

1 **Type of article- Original research paper**

2 **A pilot, randomized sham control trial of autologous bone marrow derived**
3 **mononuclear CD34+ cells in acute ischemic central retinal vein occlusion**

4 **Summary**

5 In this pilot, sham controlled randomized control trial (RCT) in patients with ischemic central
6 retinal vein occlusion (CRVO) we studied the safety and efficacy of intravitreal injection of
7 autologous bone marrow derived mononuclear CD-34+ cells and found that both patients
8 who received stem cell injections did not develop anterior segment neovascularization at 1
9 year follow up. Except for some sterile inflammatory reaction in the initial follow up, no long
10 term injection related serious adverse events (SAEs) were observed . Based on our
11 observations we recommend a larger, multicentric study to further establish the safety and
12 efficacy of this treatment in patients with ischemic CRVO.

13 **Abstract**

14 **Purpose:** To study the safety and efficacy of autologous bone marrow derived mononuclear
15 cells injected intravitreally in patients with ischemic CRVO

16 **Study design:** Randomized sham controlled trial

17 **Method:** 4 cases with ischemic CRVO were recruited into the study. 2 cases were
18 randomized into intervention group and 2 into control group. After baseline investigations
19 including best corrected visual acuity (BCVA), intra ocular pressure (IOP), fundus
20 fluorescein angiography (FFA) and optical coherence tomography (OCT). Patients in the
21 intervention group received intravitreal injection of autologous bone marrow derived stem

22 cells and in control group received sham injection. Patients were followed up over a 12
23 month period.

24 **Main outcome measures:** Development of anterior segment neovascularization.

25 **Results:** Both patients in the intervention group did not develop anterior segment
26 neovascularization over a follow up period of 12 months. 1 patient in control group
27 developed neovascularization of iris and raised intra ocular pressure over a follow up period
28 of 6 weeks and required trabeculectomy for control of IOP. The other patient in control group
29 lost follow up after 2 weeks.

30 **Conclusions:** Our initial observations suggest that intravitreal injection of mononuclear cells
31 may reduce the risk of developing anterior segment neovascularization in patients with
32 ischemic central retinal vein occlusion. A larger, multicentric study would be valuable to gain
33 further evidence to our preliminary observations.

34 **Key words**

35 Stem cells, mononuclear cell, CD-34 cells, vascular occlusion, retina, intravitreal injection,
36 neovascular glaucoma

37 **INTRODUCTION**

38 Retinal vein occlusion is the most common vascular cause of visual loss after diabetic
39 retinopathy.¹ Central retinal vein occlusion (CRVO) is the occlusion of central retinal vein at
40 or just behind the lamina cribrosa.²⁻⁴ Based on the studies of May and colleagues CRVO is
41 classified as ischemic CRVO and non-ischemic CRVO. Ischemic CRVO is defined as central
42 retinal vein occlusion characterized by more than 10 disc areas of retinal non perfusion or
43 more than 50% of capillary non perfusion areas in a 30 degree fundus photograph by fundus
44 fluorescein angiography.⁵ Major complications of ischemic CRVO are macular edema and

45 anterior segment neovascularization with subsequent development of neovascular glaucoma.
46 Treatment options available for the management of ischemic CRVO include pan retinal
47 photocoagulation, intravitreal anti-VEGF (vascular endothelial growth factor) agents and
48 corticosteroids. CRVO study has shown that pan retinal photocoagulation causes regression
49 of neovascularisation in 56% of cases if instituted after early evidence of NVI. But neither
50 pan retinal photocoagulation nor macular grid can help in improvement of vision.⁶ RAVE
51 trial (Rubeosis Anti-VEGF trial) has shown that after intravitreal injection of ranibizumab
52 monthly for 9 months, at 6 months of follow up 90% of cases had resolution of macular
53 edema, 60% of cases had improvement in visual acuity by four lines and none of the patients
54 developed neovascularization of iris. At 3 years of follow up however, patients had
55 deterioration of visual acuity and 30% of cases developed neovascular glaucoma.⁷ So far
56 there is no established treatment algorithm for ischemic CRVO.

57 Studies have shown that injection of autologous bone marrow derived mononuclear stem
58 cells in ischemic stroke results in axonal plasticity and functional recovery in both
59 experimental models and patients.⁸⁻¹⁰ The neuro-protective effect of stem cells is presumably
60 due to expression of neurotrophic factors like insulin like growth factor, basic fibroblast
61 growth factor and epidermal growth factor which rescue the injured neuron. Similarly,
62 intravitreal injection of mononuclear bone marrow derived stem cells in animal model of
63 retinal ischemia have shown reduction in development of pre-retinal neovascular tufts.¹¹

64 **Methods**

65 Approval was obtained from institutional committee for stem cell research and therapy (letter
66 enclosed) and institute ethics committee (IESC/T-448/30.11.2012), AIIMS. Patients were
67 recruited from the Retina clinic services at Dr. Rajendra Prasad Centre for Ophthalmic
68 Sciences, A.I.I.M.S (All India Institute of Medical Sciences). They were evaluated by both

69 retina (PV) and glaucoma specialists (RS). Bone marrow aspiration was carried out by
70 specialists in the department of hematology (TS). Stem cell separation was performed in the
71 Department of Stem cell facility and the final product for injection was provided by the
72 faculty incharge of this facility (SM). Four cases with ischemic CRVO confirmed by fundus
73 fluorescein angiography without evidence of anterior segment neovascularization or
74 glaucoma or other concurrent ocular pathology such as cataract or diabetic retinopathy were
75 recruited into the study. All four patients were adults with two of them having hypertension
76 as a risk factor for the development of CRVO. Two patients were of the male gender. By
77 simple randomization using enclosed chits, two cases were enrolled into intervention group
78 and two others into study group. All patients underwent a thorough ocular examination
79 including measurement of best corrected Snellen visual acuity and intra ocular pressure (by
80 Goldmann applanation tonometry), slit lamp biomicroscopy for anterior segment evaluation
81 and Goldmann single mirror gonioscopy was performed to rule out anterior chamber
82 neovascularization. All patients also underwent fundus fluorescein angiography (Carl Zeiss
83 Meditec AG, FF450 plus Fundus Camera), to note the retinal perfusion status and SD-OCT
84 (Carl Zeiss Meditec AG, Cirrus 500 HD OCT) to note central macular thickness. Retinal
85 ischemia was more than 70% in all four cases.

86 Patients in intervention group underwent bone marrow aspiration by a standard technique. In
87 lateral decubitus position, skin over the iliac bone was cleaned with antiseptic solution and
88 draped. Skin and soft tissue down to periosteum was infiltrated with local anaesthetic 1%
89 lignocaine with 1:1000 adrenaline. Approximately 40 ml of bone marrow was aspirated with
90 15G bone marrow aspiration needle from posterior superior iliac spine. Patients in control
91 group underwent sham bone marrow aspiration in which patients were positioned, parts
92 cleaned and draped, and the skin over the posterior superior iliac spine was pressed with hub
93 of syringe (and no needle) to produce sensation of pain.

94 Bone marrow aspirate was immediately transferred to the Department of stem cell facility.
95 Bone marrow mononuclear cell layer was separated by Ficoll density separation method.
96 Bone marrow sample was layered over lymphocyte separation medium (Bio Whittaker) and
97 centrifuged at a speed of 1500 rpm for 25 min. Mononuclear cells were aspirated and washed
98 thrice in heparinized normal saline to remove the traces of Ficoll. All the procedures were
99 done under strict aseptic condition. The harvested mononuclear cells were evaluated for
100 viability, CD 34+ count, total count, morphology and Giemsa staining.

101 Using flow cytometry, the mononuclear cells were characterized using the following
102 antibodies- CD-34, CD-45, CD-3, CD-4 and CD-8 (all antibodies from BD PharMingen).
103 During flow cytometry, approximately 0.5 million mononuclear cells were stained with the
104 above antibodies at 4 degree Celsius for 30 minutes. Isotope controls were also stained in
105 parallel. Analysis was done using FACS LSR-II and FACS DIVA (BD Biosciences)
106 software.¹² Total cell count was done by counting in the Neubaur under microscope. Cell
107 viability was identified by using trypan blue dye exclusion test. More than 90% viability was
108 considered as acceptable. Giemsa stain was used to assess cell morphology.

109 Intravitreal injection was given within 2 hours of mononuclear cell isolation. Standard
110 procedure was followed for intravitreal injection. In brief, pupil was dilated with Tropicamide
111 1% drops and ocular surface was anaesthetized using Proparacaine 0.5% drops. Eye was
112 cleaned with povidone iodine and draped. Bone marrow derived cell preparation of 0.09 ml
113 (containing 6-8 million stem cells) and 0.01 ml of triamcinolone acetone (containing 0.04
114 mg of the drug) was drawn into a tuberculin syringe. The combination was injected at a
115 distance of 3.5-4.0 mm from limbus with a 26G needle in inferotemporal quadrant. Anterior
116 chamber paracentesis was done to normalize intraocular pressure intraoperatively. For
117 patients in control group, a sham injection was performed; eye was cleaned, draped and the
118 globe was pressed with the syringe hub to produce sensation of discomfort. Postoperatively

all patients were prescribed Moxifloxacin 0.5% eye drops to be used four times daily for one week. Patients in the intervention group received only one injection of autologous bone marrow stem cells. None of the patients received any additional intravitreal injections like bevacizumab, ranibizumab or triamcinolone. Also, no periocular injection of corticosteroids was used in the follow up period.

Patients were followed up over a period of 12 months at 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks, 6 months and 12 months. At each visit we recorded best corrected visual acuity, and intra ocular pressure. Slit lamp biomicroscopy for evidence of intra ocular inflammation, and iris neovascularization along with gonioscopy for angle neovascularization was also performed. SD-OCT was done in every follow up while fundus fluorescein angiography was undertaken only at week 4 and week 24. For detailed summary of patient baseline and follow up results please refer to supplementary data sheet.

Results

Case 1 (Intervention group): 64 year old female with symptoms of 12 week duration with baseline BCVA of 3/60 Snellen equivalent and presence of epi macular membrane with history of hypertension. On first postoperative day patient had central vitreous haze which persisted till 4 weeks. It soon cleared spontaneously without the need for any additional therapy. At the end of 12 months patient had no evidence of anterior segment neovascularization with BCVA of 6/60 and pseudohole with pre-existing epimacular membrane. Intraocular pressure remained normal throughout the follow up.

Case 2 (intervention group): 41 year old female with symptoms of 2 weeks duration with baseline BCVA of 1/60 Snellen equivalent and central macular thickness of 764 μ with no systemic risk factors. On first post-operative day there was 4+ cells in AC which resolved by 2 weeks with topical prednisolone acetate. At 6 months follow up there was no evidence of

anterior segment neovascularization and patient had a BCVA of 6/12 Snellen equivalent and central macular thickness of 262 μ . (Figure. 1) Intraocular pressure was within the normal range at all follow ups.

Case 3 (Sham group): 74 year old male with symptoms of 12 week duration with hypertension with baseline visual acuity of 1/60 Snellen equivalent and central macular thickness of 1151 μ . There was no evidence of anterior segment neovascularization on 4 week follow up but patient presented at 6 week with complaints of ocular pain. Intra ocular pressure was recorded to be 42 mm of Hg on Goldmann applanation tonometer. There was no evidence of NVI but gonioscopy revealed presence of NVA. Patient underwent pan retinal photocoagulation. Trabeculectomy with 0.02% mitomycin c was done to control intra ocular pressure as adequate control was not achieved with medical measures.

Case 4 (sham group): 70 year old male with baseline BCVA of 1/60 Snellen equivalent with central macular thickness of 760 μ and history of 10 weeks. Patient was lost to follow up after 2 weeks. At 2 weeks there was no evidence of anterior segment neovascularization and intraocular pressure was normal.

Discussion

Ischemic CRVO is a major cause of neovascular glaucoma. Pan retinal photocoagulation after the development of significant anterior chamber neovascularization involving 2 clock hours of iris is the current standard of care. Intravitreal injection of bone marrow derived stem cells has shown some benefit in mouse models of inherited retinal degenerations and retinitis pigmentosa.^{13, 14} But these are chronic disease processes and an end point cannot be determined. In contrast CRVO is an acute event, involving inner retina and an end point can be determined. Human studies involving intravenous injection of autologous bone marrow

166 derived stem cells have been done in patients with ischemic stroke which is also an acute
167 event and of vascular origin.

168 Both patients who received intravitreal injection of mononuclear cells had minimal
169 intraocular inflammation in the first week which resolved without any complication. So it
170 gives a little evidence that the risk of severe intraocular inflammation after intravitreal
171 injection of stem cells is less likely. The immediate post-injection sterile reaction was well
172 controlled due to concurrent injection of a microdose of triamcinolone. Thus the combined
173 dose of 0.09mL of autologous bone marrow stem cells and 0.01mL of triamcinolone was
174 found to be safe and well tolerated.

175 In both patients in intervention group, anterior segment neovascularization did not develop
176 over a follow up period of 12 months. The best result was observed in case 2 in whom the
177 injection was given within 2 weeks of developing symptoms. Visual acuity in this patient
178 improved from 1/60 to 6/12. This suggests that early intervention with stem cells may be able
179 to aid better functional recovery. Better visual recovery following early intervention has also
180 been observed following intravitreal anti-VEGF injection.¹⁵ In animal models of stroke and
181 some initial stem cell trial in patients with neuronal stroke too, it is believed that early
182 intervention with stem cells is likely to improve functional recovery.¹⁶

183 An important limitation of our study is the small sample size owing to which it would be
184 difficult to ascertain if the absence/ presence of anterior segment neovascularization was a
185 result of the known natural history of CRVO.¹⁷

186 **Conclusion** In this pilot study we found that a single intravitreal injection of autologous bone
187 marrow derived mononuclear cd-34+ cells produces an early sterile reaction. However, in the
188 long term it was well tolerated and may help to reduce the risk of anterior segment

neovascularization in patients with acute onset CRVO. Multicentric and large clinical trials are suggested to add further evidence to our initial observations.

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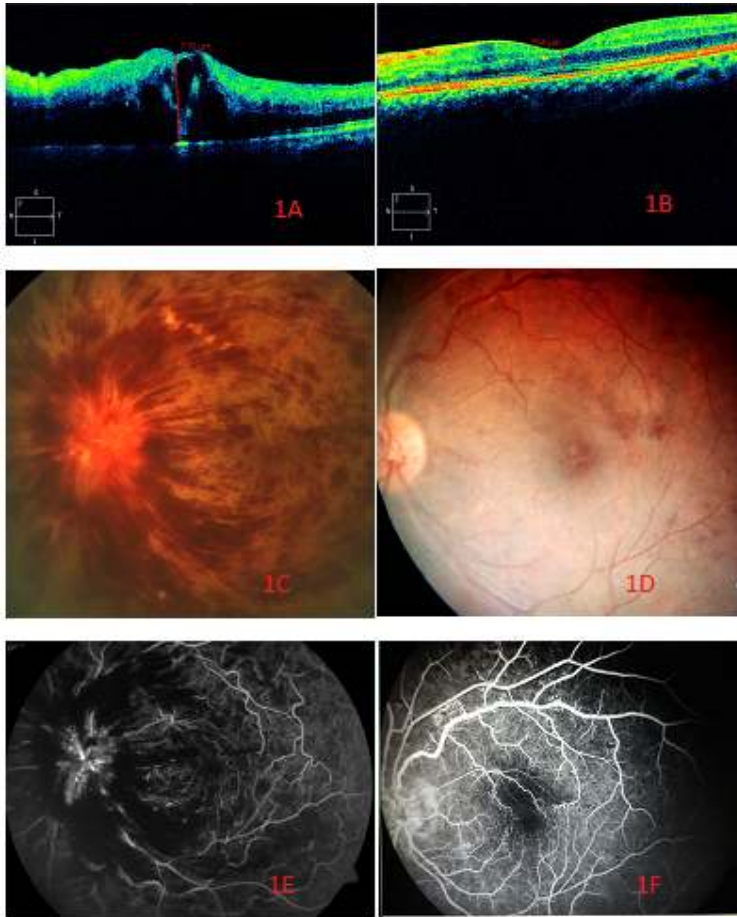
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249 **Figure legend**

250 Figure 1: 1A- Pre intervention SD-OCT showing macular edema with cystic spaces. 1B-
251 4 week post intervention SD-OCT showing resolution of macular edema. 1C- Pre-
252 intervention fundus photograph. 1D- 4 week post-intervention fundus photograph. 1E-
253 Pre intervention fundus fluorescein angiography. 1F- 4 week post-intervention fundus
254 fluorescein angiography.

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256

257

figure-1