Role of Lipid Peroxidation in the Pathogenesis of Age-Related Cataract

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<http://dx.doi.org/10.5772/45942>

1. Introduction

The occurrence and development of cataract affect the decline of visual, working and living comfort. Cataract is the leading cause of blindness, accounting for 50% of blindness worldwide [1]. Cataract is progressive lens opacity in humans of 45 years or more, occurring without any known cause such as trauma, inflammation, hypocalcemia, medications or congenital factors. Risk factors for the occurrence of cataract are numerous: aging, diabetes mellitus, UV radiation, malnutrition, smoking, hypertension, renal disease, and others. Free oxygen radicals and oxidative stress are considered to be an important factor contributing to age-related cataract [1,2]. Oxidative stress has been shown to cause cataract in in vitro models [3]. This hypothesis is supported by studies that examined the anticatarogenic effect of different nutritional and physiological antioxidants [4].

Oxygen does not manifest toxic effects on cells of aerobic organisms in molecular form, but in the form of free oxygen radicals. Free radicals occur in univalent transfer of electrons to molecular oxygen. Due to its biochemical nature, and the low activation energy, they are able to react with biomolecules of all cellular structures, thereby carrying out their chemical and physiological modification. Under physiological conditions, the level of free radicals is controlled by mechanism of antioxidant protection. The balance between the production and catabolism of oxidants by cells and tissue is essential for maintenance of the biologic integrity of the tissue. Ocular tissues contain antioxidants that prevent damage from excessive oxygen metabolites: antioxidant enzymes, proteins, ascorbic acid, glutathione, amino acids cysteine and tyrosine, and other.

Changes in the oxidation of biomolecules can be found in many human diseases of the body, but the cataract is one of the most common diseases, where oxidative modifications of proteins [5] and lipids [1,2] is a dominant metabolic substrate of pathological disorders. Oxidative modification of lens proteins, loss of protein function and the creation of protein

aggregates of high molecular mass, which increases the scattering of light, are the main features of age-related cataract [6]. These protein modifications might be caused by oxidative stress resulting in higher levels of reactive oxygen radicals.

2. Sources of reactive oxygen species (ROS) in the lens

2.1. Reactive oxygen species generated in the lens by the UV irradiation

Human lens has several systems of defense from ROS and oxidative stress, which are together responsible for the maintenance of lens transparency and prevention of cataract. But during the life the lens is exposed to multiple sources of oxidative stress, endogenous (altered mitochondrial respiration, respiratory burst of phagocytes, viral infection) and exogenous (UV light, metals,

drugs, cigarette smoke), which can lead to production of reactive oxygen species: superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and others. The lens has a protective role for the other eye structures also, because light and oxygen are synergistically involved in the pathogenesis of cataract. By absorbing the part of the ultraviolet spectrum the lens protects deeper structures of the eye from the harmful effects of the solar radiation, whereby it is only subject to photooxidative damage. Photooxidative stress and the formation of reactive oxygen species by photosensitizing mechanisms are due to absorption of light by the biomolecules of the lens. Specifically, UV irradiation can mediate damage of lens structures, due to: direct absorption of the incident light by the cellular components, resulting in excited state formation and subsequent chemical reaction, and photosensitization mechanisms, where the light is absorbed by endogenous photosensitizers that are excited to their triplet states [6]. The excited photosensitizers can induce cellular damage by electron transfer and hydrogen abstraction processes to yield free radicals or energy transfer with O_2 to yield the reactive excited state, singlet oxygen.

Experiments in organ culture have shown that cataract can be caused by photochemical production of superoxide radicals, hydroxyl radicals and H_2O_2 [7]. Other researchers [8,9] indicate that photochemical generation of reactive species of oxygen in the lens and aqueous and consequent damage to the tissue has been implicated in the genesis of age-related cataract. The fact that the incidence of cataract is higher in the population that is more exposed to sunlight [10] imposes the assumption that photocatalytic conversion of molecular oxygen from ground state to excitatory states, which are highly reactive ($O_2^{\bullet-}$, H_2O_2 , $HO^{\bullet}$ and others) occurs. High concentration of ascorbate in the aqueous humor is assumed to represent a kind of filter that prevents the penetration of UV light in the lens and thus protects tissue from oxidative damage, particularly photoinduced damage [11].

Photosensible substance, that absorbs certain wavelengths of light, activates and subtracts hydrogen or electrons from the substrate by converting them into free radicals. In the presence of O_2 , the energy is transferred from excitatory substance and produce 1O_2 , which can initiate the process of lipid peroxidation. In the ocular tissues numerous substances can initiate photodynamic reactions. These substances are riboflavin, heme derivatives,

tryptophan and its oxidation product N-formylkynurenine, lipofuscin, visible pigments (retinol), and photosensible substances of exogenous origin, such as drugs [12]. Key link between photo-oxidation and cataract is that photo-oxidation of thiol groups on lens crystalline produces disulfide bridges between molecules and, the build-up of these will lead to protein aggregation and hence cataract.

3. Mitochondria as a source of reactive oxygen species in the lens

In the ocular tissues, including the lens, as in other organ systems, ROS are formed in the mitochondria via the electron transport chain where inefficient electron coupling leads to the formation of superoxide anion. Molecular oxygen is tightly bound to the enzyme complex cytochrome C oxidase. However, the bond on the vectors of electrons in the respiratory chain in front of the system cytochrome C oxidase, on the level of NADH-coenzyme-Q reductase and the reduced forms of coenzyme Q, is not that strong and some of transferred electrons can “leak” from the system on molecular oxygen, forming $O_2^{\bullet-}$. Superoxide production is significantly increased during reperfusion of tissues, when the availability of oxygen is increased.

The human lens consists of three metabolically different zones: the epithelium, the cortex and lens nucleus. Epithelial cells and superficial cortical fibers are metabolically most active, and the greatest part of mitochondrion respiration and aerobic glycolysis in the lens occurs in them [13]. One third of total energy produced (ATP) in the lens is produced in epithelial cells under aerobic conditions, while the metabolic activity of nuclear part of the lens is at much lower level. Intense metabolic activity makes epithelial cells susceptible to oxidative damage, especially their membrane pump systems and DNA. Oxidation of unsaturated lipids in epithelial cells could be the initial step that leads to generation of oxidation products. If reactive oxygen species or secondary products of lipid oxidation from the epithelium were to migrate to the fiber cells, it is possible that prolonged accumulation of lipid oxidative products could eventually lead to alterations in fiber cell structure and increased opacity, which leads to the development of cataract.

Thiol (-SH) groups of membrane proteins, the lens epithelial cells, which are significant for regulation of ion transport, are very susceptible to oxidative attack, especially when the concentration of intracellular GSH is reduced. The optimal membrane function of lens epithelial cells depends on reduced state of protein-SH groups. The oxidation of membrane thiol (-SH) groups of the lens cells leads to breakdown of active transport through the membrane, to the increase of membrane permeability and consequently intracellular alternations, which is involved in the development of cataract. The consequence of impaired active transport is also the reduced level of ascorbic acid in the lens. Studies have confirmed that ascorbic acid (AA) levels in human lenses with the development of cataract are reduced [34], and concentration of dehydroascorbic acid (DHA) is increased [14,35]. Timely removal of dehydroascorbic acid from the lens is important because of its potential toxicity as oxidant. Increase of the current concentration of DHA/AA redox balance can be an indicator of oxidative stress in the lens [35].

4. The importance of ascorbic acid in lens

The role of ascorbic acid is important, as a strong reductant and effective scavenger of hydroxyl and superoxide anion radical. Vitamin C has antioxidant, but also prooxidant properties. In which direction will vitamin C work, depends on the concentration of vitamin C, oxygen and the presence of metal ions. Oxidation is the cause of modification of lens proteins which accumulate over a lifetime. Some believe that ascorbate can contribute to protein modifications, react as prooxidant and participate in reactions that generate radicals [14].

These reactions may be caused by light or metal-catalyzed oxidation of endogenous ascorbic acid. It is known that copper and iron are present in micromolar concentrations and that autooxidative processes can occur in the lens. Fenton-type reactions, where H_2O_2 reacts with free metal ions, iron (Fe^{2+}) or copper (Cu^{2+}) to produce the $\text{HO}^\bullet$ radical, are a major source of oxidative stress initiated by transition metals [15] and are thought to be involved in the formation of cataract [16]. In the presence of metals, especially iron and copper, and oxygen, ascorbic acid is oxidized to dehydroascorbate, which produces hydrogen peroxide and metal is reduced. Hydrogen peroxide can react with reduced metal, generating hydroxyl radical and other reactive oxygen radicals [17]. When copper and protein-bound iron is included in this reaction, the radicals cause oxidative modification of amino-acids that are near the metal. In this way ascorbate can actually become a prooxidant and lead to protein damage via both H_2O_2 and Fenton production of $\text{HO}^\bullet$. These reactions become important when cells lose their ability to remove metals, making it available for reaction and/or when cells lose their ability to maintain their vitamin C in a reduced form. It is noted that during the aging of lens, as in cataract lenses the concentration of copper and iron increases [18,19]. The data that confirm the level of iron and copper ions is lower in non-cataract lenses and study that compared cortical nuclear and mature cataracts found higher iron levels in the mature cataract [20] suggest that metal ions that mediate the production of $\text{HO}^\bullet$, may be important in the development of age-related cataract [16].

Experiments on isolated proteins showed that oxidation products of ascorbate (dehydroascorbic acid) can form cross-link with crystalline lens, producing molecules of high molecular weight, which cause light scattering typical for cataract [21]. It is assumed that similar modification of lens proteins occurs in vivo during the development of age-related cataract [22].

5. Lipid peroxidation in the lens

In physiological/controlled conditions the process of lipid peroxidation affects the permeability of cell membranes, the metabolism of membrane lipids and proteins, provides control of cell proliferation, but the adverse effects of this process occurring under conditions of oxidation stress, ie. in conditions of impaired balance of prooxidative and antioxidative factors of the cell. Lipid peroxidation (LPO) is considered a pathogenetic factor of cataractogenesis [1,2,23,24,25]. LPO in the lens may be induced by endogenous or

exogenous factors: enzymes, reactive oxygen species, metal ions, UV irradiations, heat, radical-initiating chemicals, drugs. Cell membrane lipids (phospholipids, glycolipids) are the most common substrates of oxidative attacks, and since the cell membranes have lipoprotein structure, the structure of membrane proteins is disturbed at the same time. That causes the disturbance of cell membrane barrier function, leading to a larger entry of calcium and other ions [26]. Structural changes of the cell membrane and its increased permeability change the cell volume and the configuration of the lens, leading to refractory changes that are associated with the early cataract.

Cell membranes are very sensitive to the effects of oxygen radicals, due to the presence of polyunsaturated fatty acids in lipids. Fatty acids in lipids of cell membranes contain a different number of carbon atoms (14 to 24), and the present double bonds are in *cis* configuration. The presence of double bond in the neighborhood destabilize the bond between the carbon and the hydrogen of methylene group in the chain of polyunsaturated fatty acid, and by subtracting hydrogen from such a methylene group by reactive oxidant, begins the process of oxidative modification of fatty acids - lipid peroxidation [27]. Non-enzymatic peroxidation of polyunsaturated fatty acids is a process that takes place in three stages: initiation, propagation and termination. The intensity of this process, as well as the ability of partial or complete repair of damage, depends on pro/anti-oxidative environment in which this process occurs.

Free radicals formed during lipid peroxidation have a local effect due to short life, but the degradation products of lipid peroxidation can be second messengers of oxidative stress, because of their longer half-life and ability to diffuse from the place of formation. These degradation products, mainly aldehydes, such as malonaldehyde, hexanal, 4-hydroxynonenal or acrolein, have biological roles in cellular signaling, in normal and pathological conditions and in regulation of cell cycle [28]. Due to their chemical reactivity these products can covalently modify macromolecules as nucleic acids, proteins and lipids and consequently exhibit different biological effects. They are also biomarkers of lipid peroxidation and oxidative stress.

During the development of cataract, non-enzymatic lipid peroxidation occurs. It is a nonspecific and uncontrolled process in which the resulting reactive oxygen species readily react with surrounding molecules, leading to intensification of the process, and the damage of the cell membrane. The process LPO in the lens can be initiated by hydroxyl radical, singlet oxygen, peroxy radical. These radicals seize from the unsaturated fatty acids H^+ from the methyl group ($-CH_2$) in the α - position of the double bond, to form unsaturated fatty acid lipid radical ($L^\bullet$).

By intramolecular rearrangement of double bonds in lipid radicals, conjugated dienes are formed. By adding the molecular oxygen to conjugated dienes, lipoperoxyl radical ($LOO^\bullet$) is formed. Lipoperoxyl radicals have significant oxidative potential, they can further initiate the seizure of hydrogen from the neighbouring unsaturated fatty acid, by which lipid peroxidation enters the phase of propagation and autooxidation, resulting in formation of lipid hydroperoxide ($LOOH$) and new lipid radicals ($L^\bullet$).

Lipid hydroperoxydes (LOOH), as primary molecular products of lipid peroxidation process, are well-soluble, have ability to migrate from the site of development and are important potential sources for the formation of reactive hydroxyl radical ($\text{HO}^\bullet$). In the presence of metals with variable valence they may reopen a cascade of lipid peroxidation [27]. In the presence of Fe^{+2} in the classical Fenton-type reactions, by decomposing of LOOH, $\text{HO}^\bullet$ and alkoxy radical ($\text{LO}^\bullet$) occur. In the presence of Fe^{+3} , LOOH is decomposed to peroxy radical ($\text{LOO}^\bullet$). Alkoxy and peroxy radical are responsible for initiation and propagation of LPO processes.

By removing hydroperoxides (LOOH), which occur during the first phase of lipid peroxidation, by activity of glutathione-dependent peroxidase which catalyzes reduction of hydroperoxide into the corresponding alcohol, the reactions of lipid peroxidation propagation can be prevented.

Compounds that react with reactive oxygen species produced during the chain reaction, such as peroxy and alkoxy radicals ($\text{ROO}^\bullet$ and $\text{RO}^\bullet$), and lead to the formation of the species that are unable to remove the hydrogen atom from unsaturated fatty acids, are considered to be antioxidant switches of chain reactions, of which the most important is liposoluble α -tocopherol. In series of complex degradation reactions of hydro- and dihydro-peroxides of polyunsaturated fatty acids, many other aldehydes are produced: 4-hydroxy-alkenals, 4-hydroperoxy-alkenals, 4-oxo-alkenals and bis-aldehyde malonaldehyde (MDA) [28]. These aldehydes are highly reactive compounds because they have electrophilic properties, they are less volatile than hydroperoxides, and can diffuse from the place of origin and express their reactivity through biotransformation and adduction to biomolecules (proteins, DNA).

Consequences of peroxidative damage of lens cell membranes are multiple. On the one hand, a number of membrane functions may be altered due to a direct attack by reactive oxygen species on the membrane components responsible for these functions. Indirect consequences of peroxidative damage of membranes are important. Lipid peroxidation modifies the environment of not only membrane proteins and may in this way influence their functional efficiency. The consequences of adduct formation at the protein level is associated with numerous cytotoxic consequences including the disruption of cell signalling, altered gene regulation, inhibition of enzyme activity, mitochondrial dysfunction, impaired energy metabolism. Two different ways of oxidative modifications of cellular constituents have to be considered in cataractogenesis: the direct modification by reactive oxygen species and the indirect modification via reactive products of lipid peroxidation.

Lipid peroxidation products can diffuse across membranes, allowing the reactive aldehyde to covalently modify proteins localized throughout the cell and relatively far away from the initial site of reactive oxygen species (ROS) formation. LPO is implicated in human cataractogenesis because the toxic peroxidation products induce fragmentation of soluble lens proteins and damage vital membrane structures, correlating with an increase in lens opacity and changes in the refractive properties of the lens [23]. The results obtained by the authors, that caused posterior subcapsular cataract in the rabbit, by application of peroxidative products in the vitreous, were published [29].

The resulting lipid peroxidation products, such as, 4-hydroxynonenal (HNE), can form protein-HNE adducts that may result in altered protein functions and can mediate oxidative stress-induced cell death in the lens epithelial cells [30]. Also, the resulting MDA, can react with amino-groups of proteins, forming intra-molecular cross bonds and bind two distinct proteins by forming the inter-molecular bonds [31] and thus affect the structural and functional properties of proteins. MDA is linked to the Lys residues of proteins and enzymes, and forms Schiff bases, for phospholipids, nucleic acids (shown mutagenic properties), and also MDA inhibits a number of thiol dependent enzymes: glucose-6-phosphatase, Na^+/K^+ -ATP-ase, Ca^{++} -ATP-ase.

At the level of cell membranes in the lens lipid hydroperoxides induce changes of the lipoprotein structure and permeability [13], and oxidation inhibition of membrane enzymes Na^+/K^+ -ATP-ase and Ca^{++} -ATP-ase, which are responsible for osmotic regulation and transport of metabolites. Disturbance of ion transport through the cell membrane is associated with ATP hydrolysis, which increases membrane permeability to protons, as the result of LPO. The Na^+/K^+ -ATP- and Ca^{++} -ATP-ases would be directly affected because of their regulation by proton concentration. Other transport proteins would be affected in a secondary sense because they are coupled to ions whose intracellular concentrations are regulated by the ATP-ase. For example, glucose is the main source of energy for lens cells and is typically co-transported with Na^+ , and similar transport are known for the import of amino acids across cell membranes. Formation of the lens opacity follows from either osmotic imbalance or a cytoplasmic imbalance of specific cations, in particular Ca^{+2} [32]. Disturbance of function Ca^{++} -ATP-ase and the consequent increase of Ca^{+2} in the lens leads to electrostatic changes in the crystalline, which can disrupt the protein conformation and interaction. Maintenance of calcium homeostasis is critical to lens clarity and cataractous lens has elevated calcium levels. An in vitro binding study indicates that human lens lipids have the capacity to bind nearly all the calcium present in the human lens and that age and cataract diminished the capacity of lens lipids to bind calcium. It is possible that the increased concentration of intracellular Ca^{+2} and reduced ability of lens lipids to bind calcium, to initiate further disturbances that lead to an increase of light scattering from proteins and lipids [33]. Disturbance of the lens cell membrane permeability during the development of cataract, and reduced ability of active transport of substances against the concentration gradient lead to changes in concentration of intracellular compounds and metabolic changes within the cells. This is manifested by lowering the content of GSH, ATP, and other intracellular compounds and electrolytes in lens cells.

Lipid peroxides as potential causes of cataracts, lead to the changes of not only the lens cell membrane, but also of the cytosol, because it serves to reduce concentration of glutathione and cause the change of redox relationship GSH/GSSG [1].

6. Defense against lipid peroxidation in the lens

Low molecular mass compounds which act primarily against peroxy radicals involved in radical propagation, provide first line of defense against lipid peroxidation in the lens.

These compounds (GSH, ascorbic acid, α -tocopherol) can terminate the propagation of free radical mediated reactions and interrupt the autocatalytic chain reaction of lipid peroxidation. GSH is a major antioxidant in the lens, and helps to reduce proteins, contains a side chain of sulfhydryl (-SH) residue that enables it to protect cells against oxidants. GSH can directly scavenge ROS or enzymatically via two major antioxidant enzyme systems, glutathione peroxidases (GPx) and glutathione S-transferases (GST). Enzymes such as superoxide dismutase (SOD), catalase (CAT) and GPx can decompose ROS and prevent the damage to cellular constituents and initiation of lipid peroxidation. In the event of ROS induced lipid peroxidation, secondary defense enzymes are involved in the removal of LOOH to terminate the autocatalytic chain of lipid peroxidation and protect membranes. GPx and GST which catalyze GSH-dependent reduction of LOOH through their peroxidase activity are the major secondary defenses in the lens against ROS induced lipid peroxidation. Resynthesis of GSH from the oxidized form is catalyzed by glutathione reductase, where the necessary $\text{NADPH} + \text{H}^+$ is produced in pentose pathway of carbohydrates [36]. Glutathione reductase (GR) is a control enzyme glutathione-redox cycle and by maintaining of intracellular levels of GSH can affect cation transport systems, lens hydration, sulfhydryl groups of proteins, and membrane integrity. Detoxifying role of GST is reflected in its ability to catalyze reactions of conjugation of reduced glutathione with endogenous electrophiles, mostly by the products of oxidative stress, lipid hydroperoxides and final products of lipid peroxidation [37]. Class μ and π GST isoenzymes are expressed in the human lens, with the π isoenzyme predominating. The highest GST activity occurs in the peripheral and the equatorial cortexes, with the lowest activity in the nucleus [38]. Glutathione peroxidase activity is shown by the enzymes that catalyze the reduction of hydrogen peroxide, organic hydroperoxide and phospholipid hydroperoxide using GSH as a hydrogen donor. Superoxide dismutase (SOD) catalyzes the reaction of dismutation of superoxide anion radicals ($\text{O}_2^{\bullet-}$) in the presence of hydrogen donor to hydrogen peroxide and molecular oxygen. By removing the $\text{O}_2^{\bullet-}$, SOD prevents the formation of $^1\text{O}_2$ which can initiate the process of lipid peroxidation.

Aim. Our studies have focused on measuring the products of lipid peroxidation in corticonuclear lens blocks, with different type and different degrees of maturity of age-related cataract. In addition to measuring products of lipid peroxidation in cataract lenses, our study included the determination of activities and ability of lens glutathione peroxidase and glutathione S-transferase to remove hydroperoxides, which are probably involved in the early stages of cataractogenesis and development of mature cataract through oxidative stress.

7. Material and methods – patients

Clinical and biochemical researches were carried out in 101 patients with age-related cataract, 46 women and 55 men. The average age of the group was 72.5 ($\text{SD} \pm 7.9$). According to the cataract maturity degree the patients were classified into two groups as follows: age-related cataract incipient ($N=41$) and matura ($N=60$). In the group age-related

cataract incipient there were 23 patients with posterior subcapsular (PS), 9 patients with nuclear subcapsular (NP) and 9 patients with cortical nuclear (CN) cataract. In the group age-related cataract matura there were 19 patients with cataract which started as a posterior subcapsular, 15 patients which started as a nuclear subcapsular, 16 patients with matura, which started as cortical nuclear and 10 patients diagnosed with matura, which started as cortical cataract.

Samples corticonuclear blocks/parts of lens (without epithelial cells) were obtained from patients undergoing extracapsular extraction of cataracts and used as the test material. Types of cataract were estimated during ophthalmologic examination and confirmed during its extraction. Immediately after sample acquisition, samples were closed in individual capsules and deeply frozen. This research has been conducted following the tenets of the Declaration of Helsinki and approved by the ethics committee of Medical Faculty. Informed consent was provided from all patients after a careful explanation of the aims of the study.

Homogenate of lenses from each group was prepared in 0.2 mol/L potassium phosphate buffer (pH 7.2). For analysis we used supernatant obtained by centrifugation of homogenates at 5000 rpm for 15 min at 4°C.

The concentration of conjugated dienes was measured spectrophotometrically at 233 nm [39].

Lens MDA concentrations were measured as the product of the reaction with thiobarbituric acid (TBA) using a modification of the method Ledwozyw et al [40].

Fluorescent products (lipid- and water-soluble) of lipid peroxidation were determined by spectrofluorimetric analysis at 360/430 (excitation/emission) nm [41].

The concentration of GSH in the sample was determined in the reaction 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman's reagent), after removal of proteins by perchloric acid [42].

8. Enzyme assays

The activity of glutathione peroxidase was determined at 412 nm, by the method Chin et al. [43]. The conjugation of GSH with 1-chloro,2,4 dinitrobenzene (CDNB), a hydrophilic substrate, was examined spectrophotometrically at 340 nm to measure glutathione S-transferase activity [44]. One unit of GST was defined as the amount of enzyme required to conjugate 1 μ mol of CDNB with GSH/min. The activity of glutathione reductase was assayed by the procedure of Glatzle et al. [45]. Superoxide dismutase (SOD) activity was determined by the method Misra and Fridovich [47], based on the inhibition of the adrenochrome during the spontaneous oxidation of adrenaline in basic conditions. The change in absorbance was read at 480 nm on a spectrophotometer. The SOD activity was expressed as kU/g protein (one unit was considered to be the amount of enzyme that inhibited adrenaline auto-oxidation by 50%).

To calculate the specific enzyme activity, protein in each sample was estimated by the method of Lowry et al [46].

9. Results and discussion

Senile cataract is manifested in the later years of life, so it is estimated that the costs of operation would be reduced by 45% if the incidence of age-related cataract could be delayed for ten years [48]. By studying the oxidation changes of lens structures during the development of cataract, we attempted to contribute to clearing up the process of cataractogenesis, of which even today there are many unknowns.

Lipid peroxidation is one of the possible mechanisms of cataractogenesis, caused by excessive production of reactive oxygen species in aqueous environment and reduced antioxidant defense of the lens. By studying the corticonuclear lens block of the patients with age-related cataract were detected increased concentrations in primary molecular products LPO (diene conjugates and lipid hydroperoxides) and end fluorescent LPO products (table 1).

	Age-related cataract incipient (n=41)	Age-related cataract matura (n=60)
Conjugated diens (nmol/g weight of lens)	$2.48 \pm 0.84^*$	1.57 ± 0.49
Fluorescent products/ g protein (lipid soluble)	46.59 ± 14.40	$70.94 \pm 13.21^*$
Fluorescent products/ g protein (water soluble)	57.53 ± 18.23	$103.08 \pm 27.81^*$
MDA (nmol/g weight of lens)	1.81 ± 0.67	$3.17 \pm 0.78^*$

Data is presented as means $\pm$ SD * $p < 0.001$

Table 1. Lipid peroxidation products in cataractous lenses

In the group of patients with the incipient cataract, we obtained significantly higher concentration of diene conjugates in the lenses compared to matura cataract ($p < 0.001$) (table 1). This can be explained by the fact that at the early stages of the development of cataract the most intense is the proces of lipid peroxidation, which is either the initiator of cataractogenesis proces or initiated by creation of reactive oxigen types, and continues to affect changes in the lens by its propagation.

In the lenses with the incipient cataract, the concentration of conjugated diens is significantly higher in cortical nuclear cataract (CN) compared with the lenses with posterior subcapsular cataract (PS) ($p = 0.001$) (table 2). Also, in matura cataract the concentration of conjugated diens is the highest in the lenses diagnosed with CN cataract ($p < 0.05$) (table 3).

Cataract incipient (type)	PS (n=23)	NP (n=9)	CN (n=9)
Conjugated diens (nmol/g lens)	2.12 ± 0.55	2.63 ± 0.97	3.24 ± 0.84*
Fluorescent products/g protein (lipid soluble)	40.09 ± 10.18	52.34 ± 13.92‡	57.47 ± 16.33‡
Fluorescent products/g protein (water soluble)	50.17 ± 12.88	61.99 ± 14.58	71.90 ± 24.12‡
MDA (nmol/g weight of lens)	1.60 ± 0.56	1.74 ± 0.73‡	2.41 ± 0.55*

Data is presented as means ± SD *p<0.001, ‡p<0.05, †p<0.01.

PS - posterior subcapsular, NP - nuclear subcapsular, CN - cortical nuclear

Table 2. Products LPO in lenses with cataract incipient

Cataract matura	Cataract matura began as PS (N=19)	Cataract matura began as NP (N=15)	Cataract matura began as CN (N=16)
Conjugated diens (nmol/g lens)	1.41 ± 0.30	1.46 ± 0.67	1.91 ± 0.39†
Fluorescent products/g protein (lipid soluble)	67.32 ± 15.45	76.69 ± 14.65‡	74.74 ± 7.49
Fluorescent products/g protein (water soluble)	94.83 ± 25.43	105.29 ± 24.72	120.68 ± 27.08‡
MDA (nmol/g lens)	3.32 ± 1.07	3.23 ± 0.80	3.06 ± 0.48

Data is presented as means ± SD †p<0.01, ‡p<0.05.

Table 3. Products LPO in lenses with cataract matura

In the presence of free metal ions with variable valence (Fe^{+2} or Cu^{2+}) hydrogen peroxide in Fenton's reaction is translated into highly reactive hydroxyl radical, while the lipid peroxides are translated into peroxy and alkoxyl radicals. Because of the longer half-life time compared to the alkoxyl radical, peroxy radical is ideal for propagation of oxidative chain reactions, while the oxidation of alkoxyl radicals produce dihydroperoxide, which is degraded to toxic aldehydes, such as 4-hydroxy-2,3-trans-nonenal, 4-hydroxy-pentanal, short chain malondialdehyde. Such conditions may exist in humane senile lens [49]. MDA and 4-hydroxy-2,3-trans-nonenal by forming of Schiff's bases with amino groups amino acid residues of protein contribute to increase of carbonyl groups content and produce fluorescent products of lipid peroxidation. We have measured significantly higher concentration of these fluorescent products of LPO in the lenses with mature cataract (table

1), and the highest concentration was measured in the lenses with the NP and CN cataract in relation to posterior subcapsular cataract (PS) ($p < 0.05$) (table 2,3). Some authors have identified fluorescent Schiff bases in higher concentration in human cataract lenses compared to healthy lenses, resulting from the interaction of reactive carbonyl groups MDA with amino groups of lens membrane phospholipids [50].

By measuring the concentration of MDA in the homogenate of cataract lenses, we obtained significantly higher concentration in the group of patients with matura compared to incipient cataract ($p < 0.001$) (table 1). This can be explained by the fact that the malondialdehyde is one of the final products of LPO, which accumulates in the lens during the process of lipid peroxidation and the development of cataract. Author's results with the experimentally induced cataract that have measured significantly higher concentration of MDA in cataract lenses compared to the control group were published [51], as well as author's works that obtained significantly higher concentration of MDA in cataract lenses of diabetics [2], myopic lenses and senile cataract [52]. Reduced activity of glutathione peroxidase enzyme and glutathione S-transferase, that are important for the removal of malondialdehyde, contribute to the increase of its concentration in the lens.

The reasons that cause significantly higher concentration of lipid peroxidation products in lenses with cortical nuclear (CN) and nuclear subcapsular (NP) cataract, in relation to the posterior subcapsular (PS) are numerous. The results of other researchers [49] indicate that during the life time in the lens some kind of "internal" lens barrier is developed between nuclear and cortical parts, which hinders the diffusion of molecules to the nucleus. This barrier prevents the diffusion of antioxidative molecules to the nuclear part, which increases the sensitivity of central part of the lens to oxidative damage. Also, it is possible that unstable prooxidant molecules have longer residency in the central part of the lens. The endogenous lens chromophore, tryptophan metabolites (kynurenine, 3-hydroxykynurenine, 3-hydroxykynurenine glucoside), which are relatively inert photochemically, during oxidative stress and/or aging are formed photochemically active tryptophan metabolite (N-formyl-kynurenine, xanthurenic acid) that have photochemical properties, and also act as an endogenous photosensitizers in the lens [53].

Through photosensible reactions tryptophan products transfer absorbed energy to oxygen, which further leads to a series of cellular changes through the oxidation. With age, the level of free components of UV filters ie. tryptophan derivatives in the lens are reduced, and their binding to lens proteins increases [54]. Tryptophan products are subject non-enzyme deamination, at physiological pH, resulting in α,β -unsaturated ketone, reactive intermediates [55], which can covalently bind to amino acids, usually His, Cys, or Lys residues in proteins of human lenses, or react with the Cys residue of glutathione (GSH) to form GSH-3OHKynG [54]. GSH that is present in the lens in relatively high concentrations may compete with the amino acid residues for the unsaturated ketone derivative of kynurenine, thereby protecting the crystalline from modification. This covalent modification is particularly expressed in the nucleus of the lens containing the older proteins, causing

altered transport/diffusion of small molecules in the lens. Specifically, it develops a barrier in to the movement of molecules between metabolically active cortex and inert nucleus. The barrier also restricts the flow of GSH from the cortex, which reduces the concentration of GSH in the nucleus of the lens, so the response to oxidative damages in this part of the lens is also reduced [49]. During the life time, lens fibers are very compactly arranged in nuclear part, with minimal presence of extracellular space. Nuclear plasma of the membrane undergo oxidation damage, whereas the phospholipid molecules modified by oxygen accumulate in the lipid layer, leading to changes in the structure and violate lipid-lipid and protein-lipid interactions in membranes of the lens fibers. This probably contributes to concentration of the lipid peroxidation products to be the highest in the lenses with early nuclear cataract.

Reduced glutathione (GSH) and GSH-dependent enzymes, glutathione peroxidase (GPx) and glutathione S-transferase (GST), are very important in defending the lens structures of the products of lipid peroxidation. One reason for increased production and accumulation of lipid hydroperoxides in the lens with cataract may also be reduced activity of GPx and GST.

The primary biological role of superoxide dismutase (SOD) is to catalyze reaction of dismutations of superoxide anion radicals ($O_2^{\cdot-}$) in the presence of hydrogen donor to hydrogen peroxide and molecular oxygen. Superoxides can first be degraded into H_2O_2 by SOD, and subsequently, catalyzed into ground-state oxygen and water by catalase and enzymes of the glutathione redox cycle, including glutathione reductase and glutathione peroxidase. At low levels of hydrogen peroxide, the glutathione redox cycle is responsible for protecting against H_2O_2 -induced damage and maintaining high levels of GSH in the lens, whereas, at a higher concentration, the principal mechanism for the removal of hydrogen peroxide is catalase. The study of the lens cell culture where the expression of DNK for superoxide dismutase was performed on intact lens cells, showed that the cells with higher activity of SOD resistant to oxidative damage, caused by hydrogen peroxide, superoxide anion radical and UV radiation. Expression of the superoxide dismutase enzyme prevented the beginning of the cataract in lens cells [56].

GSH and other sulfhydryls are particularly important in the protection of thiol (-SH) groups of crystalline and prevent the formation of aggregates which reduce transparency of the lens [57]. The oxidized glutathione (GSSG) is reduced back to GSH by a NADPH-dependent glutathione reductase, which in physiological conditions maintains a high ratio of GSH/GSSG in the lens and other ocular tissues. Oxidative stress, induced by accumulation of LPO products in the lens during the development of cataract, causes consumption of GSH and disruption of redox balance in the lens, so the age-related cataract is associated with progressive reduction of GSH concentration in the lens. Probably with the progression of the cataract than the consumption of GSH against toxic compounds, the synthesis of GSH is reduced, as the result of deficient availability of substrates and reduced activity of enzymes for its synthesis (γ -glutamyl-cysteine synthetase). This reduces the amount of available GSH for optimal function GPx and GST, which causes the peroxide metabolism disorder.

Figure 1. Lens glutathione and glutathione reductase in cataract.

Figure 2. Lens glutathione and glutathione peroxidase in cataract.

The significance of GSH, as an important compound for the function of GPx and GST in the tested lenses, is confirmed also by the positive correlations between the concentration of GSH and glutathione reductase ($r = 0.7011$, $p < 0.001$) (figure 1), between the concentration of GSH and glutathione peroxidase activity ($r = 0.6749$, $p < 0.001$) (figure 2), and between activities of glutathione S-transferase and the concentration of GSH ($r = 0.6379$, $p < 0.001$) (figure 3).

Figure 3. Lens glutathione and glutathione S-transferase in cataract.

Experiments showed that applying the injections of buthionine-sulphoximine as inhibitors of reduced glutathione synthesis, on the newborn rats, causes the development of cataract [58].

	Cataract incipient (n=41)	Cataract matura (n=60)
GSH	2.55 ± 0.9	$0.88 \pm 0.26^*$
GPx	3.40 ± 1.47	$2.09 \pm 0.90^*$
GR	3.03 ± 1.29	$1.61 \pm 0.71^*$
GST	2.46 ± 1.14	$1.50 \pm 0.67^*$
SOD	4.13 ± 2.14	$2.14 \pm 0.91^*$

Data is presented as means $\pm$ SD * $p < 0.001$

GSH ($\mu\text{mol/g}$ weight of lens), GPx, GR, GST (U/g protein), SOD (kU/g protein).

Table 4. Antioxidative defense factors in cataractous lenses

In tested corticonuclear lens blocks with mature cataract, we measured lower activity of GR, GPx and GST enzymes in relation to the incipient ($p < 0.001$) (table 4).

Cataract incipient (type)	PS (n=23)	NP (n=9)	CN (n=9)
GSH	2.99 ± 0.90	$2.16 \pm 0.55^\dagger$	$1.81 \pm 0.42^*$
GPx	4.58 ± 0.70	$2.17 \pm 0.53^*$	$1.64 \pm 0.17^*$
GR	4.03 ± 0.66	$1.91 \pm 0.73^*$	$1.61 \pm 0.27^*$
GST	3.31 ± 0.60	$1.61 \pm 0.75^*$	$1.14 \pm 0.29^*$
SOD	5.77 ± 1.25	$2.34 \pm 0.59^*$	$1.71 \pm 0.58^*$

Data is presented as means $\pm$ SD * $p < 0.001$, $^\dagger p < 0.01$.

GSH ($\mu\text{mol/g}$ weight of lens), GPx, GR, GST (U/g protein), SOD (kU/g protein)

Table 5. Antioxidative defense factors in lenses with cataract incipient

The lowest activity of these enzymes was measured in the lenses with the CN and NP in relation to the PS cataract (table 5). Reduced activity of GR, GPx and GST enzymes is followed by a significant decrease in the concentration of GSH in lens homogenates with matura ($0.88 \pm 0.26 \mu\text{mol/g weight}$), compared to the initial cataract ($2.55 \pm 0.9 \mu\text{mol/g weight}$) ($p < 0.001$) (table 4), with lower concentrations of GSH measured in CN and NP compared to the PS cataract (table 5,6).

Cataract matura	Cataract matura began as PS (N=19)	Cataract matura began as NP (N=15)	Cataract matura began as CN (N=16)
GSH	0.98 ± 0.19	0.78 ± 0.20	0.81 ± 0.35
GPx	2.96 ± 0.99	$1.89 \pm 0.57^*$	$1.44 \pm 0.31^*$
GR	2.26 ± 0.60	$1.31 \pm 0.49^*$	$1.06 \pm 0.47^*$
GST	2.13 ± 0.50	$1.13 \pm 0.43^*$	$1.07 \pm 0.49^*$
SOD	2.96 ± 0.65	$1.77 \pm 0.52^*$	$1.25 \pm 0.36^*$

Data is presented as means $\pm$ SD * $p < 0.001$.

GSH ($\mu\text{mol/g weight of lens}$), GPx, GR, GST (U/g protein), SOD (kU/g protein)

Table 6. Antioxidative defense factors in lenses with cataract matura

Also the results of other researchers show that the level of reduced glutathione in the lens decreases with the development of the cataract [24]. Such changes are probably a reflection of oxidative processes, increased by the formation of toxic products of lipid peroxidation, compared to a weakened antioxidative capacity of cataract lenses. In cataract lenses the concentration of GSH is being reduced, since, as a main representative of non-protein thiols, it is included and consumed in oxido-reduction processes in terms of excess oxidized substrates. A possible reason for the consumption of GSH during oxidative stress is its conversion into oxidized form, which can be conjugated with protein thiol groups to form mixed disulphides (PSSG), via a process called protein-S-thiolation [59].

Results of other researchers also show that the human lens with age-related cataract glutathione peroxidase activity was significantly reduced compared with normal lenses. The kinetic study of GPx showed that lipid hydroperoxides achieve saturation of enzymes at a concentration that is approximately 1 mmol ie. that K_m GPx is achieved at a concentration of lipid hydroperoxides of 0.434 mmol [60]. Because of these kinetic properties, GPx activity was probably inhibited in age-related cataract by products of lipid peroxidation, using non-competitive inhibition principle. GPx activity, aside from availability of GSH, is affected by other factors such as glutathione reductase activity, the amount of produced $\text{NADPH}^+ + \text{H}^+$ in the pentose pathway and availability of selenium. The authors who have examined the activity of GPx in the lenses of experimental mice and compared the degree of lens blur, and age of mice with specific activity of GPx, showed significant correlation between decreased activity of GPx and the level of lens blur, as well as the age of mice [61].

Considering the function of GST to catalyze reactions of conjugation of lipid peroxidation products with GSH [62], thereby reducing the toxicity of electrophilic compounds and their reactivity towards nucleophilic groups in biomolecules, it is logical that the activity of the GST measured in incipient cataract is higher (2.46 ± 1.14 U/g protein), because at the beginning of the development of the cataract the most intense is the process of lipid peroxidation. With advancing of the process of cataractogenesis, the amount of GSH is reduced for glutathione-S-transferase, and the enzyme activity is significantly decreased in the lenses with mature cataract (1.5 ± 0.67 U/g protein) ($p < 0.001$) (table 4).

In the study of GST activity in epithelial cells of operated cataract lenses, the group of authors showed that lens epithelial cells with cortical nuclear and cortical cataract show complete loss of activity of glutathione-S-transferase [63].

By analyzing the activity of glutathione reductase, significantly higher activity was found in the lenses with the incipient cataract (3.03 ± 1.29 U/g protein) compared to the lenses with mature cataract (1.61 ± 0.71 U/g protein) ($p < 0.001$) (table 4). Other researchers have obtained similar results after comparing the activities of GR in cataract lenses and intact lenses of older persons [64]. Glutathione reductase plays a key role in maintaining thiol (-SH) groups in the lens, and this is probably the most important role of this enzyme in maintaining lens transparency. It is possible that lower GR activity in comparison to normal activities, can be one of the causes of lens blur. In addition to its predominantly cortical distribution within lens fiber cells, high susceptibility of GR to post-translational modifications [65] could also be of critical importance for the early dysfunction of GPx and GST under oxidative stress.

Based on the fact that the activity of GPx and GST is focused on degradation of lipid peroxidation products, we tested the relationship between the activity of the antioxidant defense enzyme and products of LPO in cataract lenses.

Figure 4. Lens conjugated diens and glutathione peroxidase relationship in cataract.

Reduced specific GPx activity (U/g protein) in the lenses shows significant correlation with the increased concentration of conjugated diens ($r= -0.476$, $p<0.01$) (figure 4), lipid-soluble fluorescent products ($r= -0.429$, $p<0.01$) (figure 5), water-soluble fluorescent products ($r= -0.367$, $p<0.05$) (figure 6) and MDA ($r= -0.328$, $p<0.05$) (figure 7).

Figure 5. Lens lipid-soluble fluorescent products and GPx relationship in cataract.

Figure 6. Lens water-soluble fluorescent products and GPx relationship in cataract.

Figure 7. Lens malondialdehyde and glutathione peroxidase relationship in cataract.

Also, the reduced GST activity (U/g protein) shows the significant correlation with the increased concentration of total hydroperoxides ($r = -0.313$, $p < 0.05$), conjugated diens ($r = -0.465$, $p < 0.01$) (figure 8), lipid-soluble fluorescent products ($r = -0.398$, $p = 0.01$) (figure 9), water-soluble fluorescent products ($r = -0.347$, $p < 0.05$) (figure 10) and MDA ($r = -0.345$, $p < 0.05$) (figure 11) in homogenates of the lenses with the incipient cataract.

Figure 8. Lens conjugated diens and glutathione S-transferase relationship in cataract.

Probably, the consumption of GSH and other antioxidants in reactions of degradation of lipid peroxidation products, affects the decrease of GPx and GST enzymes activity, and as the process of cataractogenesis progresses towards mature cataract, all lens structures become affected by the changes, which probably leads to the change of enzyme molecules themselves.

Figure 9. Lens lipid-soluble fluorescent products and GST relationship in cataract.

Figure 10. Lens water-soluble fluorescent products and GST relationship in cataract.

Figure 11. Lens malondialdehyde and glutathione S-transferase in cataract.

We measured higher activity of SOD (4.13 ± 2.14 kU/g protein) in lenses with incipient cataract in comparison to mature cataract (2.14 ± 0.91 kU/g protein) ($p < 0.001$) (table 2). Such changes are probably a reflection of oxidative processes, increased by formation of toxic products of lipid peroxidation in relation to the weakened antioxidant capacity of cataract lenses. Also, other researchers have measured decreased SOD activity in the lenses of patients with senile and diabetic cataract [66]. The decreased activity of superoxide dismutase in the lenses with age-related cataract may be due to denaturation of enzyme molecules, and/or slow enzyme synthesis. Reduced SOD activity and consequently, the increase of concentration of H_2O_2 lead to the formation of hydroxyl radical from Fenton's type reaction. Subsequently, hydroxyl radical induces the formation of superoxide radical, which may initiate the process of lipid peroxidation in the lens.

10. Conclusion

Based on the results of measuring products of lipid peroxidation and antioxidant enzyme activity in corticonuclear lens blocks with age-related cataract, we can say that the lens structure changes induced by lipid peroxidation may with other risk factors present, affect the beginning or the development of cataract. The changes in redox system are particularly pronounced in cortical nuclear cataract, but are reflected in all parts of the lens, regardless of the initial localization of the lens blur. The lowest level of oxidative stress was detected in posterior subcapsular cataract, so it is possible that it has less importance in the

development of PS cataract. The development of cataract can probably be prevented/slowed down by preventing the accumulation of products of lipid peroxidation in the lens and maintaining adequate level of GSH and function of GSH-dependent antioxidant enzymes.

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