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Pathophysiology of Cataracts: Copper Ion and Peroxidation in Diabetics

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Abstract: The concentration of copper ion in cataractous lenses was higher than that in clear lenses. The concentration of copper ion was significantly higher in subjects with diabetes (DM group) than in subjects without diabetes (control group). The concentration of Cu^{2+} ion was significantly greater than that of Cu^{+} ion in the DM group. Furthermore, the concentration of copper ion unconjugated to protein was higher than that of protein-conjugated copper ion. It is assumed that in the cataractous lenses of the DM group, a decrease in the reactivity of the copper-containing enzyme superoxide dismutase and an increase in hydrogen peroxide concentration leads to the generation of hydroxyl radicals from the Fenton's-type reaction. In diabetes, an increase in lenticular glucose induces glycation with the release of copper ion from copper-containing enzymes, thus increasing the concentration of lenticular copper ion. As a result, superoxide scavenging activity is reduced and peroxide lipid concentration is increased. This is assumed to result from the hyperactivity of the peroxidation cascade. **Jpn J Ophthalmol 1997;41:130–137** © 1997 Japanese Ophthalmological Society

Key Words: Cataract, copper ion, diabetes mellitus, peroxidation.

Introduction

Trace metals are essential for maintaining bodily functions and changes in the amounts of trace metals present in the body may be related to the onset of disease. Copper, an essential trace metal, conjugates with certain enzymes, affecting their reactivities and stability; the concentration of copper changes when disease occurs.[1] In rats with streptozocin-induced diabetes, copper concentration increases in blood, liver, kidney, and duodenum.[2–4] Copper concentration also increases in the blood of human diabetics.[5,6]

An increase in the copper concentration in cataractous lenses has been reported.[7] We also have previously reported that copper concentration increased in the cataractous lenses and vitreous bodies of diabetics.[8] In this study, the amount and composition of copper ion in the cataractous lenses of diabetic patients were measured and compared to those of nondiabetic patients. The effect of the increased copper ion on the maintenance of lenticular clearance via the generating and scavenging systems of active oxygen and peroxidation was also studied.

Materials and Methods

Sample Collection

Lenses. In this study, one intracapsular-extruded lens (the dislocated lens of a 62-year-old trauma patient) was used as the control clear lens. Two intracapsular-extruded lenses of senile cataract (from patients who were 68 and 77 years old) were used as control cataract lenses. Twenty-eight phacoemulsification-indicated cataractous lenses of diabetics (from ages 54 to 78 years old, average 68.1 ± 0.7 years old) were investigated as the DM group. Thirty-two phacoemulsification-indicated cataractous lenses from nondiabetics were grade- and age-matched (from ages 50 to 81 years old, average 67.9 ± 0.8 years old) to the DM group and were investigated as the non-DM group. Mature cataracts were excluded from both DM and non-DM groups.

All patients with hyperlipidemia, nephropathy, hypertension, and insulin-dependent diabetes mellitus, and patients who were receiving hemodialysis were

Received: April 22, 1996
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Jpn J Ophthalmol 41, 130–137 (1997)

Published by Elsevier Science Inc.

0021-5155/97/$17.00
PII S0021-5155(97)00030-0

excluded. All subjects were fully informed of the details of our study and gave their written consent.

Two intracapsular-extruded lenses were used for detecting copper contained in the lenticular tissue by histopathologic stain. One was a brown cataractous lens from a 72-year old nondiabetic patient and the other was a clear dislocated lens from a 62-year-old trauma patient.

Blood, aqueous humor, and vitreous body. To quantify the amount of copper in ocular tissue, blood and aqueous humor samples were collected from patients in the DM, non-DM, and control groups. In addition, vitreal samples were collected by vitrectomy from 25 patients with diabetic retinopathy (in the DM group, from ages 41 to 75 years old, average 54.4 ± 10.4 years old) and 11 patients with rhegmatogenous retinal detachment or trauma (in the non-DM group, from ages 34 to 79 years old, average 53.3 ± 13.4 years old).

Sample Processing

All the instruments were cleaned by the following procedure: (1) cleaning with ordinary detergent; (2) soaking in acid cleaner overnight; (3) rinsing with ion-exchanged water; and (4) drying prior to storage away from sunlight.

Lenses. Intracapsular-extruded lenses were homogenated with ion-exchanged water and immediately filled with N_2 gas. Cataractous lenses from the DM and non-DM groups were collected directly from the aspiration tube after emulsification and kept at 4°C until the phacoemulsification had been completed. The cataractous lenses had already been homogenated with infusion fluid (Opegard® MA, Senju Pharmaceutical, Osaka, Japan) during phacoemulsification. Following collection, the lens samples were filled with N_2 gas. The homogenated lenses were centrifuged at 22 600 xg at 4°C for 1 hour. The supernatant was used to measure the $O_2^{\cdot-}$ scavenging activity and the amounts of copper ion, hydrogen peroxide, superoxide dismutase (SOD), glucose, and glycated protein. The pellet was used to quantify the peroxide lipid.

Blood, aqueous humor, and vitreous bodies. A 7-mL fasting blood sample was collected from each patient on the morning of his cataract operation. The blood sample was centrifuged at 22 600 xg at 4°C for 10 minutes and the supernatant was immediately filled with N_2 gas.

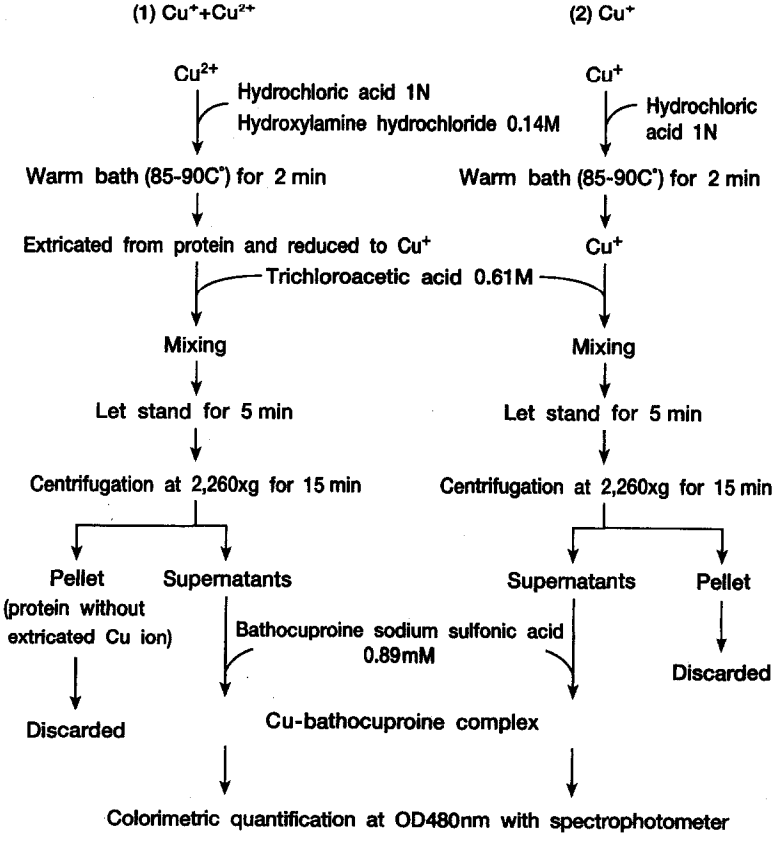

Figure 1. Bathocuproine method—Protocol of copper ion quantification.

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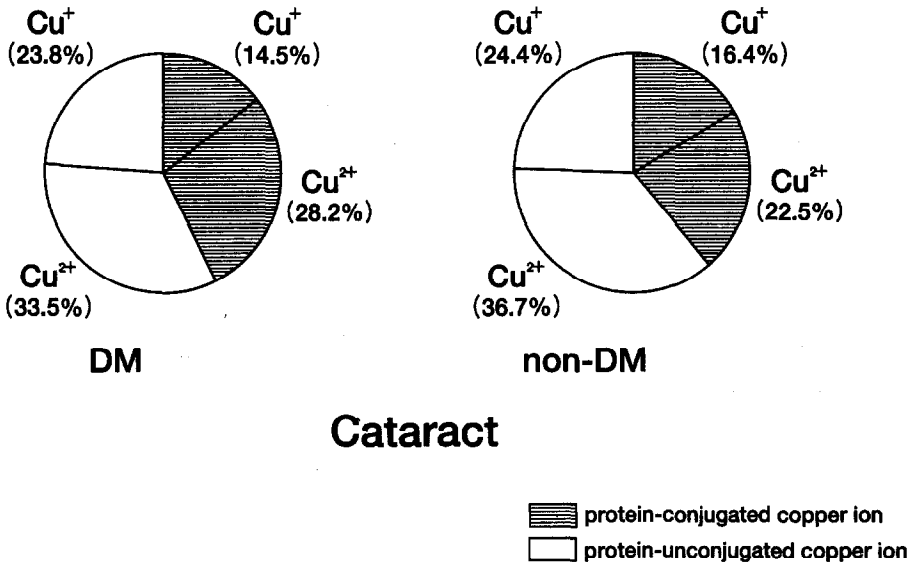

Figure 2. Composition of copper ion in human cataractous lens.

A 100-mL sample of aqueous humor was collected from each patient during his cataract operation. This sample was immediately filled with N_2 gas.

A vitreous body sample was collected from each patient during his vitrectomy. A chilled sample was immediately centrifuged at 2260 xg at 4°C for 10 minutes to collect the blood cells that were mixed in the sample. The supernatant was then isolated and centrifuged at 22 600 xg at 4°C for 1 hour. Because Opegard® MA solution was used as perfusion fluid during the vitrectomy, it was also used as a blank in subsequent measurements.

Assays

Quantification of copper ions. The supernatant from the lens sample contained copper ions; some were conjugated to protein. The copper ion content of the supernatant was regarded as the total amount of copper ion in the lens sample. Filtrate was obtained by centrifugation at 3000 xg at 4°C for 1 hour with a Centricon SR-3 centrifugal microfilter that fractionates at a molecular weight of 3000; this filtrate was regarded as a sample copper ion unconjugated to protein. The amount of protein-conjugated copper ion was determined by subtracting the amount of copper ion unconjugated to protein from the total amount of copper ion. Because the membrane of the Centricon SR-S centrifugal microfilter contained a very small amount of glycerin, it was used only after the following pretreatment: the entire reservoir, including the membrane, was immersed in deionized water for several hours within a closed container; the entire microfilter unit was then covered with a piece of absorbent tissue paper, put into a centrifugal tube that was slightly larger than itself, and centrifuged at 2260 xg for 2 minutes to remove the water.

Because monovalent copper ion (Cu^+) binds specifically to bathocuproine,[9] copper ions was extracted from proteins with hydrochloric acid, reduced to Cu^+ with hydroxylamine hydrochloride, and measured as the total amount of copper ion. The amount of Cu^+ contained in the original sample was measured without the addition of hydroxylamine hydrochloride. The amount of divalent copper ion (Cu^{2+}) was obtained by subtracting the amount of Cu^+ from the total amount of copper ion (Figure 1).

Histopathological staining of copper ions. The rhodanine method[10,11] was used to detect the copper in the brown nuclear cataractous lens removed by intracapsular cataract extraction and the traumatically dislocated clear lens. After fixation with a 10% formalin solution, the lenses were embedded in paraffin and sliced 3–5 μm thick; the slices were deparaffinized, immersed in rhodanine stain for 24 hours at 37°C, dehydrated with ethanol, cleared with xylol, and finally mounted in Permount.

Measurement of substances involved in peroxidation. Hydrogen peroxide was measured by the titanium-hydrogen-peroxide colorimetric method.[12,13] Peroxide lipid was measured by Yagi's fluorescent method.[14] Superoxide dismutase (Cu,Zn-SOD) was measured by the ELISA (enzyme-linked immunosorbent assay) method.[15] The scavenging activity of $O_2^{\cdot-}$ was measured by the NBT (nitroblue tetrazo-

Table 1. Composition of Copper Ion in Human Cataractous Lens—Classified by Conjugation of Protein

	Cu^{2+} Amount (μg/lens)	Cu^{+} Amount (μg/lens)	Total Copper Ion Amount (μg/lens)
DM group			
Protein-conjugated type	3.18 ± 2.47	1.64 ± 1.15	4.20 ± 2.84[a]
Protein-unconjugated type	3.77 ± 3.23	2.69 ± 1.87	7.78 ± 7.11
Total	6.59 ± 2.65[b]	4.00 ± 2.65[a]	10.59 ± 6.25[b]
Non-DM group			
Protein-conjugated type	1.07 ± 0.94	0.78 ± 0.73	1.84 ± 1.52[a]
Protein-unconjugated type	1.75 ± 1.11	1.16 ± 0.83	2.82 ± 0.98
Total	1.89 ± 1.64[b]	1.98 ± 1.89[a]	3.84 ± 1.67[b]

[a] $P < 0.05$.
[b] $P < 0.01$.

lium) reduction method.[15] Glycated protein was measured by the fructosamine method.[16] Assay data was analyzed with the Student's *t*-test.

Results

Quantification of Copper Ions

The amount of copper ion in the homogenated sample of cataractous lenses removed by intracapsular extraction was 2.56 ± 0.44 μg/lens; the amount of copper ion in the sample of cataractous lenses collected after phacoemulsification was 3.84 ± 1.67 μg/lens. There was no statistically significant difference with these two methods of sample collection. The amount of copper ion in cataractous lenses, 3.84 ± 1.67 μg/lens, was significantly higher than the amount of copper ion in the traumatically dislocated clear lens: 0.51 μg/lens. The amount of copper ion in the cataractous lenses was significantly higher in the DM group (10.59 ± 6.25 μg/lens) than in the non-DM group (3.84 ± 1.67 μg/lens) ($P < 0.01$).

The amounts of each copper ion valence are shown in Figure 2 and Table 1. The amount of divalent ion was significantly higher in the DM group (6.59 ± 2.56 μg/lens) than in the non-DM group (1.89 ± 1.64 μg/lens) ($P < 0.01$). The amount of monovalent copper ion was also significantly higher in the DM group (4.00 ± 2.65 μg/lens) than in the non-DM group (1.98 ± 1.89 μg/lens) ($P < 0.05$).

The amounts of both protein-conjugated and unconjugated copper ions were higher in the DM group than in the non-DM group, with the greatest difference seen in the amount of unconjugated Cu^{2+}. The total amount of protein-conjugated copper ions, regardless of valence, was significantly greater in the DM group than in the non-DM group ($P < 0.05$).

The amounts of copper ion in the sample of blood and aqueous humor were higher in the DM group than in the non-DM group (Table 2). The amount of copper ion in the samples of vitreous body was significantly higher in the DM group than in the non-DM group ($P < 0.05$).

Histopathological Staining of Copper Ions

A photograph of the brown nuclear cataract stained with rhodanine is shown in Figure 3. Subepithelial and superficial cortical regions of the cataractous lens appear brown with stain, indicating the presence of copper. These same regions of the clear lens did not accept stain.

Measurement of Substances Involved in Peroxidation

As shown in Figure 4, the amount of hydrogen peroxide tended to be higher in the DM group (30.46 ± 0.51 nmol/lens) than in the non-DM group (26.67 ± 7.97 nmol/lens). The amount of peroxide lipid was slightly higher in the DM group (8.3 ± 3.7 × 10^{-2}

Table 2. Copper Content of the Human Lens, Blood, Aqueous Humor, and Vitreous Body

	Lens (μg/lens)	Blood (μg/dL)	Aqueous Humor (μg/dL)	Vitreous Body (μg/vit)
DM group	10.59 ± 6.25[b]	105.64 ± 13.90	8.54	26.11 ± 18.79[a]
Non-DM group	3.84 ± 1.67	102.43 ± 8.38	7.65 ± 1.07	9.71 ± 7.77

[a] $P < 0.05$.
[b] $P < 0.01$.

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Figure 3. Histopathological staining of copper ion by the rhodanine method.

nmol/lens) than in the non-DM group (6.8 ± 2.8 × 10^{-2} nmol/lens). The amount of Cu,Zn-SOD was slightly lower in the DM group (8.3 ± 1.4 μg/lens) than in the non-DM group (10.2 ± 3.5 μg/lens). The scavenging activity of O_2^- was lower in the DM group (42.20 ± 36.89 U/mg lens protein) than in the non-DM group (68.25 ± 25.72 U/mg lens protein). The amount of glycated protein also tended to be higher in the DM group (9.33 ± 8.15 μmol/lens) than in the non-DM group (7.55 ± 5.47 μmol/lens.

Discussion

Trace metals such as copper are essential in the maintenance of normal biophysiological function. Since copper is able to react with peroxide (in the Fenton's-type reaction),[17] it is strongly associated with peroxidation. In addition, it is known to accelerate lenticular opacification by damaging protein, lipid, and membranous structures. The change in the amount of copper ion in the lens may be the key to revealing the mystery of cataract formation. Liver and brain tissues have the highest natural concentrations of copper in the body and the brain is the only organ in which copper concentration is known to increase with aging. Pigmented ocular tissues contain copper in very high concentrations.[1] Copper concentration in the blood is known to increase in diabetes mellitus,[5,6] which may relate to the increase of copper in cataractous lenses.[2,7,18,19] To prove the location of copper in a lens histologically, we stained lenses with rhodanine. No staining occurred in the clear lens, but copper was stained in the superficial cortex of the brown cataractous lens. This is the first histo-

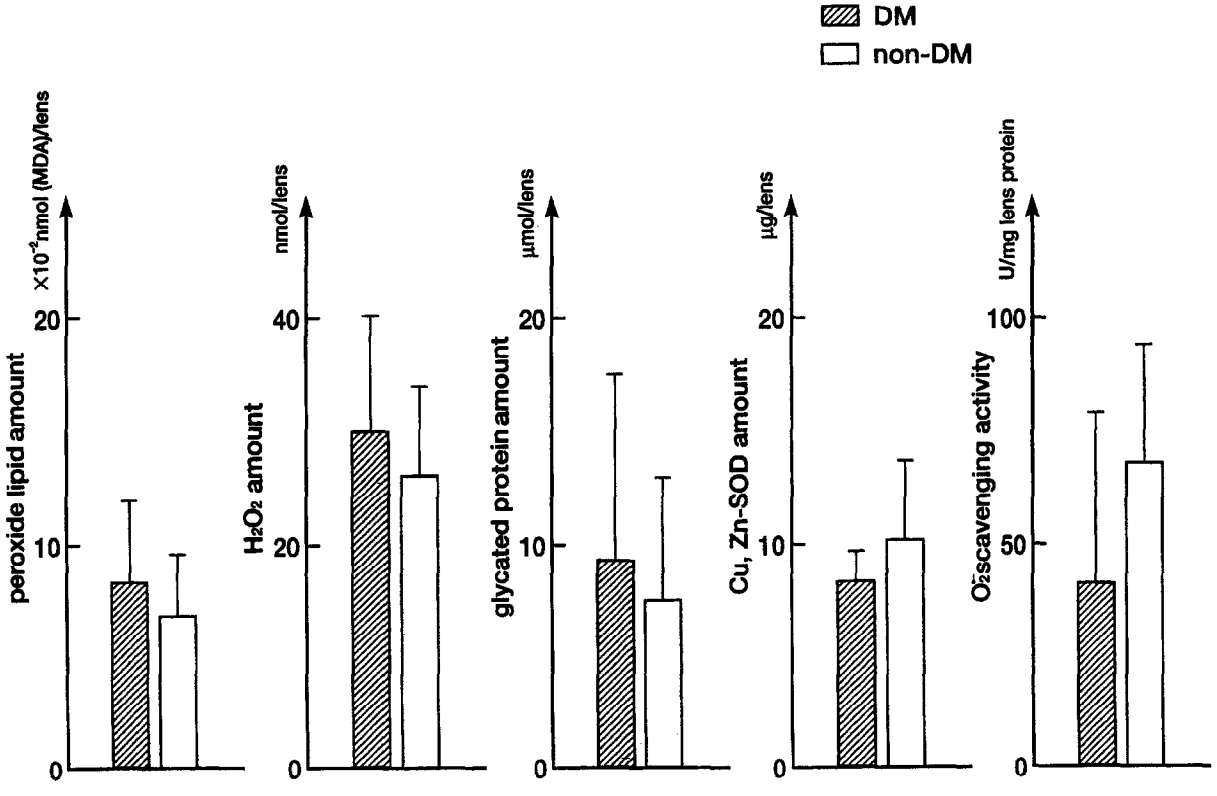

Figure 4. Substances involved in peroxidation in human cataractous lens.

logical proof of the location of copper ions in human cataractous lenses.

The results of this study have demonstrated a copper ion content of 0.51 μg/lens in the clear traumatically dislocated lens. Rácz et al[7] reported measuring the copper content in clear lenses by the atomic absorption spectrometry. Although their method differed from the one used in this study, they calculated a copper content of 0.045 μg/lens. Copper content in cataractous lenses of the non-DM group was 3.84 ± 0.67 μg/lens, higher than that in the clear lens investigated in these two studies. The amounts of both Cu^{+} and Cu^{2+} ions were significantly higher in the cataractous lenses of the DM group than in those of the non-DM group. The higher amount of Cu^{2+} ion was particularly significant.

In cataractous lenses, the amount of copper ion unconjugated to protein was higher than the amount of protein-conjugated copper ion. Copper ions that are unconjugated to protein are regarded as free copper ions, amino acid-conjugated ions, and peptide-conjugated copper ions under 3000 in molecular weight. It may be assumed that copper ion is continuously released from proteins containing copper, such as copper-containing enzymes. Although the amount of copper ion in cataractous lenses was higher in the DM group than in the non-DM group, the percentage of total lenticular copper ion that was unconjugated to protein was 57.3% for both groups.

Two factors are assumed to cause the copper increase in cataractous lenses. The first, found in diabetes mellitus, is increased vascular permeability, with resulting damage to the blood–aqueous barrier; this induces the transfer of copper from the blood to the aqueous humor, allowing copper to accumulate in the lens. Studies of cultured lenses[20,21] have also demonstrated that copper ion increases the permeability of the lenticular capsule. It is assumed that even a slight increase in the copper concentration of the blood will induce the accumulation of lenticular copper by a transfer of ions through the aqueous humor or vitreous body. In addition, the amount of copper ion measured in the vitreous body was found to be significantly higher in patients with diabetic proliferative retinopathy (DM group) than in patients with proliferative vitreoretinopathy and traumatic retinal detachment (non-DM group). It is conceivable that the amount of copper in the lens increased as a result of ion transfer through the vitreous body.

The second factor assumed to cause the copper increase in cataractous lenses is the release of copper ion from copper-containing enzymes. Copper-containing enzymes such as cytochrome-c oxidase, superoxide dismutase (Cu,Zn-SOD), and tyrosinase are present in ocular tissue.[1] In the diabetic patients, hyperglycemia induces the glycation of copper-containing proteins. When glycation occurs in Cu,Zn-SOD and other proteins that have histidine within 8 Å of lysine, the peptide bond of this histidine residual breaks and the Cu^{2+} ion conjugated to the residual is released.[22,23] As the free Cu^{2+} ion releases an electron and becomes a Cu^{+} ion, it is conceivable that the free copper ion concentration increases in the diabetic patient.

The free Cu^{+} ion induces a Fenton's-type reaction with hydrogen peroxide, a compound which also increases in concentration in the diabetic patient. This reaction generates HO· (a hydroxyl radical),[17,24] which is extremely reactive, denaturing lipids, proteins, and nucleic acids.[25] Furthermore, as Cu,Zn-SOD undergoes glycation, the subsequent HO· induces the generation of $O_2^{\cdot -}$, leading to peroxidation.[22,23]

The amounts of hydrogen peroxide and peroxide lipid both tended to be higher in the DM group than in the non-DM group. It became clear that peroxidation progresses in diabetes mellitus. It is possible that glycation in diabetes accelerates peroxidation,[26] with hyperglycemia as a key factor. However, we found that the concentration of copper ion in cataractous lenses of the non-DM group was higher than that in the clear lens. It is assumed that aging and long-term exposure to ultraviolet irradiation induce sugar hypotolerance and disrupt the body's defense against oxidation. These conditions, in turn, may cause the progressive accumulation of copper ion in lenses.

Moreover, when copper ion reacts with peroxide lipid, lipid-free radicals are generated and a cascade of peroxidation is initiated. As a result, membranous lipids are denatured and lose their functionality.

In this study, we examined the role of copper ion in the Fenton's-type reaction, in the glycation of superoxide dismutase and in combination with peroxide lipid. Copper ion can also catalyze other reactions.[27] For example, copper ion is associated with generation of an ascorbic acid free radical, and it inhibits enzymes of glycolysis and the pentose phosphate cycle.[1,28–31] (Figure 5).

Several mechanisms are essential for maintaining the transparency of the lens, including peroxide scavenging systems, glycolysis, and the pentose phosphate cycle. Disruption of the normal function of these mechanisms will impair their ability to keep the lens transparent. Because an increase in the amount of lenticular copper disturbs both the sugar

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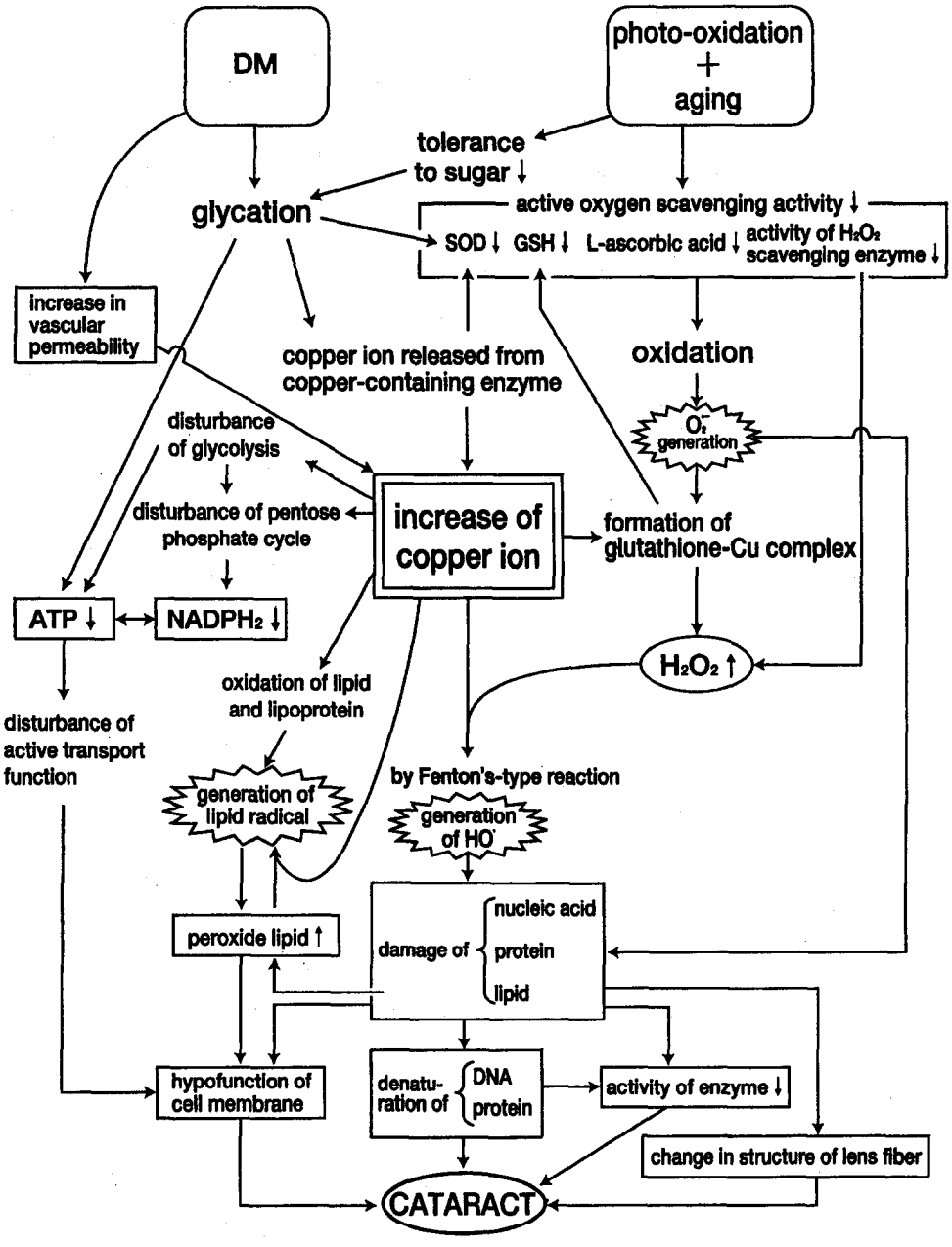

Figure 5. The influence of copper ion upon the lens and the generation of active oxygen.

metabolism and the peroxide scavenging function, it is reasonable to assume that an increase in the amount of lenticular copper will also have a strong effect on the lens opacity of diabetic patients.

Professor Naohito Taniguchi of the Department of Biochemistry, Osaka University School of Medicine provided insight into the relationship between copper ion and glycation. Dr Yasushi Tanaka, Mrs Kiyomi Arai, and Professor Yoshitaka Obara of the Department of Ophthalmology, Koshigaya Hospital, Dokkyo University School of Medicine provided valuable and continuous guidance.

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