Homocysteine Levels in the Vitreous of Proliferative Diabetic Retinopathy and Rhegmatogenous Retinal Detachment: Its Modulating Role on Lysyl Oxidase

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Purpose. Homocysteine (Hcys), a well-known inducer of vascular endothelial cell damage has been associated with extracellular matrix changes. Lysyl oxidase (LOX) is a copper-dependent amine oxidase that initiates the covalent cross-linking of collagen and elastin in the extracellular matrix (ECM). LOX contributes to the structural integrity of the ECM, and low LOX activity could promote ECM disorganization. Hydroxyproline levels are used to predict collagen turnover status, and most of the endogenous hydroxyproline present in biological fluids is derived from the degradation of various forms of collagen. As Hcys is known to regulate ECM turnover and also inhibit LOX activity, the purpose of this study was to estimate the vitreous levels of Hcys in eyes with proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD) and to correlate the effect of Hcys, if any on LOX activity.

Methods. Undiluted human vitreous specimens obtained during vitreoretinal surgeries for PDR ($n = 18$) and RRD ($n = 17$) were used. Vitreous specimens from donor eyeballs were used as control ($n = 19$). Hcys was estimated by HPLC using a fluorescent detector. Hydroxyproline was estimated spectrophotometrically.

Results. The total vitreous Hcys level was found to be increased significantly in PDR ($P = 0.011$) and in RRD ($P = 0.001$) compared with that in control samples. Hydroxyproline was significantly increased in PDR ($P = 0.049$) and RRD ($P = 0.007$) compared with the level in control samples. There was a significant negative correlation between the Hcys level and the specific activity of LOX in PDR ($P = 0.040$) and in RRD ($P = 0.029$)

Conclusions. This report shows that increased vitreous Hcys in PDR and RRD is associated with a significant decrease in LOX-specific activity along with an increase in collagen turnover. (*Invest Ophthalmol Vis Sci.* 2009;50:3607–3612) DOI:10.1167/iovs.08-2667

Homocysteine (Hcys) is a sulfur-containing amino acid with a free thiol (sulfhydryl; SH) group, formed from methionine through *S*-adenosyl methionine in blood.[1] Elevated plasma Hcys has been associated with vascular remodeling in the context of both cardiovascular[2] and cerebrovascular diseases[3] through the activation of matrix metalloproteinases (MMPs).[4] Ocular complications associated with Hcys include ectopia lentis,[5] secondary glaucoma,[6] optic atrophy,[7] age-related macular degeneration (ARMD),[8] central retinal vein occlusion (CRVO),[9] and diabetic retinopathy.[10] The consequences of elevated levels of Hcys on retinal function in in vitro and in vivo models, has shown that Hcys induces apoptotic retinal ganglion cell (RGC) death.[11,12] Poloschek et al.[13] reported in a case study that hyperhomocysteinemia caused by methionine synthase deficiency demonstrated decreased rod response and RGC loss, as analyzed by ERG and visual evoked potential. However, less is known about the effects of Hcys on retinal function. Plasma total Hcys concentration has been suggested to be a useful biomarker and a risk factor for diabetic retinopathy in people with type 2 diabetes.[10] This cross-sectional study reported that a difference in Hcys concentration of 2 μM separated subjects with and without retinopathy and a relatively small increase in the plasma Hcys concentration, on the order of 1 μM, may be useful as a trigger for intensification of treatment of the major risk factors for diabetes complications.[10]

Proline and hydroxyproline together comprise 23% of the collagen molecule. Hydroxyproline levels are used to predict collagen turnover status and most of the endogenous hydroxyproline present in biological fluids is derived from the degradation of various forms of collagen.[14,15] It has been reported that in the early phase of wound healing, increased hydroxyproline is associated with increased collagen deposition.[16,17] Hcys has also been shown to have a role in collagen synthesis and cross-linking.[14] Collagen turnover in the vitreous has been shown to be associated with ageing and vitreoretinal diseases, which predisposes to posterior vitreous detachment.[18]

Vitreoretinal diseases, such as proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD), show extensive ECM disruption.[19] PDR is a common complication of diabetes mellitus characterized by preretinal neovascularization and development of epiretinal fibrovascular traction and retinal detachment.[20] RRD is a complex wound-healing pathobiology of proliferative vitreoretinopathy (PVR), which involves inflammation, ECM deposition, and tissue remodeling.[21]

The covalent cross-linking of collagens and elastin in the ECM is performed by LOX, a copper-dependent amine oxidase enzyme.[22] Inhibition of LOX activity has been related with hyperhomocysteinemia and has been studied in terms of its molecular mechanisms in vascular diseases.[23,24] The levels of Hcys has rarely been studied in the vitreous during vitreoretinal disease except for a recent report by Aydemir et al.[25] on the elevated levels of Hcys in the vitreous and plasma of patients with PDR. In a recent study, we reported that the specific activity of LOX is decreased in the vitreous of patients with PDR or RRD with a concomitant increase in MMPs.[26] LOX inhibition favors the presence of soluble forms of collagen that are highly susceptible to degradation by MMPs.[27] The purpose

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Table 1. Clinical Details of Patients with PDR

Patient No.	Sex	Age (y)	Duration of Diabetes (y)	Eye Involved	Other Systemic Illness	Vitreous Hemorrhage	Quadrant Involved	Retinal Detachment	Patent Vessels
1	M	55	10	OD	Sys HT	Present	4	TRD	FVP over retina, active
2	M	63	30	OS	Sys HT, IHD	Present	4	CRD	FVP over retina, active
3	M	34	10	OS	Sys HT, CRF	Present	4	No RD	FVP over retina, active
4	M	53	24	OD	Sys HT, diabetic nephropathy	Present	4	No RD	No
5	M	64	6	OD	Sys HT, diabetic nephropathy	Present	4	No RD	FVP over disc, active
6	M	37	7	OD	None	Absent	4	TRD	FVP present
7	M	54	15	OS	Sys HT	Absent	4	No RD	No
8	F	65	10	OS	Sys HT	Absent	4	No RD	FVP over retina, active
9	M	65	5	OD	Sys HT	Absent	4	No RD	FVP over disc
10	F	58	17	OS	Sys HT	Absent	4	TRD	FVP over retina, active
11	M	39	6	OS	Sys HT, diabetic nephropathy	Present	4	TRD	FVP over retina, active
12	F	46	15	OD	None	Present	4	TRD	FVP, active
13	F	62	12	OD	Sys HT	Present	4	TRD	FVP over retina, active
14	M	53	1	OS	None	Present	4	TRD	FVP over retina, active
15	M	45	38	OS	None	Present	4	TRD	FVP over disc, active
16	M	61	10	OD	IHD	Present	4	TRD	FVP of disc
17	M	54	15	OD	Sys HT	Present	4	TRD	FVP, active
18	M	38	3	OD	None	Present	4	TRD	FVP over retina, active

TRD was present in 11 patients, CRD in 1, and vitreous hemorrhage in 13. Active PDR was graded in 13 patients. TRD, tractional retinal detachment; CRD, combined retinal detachment; FVP, fibrovascular proliferation, Sys HT, systemic hypertension; IHD, ischemic heart disease.

of the present study was to estimate vitreous levels of Hcys in eyes with PDR and RRD to see whether there is any correlation in the activity of LOX and collagen turnover.

Materials and Methods

All experiments involving human subjects adhered to the tenets of the Declaration of Helsinki. Undiluted vitreous samples from 18 patients (mean age, 52 ± 10; 14 men and 4 women) with PDR and 17 patients (age, 46 ± 15; 14 men and 3 women) with RRD were collected by the vitreoretinal surgeon at the time of vitreoretinal surgery. In patients with PDR, the clinical ocular findings were graded at the time of vitrectomy for the presence of hemorrhage, tractional retinal detachment, and presence or absence of patent new vessels in the retina or optic disc. Active PDR was graded in patients on the basis of visible patent new vessels in the retina or optic disc, and their absence was deemed inactive PDR. In patients with RRD, the clinical ocular findings were graded at the time of vitrectomy for the presence or absence of hemorrhage, duration of vision loss before surgery, quadrant involvement, macular status, and PVR grading. Clinical details of the patients are given in Tables 1 and 2. The samples were transported on ice and centrifuged at 3000 rpm for 10 minutes at 4°C. The centrifuged samples were frozen at −80°C until they were assayed with correspondingly stored control specimens. Human donor eyeballs from CU

Table 2. Clinical Details of Patients with RRD

Patient No.	Age (y)	Sex	Eye Involved	Duration of Vision Loss before Surgery	Other Systemic Illness	Quadrant Involved	Macular Status	PVR Grade	Vitreous Hemorrhage	Anterior Uveitis
1	56	M	OD	12 d	Sys HT	4	Detached	B	Present	Absent
2	38	M	OD	18 d	None	3	Detached	A	Present	Absent
3	50	M	OD	Not mentioned	None	3	Detached	Not done	Absent	Absent
4	22	M	OD	14 d	None	4	Detached	B	Absent	Present OD
5	20	M	OS	2 y	None	4	Detached	D2	Absent	Absent
6	52	M	OS	20 d	None	4	Detached	B	Absent	Present OS
7	56	F	OD	14 d	None	1	Attached	A	Absent	Absent
8	72	M	OS	4 mo	None	3	Detached	B	Absent	Absent
9	32	M	OD	22 d	None	4	Detached	B	Present	PE+IOL done
10	54	M	OS	2 mo	None	4	Detached	B	Absent	Absent
11	26	M	OD	6 d	None	4	Detached	B	Absent	Absent
12	56	F	OD	Not available	None	—	—	—	—	—
13	36	M	OD	Not mentioned	None	4	Detached	A	Absent	Absent
14	38	M	OD	19 d	None	3	Detached	A	Absent	Absent
15	59	M	OD	7 d	IHD	3	Detached	B	Absent	Absent
16	70	F	OS	1.5 y	Sys HT	2	Detached	C2	Absent	Absent
17	55	M	OS	9 d	None	4	Detached	B	Present	Absent

The macula was detached in 16 patients. Vitreous hemorrhage was present in 4. PVR grading was performed in 15 patients. Anterior uveitis was present in 2. One patient had undergone phacoemulsification with intraocular lens implant.

Shah Eye Bank (Sankara Nethralaya, India), were used as control specimens after light microscopic examination and removal of the cornea (mean age, 73 ± 22 years; 12 men; 7 women). Donors with a history of diabetes, hypertension, carcinoma, and sepsis were not accepted for the study. The donors had no history of ocular diseases. The vitreous was aspirated with a needle and syringe similar to the ones used for collecting vitreous samples. Care was taken to collect the vitreous within 5 hours of death. All fine chemicals used in the study were procured from Sigma-Aldrich (St. Louis, MO) unless specified. HPLC-grade solvents were purchased from E-Merck Chemicals (Mumbai, India).

HPLC Analysis of Hcys

Hcys was analyzed with HPLC, as described by Tcherkas and Denisenko,[28] with slight modification. The HPLC system (model 1100; Agilent Technologies, Palo Alto, CA) consisted of two pumps fitted with a 50-μL loop rheodyne injection valve and fluorescence detector. Separation was performed on a 150 × 4.6-mm column (internal diameter, 5 μm; ODS; Phenomenex, Torrance, CA). The elution procedure was performed with 0.05 M acetate buffer (pH 7.0) containing 75% methanol in isocratic mode, at 26°C with a flow rate of 0.7 mL/min. A fluorescence detector with excitation at 330 nm and emission at 450 nm was used for detection. Before analysis, the system was calibrated with authentic DL-homocysteine standards in the range of 25 to 100 ng. Hcys eluted at a 2.9-minute retention time. The interassay coefficient variation (CV) was 2.8% and the intra-assay CV was 12.6%.

Processing of the Vitreous Samples

In brief, 2 μL of 2-mercaptoethanol was added to 20 μL of vitreous sample for reduction of disulfide bonds, followed by 40 μL of 0.8 M iodoacetic acid and 120 μL of 0.1 M borate buffer (pH 11.5). After incubation for 30 seconds at room temperature 20 μL of OPA-2-ME reagent was added and 50 μL was injected onto the HPLC system. Specific activity of LOX was determined according to the method described elsewhere by Coral et al.[26]

Hydroxyproline Estimation

Hydroxyproline was measured by the method of Woessner.[29] Briefly, 1.0 mL of 0.05 M chloramine T was added to 50 μg of vitreous and incubated at room temperature for 20 minutes. Then, 0.5 mL of 3.5 M perchloric acid and 0.5 mL of 20% *p*-dimethylamino benzaldehyde solution was added to the oxidized sample, and the chromophore was developed by incubating the samples at 70°C for 30 minutes. The absorbance was read at 550 nm using a Spectrophotometer (DU640 UV/visible; Beckman, Fullerton, CA). The hydroxyproline concentration in the vitreous was calculated and based on the standard graphically (0.2–1.0 micrograms; $R^2 = 0.9853$) and expressed as micrograms hydroxyproline/milligram protein.

TABLE 3. Comparative Levels of Hcys, LOX, and Hydroxyproline in Control versus Diseased Vitreous

Conditions	Protein (mg/mL)	Homocysteine (μM)	Hydroxyproline (micrograms/ mg protein)
Control	1.12 ± 0.18	1.49 ± 0.37 (n = 19)	4.55 ± 0.74 (n = 14)
PDR	3.82 ± 0.80* (P = 0.002)	2.76 ± 0.28* (P = 0.011) (n = 18)	14.25 ± 4.40* (P = 0.049) (n = 13)
RRD	8.82 ± 2.00* (P = 0.000)	4.68 ± 0.74** (P = 0.001) (n = 17)	14.93 ± 3.23* (P = 0.007) (n = 15)

Data are expressed as the mean ± SEM.
* $P < 0.05$; ** $P < 0.01$.

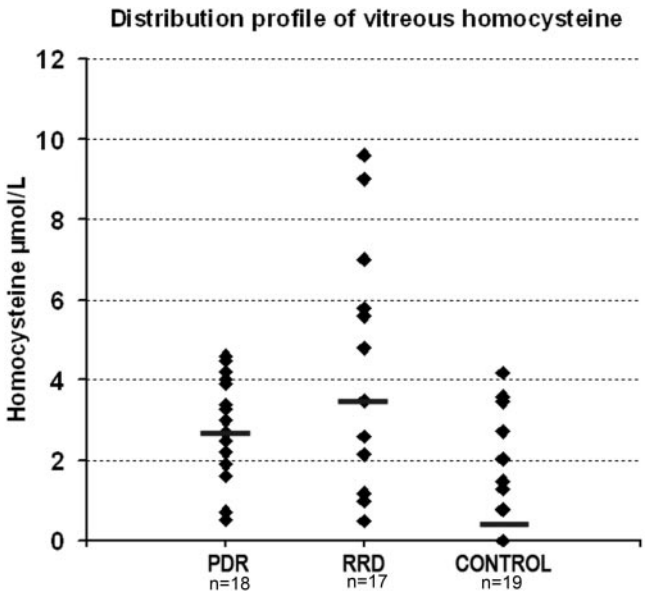

FIGURE 1. Distribution profile of vitreous Hcys in eyes with PDR, RRD, and the donor eye control. *Solid lines*: the median. The median Hcys levels were 2.6 μM in PDR, 3.5 μM in RRD, and 0.78 μM in the control.

Statistics

With commercial software (SPSS software; ver.:14.0; Chicago, IL) the raw data were analyzed for statistical significance with the independent-sample *t*-test and Pearson's correlation. $P < 0.05$ is considered significant.

RESULTS

HPLC Analysis of Hcys in the Vitreous

The mean total Hcys level in PDR was found to be 2.76 ± 0.28 μM ($P = 0.011$), showing a twofold increase compared with that in the control samples. In RRD, there was a 3.5-fold increase with a mean of 4.68 ± 0.74 μM ($P = 0.001$) compared to 1.28 ± 0.31 μM in the control (Table 3). The distribution profile of Hcys in the vitreous was found to be in the range of 0.53 to 4.6 in PDR, 0.50 to 9.6 in RRD and 0.0 to 4.18 in the control. There was an undetectable Hcys level in 7 of 19 control donor vitreous samples (Fig. 1). Within the two disease conditions, Hcys was found to be significantly increased in RRD compared with PDR ($P = 0.019$).

Since plasma Hcys is a known risk factor in hypertension, the PDR and RRD cases were analyzed clinically, as having or not having hypertension. There was no significant difference in the vitreous levels of Hcys in both the groups. However, larger sample sizes must be examined before any conclusions are drawn on these results. Among the 17 RRD eyes, 3 of them showed anterior segment disease associated with inflammation, and all cases had relatively higher Hcys level in the vitreous compared with other eyes. The effects of any medication on the outcome of the results have been ruled out.

Correlation between LOX and Hcys in PDR and RRD

To determine whether there is a statistically significant inverse relationship between LOX and Hcys, we analyzed the data by Pearson's correlation. We found that there was a statistically significant negative correlation between vitreous Hcys and LOX in PDR cases ($P = 0.040$, $r = -0.534$) and in RRD ($P =$

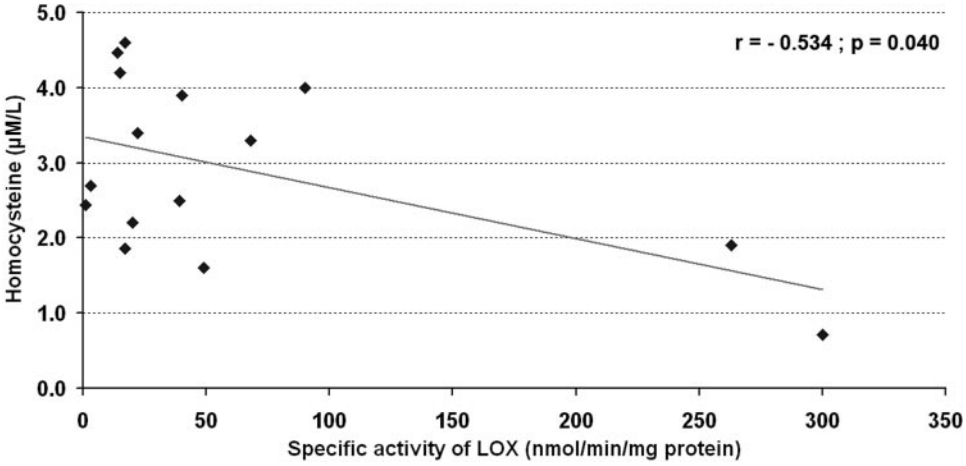

FIGURE 2. Pearson's correlation between Hcys and LOX specific activity in the vitreous of eyes with PDR ($n = 15$). There is a statistically significant negative correlation ($r = -0.534$; $P = 0.040$).

0.029, $r = -0.583$; Figs. 2 and 3). Thus, the detailed analysis of individual data show a statistically significant correlation between decreased specific activity of LOX and increased Hcys in PDR and RRD. There was no correlation between LOX and Hcys in the control samples ($P = 0.46$, $r = -0.17$).

Hydroxyproline Level in the Vitreous

Hydroxyproline levels which were measured to predict collagen turnover was found to be significantly increased in PDR, with a mean of 14.25 ± 4.40 micrograms/mg protein ($P = 0.040$), and in RRD, with a mean of 14.93 ± 3.23 micrograms/mg protein ($P = 0.024$), compared with the control of 4.55 ± 0.74 micrograms/mg protein (Table 3). However, there was no correlation between hydroxyproline levels and Hcys in subjects with PDR, RRD, and the control. The distribution profile of hydroxyproline in the vitreous was found to be in the range of 2.97 to 49.98 micrograms/mg protein in PDR, 1.94 to 49.80 micrograms/mg protein in RRD and 1.23 to 9.27 micrograms/mg protein in the control (Fig. 4). The decrease in LOX is reflected as increased collagen turnover, as measured by hydroxyproline content in PDR and RRD.

DISCUSSION

Increased Hcys at the level of plasma has been shown as a risk factor in ocular diseases such as CRVO, ARMD, optic neuropathy, and diabetic retinopathy.[6–10] In these studies, the plasma Hcys levels were correlated with the disease condition. Aqueous humor Hcys has been associated with pseudoexfoliation (PEX) and glaucoma.[30–32] The tear Hcys level in glaucoma has been reported by Roedl et al.[33] However, we found only one study on Hcys levels in the vitreous. Aydemir et al.[25] showed elevated Hcys in the vitreous of eyes with PDR that correlated with plasma Hcys levels in comparison with vitreous from eyes with nonproliferative ocular diseases. In the present study, we report vitreous Hcys levels to be increased in eyes with PDR, a condition associated with neovascularization, and in RRD, in which there is no neovascularization. In both these conditions, however, there are extensive ECM changes. In our previous study we showed that LOX, the collagen cross-linking enzyme, has significantly lowered activity with an increase in MMP-2 in RRD and MMP-9 in PDR,[26] indicative of the altered ECM activity in these two vitreoretinal diseases.

Although the etiology of PDR and RRD is different, the increased level of Hcys in PDR may be due to an Hcys-mediated change in inner retinal barrier permeability, whereas in RRD it could be due to an outer blood–retinal barrier breakdown. Loss of blood–retinal barrier permeability and blood–retinal barrier breakdown (outer and inner) may augment the diffusion of Hcys into the vitreous. The fact that in RRD there is a significantly higher Hcys level compared with PDR, indicates that the varying degree of outer blood–retinal barrier breakdown that can increase the vitreous Hcys levels.

Sen et al.[34] suggested that increased Hcys accumulation is associated with matrix remodeling, such as collagen type-1 synthesis and matrix metalloproteinase (MMP)-9 activity. Studies have shown that Hcys increases MMP-2 and -9 synthesis in endothelial cells and vascular smooth muscle cells.[35,36] Characteristic changes in the levels of MMP-2 and -9 activity attributed to ECM remodeling have been reported in both PDR[37–39]

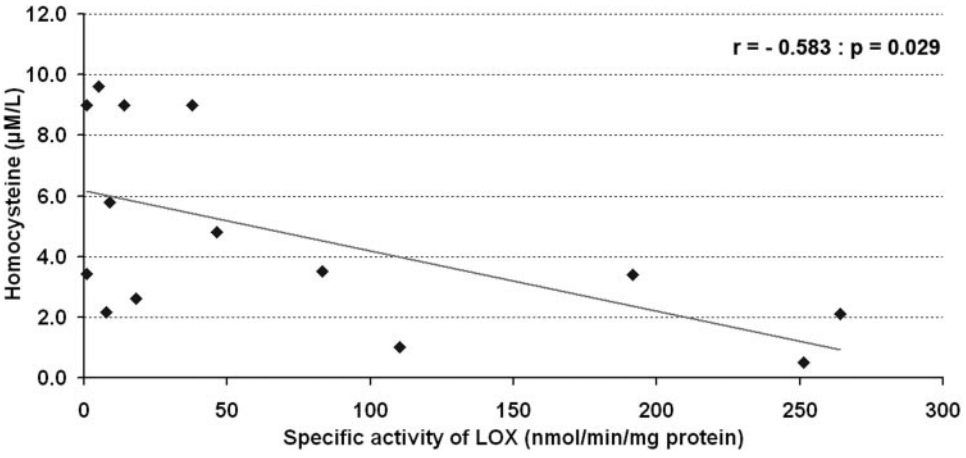

FIGURE 3. Pearson's correlation between Hcys and LOX specific activity in the vitreous of eyes with RRD ($n = 14$). There is a statistically significant negative correlation ($r = -0.583$; $P = 0.029$).

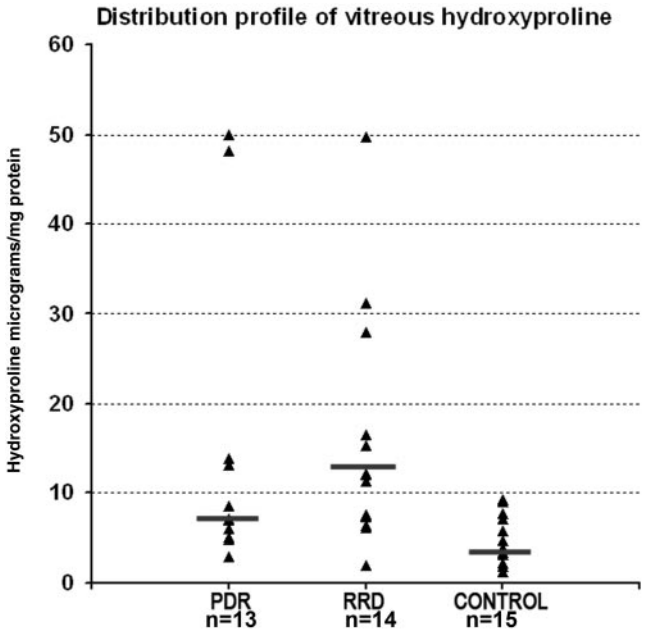

FIGURE 4. Distribution profile of vitreous hydroxyproline in eye with PDR or RRD and the donor eye control. *Solid lines*: the median. The median hydroxyproline level was 7.05 micrograms/mg protein in PDR and 12.0 micrograms/mg protein in RRD compared with 3.58 micrograms/mg protein in the control.

and RRD.[40,41] Hence, the increased Hcys levels in the vitreous could be a causative factor in the increased activity of MMP-2 and -9, thereby degrading the ECM.

Hcys modulates ECM changes not only by activating MMPs but also by downregulating LOX.[23] The present study shows a significant negative correlation between Hcys and LOX, both in RRD and PDR which is substantiated by the higher mean Hcys level in the vitreous of eyes with RRD, compared with those with PDR. Raposo et al.[23] have reported that pathophysiological concentrations of Hcys (35 μM) inhibited LOX activity and mRNA expression in cultured porcine aortic endothelial cells. They observed that the Hcys thiol group and the oxidative stress triggered by the auto oxidation of this amino acid are involved in LOX inhibition. Rodríguez et al.[42] have also reported that vascular LOX is downregulated in the early stages of atherosclerosis and increases in the advanced stages. Decreased vascular expression of LOX has also been reported in diabetic rats with a concomitant decrease in the ECM components.[43]

Apart from activating MMPs and downregulating LOX, a recent review by Toohey[14] proposes that Hcys toxicity can cause connective tissue disease. He proposed that mercaptoaldehyde formed from Hcys thiolactone may affect collagen metabolism by depleting the ascorbic acid necessary for collagen synthesis or may cause abnormal collagen cross-linking.[14] Although there is a continuous synthesis and breakdown of collagen, the regulation of collagen turnover in the vitreoretinal diseases such as PDR and RRD is still not very clear.[44,45] Studies are suggestive of vitreous collagen synthesis that occurs mainly prenatally, although there is a low level production, even in adulthood.[18,46] The present study showed significantly elevated levels of hydroxyproline in the vitreous of eyes with PDR and RRD compared with donor eye ball vitreous showing increased collagen turnover. However, no significant correlation between the hydroxyproline levels and LOX activity was observed in this study. LOX inhibition favors the presence of soluble forms of collagen that are highly susceptible to degradation by MMPs.[27] Thus, increased collagen turnover is reflective of an altered ECM. Whether the increased Hcys correlating with decreased LOX can lead to inadequate collagen cross-linking should be investigated.

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