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Failure to withstand oxidative stress induced by phospholipid hydroperoxides as a possible cause of the lens opacities in systemic diseases and ageing

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Abstract

Lipid peroxidation (LPO) is a causative factor of cataract. The increased concentrations of primary molecular LPO products (diene conjugates, lipid hydroperoxides) and end fluorescent LPO products, were detected in the lipid moieties of the aqueous humor samples obtained from patients with senile and complicated cataracts as compared to normal donors. The degrees of lens clouding were assessed quantitatively by measuring the optical density indices and areas of equidensities using digital image analysis. Human cataractous lenses showed decreased activity of glutathione peroxidase (GPX, catalyzing reduction of organic hydroperoxides including hydroperoxides of lipids). The apparent K_m for tert-butylhydroperoxide was 0.434 mM for human normal and cataractous lens GPX. When lenses were exposed for 1 h at 37°C to linoleic acid hydroperoxide (LOOH, 0.5 mM) or egg phosphatidyl-choline hydroperoxide (PLOOH, 1 μmol per 112 μmol of phospholipid) in liposomes suspended in the incubation medium, normal, immature and mature human cataractous lenses showed a significant loss in the residual content of liberated LOOH to 62%, 38% or 17%, correspondingly, but little or no reduction was observed with PLOOH in liposomal membranes. Human, rabbit or mice transparent or immature cataractous lenses induced significantly more absorbance changes in conjugated diene, iodometric and TBA-reactive substance measurements when incubated with liposomal membranes which were decreased in the presence of free radical scavengers and antioxidant enzymes (EDTA, SOD, L-carnosine, chelated iron, catalase). Injection into the vitreous body of the rabbit eye of a suspension of liposomes prepared from phospholipids containing LPO products induced the development of posterior subcapsular cataract. Saturated liposomes did not cause clouding of the lens. This modelling of cataract was accompanied by accumulation of fluorescing LPO products in the vitreous body, aqueous humor and the lens and also by a fall in the concentration of GSH in the lens. The peroxidative damage to the lens cell membranes and biomolecules induced in the lack of reductive detoxification of phospholipid hydroperoxides is proposed as the triggering mechanism of cataractogenesis.

Keywords: Eye lens; Digital image analysis; Aqueous humor; Liposome; Lipid peroxidation; Cataract; Systemic, retinal disease and ageing

1. Introduction

It is well established that a major factor involved in the development of cataract is oxidative insult [1–12]. Oxidative stress associated with the formation of lipid peroxides is suggested to contribute to pathological processes in ageing and systemic diseases, such as diabetes, atherosclerosis, chronic renal failure, inflammation and retinal degenerative diseases known as statistically significant risk factors for cataract [13–18]. The observation that lipid peroxides are elevated in the lens membranes of some patients with cataract has drawn attention to these toxic oxidants [19–21]. Lipid peroxides can cause cataract, producing damages to both cell membrane and cytosol regions [18,20,22–26]. At the membrane, lipid hydroperoxides induce changes in permeability [27–30], refashion the microviscosity (order) of its lipid-protein environment [31–33], cause an uncoupling of the membrane-bound enzyme Na, K-ATPase and oxidative inhibition of Ca^{2+}-ATPase in several tissues including the lens [34–36]. Within the cell, lipid peroxides can damage DNA [37], induce a drop in total glutathione and dramatic change in the redox ratio of glutathione, lead to the appearance of new fluorophores

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and large protein aggregates with low solubility (clouding matrix) in the lens matter [12,37–41].

The aqueous humor contains about 4 μg/ml of high density lipoproteins, which evidently take part in the renewal of lipid compositions of the lens [42,43]. The oxidative modification of lipoproteins in the presence of trace amounts of transition metals (copper or iron) is variously associated with lipid peroxidation (LPO), an increase in net negative charge, hydrolysis of phospholipid and fragmentation of apoprotein B (for review see Ref. [44]) and the oxidized lipid moieties of lipoprotein particles can be implicated to the lens toxicity triggering cataractogenesis [45,46]. Supposedly, this event is controlled because of the ability of reduced glutathione (GSH) of the lens to detoxify hydroperoxides via glutathione peroxidase (GPX) [47,48]. The enzymatic detoxification of lipid-derived hydroperoxides in which GSH-dependent selenoperoxidase of the lens is thought to play a key role, leads to reduction of hydroperoxides (LOOH) to alcohols at the expense of GSH (Eq. 1) which regeneration to the reduced form is catalyzed by GSSG reductase (Eq. 2), utilizing NADPH as a co-factor [49,50]:

$$LOOH + 2GSH \rightarrow LOH + GSSG + H_2O \quad (1)$$

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH \quad (2)$$

Studies with phospholipid membrane vesicles (liposomes) which lacked cholesterol and lipoproteins have indicated that classical GPX does not act directly on phospholipid hydroperoxides either membrane-bound or detergent-dispersed [51]. The phospholipid hydroperoxides present in the human erythrocyte ghost natural membrane rich in cholesterol comparably to the lens membranes [52], are also unreactive with GPX unless first hydrolyzed by phospholipase A_2 [53]. At last, cholesterol hydroperoxides were found to be totally resistant to GPX, even after extraction from the membrane [53]. These findings raised an important question of how the lens might detoxify phospholipid hydroperoxides which mediate oxidative stress implicated in development of maturity onset cataract. The ability of the lens to catalyze directly in situ reduction of phospholipid hydroperoxides in model membranes and the lens GPX ability to remove certain organic (including liberated fatty acid) hydroperoxides were quested in this study.

2. Materials and methods

2.1. Patients

Aqueous humor and human lenses were obtained from patients undergoing intracapsular cryoextraction of senile or complicated cataracts and used as the test material. This project was approved by the ethic commission of Helmholtz Research Institute for Eye Diseases (no. 008/136/30). The control aqueous and transparent human lenses were obtained from eyes donated for corneal grafting supplied from a corneal transplant bank within 15 h post mortem. The mean age at surgery and the average donor age was 59 years ranging from 16 to 89 years. Both groups contained an almost equal number of males and females.

2.2. Aqueous humor sampling

After retrobulbar and lid anesthesia the two vertical recti muscles were fixed. A stab incision was performed transcorneally 1 mm from the limbus in the temporal superior quadrant. Aqueous humor from human eyes (about 0.1–0.2 ml per eye) was aspirated from the anterior chamber of each eye with a 25-gauge needle connected to a

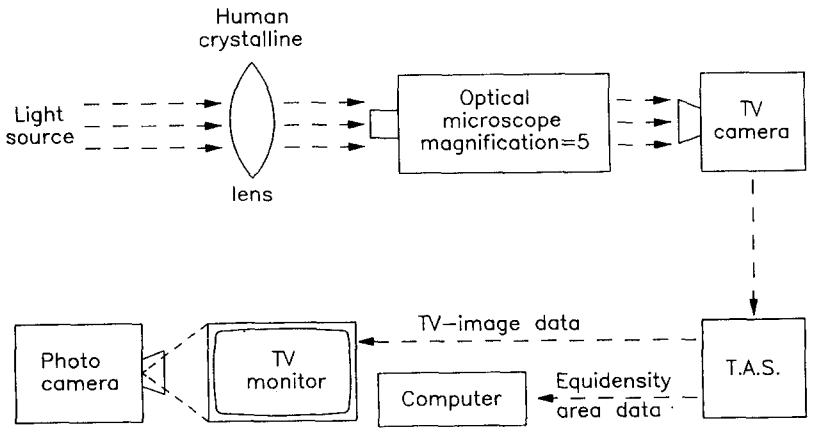


Fig. 1. Image analysis procedure which makes it possible to obtain an image of the lens, to determine values of the optical density in different parts of the lens, to divide the lens surface into zones of defined density range, to measure the areas of equidensities and to evaluate the precise topography of every zone. All parameters are estimated quantitatively by use of a computer. The lens is illuminated in a frontal projection, adjusting the intensity of the illuminating light to make the transmission through each lens constant. An electronically produced measuring field is determined by a circular mask whose position is under the control of the analyser. To control its position, the coordinates of the central point of the mask are automatically digitally defined. The image is then scanned and the density at each coordinate stored in the computer.

tuberculin syringe immediately before surgery and shipped on wet ice under sterile conditions. Human aqueous humor was then briefly centrifuged at low speed to remove any cellular debris and the aliquots of fresh aqueous humor from each patient were immediately used for lipid extraction.

2.3. Lenses

The test and control material consisted of opaque human lenses at different stages of cataract or normal human lenses. Before surgery all the lenses were studied by biomicroscopy and assigned to cataractous or transparent lenses in line with the clinical characteristics of clouding. Rabbit transparent lenses were obtained from freshly enucleated eyes of the Chinchilla race animals. Normal mouse lenses were extracted from the eyeballs by the posterior approach from mice of the strain C57BL or hybrids F_1 (CBA × C57BL) resistant to cataract formation. In all cases the integrity of the lens capsule was preserved. The lenses removed were briefly rinsed in Hanks' medium for 20–30 s and then immediately placed into a moist chamber. They were handled with the greatest care when being placed inside and being removed from their individual containers for measurements. The lenses were either used directly after the extraction procedure, or surgery, or at least 1–3 h were allowed to elapse between the dissection from the eye and the start of the measurement. This delay did not influence the results. The development of quantitative morphometric criteria for evaluation of the lens opacities was presented by us [54,55]. All opacities were graded by their area and density, as judged by the degree of clouding (Fig. 1). The integral degree of lens clouding (IDLC) was estimated as the ratio between the opacity zone area (corresponded to maximum levels of the lens optical density values) and the whole lens surface area expressed as a percentage. This measuring procedure was brief (5–10 min) and did not influence the further lens behaviour.

2.4. Incubation of lenses with lipid hydroperoxides

The lenses were incubated in Hanks' balanced salt solution (without bicarbonate, pH 7.0) containing 7 mM glucose. Since most incubation media or balanced salt solutions comprise phosphate buffer of different molarity with different iron contaminations in all phosphate salts involved in metal-catalyzed LPO reactions, a glucose-containing medium nutritious to the lens comprising 50 mM Tris-HCl, 5.5 mM glucose, 4.0 mM KCl, 102.5 mM NaCl, 1.0 mM K_2HPO_4 (pH 7.2–7.4) (medium B) was used. Both media were adjusted to 290–300 mOsm with NaCl and equilibrated with 95% air and 5% CO_2. When significant concentrations of oxygen scavengers or other agents were added, the change in osmolarity was compensated by decreasing the amount of NaCl used to adjust the media back to the required osmolarity following modification. The human or rabbit lenses were incubated in 3.0 ml of medium and the mouse lenses were placed in a cluster dish containing 200.0 μl of medium/lens. The tests of lens integrity during incubation, to indicate that the lens is functioning normally, were performed as previously [56]. The incubations were carried out at 37°C or at room temperature. For incubation 10 μl of linoleic acid 9-monohydroperoxide (LOOH), dissolved in methanol/water (9:1, v/v) mixture, was suspended in the lens incubation medium to reach 0.5 mM concentration and incubated with the lens (or without a lens, in control). When necessary, the lens incubation media contained the liposome suspension (0.5 mg/ml) or phosphatidylcholine hydroperoxide peroxide (PLOOH) in the contents of liposomes (1 μmole PLOOH/112 μmole PL). The time of incubations varied from 1 to 3 h. Fixed aliquots of media (50–500 μl) were taken out at different times from the tissue cultures of lenses for the proper measurements of products.

2.5. Modelling of cataract

Experiments were carried out in 35 Chinchilla rabbits (70 eyes) aged 4 months weighing on average 2.5 kg. Cataract was induced by injection into the vitreous body of the rabbit eye of a suspension of liposomes prepared from the egg yolk phosphatidylcholine containing LPO products, exactly as originally described [18]. Two months postinjection, rabbits were sacrificed by air embolism under general anesthesia, according to the resolution of the ARVO, Inc., on the Use of Animals in Research, and the ocular tissues were taken for biochemical analyses. Aqueous humor was drawn off by paracentesis and then the lens and vitreous body were isolated. The lipids were extracted and the content of reduced thiols in the lenses were determined immediately after obtaining the material.

2.6. Lipid extraction and quantification

Aliquots of fresh aqueous humor from each patient or animal were immediately extracted into 40 vol. of chloroform-methanol (2:1, v/v) with 4-methyl-2,6-ditert-butylphenol (BHT) antioxidant addition (0.5 mg/100 ml) during 10 min. The obtained extract was washed according to the modified Folch procedure, and the chloroform layer was recovered and evaporated under argon [12]. Aliquots of the lipid residue were analysed for total phospholipid phosphorus and fractioned to discriminate cholesterol, free fatty acids and the individual spots of the polar lipids as described previously [45]. The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) were reported [39,45].

2.7. Peroxidation reaction techniques

Linoleic acid 9-monohydroperoxide (LOOH) was prepared by use of potassium linoleate and lipoxydase (EC

1.13.11.12, Type IX, potatoes) as described [57]. The reaction product was extracted by ether and purified by column chromatography on a Silica Gel L 100/250 (4 × 30 cm) column with elution by the mixture of hexane-acetone (95:5). The purity of the hydroperoxide was 100 ± 5% when estimated in methanol on the basis of its molecular extinction coefficient at 234 nm:2.8×10^4 M^{-1} cm^{-1} and also determined using the established iodometric technique [58]. Peroxidation of the phosphatidylcholine (derived from egg yolk) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbate in 0.1 M Tris-HCl buffer (pH 7.4) to the suspension. The incubations were performed at 37°C. The concentration of LPO products in the oxidized lipid substrates was measured by the reaction with thiobarbituric acid (TBA). The peroxidation reaction was arrested by adding EDTA to an end concentration of 50 μM or by the addition of 2.0 ml of ice-cold 0.25 N HCl containing 15% TCA. TBA (0.125%) was then added to the mixture and followed by boiling for 15 min. The separately utilised TBA assay was described previously [45]. The absorbance of the pink-coloured trimethine condensation product of 1 mol malonyldialdehyde (MDA) with 2 mol TBA was calculated from ε 535 nm MDA = 1.56×10^5 M^{-1} cm^{-1}. MDA, synthesized from 1,1,3,3-tetramethoxypropane by hydrolysis with 0.1 M HCl for 24 h at room temperature, was used as standard. The TBA reaction per se was not affected by the components of the radical generators or scavengers used in the study. PLOOH was prepared by use of egg yolk phosphatidylcholine and the peroxidation technique described above. The product was purified by preparative TLC using Silica Gel-60H (Merck) plates with methanol/chloroform/water (10:1.5:0.5) as a developing solvent. PLOOH showed an absorption maximum at 233 nm in methanol-heptane (5:1,v/v) and a peroxide value of 2600 ≈ 3200 meq/kg. The content of lipid hydroperoxide was measured after the chloroform-methanol (2:1,v/v) extraction with addition of BHT (0.5 mg/100 ml) by iodometric titration and measuring the absorption maximum at 233 nm after the dissolution of a dry residue in 2.5 ml of the methanol-heptane (5:1,v/v) mixture. This protocol removes any water-soluble secondary oxidation products, leaving them in the methanol-aqueous phase. The equalization of the extracted lipid concentrations to the measured phosphorus was done using the characteristic absorption in 206–210 nm area of the lipid sample. Accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides was assessed from characteristic absorbance of diene conjugates in the UV region in a Shimadzu UV-260 spectrophotometer (Japan). Absorbance of the secondary molecular LPO products at ~ 274 nm, corresponding to the concentration of conjugated trienes and ketodienes was also measured spectrophotometrically from the lipid spectra [12]. An average MW of phospholipid was assumed as ~ 730 D. Lipid fluorochromes were determined in the same probes at 365 nm excitation and 420- to 440-nm emission wavelengths. The fluorescence data were normalised to the characteristic absorption in 206–210 nm area and to the phospholipid content. The spectrofluorophotometer (Shimadzu RF-540, Japan) was calibrated at the beginning of every working day with a solution of quinine-sulphate (1 μg/ml in 0.1 N H_2SO_4) standard, at 435 nm fluorescence emission and 365 nm excitation wavelengths.

2.8. Antioxidant enzymes

For the assays of enzyme activities, the lenses were weighed and homogenized in double glass distilled water. GPX (catalyzing reduction of organic hydroperoxide tert-butyl hydroperoxide (TBHP)) activity was assayed exactly as described previously [56]. GSH levels in the lenses were determined from the reaction with 5,5′-dithiobis-2-nitrobenzoic acid [59].

2.9. Reagents

All chemicals were of reagent grade. SOD was obtained from Sigma Chemical Co. (St. Louis, USA). Catalase was from Serva (Germany). L-Carnosine was purchased from Neosystem Laboratories (France).

3. Results

3.1. Aqueous humor

Lipid and phospholipid contents in aqueous humor samples obtained from human eyes were reported recently [45]. Typical UV absorption spectra of lipids have their maximum in the 206-nm regions related to absorption of isolated double bonds of hydrocarbon phospholipid chains (Fig. 2). Lipid extract from normal aqueous humor exhibits a modest shoulder of absorption at about 230 nm and a

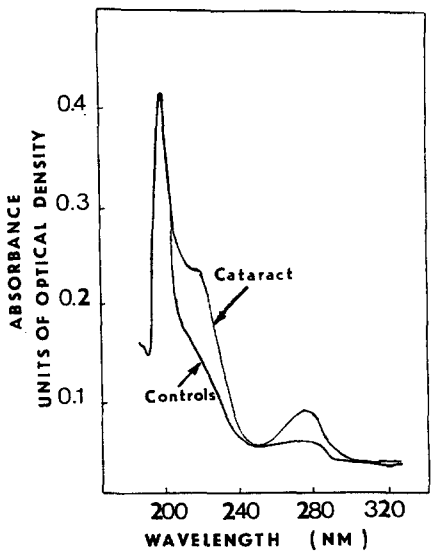


Fig. 2. UV absorption spectra of lipid extracts (methanol-heptane 5:1, v/v) from the aqueous humor samples, obtained from human eyes: normal (control, lower curve) and mature cataract (upper curve). For details of processing procedure, see Section 2.

Table 1
Lipid peroxidation products in aqueous humor from the eyes of patients with cataract

Lipid peroxidation products	State of the lenses/degree of lens clouding, %		
	Control (n = 10) (0.0–10)	Immature cataract (n = 12) (10–64)	Mature cataract (n = 16) (64–100)
I. Diene conjugates			
1. OD_{232}/mg phospholipids	5.86 ± 0.44 [a]	7.93 ± 0.65 [b]	9.48 ± 0.34 [c]
2. OD_{232}/OD_{206}	0.307 ± 0.024	0.425 ± 0.035 [d]	0.518 ± 0.020 [c]
3. mole/100 mole phospholipids	2.37 ± 1.14	5.40 ± 1.70	9.44 ± 0.89 [c]
II. Triene conjugates, ketone and aldehyde products			
1. OD_{274}/mg phospholipids	2.33 ± 0.13	2.39 ± 0.23	2.86 ± 0.40
2. OD_{274}/OD_{206}	0.122 ± 0.007	0.133 ± 0.012	0.163 ± 0.022
III. Fluorescence products			
Fl/μg phospholipids	17.2 ± 6.4	42.0 ± 7.3 [b]	71.7 ± 20.2 [b]

[a] Mean ± S.D. (n, number of the examined samples). Significant differences with control: [b] $P < 0.05$, [c] $P < 0.001$, [d] $P < 0.02$.

certain absorption peak around 280 nm in the UV region. However, lipid extract of patients with cataract usually exhibits the stronger absorption shoulder at 230 nm, characteristic of conjugated double bonds in the fatty acids (primary LPO products) and an absorption peak around 280 nm (characteristic of ketones, secondary molecular LPO products) (Fig. 2, upper curve). When the level of secondary products not removed into the water-soluble phase was not significantly increased in UV absorption spectra of lipid extracts of aqueous humor from the cataractous patients (Table 1), the distinct increase of the contents of the end fluorescent LPO products expressed in terms of arbitrary fluorescence units/μg phospholipids (PL) was about 2.4-fold and 4.2-fold higher correspondingly for immature or mature cataracts as compared to normal lenses (Table 1). In the samples of aqueous humor aspirated from donor eyes with normal lens, one in about 42 PL molecules contains a conjugated diene, while in case of immature cataract (IDLC 10–64%), the average amount of conjugated dienes reaches one in 19 aqueous PL molecules, whereas in mature cataract (IDLC 64–100%) this estimation averages one conjugated diene per 11 PL molecules. Net concentrations of primary (diene conjugates, phospholipid hydroperoxides) and end (fluorescent) LPO products were found to be increased in aqueous humor of the anterior chamber in cases of cataract irrespective of its genesis comparatively to donor (control) eyes and in line with the maturity stage measured by degree of clouding (Table 1).

Table 2
Glutathione peroxidase (substrate: organic hydroperoxide) activity of transparent lenses and lenses affected by cataract (M ± m)

Lens	Lens area of opacity [a]	Lens homogenate
		Glutathione peroxidase (substrate: TBHP) [b]
Transparent human (n = 10)	0–10	0.51 ± 0.04
Immature human cataract (n = 9)	10–70	0.404 ± 0.049 **
Mature human cataract (n = 8)	80–100	0.195 ± 0.047 *

[a] Ratio of area of zone of opacity to total area of lens, %; [b] in μmol NADPH/min per lens with tert-butyl hydroperoxide (TBHP) as the substrate. * $P < 0.001$, ** $P < 0.1$ compared with transparent lens. Measurements were performed at 37°C.

3.2. Lipid hydroperoxide metabolizing activities of lenses

In human lenses with mature cataract the activity of GPX (catalyzing reduction of organic and lipid hydroperoxides) was sharply reduced as compared to normal lenses (Table 2). Kinetic studies showed that lipid hydroperoxide approached saturation at concentration of ≈ 1 mM, and the apparent K_m value was 0.434 mM (Fig. 3). At the stage of mature cataract glutathione lipoperoxidase activity was significantly inhibited (Table 2, Fig. 3: maxi-

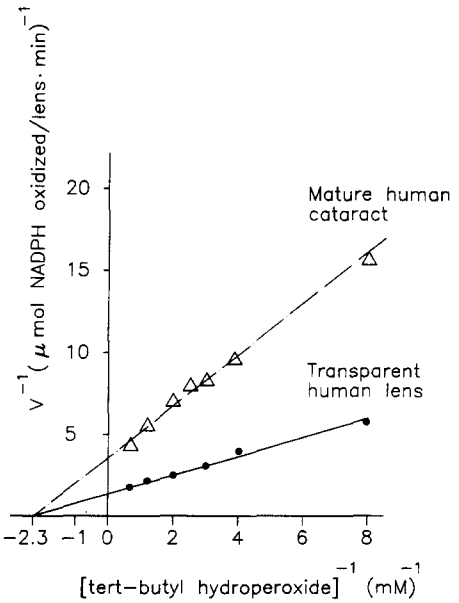
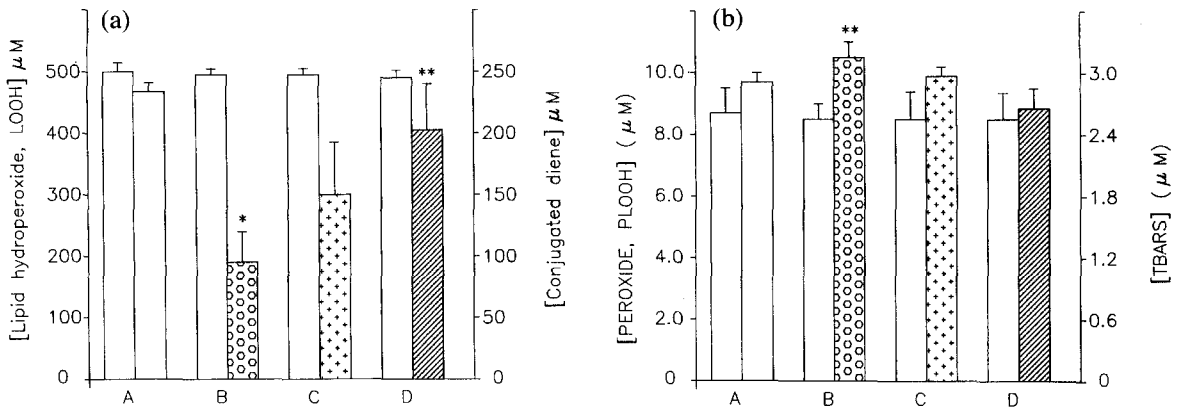
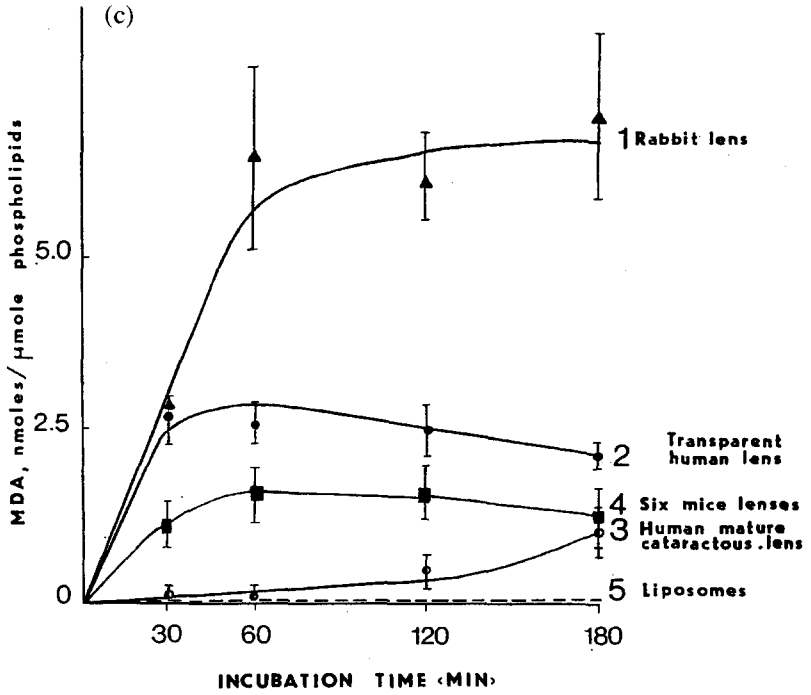
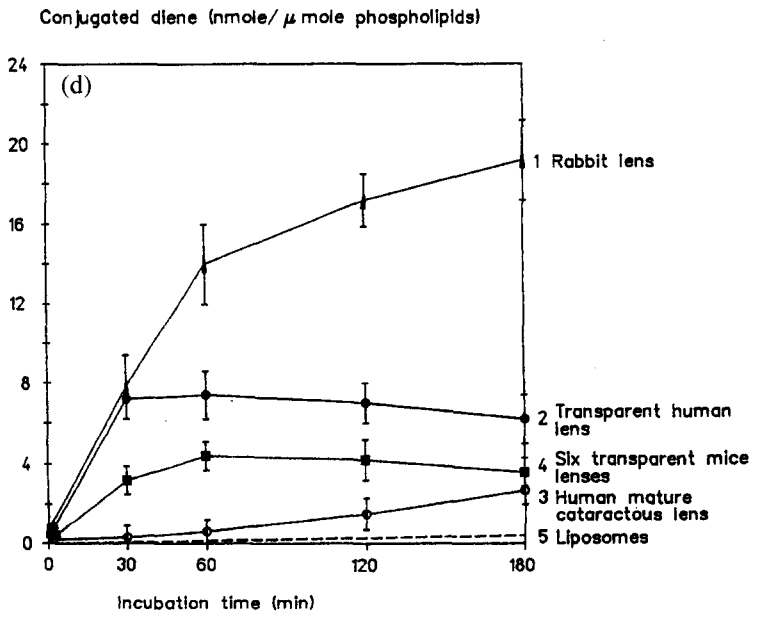


Fig. 3. Reciprocal velocity of glutathione peroxidase of normal and cataractous human lenses as a function of reciprocal concentration of tert-butylhydroperoxide. Tert-butylhydroperoxide was varied from 0.13 to 1.52 mM with a constant glutathione concentration of 5 mM. The crossing point with the abscissa axis $(-K_m)^{-1}$ indicates the K_m value for normal and cataractous human lenses of 0.434 mM. The crossing points with the ordinate axis outline $(V_{max})^{-1}$. At the concentration of tert-butyl hydroperoxide of 0.74 mM, the share of the occupied active centres of GSH peroxidase is calculated as: $f_{ES} = ([S])/([S] + K_m) = 0.63$.


(a)
[Lipid hydroperoxide, LOOH] μM
[Conjugated diene] μM
A
B
C
D
(b)
[PEROXIDE, PLOOH] (μM)
[TBARS] (μM)


(c)
MDA, nmoles/μmole phospholipids
1 Rabbit lens
2 Transparent human lens
4 Six mice lenses
3 Human mature cataractous lens
5 Liposomes
INCUBATION TIME (MIN)


Conjugated diene (nmole/μmole phospholipids)
(d)
1 Rabbit lens
2 Transparent human lens
4 Six transparent mice lenses
3 Human mature cataractous lens
5 Liposomes
Incubation time (min)

mum enzymic rate V_{max} is decreased), however K_m was unchanged. This indicates that during cataract formation a deficiency of GPX activity occurs due to the non-competitive inhibition of enzyme.

Human normal or cataractous lenses of different ethiology or degrees of opacity were exposed at 37°C for 1 h to linoleic acid 9-monohydroperoxide (LOOH) or PLOOH in the contents of 0.5 mg/ml liposomes suspended in the lens incubation medium. Reducibility of LOOH or membrane PLOOH was studied by spectrophotometric (conjugated diene) or iodometric assays of the residual LOOH(PLOOH) levels during the incubation of lenses (or without a lens in a background study), and MDA levels were determined as TBA-reactive substances (TBARS). Results of a typical experiment are shown in Fig. 4a,b. The starting level of LOOH in the incubation medium was of 0.5 mM and 1 μmol PLOOH per 112 μmol of membrane PL. Incubations with normal human, immature or mature cataractous lenses for 1 h at 37°C caused a decrease of total liberated fatty acid hydroperoxide (LOOH), correspondingly, to 310 ± 50 nmol/lens (mean ± S.E.M., $n = 10$) at IDLC of 0–10% (ages 17–64 years); to 192 ± 85 nmol/lens ($n = 4$) at IDLC of 10–65% (ages 60–89 years) and to 85 ± 70 nmol/lens ($n = 4$) at IDLC of 66–100% (ages 71–86 years) in 1.0 ml volume of incubation medium. Within the same time interval of incubation and temperature, human transparent or cataractous lenses showed little or no reduction of the liposome membrane PLOOH (Fig. 4b) but, instead, the assay system detected more absorbance changes of conjugated diene, iodometric and TBARS measurements in incubations of transparent or immature cataractous human lenses, indicating the PLOOH-dependent propagation of lipid hydroperoxides and that the assay system was fully operational. Incubation with 0.5 mM EDTA produced no net reducing effect on the LOOH(PLOOH) level over that observed in control incubations (LOOH or PLOOH alone). The reducing agent 10 mM $NaBH_4$ showed the significant reactivity (62%) for the measurable LOOH reduction relative to the background decay.

Human lenses with different degrees of opacity and transparent rabbit or mice lenses were incubated in the tissue medium B containing 0.5 mg/ml liposome suspension as the oxidation substrate for 3 h at room temperature, and the kinetics of LPO reaction were estimated by measuring MDA, liposomal-conjugated dienes and trienes making appropriate corrections for liposome autooxidation (Fig. 4c–e). In the absence of the lens, virtually no oxidation of liposomes took place during 180 min (Fig. 4c–e, curve 5). In the presence of the lens, a marked increase in concentration of different molecular LPO products was demonstrated for the proper time of incubation. The rate of MDA accumulation during the first 30 min of incubation was significantly higher in the presence of transparent human lenses (and also of lenses at the initial stage of cataract) than in the presence of lenses with a mature cataract and the final MDA level within 3 h of incubation was 2.5- to 4.5-fold higher for transparent lens than for the lens with a ripe cataract, integrally indicating that human mature cataractous lenses peroxidized liposomes less than the transparent lenses. The level of MDA accumulation after incubation for 3 h of rabbit lenses was 3.5- and 5.3-fold higher than of normal human or mouse lenses. The larger normal lenses (rabbit or human) are metabolically active and generate the reactive oxygen species and

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Fig. 4. Reactivity of hydroperoxides with human normal and cataractous lenses measured by iodometric, conjugated diene and TBARS assays. The lenses were treated with either (a) 0.5 mM linoleic acid 9-monohydroperoxide (LOOH) or (b) membrane phospholipid hydroperoxide (PLOOH) (1 μmol PLOOH per 112 μmol of membrane phospholipid in 0.5 mg/ml liposomes) suspended in the incubation medium of the lens (Hanks' balanced salt solution, pH 7.0) and incubated for 1 h at 37°C. At the end of incubation, samples were aspirated and the content of lipid hydroperoxide was determined (see Section 2). Initial levels of peroxide in the reaction mixture and at the end of incubation are shown. (a): A, background incubation of LOOH without lens; B, LOOH incubation with transparent human lens (IDLC 0–10%, ages 17–64 years, $n = 10$); C, LOOH incubation with immature cataractous human lens (IDLC 10–65%, ages 60–89 years, $n = 4$); D, LOOH incubation with mature human cataract (IDLC 66–100%, ages 71–86 years, $n = 4$). Significant differences from transparent lens ** $P < 0.05$ and from the background incubation * $P < 0.01$ are indicated. (b): A, background incubation of PLOOH in liposomes without lens; B, PLOOH with transparent human lens (IDLC 0–10%, ages 20–45 years, $n = 3$); C, PLOOH incubation with immature human cataractous lens (IDLC 10–65%, ages 55–70 years, $n = 3$); D, PLOOH incubation with mature human cataractous lens (IDLC 66–100%, ages 76–89 years, $n = 3$). Significant difference from the initial level of PLOOH in the reaction mixture ** $P < 0.05$ is indicated. Normal lenses obtained from the cases of incidental death and cardiovascular shock were included in the group of transparent lenses. The lenses obtained during surgery from the patients with senile (9 lenses) and complicated (5 lenses) cataracts were included in the C and D groups on the basis of IDLC measurements (see Section 2). Data columns shown with error bars are means ± S.E.M. of 3–10 lenses. (c–e): The accumulation of lipid peroxides: TBA-reactive substances (c), conjugated diene (d); triene conjugates, ketone and aldehyde products (274 nm absorbing material) (e) in the liposomes (0.5 mg/ml) incubated alone (control) or in the lens-containing medium B at room temperature for 3 h. Samples were taken at zero time and at varying time intervals as indicated in the figures. The above fixed aliquots of samples (50–500 μl) were directly used for the measurement of TBA-reactive substances (for details, see Section 2). Also, a similar amount of incubated sample was partitioned through chloroform as described in the lipid extraction procedure and after dissolution in 2–3 ml of methanol-heptane mixture (5:1, v/v) was used for detection of conjugated diene and triene conjugate/ketones (274 nm absorbing material). (1) Transparent rabbit lenses (IDLC 0–8%), Mean ± S.E.M., $n = 5$; (2) transparent human lenses (IDLC 0–10%), $n = 4$, initial cataract (IDLC 10–40%), $n = 3$; (3) human mature cataractous lenses (IDLC 64–100%), $n = 5$; (4) six normal mice lenses (IDLC 0–10%), $n = 3$; (5) (control) liposomes, $n = 5$. Experimental details are given in the text. (f): Effect of various oxygen radical scavengers on lipid peroxide formation in liposomes added to the incubation medium of the normal rabbit lens (IDLC 0–8%). In a total vol of 3.0 ml the incubation mixture of the lens contained medium B, 0.5 mg/ml liposome suspension and the appropriate concentrations of scavenger as indicated. Mean values for the MDA concentrations are given for a representative experiment, with the error bars indicating the standard deviation obtained for the group of 3–5 lenses.

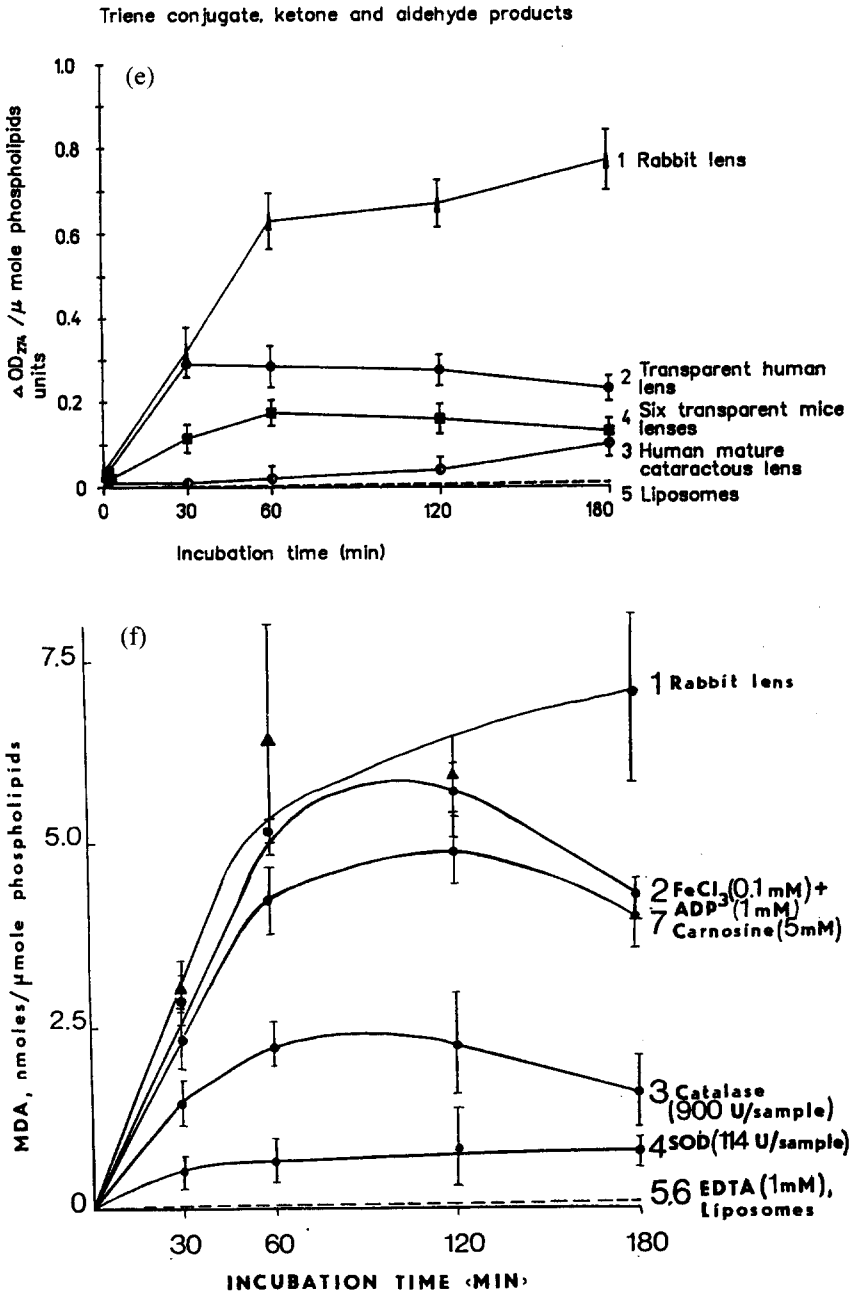

Fig. 4 (continued).

lipid peroxides more rapidly than cataractous lenses with the exhausted pool of reductants or tiny mouse lenses [45]. Fig. 4c and 4f document in some cases a small decrease in the liposomal MDA concentration after incubation for 2 h. This might be connected with MDA utilization by the lens itself (interaction of MDA with amino group, or its lowering by lenticular aldehyde dehydrogenase [60]). A considerable reduction of the accumulation rate of the liposomal LPO products was found after the addition of catalase (900 U/sample) (Fig. 4f, curve 3). This suggests a role of H_2O_2 in promotion of LPO by the lens. Addition of SOD (114 U/sample) to the incubation medium of the lens led to a marked reduction of the liposomal MDA level (Fig. 4f, curve 4), suggesting the ability of the lens to generate $O_2^{\cdot-}$ in the surrounding medium. Addition of the ADP-Fe complex to the incubation medium of the lens decreased accumulation of TBA-reactive material in liposomes by 33–50%, indicating a decomposition of the accumulated TBA-reactive material. The LPO is resulted from free-radical oxidation reactions induced by active oxygen forms

with participation of transition metal ions in the free catalytically active state. This is supported by the virtually total inhibition of TBA-reactivity in the liposomes after the addition of the chelating agent 1 mM EDTA eliminating the free and accessible metal ions from the peroxidizing system (Fig. 4f, curve 5). In the presence of 5 mM L-carnosine in the rabbit lens/liposome-containing medium, a decrease in the TBA-reactivity by approx. 25% for 2 h of incubation was revealed (Fig. 4f, curve 7). Since the reactivity of L-carnosine with $O_2^{\cdot-}$, H_2O_2 or hypochlorous acid at biologically significant rates was neglected as well as its binding ability with iron ions excluded [61], the specific inhibitory effect of L-carnosine in the incubation system detects $OH^{\cdot}$ or lipid peroxy radicals ($LOO^{\cdot}$) generated in the medium surrounding the lens in organ culture [62].

3.3. Induction of cataract by phospholipid hydroperoxides

The introduction into the vitreous body of the rabbit eye of a liposome suspension in an amount of 0.4 mg (content of MDA 2.0 nmol/μmol PL) induced the development of typical posterior subcapsular cataract within 24 h. The anterior cortex and the nucleus of the lens remained transparent but clouding was noted in the region of the posterior pole of the lens and in the vitreous body. Injection into the vitreous body of the rabbit eye of the same quantity of more oxidized liposomes (22.2 nmol MDA/μmol PL)

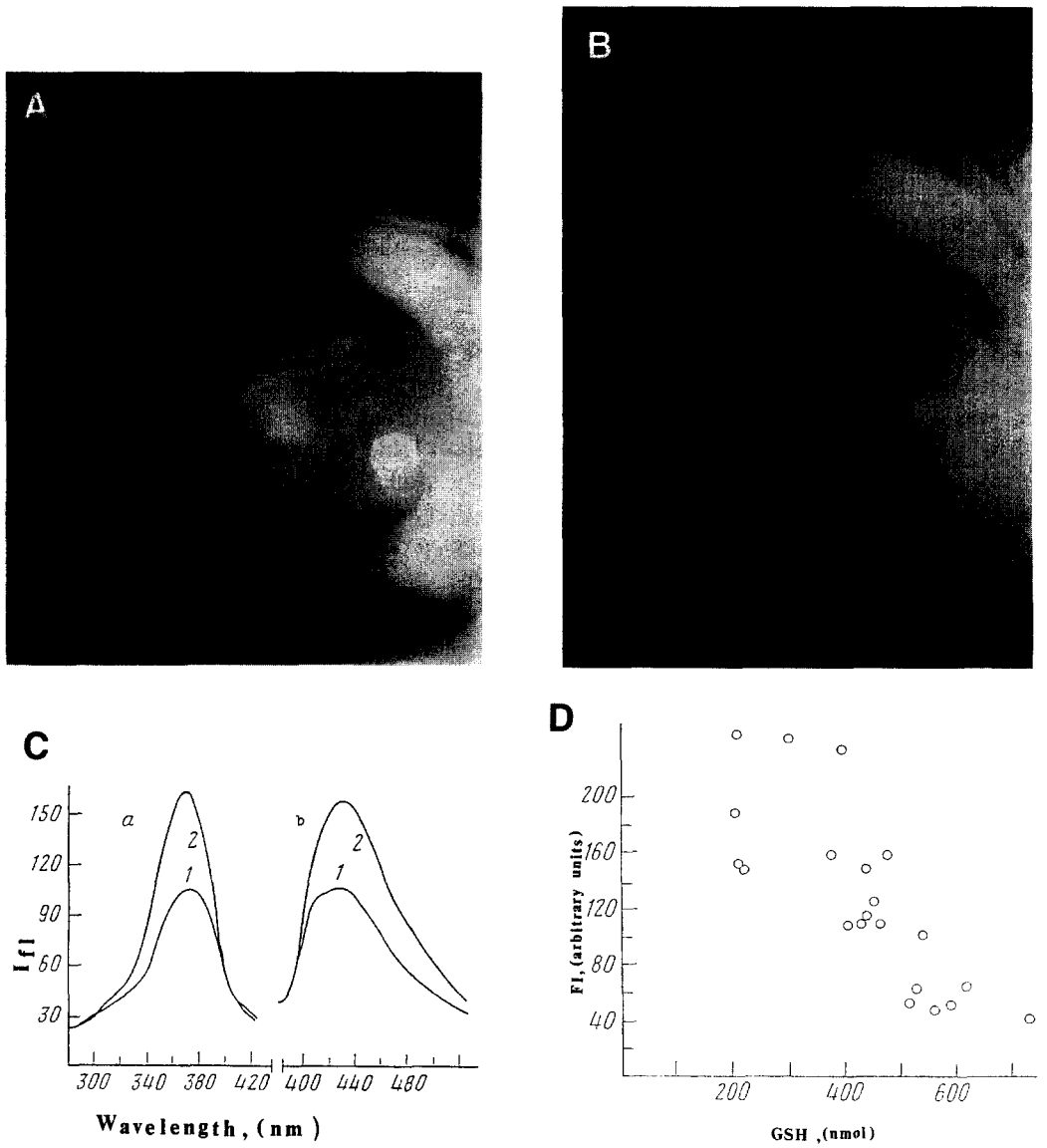

Fig. 5. Type of clouding on modelling cataract by liposomes (0.4 mg phospholipids) within 24 h (10 ×). Injection into vitreous body of (A) oxidized phospholipids (22.2 nmole MDA/μmol phospholipids); (B) liposomes prepared from β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine. Photographs obtained with an ZEISS SL-30 photoslit lamp. (C) Spectra of excitation with λ_{fl} = 438 nm (a) and emission with λexcit = 365 nm (b) of the fluorescence of lipid extracts (methanol/heptane, 5:1) from the lenses after introduction into the eye of saturated liposomes (1) and liposomes from oxidized phospholipids (2). (D) Level of GSH in lens as a function of the accumulation of LPO products, Fl is level of fluorescing LPO products in lens.

Table 3
Intensity of fluorescence (relative units) of lipid peroxidation products in eye tissues on modelling cataract by non-oxidized, oxidized and saturated liposomes ($\lambda_{excit.}$ = 365 nm; λ_{emis} = 438 nm)

Tissue	Norm	Non-oxidized liposomes 2.0 nmol MDA/μmol of phospholipids	Oxidized liposomes: 22.2 nmol MDA/μmol of phospholipids	Saturated liposomes: 1.2 nmol MDA/μmol of phospholipids
Lens	96 ± 20 (n = 10)	150 ± 20 (n = 7)	170 ± 30 (n = 10)	100 ± 10 (n = 6)
Vitreous body	80 ± 30	300 ± 100	173 ± 40	80 ± 10
Aqueous humor	130 ± 30	230 ± 50	240 ± 14	170 ± 8

The data obtained on introducing 0.4 mg phospholipids into the eye are presented. The concentration of the material in the specimens was equalized for the concentration of lipids (calculated from the characteristic absorption of the lipid extract at 206 nm). The relative fluorescence of quinine sulfate (1 μM in 0.1 M H_2SO_4) standard was 375 units at emission 460 nm on excitation at 365 nm. The measurements were made on day 60 after liposome injection.

Table 4
The content of total and non-protein thiols in rabbit lenses on modelling cataract by non-oxidized, oxidized and saturated liposomes

Liposomes	C = 0.4 mg			C = 1.5 mg		
	n	C_o	GSH	n	C_o	GSH
Non-oxidized	7	2.92 ± 0.34	450 ± 116	8	2.99 ± 0.37	251 ± 75
Oxidized	10	2.96 ± 0.24	403 ± 63	8	3.10 ± 0.47	121 ± 52
Saturated	6	3.2 ± 0.5	520 ± 71	3	3.1 ± 0.6	500 ± 102
Control	10	4.1 ± 0.4	705 ± 106			

The mean molecular weight of protein taken as equal to 20000.
C, content of phospholipids; C_o, the total content of thiols (μmol/μmol protein); GSH, reduced glutathione (nmol/μmol initial protein). The majority of the reduced non-protein-bound thiols is represented as reduced glutathione.

(Fig. 5A) and also of a higher dose of liposomes containing LPO products led to more marked clouding of the lens, which, however, was localized to a high degree in the middle cortex. The introduction into the vitreous in the control series of solvent (in which the liposome suspension was prepared) contained 2.5 μM Fe^{2+}, 200 μM ascorbate, 50 μM EDTA and also liposomes containing 0.1 mg phospholipids did not cause appreciable clouding of the lens. Injection into the rabbit vitreous body of 0.4-1.5 mg β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine liposomes prepared from saturated phospholipids (level 1.2 nmol MDA/μmol PL) did not lead to clouding of the lens (11 eyes examined) (Fig. 5B). However, in some cases, the presence of single cells of inflammation in the vitreous body was noted. Observation for 2 months of the rabbits with cataract did not reveal its regression. The induction of posterior subcapsular cataract was accompanied by the accumulation of fluorescing LPO products in the vitreous body, aqueous humor and lens (Table 3, Fig. 5C). On day 60, the level of these products was maximum in the vitreous body in the case of introduction of non-oxidized (2.0 nmol MDA/μmol PL) but prepared from unsaturated (potentially auto-oxidized) phospholipids and the aqueous humor and lens on injection of already highly oxidized (22.2 nmol MDA/μmol PL) liposomes. Apparently this reflects the diffusion of the polar secondary LPO products from the vitreous body towards the lens. The clouding of the lens was accompanied by a regular drop in the concentration of GSH with a concomitant accumulation of fluorescing LPO products (Table 4, Fig. 5D). This confirms the hypothesis that LPO products induce oxidation of the cell thiols leading to clouding of the lens. However, the insignificant fall in the concentration of the total thiols in lens (Table 4) indicates that in this type of posterior subcapsular cataract the main cause of polymerization of the crystallins is their interaction with the intermediate LPO products-bifunctional compounds of the dialdehyde type.

4. Discussion

The primary molecular LPO products, i.e., phospholipid hydroperoxides and their in vivo indicator, diene conjugate, represent the major change in the lipid composition of aqueous humor during cataract formation. At the stages of ripe cataract, end fluorescent LPO products are distinctly detected. Crystalline lens is well equipped with antioxidant defences showing some specificity in their action. Primary defence prevents the initiating reactions of LPO and is achieved by the enzyme scavengers of reactive oxygen species, e.g., SOD, catalase and GPX utilizing H_2O_2; chemical antioxidants, e.g., α-tocopherol, β-carotene, ascorbate and GSH; structural antioxidants, e.g., cholesterol and membrane protein; transition metal-sequestering proteins including aqueous and plasma coeruloplasmin and transferrin. A second mode of the lens protection involves enzymatic removal of lipid-derived hydroperoxide intermediates. These reactions are typically catalyzed by Se-dependent GPX of the lens in addition to

primary lines of defence that involve iron inactivation or oxyradical and H_2O_2 scavenging. Mature human cataractous lenses showed the decreased activities of GPX (reducing organic hydroperoxides, including hydroperoxides of lipids), glutathione reductase, SOD, but no signs of deficiency in activities of catalase or GPX (utilizing H_2O_2) (this study, and Ref. [56]). The larger sizes of a lipid hydroperoxide molecule, its highly hydrophobic character and lower solubility in aqueous system than of the H_2O_2 molecule are responsible for different accessibility of the hydroperoxide substrates for Se-dependent lens GPX. Kinetic studies revealed that for the single lens, GPX activity towards H_2O_2 (GSH-H_2O_2 oxidoreductase, EC 1.11.1.9) was significantly higher than GPX activity towards TBHP [56]. The reaction rate was too rapid with 1 mM H_2O_2 to be compared with equimolar concentration of TBHP. The reaction rate with 0.074 mM H_2O_2 was similar to that with 0.74 mM TBHP and considerably more rapid than 0.74 mM concentration of this substrate. The apparent K_m for H_2O_2 was 0.045 mM, an order of magnitude lower than those for TBHP. So lens GPX has a greater affinity for the natural substrate H_2O_2 and is able to remove the cytosolic hydroperoxides. The lipid peroxidase activity of the lens is critical in the prevention of LPO in cell membranes and can play a role in the maintenance of its clarity. Exposure of transparent human lenses to LOOH (0.5 mM) resulted in significant decrease (62%) in the concentration of hydroperoxide in the culture medium after 1 h of incubation. Immature senile or complicated cataractous lenses detoxified correspondingly, 62% and 91% of the LOOH or H_2O_2 amounts reduced by transparent human lenses upon the hydroperoxide has been pulsed in the incubation medium. Thus the peroxidase ability of lenses is not diminished significantly in early cataracts (see also Ref. [56]). Mature human cataractous lenses cause a 17% decrease of total LOOH per lens when incubated for 1 h at 37°C. This stage (IDLC 66–100%) of cataract differs significantly in the lower GSH lipoperoxidase activity and decreased GSH level in the lens (this study and Ref. [63]. The level of lipid hydroperoxide in the medium was decreased by incubation with lenses due to the fact that the lipid hydroperoxide was taken up by the lens or was scavenged by factors (enzyme and/or non-enzyme antioxidants) released from the lenses. GPX is known to catalyze the reduction of a wide range of hydroperoxides including hydroperoxides of free fatty acids. However, the lens lipid peroxidase showed failure to decrease the residual membrane-bound PLOOH level during the incubation of normal or cataractous lenses for 1 h at 37°C. The negligible loss of peroxide incorporated in the natural membranes (liposomes) bathed in an aqueous environment of lenses is anticipated due to steric inaccessibility of the non-stripped from the lipid bilayer hydroperoxide substrates of a heterogeneous population, the composition of which is dependent on the nature of unsaturated fatty acyl groups in the sn-2 position. When the intact human transparent and cataractous lenses or normal mouse and rabbit lenses were incubated with liposomes in organ culture, the level of liposomal hydroperoxides was significantly (2.5–4.5-fold) higher after 3 h of incubation of the transparent lenses (or the lenses at initial stage of cataract) at room temperature than after the proper time of incubation of human mature cataractous lenses and no oxidation of prior untreated liposomes was detected in the absence of the lens. Metabolic devices in the normal lens are thus adjusted not just with destruction of oxidants, but rather specialized reactions also exist for the production of free radicals. It would be anticipated that the mature cataractous lenses maintain more peroxides in aqueous humor and the lens tissues [12] because of their reduced detoxification potential, e.g., reduced ability to detoxify H_2O_2 due to depletion of GSH, decreased activities of GSH reductase, SOD and an impairment of GPX (catalyzing reduction of organic/lipid hydroperoxides) [45]. LPO in the lens incubation system was decreased in the presence of free radical scavengers and enzymes that degrade H_2O_2 (EDTA, SOD, catalase, L-carnosine and chelated iron). The most effective agent was EDTA which chelates the free metal cations required to generate $O_2^{\cdot-}$ radicals that initiate the free radical process culminating in LPO. The mechanism of metal-catalysed oxidation reactions of the lens reductants (GSH, ascorbate, lens crystallins containing SH groups) was proposed as a basic for release of free radicals or lipid hydroperoxides in the lens medium and the rates and final levels of oxidants formation by lenses were found inversely dependent to their integral degrees of clouding [45].

Since lipoproteins are present in aqueous humor, a possibility cannot be ruled out that their lipid moiety may undergo oxidation in systemic diseases (chronic renal failure, anemia, diabetes, atherosclerosis, inflammation, retinal diseases) and ageing [64] and that phospholipid peroxides can contribute to the oxidative insult triggering the onset risk of cataract. In the anterior segment of the eye, the iris-ciliary body is the source of phospholipases A_2, C and arachidonic acid contributing to protective capacity to synthesize prostaglandins, other cyclooxygenase and lipoxygenase products in ocular fluids and tissues including the lens cells [65–68]. The activity of intrinsic phospholipase A_2, able to attack both non-oxidized and oxidized phospholipids in subcellular fraction of the lens, arranges lipid environment in the lens membrane increasing the cholesterol/phospholipid ratio [69] and hydrolyzing phospholipid hydroperoxides for accessible reduction with GPX. Phospholipid peroxidation products are a significant cataract-forming factor, since their entrance into the vitreous body of the eye induces the development of posterior subcapsular cataract with the concomitant fall in the concentration of GSH in the lens. Such mechanism of cataract induction was proposed on degeneration of retina [18].

The hydrophobicity of phospholipid amphiphilic

molecules is determined by their hydrocarbon tails, so their shortening by oxidative destruction of hydroperoxides or their chemical modification leading to the appearance of polar (hydroperoxide, carbonile) groups in fatty acyl, inevitably effects their hydrophilic-hydrophobic balance. Such modified molecules acquire properties of detergents similar to lisophospholipids forming under the action of phospholipase A_2. As a result of LPO, phospholipids modified with oxygen appear in lenticular fibre membranes able to increase the plasma membrane hydrophilicity and to impair lipid–lipid and protein–lipid interactions. The structural modifications of phospholipids of the lens fibre cell membranes at the carbonyl and phosphate-oxygen sites have been recently demonstrated upon metal oxidation using vibrational Fourier transform IR spectroscopy [70]. These effects form a basis of membrane fragmentation, interdigitations and foldings in the opaque and aged regions of the lens matter [12]. Leakage of water-soluble lens proteins (γ- and α-crystallines) into both aqueous and vitreous humors might be expected to occur [71–74] as a result of hydroperoxide, lipid peroxy radical and free radical oxygen toxicity to the lens in senile and complicated cataracts. The development of cataracts might be due to a disturbance of the lens cation permeability through cell membranes caused by phospholipid hydroperoxides. The lens fiber cell membrane is the site of oxidation-induced precipitation of cytoplasmic proteins, eventually producing aggregates of proteins around membrane micelles and fragments that serve as scattering centers. Cross-linking of biomolecules by the reaction of the carbonyl groups of MDA and amino groups of amino acids, proteins, nucleic acids and their bases, and phospholipids produces lipofuscins, fluorescent Schiff-base conjugates resulting in formation of high molecular weight aggregates chronically accumulated in cataracts and increased in ripe cataracts as well as inactivating enzymes in the lens [11,38,21]. It is apparent from the findings that membrane-bound phospholipid hydroperoxides escape detoxification by the lens enzymatic reduction. The lens cells containing these species would still be vulnerable to peroxidative attack which trigger the PLOOH-dependent chain propagation of LPO and other damages in membrane (lipid and protein alterations). Since LPO is clinically important in many of the pathological effects and ageing, new therapeutic modalities should treat the incessant infliction of damage to the lens cells and biomolecules by reactive lipid peroxides and oxygen species and 'refashion' the affected lens membranes in the lack of important metabolic 'detoxification' of phospholipid peroxides.

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