·Clinical Research·

Aqueous levels of erythropoietin in acute retinal vein occlusion with macular edema

Hyun Jin Shin [1], Hyung Chan Kim [1], Jun Woong Moon [2]

[1]Department of Ophthalmology, Konkuk University Medical Center, Konkuk University School of Medicine, Seoul 143-729, Korea
[2]Gongdeok Seoul Eye Clinic, Seoul 121-706, Korea
Corrspondence to: Jun Woong Moon. Gongdeok Seoul Eye Clinic, Renaissnace Tower Bldg., Gongdeok-dong, Mapo-gu, Seoul 121-706, Korea. imoon58@netsgo.com
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Abstract

• **AIM: To investigate the aqueous erythropoietin (EPO) levels and associated factors in patients with acute retinal vein occlusion (RVO).**

• **METHODS: The aqueous EPO level was measured in patients with macular edema (ME) secondary to acute branched retinal vein occlusion (BRVO) or central retinal vein occlusion (CRVO). Aqueous fluid from cataract patients served as the control. We also evaluated whether aqueous level of EPO was associated with factors such as serum EPO level, non-perfusion area, central macular thickness (CMT), and arterio-venous (AV) transit time**

• **RESULTS: Twenty-seven RVO patients (16 BRVO, 11 CRVO) and 9 control subjects were enrolled in the study. The aqueous EPO level (mU/mL) was higher in RVO (68.2±54.3) than that in the control subjects (12.9±5.9). More specifically, the aqueous EPO level was higher in CRVO (118.9±52.1) than that in BRVO (33.3±10.8). However, no differences were found in serum EPO levels among three groups. CMT in RVO patients had a positive correlation with the aqueous EPO level (r=0.66). Also, in terms of non-perfusion area, the aqueous EPO levels were more elevated in the ischemic subgroup than in the non-ischemic subgroup in both BRVO and CRVO.**

• **CONCLUSION: Aqueous EPO levels are elevated in patients with macular edema secondary to recent onset RVO. Patients with CRVO have higher EPO levels than those with BRVO. The aqueous EPO level in RVO has a positive correlation with CMT and is associated with non-perfusion area. These results suggest that the aqueous EPO level could be associated with retinal ischemia and may be involved in the pathogenesis of macular edema secondary to RVO.**

• **KEYWORDS:** aqueous level of erythropoietin; acute retinal vein occlusion; retinal ischemia; macular edema
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INTRODUCTION

Retinal vein occlusion (RVO) is one of the most frequently occurring retinal vascular disorders that can result in macular edema (ME), which is the most common cause of visual loss in patients with RVO [1,2]. Thus, it is important to determine the factors that correlate with the pathogenesis of ME in RVO. Recently, several studies have tried to clarify the expression of various cytokine levels in ocular fluids. Noma *et al* [3,4] reported that vitreous vascular endothelial growth factor (VEGF) increased in patients with ME secondary to RVO. Also, Chen *et al* [5] reported an increase in interleukin-6 in the aqueous humor with neovascular glaucoma secondary to central retinal vein occlusion (CRVO).

Erythropoietin (EPO) is a circulating glycoprotein that consists of 165 amino acids. EPO is mainly produced in the kidneys and has been reported to be over-expressed in the brain, liver, and retina under ischemic conditions [6]. Beyond its erythropoietic function, researchers have suggested that EPO is related to thrombosis, hypertension, and oncogenic effects[7]. In the eye, intraocular EPO is known to be a potent ischemia-induced retinal angiogenic factor in proliferative diabetic retinopathy and is elevated in patients with diabetic macular edema [8,9]. However, little information is available regarding EPO in RVO. As RVO also causes retinal ischemia, it seems reasonable that intraocular EPO would be elevated in RVO.

We investigated the aqueous levels of EPO in treatment-naïve patients with macular edema associated with acute RVO [branch retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO)]. Additionally, we evaluated the correlations of aqueous level of EPO with various factors such as serum level of EPO, non-perfusion area, central macular thickness (CMT), best corrected visual acuity (BCVA) in RVO, and arterio-venous (AV) transit time in CRVO.

SUBJECTS AND METHODS

Subjects Twenty-seven RVO patients (16 BRVO, 11 CRVO) and 9 control subjects were enrolled in the study. Informed consent was obtained from each patient following an explanation of the purpose and potential adverse effects of the procedure. This study was approved by the Ethical Committee of Kunkuk College of Medicine and was performed in accordance with the ethical standards in the 2008 Declaration of Helsinki.

Methods The study included non-diabetic, treatment-naïve patients with BRVO or CRVO with macular edema. The control group consisted of non-diabetic cataract patients requiring cataract surgery. Patients were enrolled in the study after undergoing systemic evaluation including medical history, blood pressure, serum hemoglobin levels, renal profiles, and ocular examination. Ocular examination included BCVA, intraocular pressure, biomicroscopic evaluation, and fundus examination using a +90 diopter noncontact lens (Volk Optical Inc., Mentor, Ohio, USA).

Exclusion criteria included uncontrolled hypertension (systolic and diastolic blood pressure greater than 160 mm Hg or 100 mm Hg, respectively), anemia (hemoglobin level <12 g/dL), pregnancy, mailignacy, chronic renal failure, diabetic mellitus, or having only a single kidney. Ocular exclusion criteria were prior intraocular surgery or injection of steroid or anti-angiogenic factors, longstanding (more than 3mo) BRVO or CRVO, collateral formation detected on fluorescein angiography (FAG), massive vitreous hemorrhage, significant cataracts, graded at more than NO3 or NC3 according to the Lens Opacity Classification Scheme, and other coexisting retinal pathologies affecting retinal EPO level (*e.g.* uveitis, exudative age-related macular degeneration). Also we excluded patients with taking systemic or topical drug potentially affecting serum and aqueous EPO level such as steroid, any hormonal drug, anti-hypertensive medication with an angiotensin converting enzyme inhibitor (ACEI) or angiotensin II receptor blocker (ARB) and antiglaucoma eyedrops.

Harvesting aqueous fluid Undiluted aqueous fluids (0.1-0.2 mL) were aspirated into a 1 mL syringe attached to a 30 G needle just before intravitreal bevacizumab (IVB) injection or cataract surgery. The specimens were transferred to sterile Eppendorf tubes and stored at -80℃ until assayed. Since EPO presents a diurnal rhythm in its circulating serum level, aqueous fluids were harvested at similar times (9 to 10 o'clock in the morning) to minimize the confounding effects of the serum diurnal rhythm[10].

EPO assessment Aqueous level of EPO was measured by chemiluminescent immunoassay. The IMMUNLITE 2000 EPO assay (Diagnostic Products Corporation, Siemens, USA) consists of a ligand-labeled monoclonal anti-EPO antibody, an alkaline phosphatase-labeled polyclonal conjugate antibody, and solid-phase anti-ligand-coated polystyrene beads. Patient samples and ligand-labeled anti-EPO antibodies were incubated with solid phase, and the EPO in the patient sample was bound to the monoclonal antibody and immobilized onto the solid phase. Alkaline phosphatase-labeled polyclonal anti-EPO antibody was then introduced and bound to the EPO attached to the solid phase; the un-bound enzyme conjugate was removed by a centrifugal wash step. A chemiluminescent substrate, alkaline phosphate, was added. The photon output, which is proportional to the EPO concentration in the sample, was measured by a luminometer [11,12]. Serum EPO was analyzed from venous blood samples using the same technique. The limit of detection of this method was 0.1 mU/mL.

Outcome measures The main outcome measured was the aqueous level of EPO (mU/mL) among the three groups (control, BRVO, CRVO). Serum level of EPO (mU/mL), non-perfusion area (ischemic and non-ischemic type), CMT (μm), BCVA (logMAR) in RVO, and AV transit time (s) in CRVO were studied in order to analyze the correlation with the aqueous level of EPO.

Non-perfusion area and AV transit time were evaluated with fluorescein angiography (FAG; TRC-50IX, Topcon, Tokyo, Japan). Non-perfusion area was defined as disc diameter in BRVO and a disc area in CRVO. RVO patients were divided into two subgroups according to non-perfusion area. A non-perfusion area greater than 5 disc diameters (DD) in BRVO and a 10 disc area (DA) in CRVO were considered the standard for the ischemic type in each group. AV transit time from the retinal arterial emergence of the dye to the appearance of venous laminar flow was measured in the CRVO group. Central macular thickness (CMT) was measured based on images from Spectralis HRA+OCT ® (Heidelberg Engineering, Heidelberg, Germany) and was automatically calculated as an average retinal thickness within a circle having a 500-μm radius centered on the fovea.

Statistical Analysis Statistical analysis was performed using SPSS v.17.0 for Windows (SPSS, Chicago, IL, UAS). Results are expressed as mean (standard deviation: SD). Continuous variables (aqueous EPO, serum EPO, AV transit time, CMT, age, blood pressure, serum hemoglobin, and creatinine) were analyzed with a normality test (Shapiro-Wilke test), and all the variables showed a normal distribution. Concentration differences (aqueous and serum EPO, age, blood pressure, serum hemoglobin, and creatinine) among groups (CRVO, BRVO, and control) were evaluated by an ANOVA test with Bonferroni's method for multiple comparisons. Chi-square test was used for comparison of discrete variables (gender) among groups. Concentration differences according to the non-perfusion area within the groups (CRVO and BRVO) were evaluated

by Student's *t*-test. Bivariate relationships between aqueous EPO and associated factors (CMT, BCVA and AV transit time) were analyzed using the Pearson correlation coefficient. Then, multivariate analysis was performed with linear logistic regression over the specific predictor. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

The study included 36 patients with 27 treatment-naïve RVO patients (16 BRVO, 11 CRVO) and 9 control subjects. There were 17 women and 19 men. The mean age (y) of each group was 65.2±15.2 for BRVO, 60.0±12.3 for CRVO, and 65.2±15.2 for control. Demographic characteristics (age and sex) and systemic conditions (systolic/diastolic blood pressure, serum creatinine, and hemoglobin) were not statistically different among the groups. Demographic characteristics among the groups are summarized in Table 1. At the initial visit, all eyes, except in the control group, showed marked ME associated with RVO; mean CMT was 389.1±88.0 μm in the BRVO group and 617.3±176.7 μm in the CRVO group (*P*=0.002).

EPO Concentration (Aqueous and Serum) For the aqueous level of EPO, there were significant differences among the three groups (*P*=0.0001). Aqueous level of EPO was significantly elevated in the RVO group compared with that in the control group. Also aqueous level of EPO was higher in the CRVO group than in the BRVO group (BRVO, 33.3±10.8 mU/mL *vs* control, 12.9±5.9 mU/mL, *P*=0.032; CRVO, 118.9±52.1 mU/mL *vs* control, 12.9±5.9 mU/mL, *P*=0.0001; CRVO, 118.9±52.1 mU/mL *vs* BRVO, 33.3±10.8 mU/mL, *P*=0.0001) (Figure 1).

However, serum levels of EPO were within normal limits, and there were no significant differences among the three groups (BRVO, 11.4±2.9 mU/mL, CRVO, 11.7±2.9 mU/mL, control, 9.2±2.4 mU/mL, *P*=0.132) (Figure 2).

Non-perfusion Area In BRVO, the aqueous levels of EPO in the non-ischemic (10 patients) and ischemic (6 patients) subgroups were 28.4±7.2 mU/mL and 41.4±11.5 mU/mL, respectively. In CRVO, the aqueous levels of EPO in the non-ischemic (6 patients) and ischemic (5 patients) subgroups were 83.7±39.2 mU/mL and 161.1±28.1 mU/mL, respectively.

In both BRVO and CRVO, the aqueous level of EPO was more elevated in the ischemic subgroup than in the non-ischemic subgroup (*P*=0.015 and 0.005, respectively) (Figure 3).

Central Macular Thickness The average CMT in RVO was 482.1±171.34 μm. There was a significant positive correlation between CMT and aqueous level EPO (*R*=0.66, *P*=0.0002). In accordance with an increase in the aqueous level of EPO, the CMT increased (Figure 4).

Best Corrected Visual Acuity The average BCVA in RVO was 0.68±0.62 (logMAR). There was a significant positive correlation between logMAR BCVA and aqueous level EPO (*R*=0.40, *P*=0.013). In accordance with an increase in the aqueous level of EPO, the BCVA (logMAR) increased (Figure 5).

Arterio-venous Transit Time in Central Retinal Vein Occlusion Average of AV transit time in CRVO was 24.0±6.9s. The range was 17-37s. There was no significant correlation between AV transit time and aqueous level of EPO (*P*=0.746) (Figure 6).

Table 1 Clinical characteristics of all study eyes with retinal vein occlusion (RVO), and controls

Parameters	BRVO (*n*=16)	CRVO (*n*=11)	Controls (*n*=9)	*P*
Mean age (a)	65.2±15.2	60.0±12.3	65.2±15.2	[1]0.531
Gender (M/F)	9/7	6/5	4/5	[2]0.590
Systolic blood pressure	142.3±18.4	139.6±12.3	135.2±16.1	[1]0.227
Diastolic blood pressure	82.4±10.1	81.9±11.2	79.5±8.1	[1]0.529
Hemoglobin (%)	14.1±1.0	13.4±1.2	13.2±1.7	[1]0.461
Serum creatinine (mg/dL)	1.1±0.1	1.1±0.1	1.0±0.2	[1]0.792
Symptom duration (wk)	2.1±1.12	2.64±1.16	-	[3]0.567
BCVA (logMAR)	0.64±0.73	0.75±0.51	0.51±0.63	[1]0.712
IOP (mm Hg)	14.5±2.7	15.9±1.8	15.4±2.0	[1]0.671
CMT (μm)	389.1±88.0	617.3±176.7	-	[3]0.002

BCVA: Best corrected visual acuity; BRVO: Branched retinal vein occlusion; CMT: Central macular thickness; CRVO: Central retinal vein occlusion; IOP: Intraocular pressure; logMAR: Logarithm of the minimum angle of resolution; [1]*P* values relate to ANOVA test; [2]*P* values relate to Chi-square test; [3]*P* values relate to Student's *t*-test.

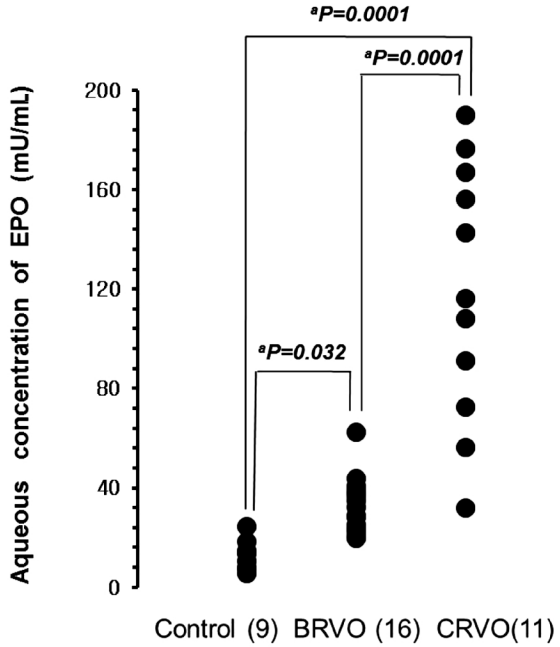

Figure 1 Aqueous level of erythropoietin (EPO) in control, branched retinal vein occlusion (BRVO), and central retinal vein occlusion (CRVO). The aqueous level of EPO was significantly higher in patients with BRVO or CRVO than in control patients (*P*=0.032 and 0.0001, respectively). The EPO level was also significantly higher in patients with CRVO compared with patients with BRVO (*P*=0.0001).

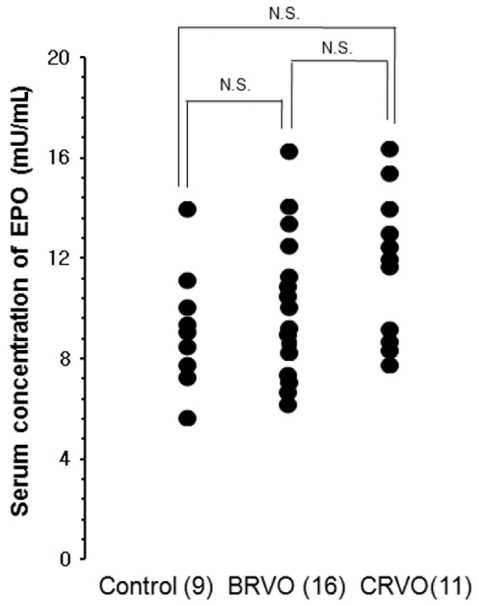

Figure 2 Serum level of EPO in control, BRVO, and CRVO. There were no significant (N.S) differences in serum levels of EPO among groups. P=0.132.

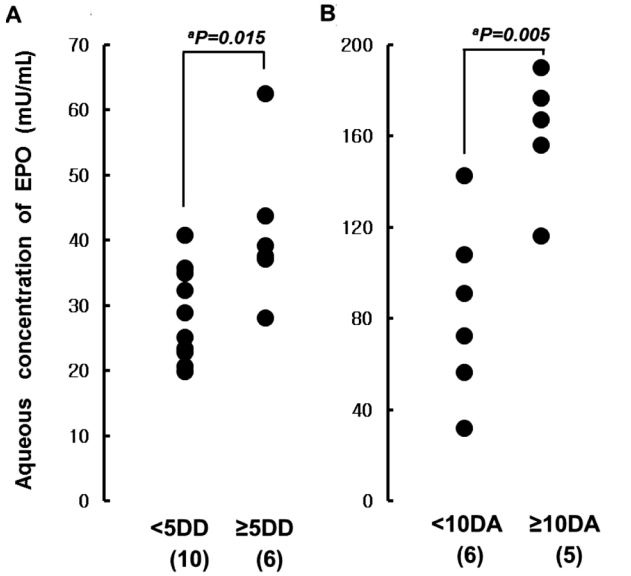

Figure 3 Aqueous levels of EPO according to the non – perfusion area in retinal vein occlusion (RVO) A: Non-perfusion area in BRVO. There were significant differences in aqueous levels of EPO in non-perfusion area more than 5 disc diameter (DD) and in non-perfusion area less than 5 DD; B: Non-perfusion area in CRVO. There were significant differences in aqueous levels of EPO in non-perfusion area more than 10 disc area (DA) and in non-perfusion area less than 10 DA. In both group (BRVO, CRVO), the aqueous EPO level was significantly higher in patients with broad non-perfusion area subgroup (P=0.015 and 0.005, respectively).

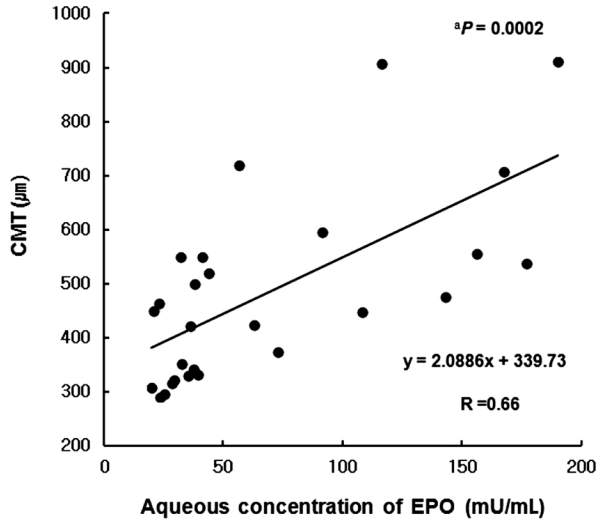

Figure 4 Aqueous levels of EPO according to central macular thickness (CMT) in RVO (27 eyes). There was a significant ositive correlation between CMT and aqueous EPO. In accordance with an increase in the aqueous level of EPO, the CMT increased. P<0.001.

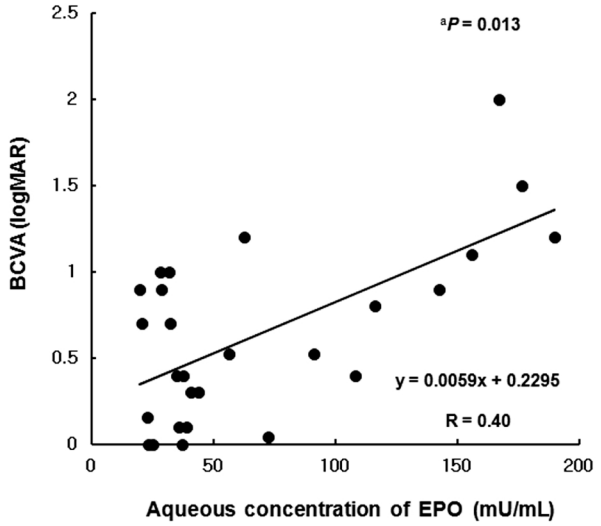

Figure 5 Aqueous levels of EPO according to best corrected visual acuity (BCVA) in RVO (27 eyes). There was a significant positive correlation between BCVA (logMAR) and aqueous EPO. In accordance with an increase in the aqueous level of EPO, the BCVA (logMAR) increased. P=0.013.

DISCUSSION

The aim of our study was to investigate the aqueous level of EPO in the acute phase of RVO. Additionally, the associations between aqueous level of EPO and ischemia-related factors were analyzed. The major findings in this study were as follows. First, the aqueous level of EPO increases early in the onset of RVO. Second, the aqueous level of EPO is associated with retinal ischemia such that the aqueous EPO level is significantly higher in recent onset RVO than in the normal population. Also, EPO is significantly elevated in CRVO (which is theoretically a more ischemic disease than BRVO) compared with BRVO. The aqueous level of EPO is more elevated in patients with a broad non-perfusion area (more than 5 DD in BRVO and 10 DA in CRVO). Third, EPO could be involved in the pathogenesis of macular edema secondary to RVO. The aqueous level of EPO had a positive correlation with CMT.

This is the first study that investigates the aqueous EPO level in acute RVO. Previous studies investigated vitreous samples

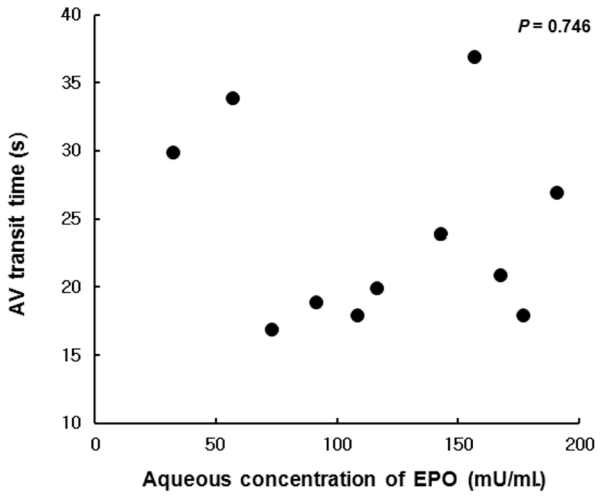

Figure 6 Aqueous levels of EPO according to the arterio-venous(AV) transit in CRVO (11 eyes). There was no significant correlation between AV transit time in CRVO and aqueous EPO concentration. P=0.746.

during surgery. However, for the accurate and innate investigation of EPO levels in RVO, we focused on recent onset, treatment-naïve patients with RVO. We excluded patients taking anti-hypertensive medication with ACEI or ARB because it is possible that aqueous the EPO level is blunted by long-standing RVO, ACEI or ARB and by previous treatments such as intravitreal bevacizumab injection, which decreases aqueous VEGF levels in RVO[13,14].

Previous studies investigating vitreous samples included only patients with RVO with need for vitreoretinal surgery. In other words, patients with milder forms of RVO who usually do not require vitreal surgery were excluded. This criterion represents a selection bias for more severe cases of RVO. On the other hand, aqueous sample could be yielded to all patients from mild to severe form of RVO. Also, considering of acute nature of the RVO, aqueous sample could be yielded to acute phase and treatment-naïve patients. Although, erythropoietin levels in vitreous have a close relationship with the erythropoietin level in anterior chamber, aqueous sample could represent more accurate levels of EPO in RVO.

The results of the present study correspond with those of the previous study by Inomata *et al*[8]comparing vitreous level of EPO between ischemic retinal diease (proliferative diabetic retinopathy, RVO) and macular hole. Inomata *et al* [8] found vitreous levels of EPO were significantly elevated in patients with proliferative diabetic retinopathy and branch retinal vein occlusion as compared to patients with macular hole. However, García-Arumi *et al* [15] reported that vitreous EPO level in patients with macular edema secondary to BRVO or CRVO was not different from BRVO, CRVO, and the control (non-diabetic patients with other condition requiring vitrectomy). These findings are in contrast to our results. However, the study by García-Arumí *et al* [15], had several differences to our study. First, in contrast to our aqueous samples, García-Arumí *et al* [15] obtained the majority of the vitreous samples from patients undergoing vitreoretinal surgery. It is possible that previous treatments, like intravitreal avastin or triamcinolone acetate injection, had been performed in some of these patients, which could have affected vitreal EPO concentration. However, we recruited acute (mean symptom duration; 2.1wk in BRVO, 2.6wk in CRVO) and treatment-naïve patients only. Second, they did not record symptom duration or collateral formation on FAG. Chronic phase patients were not excluded from their study. It is possible that, due to the acute nature of the disease, EPO production occurred in the early phase and then diminished in the chronic phase. Finally, their study included few patients with RVO (BRVO 3, CRVO 9) compared to ours (BRVO 16, CRVO11).

In this study, the aqueous level of EPO did not correlate with the serum level of EPO. This result corresponds with a previous study by Stahl *et al*[16]. They investigated EPO level in serum and vitreous sample in RVO and reported that EPO level was within normal range in serum, unlike the increasing level observed in the vitreous. This suggests that the intraocular level of EPO is regulated by a autocrime/paracrine mechanism and elaborated in retina that is responsive to retinal ischemia. The study by Hernández *et al* [17] reported that apart from kidney and liver, EPO is also produced by the brain and the retin. Also they indicated that EPO mRNA expression was detected in the human retina and was higher in the retina of diabetic than non-diabetic donors[18].

We found that the aqueous level of EPO had a positive correlation with CMT in acute RVO. This result simillar with an earlier study, which reported that, in accordance with an increase in the vitreous levels of EPO, the CMT increased in RVO patients [16]. It is possible that more severe ischemia might lead to retinal damage during the acute phase of RVO. This ischemic damage of retina might cause macular edema with increasing intraocular EPO, or that severe ischemia of retina might lead to increased production of intraocular EPO by paracrine mechanism in turn causes macular edema.

RVO groups displayed a higher tendency towards systolic blood pressure than the control group (Table 1). Hypertension is one of the risk factors of retinal vein occlusion and strongly associated with it[19]. We reasoned that although we excluded patients with uncontrolled hypertension, RVO patients group would show a tendency to high blood pressure in comparison to the control group. In this study, CMT in control group was not considered in analysis to determine factors associated with EPO levels, since fundus examination of control group was normal and CMT from some control patients was not available

Multivariate analysis shows a significant positive correlation between logMAR BCVA and aqueous level EPO (r=0.40, P= 0.013 in Figure 5). In other words, in accordance with an increase in the aqueous level EPO, the BCVA worsened.

Because aqueous level EPO could influence central macular thickness (Figure 4 shows positive correlation between CMT and aqueous EPO), BCVA could be worsened as the aqueous level EPO increased, consequently.

There are two conflicting opinions about the role of EPO in retinal disease. Some studies have found that EPO plays a beneficial role in the neuroprotective effects against ischemic-reperfusion anti-apoptotic role in the retina [20,21]. Other studies suggested that EPO has a harmful role that is associated with pathologic neovascularization in proliferative diabetic retinopathy and retinopathy of prematurity [22,23]. However, in this study, we could not demonstrate a pathophysiologic role for increased intraocular EPO.

The current study has several limitations. First, there was a relatively small number of patients in each group and a large range of EPO concentrations in CRVO patients (range 31.8-190 mU/mL). Nonetheless, statistically significant differences among groups were observed. Second, this study only investigated EPO levels in aqueous humor samples and did not analyze other angiogenic factors, such as VEGF or interleukin-6, due to an insufficient amount of aqueous sample. However, the aqueous samples from treatment-naïve patients represent a more accurate intraocular level of EPO than the vitreous samples from patients who have previous treatment history or chronic nature. Contrary to our expectation, the aqueous EPO level was not correlated with AV transit time in CRVO, which may reflect the degree of retinal ischemia. Our small sample size is postulated as one of the reasons for this seeming discrepancy, and future studies need to be performed on a larger scale.

Our study demonstrates that aqueous level of EPO increases in recent onset RVO. Aqueous level of EPO could be associated with retinal ischemia. EPO may be involved in the pathogenesis of macular edema secondary to RVO. In the future, precise role of EPO in the pathogenesis of RVO and therapeutic strategies aimed to control intraocular level of EPO should be clarified.

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Conflicts of Interest: Shin HJ, None; **Kim HC,** None; **Moon JW,** None.

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