

# Aqueous Humor Nitric Oxide in Patients with Retinal Vein Occlusion

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## Abstract

**Purpose:** To investigate aqueous humor nitric oxide (NO) levels in patients with branch retinal vein occlusion (BRVO) and central retinal vein occlusion (CRVO) and to compare these with age-matched controls.

**Methods:** Eight consecutive patients with BRVO and 16 patients with CRVO were included in this study. Aqueous humor specimens were obtained within 21 days of diagnosis. Samples of aqueous humor were also collected from 20 control patients undergoing cataract surgery. For each sample after reduction of nitrate to nitrite with vanadium chloride (VCL3), we used spectrophotometric method for simultaneous detection of nitrate and nitrite.

**Results:** Mean level of aqueous humor nitrite and nitrate were  $84.08 \pm 21.29$   $\mu\text{mol/l}$  in BRVO group,  $65.40 \pm 22.23$   $\mu\text{mol/l}$  in CRVO group, and  $55.68 \pm 11.02$   $\mu\text{mol/l}$  in control group. The difference between aqueous humor nitrite and nitrate levels of BRVO group and that of control group was statistically significant ( $P < 0.0001$ ) but not for the difference between those of CRVO and control groups ( $P = 0.10$ ).

**Conclusion:** The results may support involvement of nitric oxide in the pathogenesis of BRVO.

**Keywords:** Nitric Oxide, Branch Retinal Vein Occlusion, Central Retinal Vein Occlusion, Spectrophotometry

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## Introduction

Nitric oxide (NO), a free radical gas with a half-life of a few seconds, has been shown to play various physiological and pathophysiological roles in the nervous system.<sup>1</sup> NO is generated by the oxidation of arginine, a reaction catalyzed by the enzyme nitric oxide synthase (NOS).<sup>2</sup>

There are three NOS isoforms coded by separate genes that contribute to the production of NO to variable degrees in different tissues. Endothelial NOS (eNOS) is responsible for endothelial cell-derived NO.

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Neuronal NOS (nNOS) is differentially expressed in neurons. Inducible NOS (iNOS) has been demonstrated in many other tissues, it generally has low or undetectable basal expression levels that are increased by various cytokines and other stimuli.<sup>3</sup>

In the eye, neuronal nitric oxide synthase is thought to be responsible for producing nitric oxide in photoreceptors and bipolar cells<sup>4</sup>, whereas endothelial nitric oxide synthase is present in vascular endothelial cells.<sup>5</sup> However, inducible nitric oxide synthase may be involved in phagocytosis of the photoreceptor outer segment<sup>6</sup>; inflammatory<sup>7</sup>, and ischemic processes; and in the pathogenesis of diabetic retinopathy.<sup>8,9</sup> Nitric oxide could be protective or destructive to the retina depending on the stage of the evolving ischemic process. It can be beneficial in its role as a vasodilator, but high concentrations of nitric oxide produced by inducible nitric oxide synthase are neurotoxic.<sup>10</sup>

Branch retinal vein occlusion (BRVO) is three times as common as central retinal vein occlusion (CRVO) and second only to diabetic retinopathy as the most common retinal vascular cause of visual loss and their visual prognosis remains poor.<sup>11</sup> The abnormalities of hemostasis may result in a hypercoagulable state and thrombus formation, leading to the occurrence of retinal vein occlusion, or to the development of retinal ischemia and neovascularisation.<sup>12</sup>

So we think that nitric oxide may involve in the pathogenesis of BRVO or CRVO as an ischemic process.

However, to our knowledge, there has been no clinical human study done on NO level in patients with retinal vein occlusion. Thus we investigated whether NO is involved in retinal vein occlusion in human.

## Methods

### Study patients

Written informed consent was obtained from all the patients after complete explanation. The review board and ethical committee of Eye Research Center of *Tehran* University of Medical Sciences approved the trial; methods complied with the Declaration of Helsinki. Undiluted samples of aqueous humor were drawn by paracentesis from 20 age-matched patients with no systemic and ocular disease

undergoing cataract surgery at the beginning of their cataract surgery.

Also aqueous humor of 8 patients with BRVO and 16 patients with CRVO were drawn as a therapeutic intervention that underwent intravitreal tissue plasminogen activator injection within 30 days of visual loss. Number of patients was based on prior similar studies and seems to be sufficient.

The diagnosis of BRVO and CRVO were made based on clinical examination (visual loss, retinal hemorrhages, and edema of the macula and optic nerve). Any patient did not have iris and retinal neovascularization. All three groups did not have history of prior surgery.

### Sample collection

Specimens were collected in sterile tubes and rapidly frozen at -20°C. The samples were then labeled anonymously without identifying clinical information and shipped on dry ice to the Faculty of Pharmacology at *Tehran* University of medical science. Sample volumes ranged from 50 to 70 µl.

### Nitric oxide assay

Nitrite is the only stable end product of autooxidation of NO in aqueous solution. Nitrate is formed by reaction of NO with superoxide.<sup>13</sup> Reduction of nitrate to nitrite is achieved with vanadium chloride (VCL3). Saturated solution of VCL3 (400 mgr) was prepared in 1 M hydrogen chloride (HCL 50 ml). Then nitrite level was measured with Griess reaction.<sup>14</sup> Griess reaction entails formation of a chromophore from the diazotization of sulfanilamide (2% in 5% HCL) by acidic nitrite followed by the coupling with n-1-(naphthyl) ethylenediamine (0.1%). Then total nitrite was determined by measuring absorbance at 540 nm using the spectrophotometric method and standard curve. This method results in measurement in biological samples of total nitrogen oxide concentration from both nitrite and nitrate.<sup>15</sup>

### Statistical analysis

We used the Fisher exact chi-square test to compare nitrite levels in patients with retinal vein occlusion disease with those in controls. P values of < 0.05 were considered significant. All results are expressed as means ± standard error of mean.

## Results

The patients' ages ranged from 50 to 72 years in the BRVO group (mean 61.6 years) and from 40 to 66 years in the CRVO group (mean 57.6 years). The mean duration of BRVO and CRVO by the time of intraocular surgery was 19 days and 18 days respectively.

Of 16 patients with CRVO, 8 (50%) were female and, 8 (50%) were male. Of 8 patients with BRVO, 5 (62.5%) were female and, 3 (37.5%) were male; the corresponding figures in control group were 11 (55%) and 9 (45%), female and male respectively.

Mean level of aqueous humor nitrite and nitrate in BRVO group was  $84.0779 \pm 21.2888$   $\mu\text{mol/l}$  (range, 44.6511-115.4778  $\mu\text{mol/l}$ ), in CRVO group was  $65.3966 \pm 22.2294$   $\mu\text{mol/l}$  (range, 42.9066-110.0117  $\mu\text{mol/l}$ ) and in control group was  $55.6753 \pm 11.0228$   $\mu\text{mol/l}$  (range, 33.7189 - 75.2380  $\mu\text{mol/l}$ ). There was a statistically significant difference in aqueous humor nitrite and nitrate level between BRVO group and control group ( $P < 0.0001$ ). There was not a statistically significant difference in aqueous humor nitrite and nitrate level between CRVO group and control group ( $P = 0.10$ ).

## Discussion

Our study shows that individuals with BRVO have elevated levels of NO in the aqueous humor. To the best of our knowledge, this is a new finding.

NO is a reactive gas with various physiological functions, including relaxation of blood vessels, signal transduction and cytotoxicity.

*Donati* et al found that after branch vein occlusion there was a decrease in preretinal NO concentration and a simultaneous decrease in the diameter of the arteriole in the affected territory. These results show that experimental branch vein occlusion induces in the affected retina an impairment in the release of constitutive NO and an arteriolar constriction, which, in turn, contributes to the development of hypoxia in tissue and neuronal swelling and death in the inner retina.<sup>16-18</sup>

Another study showed that inhibition of NOS with intraperitoneal injection of NG-nitro-L-arginine (L-NNA) worsens retinal damage after ischemia-reperfusion and alters post ischemic retinal circulation. Nitric oxide may

play an important role in protecting the retina from ischemic injury, possibly by preventing post ischemic hypoperfusion.<sup>19</sup>

*Sato* et al found that increase in retinal blood flow during hypoxia is greatly suppressed by intravitreal injection of NO synthase inhibitor strongly suggests that NO in the retina is involved in the increase in retinal blood flow during hypoxia.<sup>20</sup>

*Kashii* et al showed that NO released by NO donors leads to the death of cultured retinal neurons.<sup>21</sup> Also others suggest that NO plays a pathogenic role in degenerative retinal diseases.<sup>22</sup>

The wide range of activities of NO has been attributed to the existence of different isoforms of the enzyme nitric oxide. One study determined whether nNOS and eNOS are critical in excitotoxic damage in the retina of nNOS- and eNOS-deficient mice were subjected to intravitreal injections of N-methyl-D-aspartate (NMDA) or to arterial occlusions. It found retinal ganglion cells in the nNOS-deficient mouse were relatively resistant to NMDA and to arterial occlusion. In contrast, the damage in the eNOS-deficient mouse retina was not distinguishable from that in control animals. Preinjection with an NOS inhibitor was partially protective. The presence of nNOS is a prerequisite for the full expression of excitotoxicity in the retina; eNOS does not appear to play a significant role.<sup>23</sup>

Another study has confirmed there is an early period of primary degeneration. In addition, there is a period of secondary neuronal degeneration. Nevertheless, this secondary phase is substantial and occurs over a prolonged time period. Thus continuous pharmacological treatment with inhibitors of NOS-2 activity during the 2 weeks post ischemia period provides significant neuroprotection against the loss of retinal ganglion cell.<sup>24</sup>

Several human studies have been performed about the role of nitric oxide in eye. Neufeld et al found three isoforms of NOS are present in apparently increased amounts in the optic nerve head of patients with primary open-angle glaucoma. Conversely, the increased level of NOS-3 in vascular endothelia may be neuroprotective by causing

vasodilation and increased blood flow in the tissue.<sup>25,26</sup>

Ahmed et al have suggested that Müller cells may be involved in the microvascular remodeling of the diseased retina and that high concentrations of nitric oxide produced by inducible nitric oxide synthase could contribute to neurotoxicity and angiogenesis that occur in human diabetic retinopathy. The consequences of increased levels of nitric oxide in retinas from subjects with diabetes could be twofold: neurotoxicity and angiogenesis. Nitric oxide can be beneficial in its role as a vasodilator, but high concentrations of nitric oxide produced by inducible nitric oxide synthase are neurotoxic.<sup>27</sup>

In patients with diabetes one group found that the aqueous NO levels of the active proliferative diabetic retinopathy were significantly higher than those of the background diabetic retinopathy group and the diabetics without diabetic retinopathy.<sup>28</sup> One study showed that patients with CRAO presented with elevated levels of NO in the aqueous humor. But they assayed only the amount of nitrite not total amount of nitrite and nitrate.<sup>29</sup> Biologic processes modulated by nitric oxide might extend to include angiogenesis and that nitric oxide may be an important mediator of growth factors signaling in endothelial cells.<sup>30</sup>

Our investigation reveals that patients with BRVO have elevated aqueous humor NO level. It seems that in the first hours after BRVO, eNOS expression may be beneficial and NO increase retinal blood flow during

hypoxia, although its release may be impaired in severe retinal hypoxia but in the period of prolonged ischemia/secondary neural degeneration occur with NOS-2 over expression and cause neurotoxic outcome. In our study level of increased aqueous NO in BRVO may be the result of NOS-2 over expression in adjacent healthy retina in response to ischemia. NOS-2 expression causes neurotoxicity and neovascularization in retina. But in CRVO, because of severe ischemic insult, diseased retina may not produce NO as much as BRVO. This phenomenon may explain why in patients with BRVO, retinal neovascularization occur more than CRVO (although absence of intact endothelium in CRVO also may be responsible).

### Conclusion

To our knowledge, this is the first investigation of NO level in human retinal vein occlusions; thus our results provide new information. These data provide new approaches for managing BRVO and prophylaxis against retinal neovascularization with selective iNOS inhibitors. However, further studies are required to investigate the role of NO in human retina during ischemia and management of retinal vein occlusion.

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