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ORIGINAL RESEARCH

Free radicals in the aqueous humor of patients with glaucoma

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Purpose: To clarify the presence of oxidative stress in glaucoma and discuss whether free radicals contribute to neovascular glaucoma (NVG) and non-NVG.

Methods: Two patient groups were formed: the NVG group (n = 10) and the non-NVG group (n = 17). Free radicals in aqueous humor were detected by measuring the electron spin resonance (ESR). To identify free radical species, either superoxide dismutase (SOD) or catalase was added to the aqueous humor and compared with aqueous humor in which SOD or catalase was not added.

Results: In the NVG group, free radical waveforms were detected that differed from ascorbate-free radical (AFR) in all cases. Under SOD was added, the characteristic waveforms disappeared and the AFR of a specific waveform appeared. After catalase was added, the waveforms were unchanged. In the non-NVG group, the AFR of specific waveforms were detected in all cases. In 4 cases, the waveforms detected the presence of a trace of superoxide.

Conclusions: In the NVG group, superoxides were detected, suggesting that superoxide scavenging activity was decreased markedly. L-ascorbic acid likely has an antioxidative function in the non-NVG group, suggesting that the aqueous humor in the NVG group was under higher oxidative stress compared with the non-NVG group.

Keywords: oxidative stress, free radicals, glaucoma, ascorbate

Introduction

Free radicals are highly reactive due to their extreme instability. In biologic reactions, free radicals have been reported to be involved in tissue disorders caused by peroxidation. In ophthalmology, oxidative stress has been reported to induce and facilitate progression of cataracts and diabetic retinopathy.[1,2] In glaucoma, antioxidant levels decrease in the aqueous humor in patients with primary open-angle glaucoma (POAG) compared with in the aqueous humor of patients with cataracts, suggesting that peroxidation may be involved in the development of glaucoma.[3] However, no studies have reported the results of direct measurement of free radicals in the aqueous humor in patients with glaucoma. In the current study, we measured the levels of free radicals in the aqueous humor of patients with glaucoma by electron spin resonance (ESR) to determine if there is a relationship between free radicals and neovascular glaucoma (NVG) and non-NVG.

Subjects and methods

The study included 10 eyes with NVG (mean patient age, 67 ± 10 years; men, 7 eyes; women: 3 eyes) and 17 eyes with non-NVG (mean patient age, 60 ± 14 years;

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men, 8 eyes; women: 9 eyes) examined at the Department of Ophthalmology, Dokkyo Medical University Koshigaya Hospital, Japan. The non-NVG group included 11 eyes with POAG, 5 eyes with primary angle closure glaucoma (PACG), and 1 eye with normal tension glaucoma (NTG) (Table 1A, 1B). In accordance with the 2000 World Medical Association Declaration of Helsinki (Edinburgh, Scotland, 2000) informed consent was obtained from all subjects after they had received a detailed verbal explanation of the study before aqueous humor specimens were collected.

The aqueous humor specimens were obtained using a 27-gauge needle during trabeculectomy and immediately frozen with liquid nitrogen followed by measurement of free radicals by ESR spin trapping. The ESR measurement conditions are shown in Table 2. Free radicals were measured after mixing 10 μLof 5,5-dimethyl-1-pyrroline-N-oxide, the trapping agent, in 140 μL of ultrapure water followed by 20 μL of aqueous humor.

A superoxide scavenger of Cu, Zn-superoxide dismutase (SOD) and a hydrogen peroxide scavenger of catalase were used as active oxygen scavengers to identify the free radicals species formed. When SOD or catalase were added, the measurement solution of free radicals added to SOD or catalase to make up for 140 μL of ultrapure water which in the case of non-addition of SOD or catalase to aqueous humor, and similarly measured by ESR. The measurement solution was added to 40 μL of Cu, Zn-SOD (10 U/μL) and then to 100 μL of ultrapure water, or 140 μL of catalase (22 U/μL). The volumes of DMPO and aqueous humor were the same as previously described.

Results

NVG group

Free radical waveforms were detected that differed from AFR in all 10 cases. The waveforms in all cases did not change under catalase was added but did change to AFR with specific waveforms after SOD was added (Figure 1).

Table 1A Patient demographic data

NVG group			
Case	**Disease**	**Age**	**Gender**
1	NVG	56	male
2	NVG	76	male
3	NVG	51	male
4	NVG	81	male
5	NVG	63	female
6	NVG	63	female
7	NVG	69	male
8	NVG	59	female
9	NVG	70	male
10	NVG	79	male

Table 1B Patient demographic data

Non-NVG group			
Case	**Disease**	**Age**	**Gender**
11	PACG	21	male
12	PACG	73	female
13	PACG	63	male
14	POAG	57	male
15	POAG	57	male
16	NTG	67	female
17	PACG	66	female
18	POAG	54	male
19	POAG	74	male
20	POAG	38	female
21	POAG	57	female
22	POAG	59	female
23	POAG	77	female
24	POAG	59	female
25	PACG	72	female
26	POAG	56	male
27	POAG	70	male

Non-NVG group

The ESR spectra were roughly divided into two patterns. In 13 of the 17 cases, AFR spectra were observed and the waveforms did not change after catalase or SOD was added (Figure 2). In 4 of the 17 cases, slight variations with slightly incomplete AFR waveforms were observed. These waveforms did not change after catalase was added, but did change to complete AFR waveforms after SOD was added (Figure 3).

Discussion

Fong et al[4] reported on ascorbic acid and peroxidation reactions in the aqueous humor of patients with POAG. These patients can be classified as those with only extremely low levels of ascorbic acid and those with high levels of ascorbic acid in the aqueous humor in the presence of glaucoma. Those with extremely low levels of ascorbic acid in the aqueous

Table 2 ESR measurement conditions

Power	8 mW
Magnetic field	334.1 mT
Field modulation width	0.1 mT
Frequency modulation	9.415 GHz
Sweep width	±5 mT
Sweep time	2 min
Time constant	0.1 sec
Receiver gain	7.9 × 100
Temperature	25°C

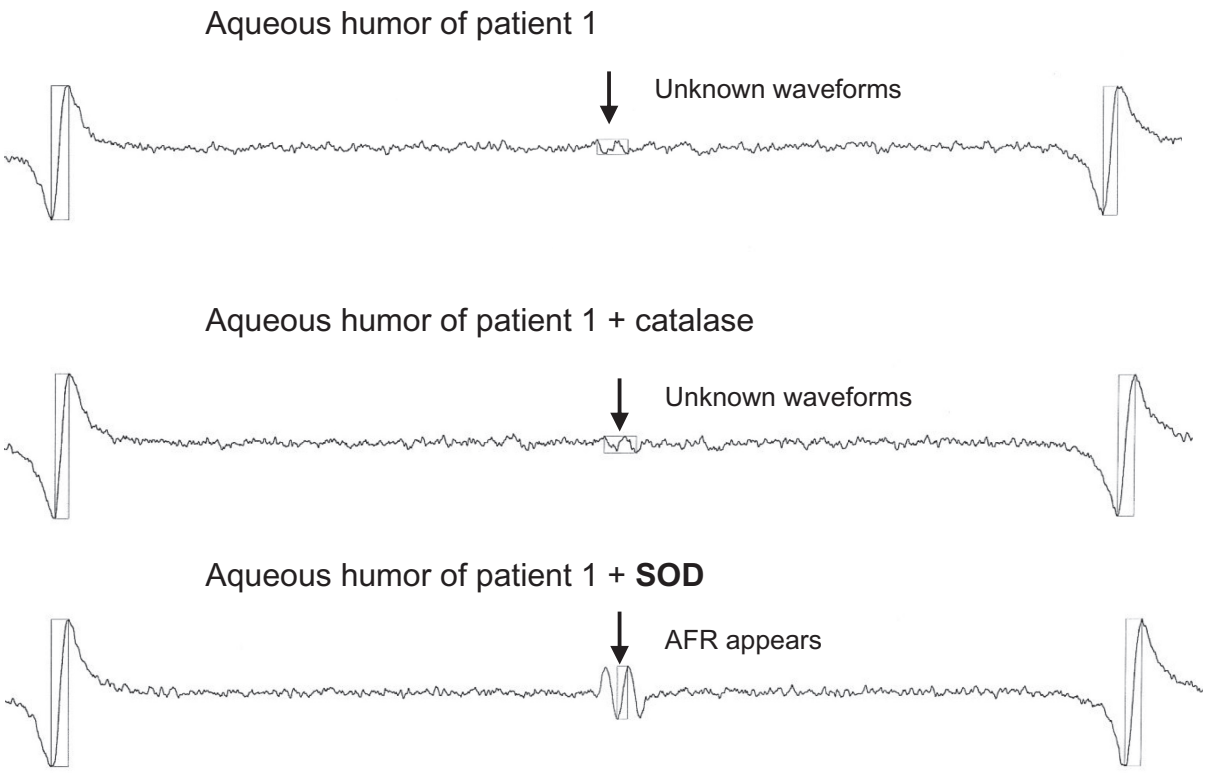

Figure 1 In the NVG group, free radical waveforms are detected that differ from AFRs in all 10 eyes. These waveforms are unchanged following addition of catalase but change to AFRs following addition of SOD.

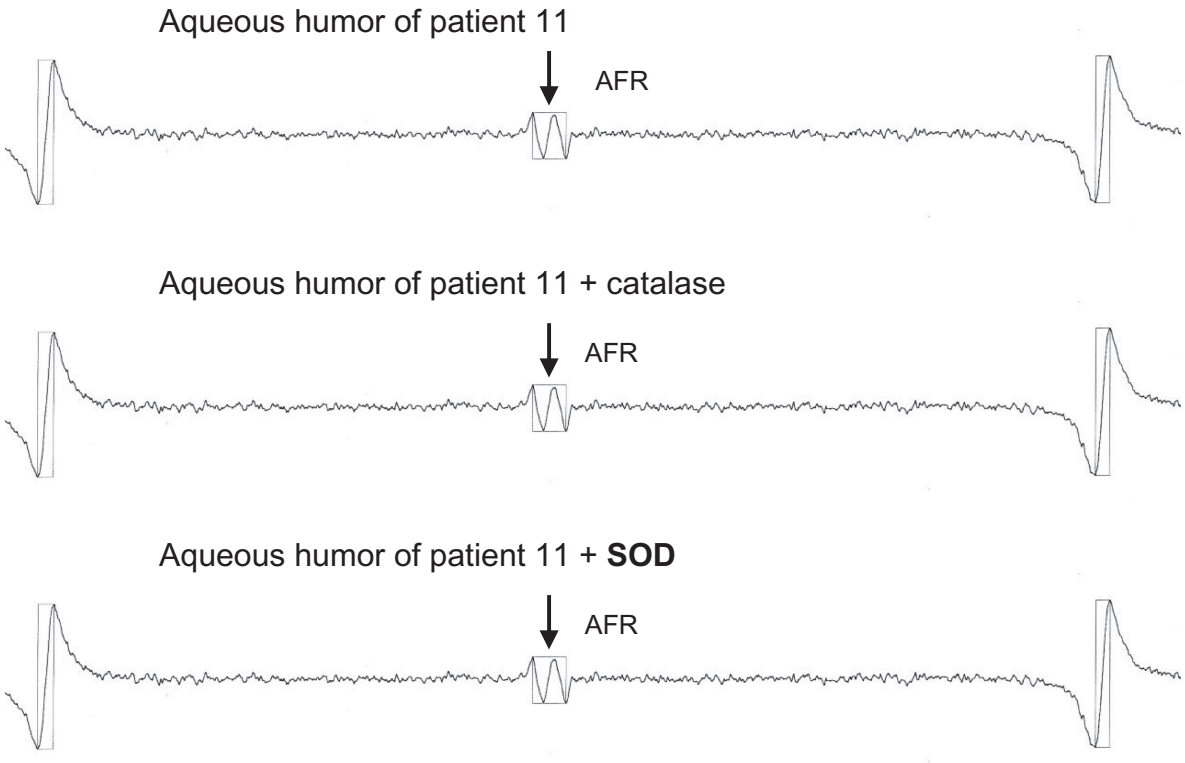

Figure 2 In 13 of the 17 eyes in the non-NVG group, AFR spectra are observed and the waveforms do not change following addition of catalase or SOD.

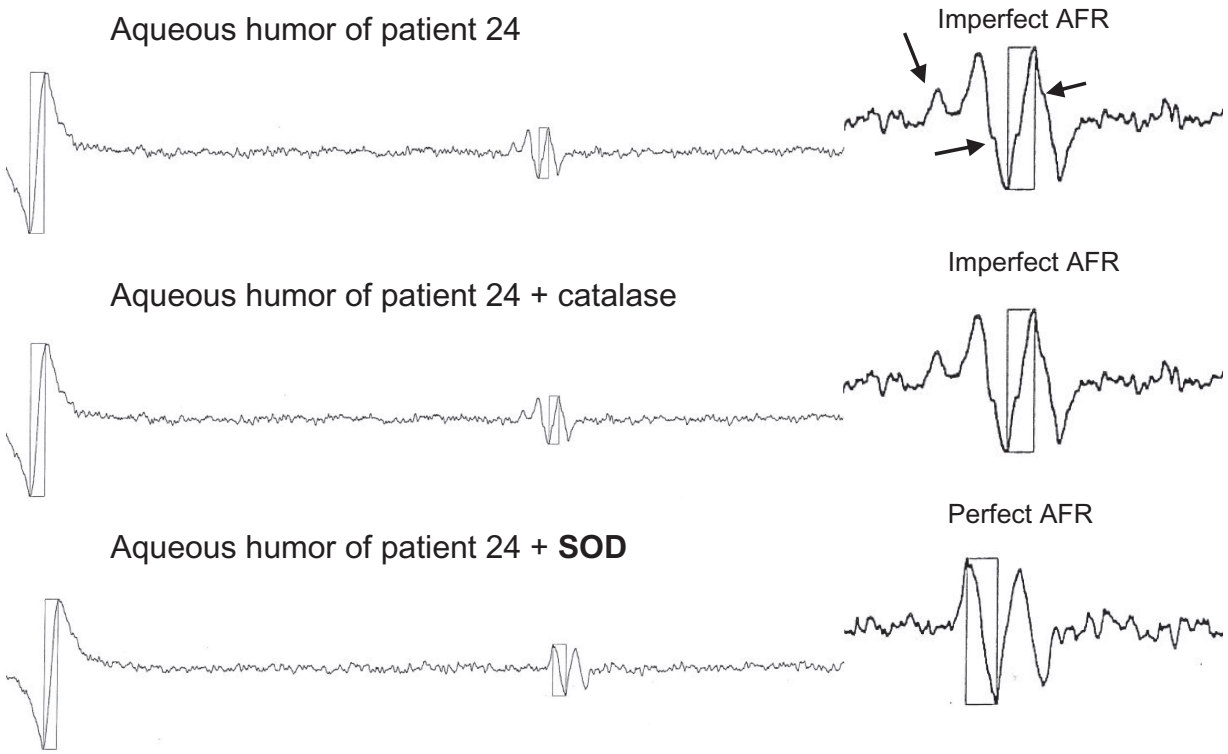

Figure 3 In 4 of the 17 non-NVG group eyes, inconclusive AFR waveforms are observed. These waveforms do not change following addition of catalase but change to a more conclusive AFR waveform following addition of SOD.

humor are susceptible to peroxidation stimulation. However, no free radicals were detected in the aqueous humor.

In the current study, we report for the first time the detection of free radicals in the aqueous humor of patients with glaucoma. AFR is formed by a reaction between L-ascorbic acid and superoxide. Moreover, since oxidized ascorbic acid is formed when superoxide reacts with AFR, AFR form intermediate products during the conversion from the reduced type to the oxidized type of ascorbic acid. Figure 4 shows ESR reaction spectra following *in vitro* formation of superoxide with a xanthine-xanthine oxidase system after different concentrations of L-ascorbic acid were added. The uppermost waveform is that of superoxide alone without L-ascorbic acid. The concentration of L-ascorbic acid increases moving downward and approaches the waveform of the AFR. The waveforms of the aqueous humor in the NVG group approximate the state of the second waveform from the top, confirming that the concentration of L-ascorbic acid is low relative to that of superoxide. The waveforms of the aqueous humor of the non-NVG group approach the state of the bottom waveform, confirming that the concentration of L-ascorbic acid is high relative to that of superoxide. The values of L-ascorbic acid measured in the aqueous humor specimens of the non-NVG group were equivalent to those in the specimens containing a high level of ascorbic acid reported by Fong et al while the values in the NVG group were equivalent to the extremely low values of ascorbic acid reported by Fong et al.[4]

Based on these results, Table 3A, 3B shows the free radicals detected in the aqueous humor of patients with glaucoma. Waveforms of the free radicals that differed from the AFR were detected in the NVG group; these waveforms did not change after catalase was added but did change to AFR after SOD was added. Since the waveforms changed as a result of SOD, these free radicals were determined to be superoxides. AFR was detected in 13 of 17 cases in the non-NVG group. Imperfect AFR waveforms were observed in the remaining 4 cases; these waveforms converted to complete AFR of specific waveforms only after SOD was added, which indicated that these free radicals contained a small amount of superoxide in the AFR (Table 3A, 3B).

AFR is generated from L-ascorbic acid as a result of oxidation of L-ascorbic acid by unpaired electrons of superoxides, and so on. Moreover, the oxidized type of dehydroascorbic acid is reduced by the reaction between glutathione peroxidase (Gpx) and reduced glutathione (GSH)

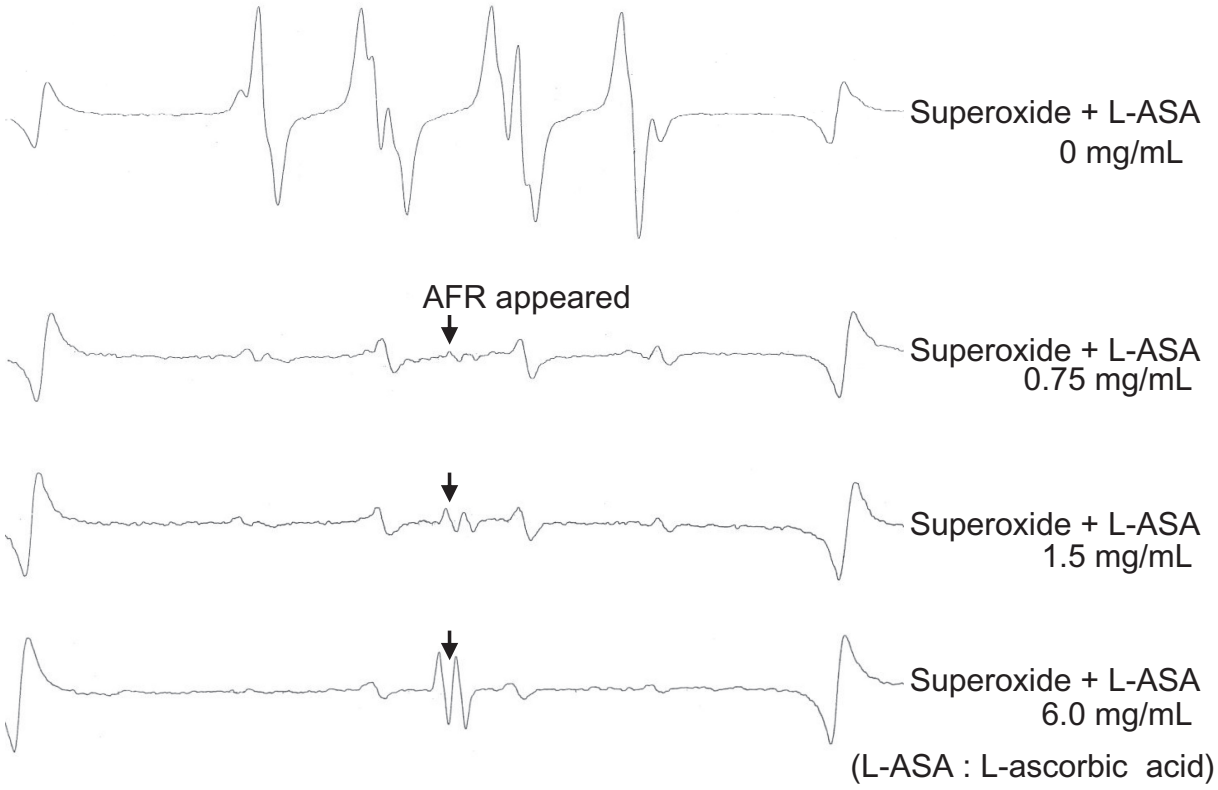

Figure 4 *In vitro* formation of superoxide with a xanthine-xanthine oxidase system followed by the addition of different concentrations of L-ascorbic acid.

(Figure 5A). Based on this, we constructed a hypothetical model of the reactivity of free radicals in the aqueous humor of patients with glaucoma based on the results of the current study. Since superoxides were detected in the NVG group, the activity of superoxide scavengers such as SOD, L-ascorbic acid, and GSH decreased markedly in the aqueous humor of this group, resulting in the presence of a superoxide. In the NVG group, we believe that the L-ascorbic acid captured a large amount of superoxides and was subjected to peroxidation to the state of oxidized ascorbic acid through the AFR stage, resulting in the superoxide detection by ESR without elimination by ascorbic acid (Figure 5B). In addition, AFR may have been detected in the NVG group after SOD was added because excessive superoxide that had formed were eliminated, causing the L-ascorbic acid to remain at the AFR stage without undergoing peroxidation to the stage of dehydroascorbic acid (Figure 5C).

Table 3A Free radicals in aqueous humor of patients with glaucoma

NVG group				
Case	**Aqueous only**	**Aqueous + catalase**	**Aqueous + SOD**	**Free radical species**
1	non-AFR	non-AFR	AFR	superoxide
2	non-AFR	non-AFR	AFR	superoxide
3	non-AFR	non-AFR	AFR	superoxide
4	non-AFR	non-AFR	AFR	superoxide
5	non-AFR	non-AFR	AFR	superoxide
6	non-AFR	non-AFR	AFR	superoxide
7	non-AFR	non-AFR	AFR	superoxide
8	non-AFR	non-AFR	AFR	superoxide
9	non-AFR	non-AFR	AFR	superoxide
10	non-AFR	non-AFR	AFR	superoxide

AFR was detected in the non-NVG group. Since superoxide scavengers such as SOD, L-ascorbic acid, and GSH function in the aqueous humor of this group, superoxide was not present to the degree they were present in the NVG group, which enabled L-ascorbic acid to eliminate superoxides, resulting in the detection of AFR by ESR (Figure 5D). Ferreira et al[3] reported that the total levels of antioxidants in the aqueous humor decreased in POAG compared with eyes with cataracts, resulting in damage due to oxidative stress. Since SOD and Gpx activities increased without change catalase, there may have been a decrease in other antioxidants in the form of L-ascorbic acid and GSH. In the current study, since AFR were detected in all subjects in the non-NVG group, a decrease in L-ascorbic acid and GSH caused by the conversion of L-ascorbic acid to an AFR agreed with the total decrease in the levels of antioxidants reported by Ferreira et al.[3] Babizhayev and Bunin[5] reported increased levels of

Table 3B Free radicals in aqueous humor of patients with glaucoma

Non-NVG group				
Case	**Aqueous only**	**Aqueous + catalase**	**Aqueous + SOD**	**Free radical species**
11	AFR	AFR	AFR	AFR
12	AFR	AFR	AFR	AFR
13	AFR	AFR	AFR	AFR
14	AFR	AFR	AFR	AFR
15	AFR	AFR	AFR	AFR
16	AFR	AFR	AFR	AFR
17	AFR	AFR	AFR	AFR
18	AFR	AFR	AFR	AFR
19	AFR	AFR	AFR	AFR
20	AFR	AFR	AFR	AFR
21	AFR	AFR	AFR	AFR
22	AFR	AFR	AFR	AFR
23	AFR	AFR	AFR	AFR
24	imperfect AFR	imperfect AFR	AFR	AFR, superoxide
25	imperfect AFR	imperfect AFR	AFR	AFR, superoxide
26	imperfect AFR	imperfect AFR	AFR	AFR, superoxide
27	imperfect AFR	imperfect AFR	AFR	AFR, superoxide

lipid peroxides in the aqueous humor, trabecular meshwork, and Schlemm's canal in POAG compared with control eyes, and suggested that lipid peroxidation was responsible for destruction of the trabecular meshwork and Schlemm's canal. In addition, Bunin et al[6] reported that destruction of the drainage system in POAG is accelerated by lipid peroxidation. Yan et al[7] reported that hydrogen peroxide (H_2O_2) of a reactive oxygen is present in the aqueous humor and affects the drainage function of the aqueous humor in various animal experiments. After examining the damage to this drainage function caused by peroxidation of aqueous humor in an experiment in which the aqueous humor in porcine eyes was refluxed with 3 mM H_2O_2, Yan et al reported that although drainage increased at a normal ocular tension of 7.5 mmHg, drainage decreased at high ocular tension of 30 mmHg.

In the current study, although the involvement of H_2O_2 can be excluded because no changes were observed after catalase was added, lipid peroxidation might have been accelerated by superoxides that formed in the NVG group, which in turn might have induced destruction of the trabecular meshwork, Schlemm's canal, and other components of the drainage system. On the other hand, active oxygen has been

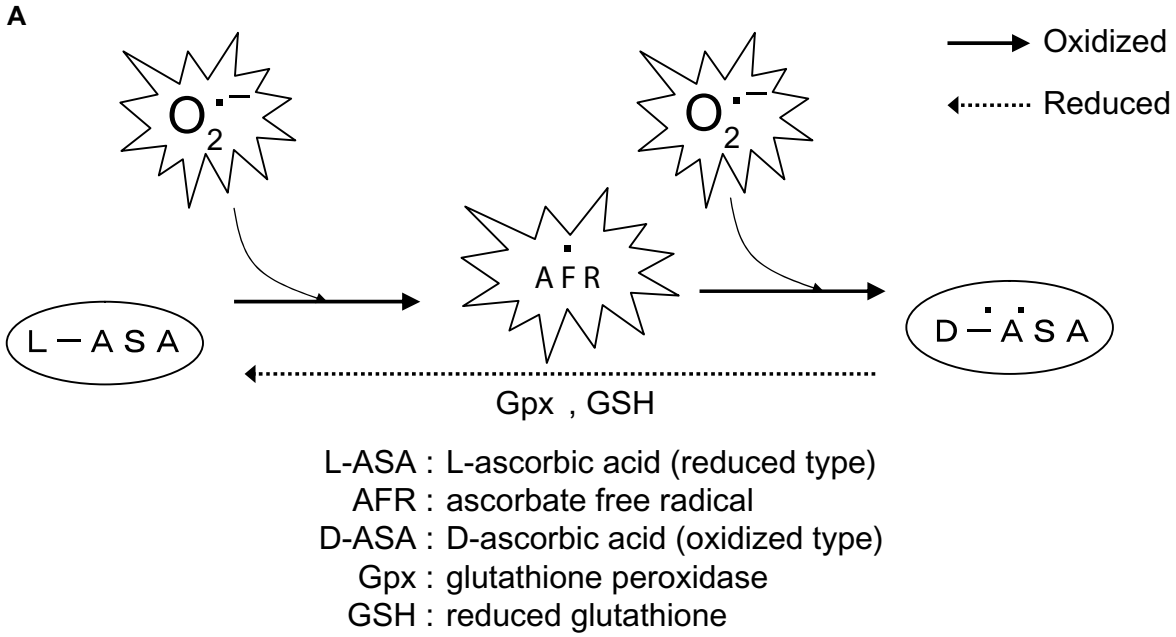

Figure 5 A) AFRs are generated from L-ascorbic acid as a result of L-ascorbic acid oxidation by unpaired electrons of superoxides. Moreover, the oxidized type of dehydroascorbic acid is reduced by the action of Gpx and GSH. **B**) In the NVG group, L-ascorbic acid captures a large amount of superoxides and is subjected to peroxidation to the state of oxidized ascorbic acid through the AFR stage, resulting in superoxides being detected without being eliminated by ascorbic acid. **C**) In the NVG group following addition of SOD, excessive superoxides are eliminated, causing the L-ascorbic acid to remain at the AFR stage without being peroxidated to oxidized L-ascorbic acid. **D**) In the non-NVG group, no superoxides are present to the degree they are present in the NVG group, enabling the L-ascorbic acid to eliminate superoxides and resulting in the detection of AFRs.

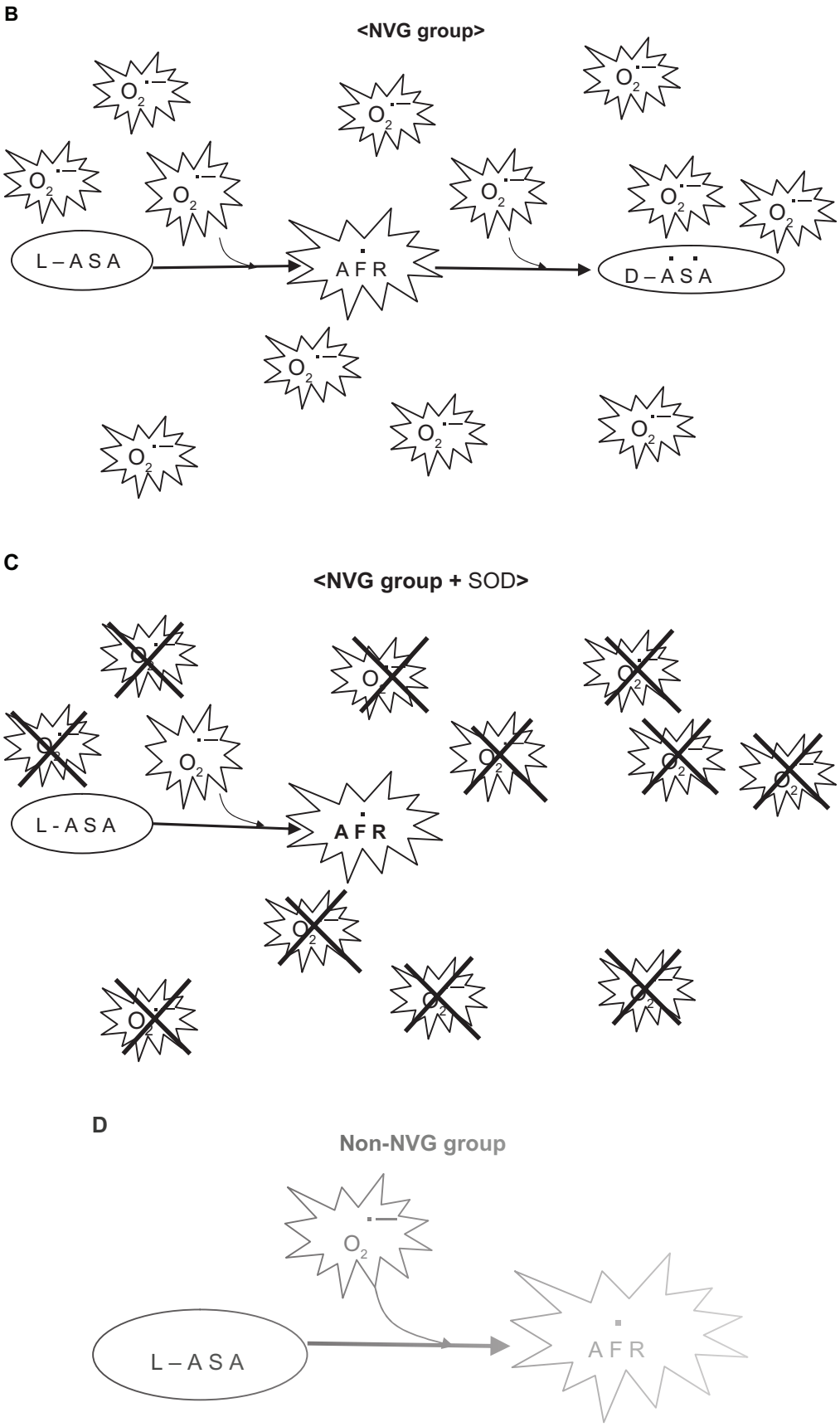

Figure 5 (*Continued*)

reported to be involved in vascularization. In the vitreous, we reported that low levels of superoxide scavenging activity, L-ascorbic acid and GSH are present, but there are high levels of lipid peroxides and H_2O_2 levels in diabetic retinopathy[2] and that there is a correlation between vascular endothelium growth factors and hydrogen peroxide in proliferative diabetic retinopathy.[8] Consequently, the superoxide detected in the aqueous humor of the NVG group in the current study might have been involved in vascularization of the anterior chamber of subjects of the NVG group as well. It is necessary to conduct additional studies that include are larger number of subjects.

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Disclosure

The authors have no proprietary interest in any aspect of this report.

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