outer canthus reference skin electrode and a Jett corneal electrode were placed. We used lubricant eye gel on the surface of Jett electrode that contact to the cornea. MfERG examination was performed on the fully dilated pupil and after adjustment of refractive error. All patients underwent a 20 minutes photopic and scotopic preadaptation. Sixty-one scaled hexagons were then displayed on a high resolution, black and white cathode ray tube (CRT) monitor with a frame rate of 75 Hz as the multifocal stimuli [14]. We recorded the amplitude (nV/deg2) and implicit times (ms) of the first order kernel responses (N1 and P1 waves). The result was grouped into five rings: ring 1 covers central to 2°; ring 2 between 2º –5°; ring 3: 5 º–10°; ring 4, 10 º–15°; ring 5 : wider then 15º.

Peribulbar secretome injection

All patients received 2 ml peribulbar secretome injection containing original growth factors, exosome, microRNA from cultured umbilical cord MSC extraction[15]. Only the worse eye was selected due to ethical consideration, determined from the visual acuity, fundus pathology, OCT and ERG examination before the injection. Peribulbar injection was done in a semi-sterile procedure room. Standard surgical septic-antiseptic and sterile eye draping procedures were carried out to ensure minimal risk of contamination, Peribulbar secretome injection was performed using a 26G injection needle under topical anesthesia. Patients evaluated for any infection, inflammation and increase of ocular pressure in the 1st and 7th day after injection.

Results

There were four patients recruited for this study. Table 1 showed the demographic data of the patients. Overall, we recorded subtle changes in N1 and P1 amplitude and implicit time in ring 1 for subject 1, 3 and 4 at 1, 3 and 6 month post secretome injection (Table 2). Subject 1 demonstrated decreased N1 amplitude within ring 1 at 1 month after injection, but subsequent increase after 3 months and6 month. In addition to N1 amplitude. There was a slight decrease in P1 amplitudealso within ring 1 at 1, 3 and 6 month after injection.Ring 2, 3, 4,5 showed flat patterns at each follow-up.(Table 2)

Subject 2 showed a very limited ring 1 electric activity of N1 amplitude before injection and stayed on a decreasing patternat 1, 3 and 6 months follow-up, then the mfERG trends ended as noise, similar to the implicit time. A different respond was shown by P1 amplitude, where a decreased of amplitude on the 1st month after injection followed by noise on the 3rd month after injection. Ring 2, 3, 4,5 showed a flat pattern.(Table 2)

Subject 3 ring 1 showed N1 wave detected noise before injection, until one month after injection. There was a slight electrical N1 activity seen at 3 month. P1 amplitude wave became noise at 1month and then re-appeared at 3 month which remained until 6 month. There was a small increase in P1 activity at 6 month. Ring 2, 3, 4,5 also showed flat pattern. (Table 2)

Subject 4 ring 1 showed a slight improvement of N1 and P1 amplitude at 1 month after injection which was relatively stable until 3 month. However at 6 month, both N1 and P1 amplitude was reduced. N1 and P1 implicit times after secretome injection were longer than before injection at all follow up examinations. Ring 2, 3, 4,5 showed flat pattern. (Table 2)

All patients showed very little visual acuity fluctuations. Most patients demonstrated an improvement at 6th month after secretome injection. Patients 1, 3 and 4 showed a increase visual acuity 6th month post injection, while patient 2 showed the same value before and after injection.(Table 2)

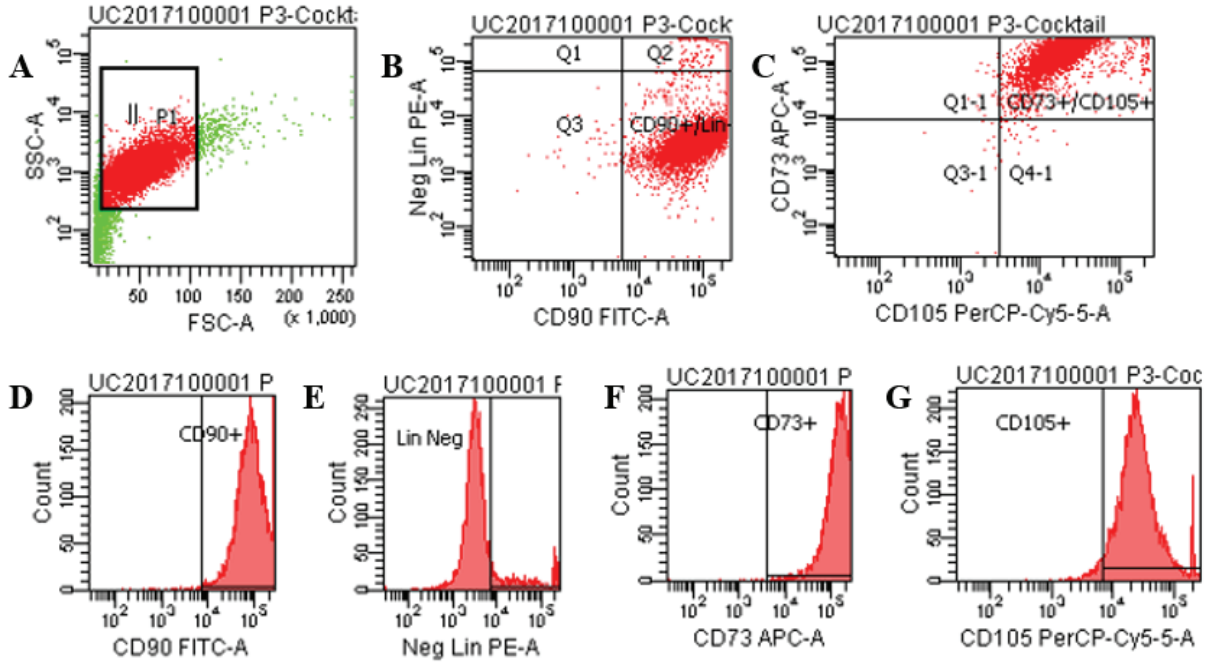

Figure 1. Mesenchymal stem cells staining

The cells were stained with specific antibody for MSC. (A) cells were initially displayed on SSC density plot (gate P1) which subgated onto identification (B) CD90 and Lin Neg (gate Q4) and (C) CD73 and CD105 (gate Q2-1). Cells positive for CD 90 FITC-A (D), Lin neg PE-A (E), CD 73 APC-A (F) and CD105 PerCP-Cy5-5-A (G)

Table 1. Patients' Demographic Data

Cases	Occupation	Injected eye	Age (y.o)	Visual Acuity in injected eye (logMAR)	IOP on Injected eye (mmHg)
Patient 1	Hospital staff	OD	31	0.5	8
Patient 2	Small village traders	OD	44	0.3	12
Patient 3	College students	OS	21	0.5	11
Patient 4	Retired	OS	51	0.7	13

IOP: Intra Ocular Pressure; y.o = years old; OD: oculus dexter; OS: oculus sinister.

Table 2. mfERG (N1. P1. and Implicit time) values on Ring 1.

Patient	Period	N1		P1		Visual Acquity
		Amplitude (nV)	Implicit time (ms)	Amplitude (nV)	Implicit time (ms)	LogMAR
1	Preinjection	-802	34.2	384	51.1	0.5
	Day 30	-162	17.6	379	50.6	0.5
	Day 90	-301	31.3	343	60.7	0.5
	Day 180	N	N	121	34.1	0.4
2	Preinjection	-176	17.2	195	57.2	0.3
	Day 30	-98.5	23.5	166	40.7	0.2
	Day 90	N	N	N	N	0.2
	Day 180	N	N	259	31.7	0.3
3	Preinjection	N	N	226	36	0.5
	Day 30	N	N	N	N	0.5
	Day 90	-28.1	50	143	63.5	0.5
	Day 180	N	N	273	43.2	0.4
4	Preinjection	-244	17.2	149	30.6	0.7
	Day 30	-341	25.1	410	48.9	0.3
	Day 90	-279	29.3	467	47.9	0.3
	Day 180	-61.1	33.3	214.0	50.4	0.2

N: noise,Normal visual acuity 6/6 = Logmar value 0

Discussion

In this study, we demonstrated subtle changes in N1 and P1 amplitude within ring 1 with prolonged implicit time, but these changes of retinal electrical activity shown by mfERG were only evident mostly within the first 3 months and did not showa specific pattern. Ring 2, 3, 4 and 5 remained flat in all follow-up period. These findings suggest that despite strong theoretical background of secretome as regenerative stimulator of photoreceptor cells, peribulbar secretome injection showed very little effect in patients with late-stage RP.

There is very little evidence in this area. A recently published study by Ozmert and associates reported an improvement of P1 amplitudes and implicit time in all rings aftersub-tenon Umbilical cord MSC injection in patient with RP[16]. Our findings with secretome only injection, however, did not show similar pattern of improvement. There are twothings that may causethis difference. Secretome alone is not sufficient to generate supporting effect to photoreceptors and variation ofretinal damage severity level in this 4 subjects may differ their respon to secretome[17]

Different stage of RP determines the amount of dying photoreceptor, thus require different amount of growth factor contained within secretome[1,3,18,19]. Cultured UC-MSC produces secretome which contain growth factor and exosome [20]. However, secretome alone only contain limited source of growth factor and exosome,when not combined with MSC. In our study, a mixture of secretome with nutrition rich conditioned media was injected to peribulbar space. Hypothetically, growth factor may activate dormant resident stem cell that lies beneath the RPE. This resident stem cell will start the regeneration process of the photoreceptor. Activated resident stem cell also send homing signal through the circulation for other stem cells from other reservoir marching in to target tissue[20,21]. This is the paracrine effect that expected to happen in secretome injection[21-23]. If stem cell was injected, the situation may be different. Stem cells will continuously produce growth factor and exosome required to repair the damage tissue[22,23]. In genetic disease like RP, revitalization of tissue will be more likely to happenthan regeneration. Mutated genes would likely initiate abnormal protein synthesis that leads to photoreceptor death but epigenetically MSC will influence the protein synthesis and support an optimal life of photoreceptor[23-25].

Strength of our study included meticulous preparation of UC-MSC and secretome, ensuring minimal contamination. However, there are few limitations noted in this study. First, RP is a rare genetic disease with no gold standard of treatment. Therefore, we were unable to provide comparison with other treatment group. Second, there were only very few patients recruited for this study because this is the first study to provide preliminary data on the effect of secretome treatment for patients with RP. Thus it is considered unethical to recruit a large number of patients when no prior beneficial or harmful effects are documented. Third, RP patients often come to visit ophthalmologist in the later stage of the disease when wide atrophy of photoreceptor already exist. It is hard to findgroup of patients with the same stage of photoreceptor damage because patients more likely unaware of peripheral visual field loss in early stage of the disease.

Conclusion

In this preliminary group of patients with late-stage RP, we demonstrated subtle changes and limited individual variation without any specific pattern of mfERG activity after peribulbar secretome injection, suggesting that peribulbar injection of allogeneic UC-MSC secretome may only haveminimal influence onphotoreceptor activity in late-stage RP patients. At the same time, our findings also suggested that there may be potential value of secretome injection in patients with RP, but the beneficial effects of secretome injection have not been well-concluded despite its strong theoretical foundation. Therefore, further studies with more subjects with less severe stage of RP are warranted.

Ethical Clearance: Ethical approval was obtained from Medical and Health Research Ethics Committee, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada (KE/ FK/ 0616 /EC/2018).

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Conflict of Interest: Bayu Winata Putera (B.W.P), Cynthia Retna Sartika (C.R.S), Andi Wijaya (A.W) are a stem cell researchers at Prodia Stem Cell Laboratory,

who involved in secretome production and stem cell isolation but has no involvement in the study design, data collection nor data analysis. None of other authors has any conflict of interest.

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