

## Revival of the Lens Transparency with N-Acetylcarnosine

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**Abstract:** The risk, cost and social requirement factors drive the investigation of pharmaceutical approaches to the management of cataracts. The role of free-radical-induced lipid oxidation (LPO) in the development of cataracts has been identified. Initial stages of cataract are characterized by the accumulation of primary (diene conjugates, cetodienes) LPO products, while in later stages there is a prevalence of LPO fluorescent end products. Reliable increase in oxiproduces of fatty acyl content of lenticular lipids was shown by a direct gas chromatography technique producing fatty acid fluorine-substituted derivatives. The lens opacity degree correlates with the level of the LPO fluorescent end product accumulation in its tissue, accompanied by SH group oxidation of lens proteins due to a decrease of reduced glutathione concentration in the lens. The injection of LPO products into the vitreous was shown to induce cataract. Peroxide damage of the lens fiber membranes may be the initial cause of cataract formation. The authors developed N-Acetylcarnosine ophthalmic drug with lubricant carboxymethylcellulose in eye drops (NAC, Can-C™, Nu-Eyes™) suitable for the non-surgical prevention and treatment of age-related cataracts. The NAC ophthalmic drug protects the crystalline lens from oxidative stress-induced damages and in a recent clinical trial it was shown to produce an effective, safe and long-term improvement in sight. When administered topically to the eye, NAC drug functions as a time-release prodrug form of L-carnosine resistant to hydrolysis with carnosinase and significantly increases the intraocular uptake of L-carnosine in the aqueous humor. The mechanisms of prevention and reversal of cataracts with NAC ophthalmic drug are considered which include prevention by the intraocular released carnosine of free-radical-induced inactivation of proprietary lens antioxidant enzymes (superoxide dismutase), prevention of carbohydrate and metal-catalysed autooxidation of ascorbic acid –induced cross-linking glycation reactions to the lens proteins, universal antioxidant and scavenging activity towards lipid hydroperoxides, aldehydes and oxygen radicals, activation with L-carnosine ingredient of proteasome activity in the lens. In this study the clinical effects of a topical solution of NAC ophthalmic drug on lens opacities were examined in patients with cataracts and canines with age-related cataracts. The positive effect on lens clarity and clarifying modification of opacification zones is demonstrated. The data suggest a potential and show the efficacy of developed NAC ophthalmic drug for a positive effect of treatment (both reversal and prevention) of age-related cataracts. Innovative Vision Products, Inc. is a holder of the worldwide patent (including PCT International Publication Number WO 2004/028536 A1) for the application of N-acetylcarnosine for the treatment of ophthalmic disorders including cataracts.

**Key Words:** Age-related cataracts, crystalline lens, epidemiology, lipid peroxidation, active oxygen species, universal antioxidant, L-carnosine, ophthalmic prodrug, N-acetylcarnosine, eye drops, lubricant, carboxymethylcellulose, medicinal cure for cataracts in human and canines, mechanisms of action.

### CATARACTS AND BLINDNESS

Cataract, the opacification of the eye lens, is the leading cause of blindness worldwide [1], accounting for approximately 42% of all blindness [1]. More than 17 million people are blind because of cataract and 28,000 new cases are reported daily worldwide [2]. In the United States, over 1.3 million cataract operations are performed annually at a cost of \$3.5 billion [1]. Cataract is the leading cause of functional impairment among the elderly in the United States and is the most commonly performed surgical procedure in people over 65 years of age in the United States [3]. Forty-three percent of all visits to ophthalmologists by Medicare patients are associated with cataract [1]. Approximately 25% of the population over 65 and about 50% over 80 have serious loss of vision because of cataract. Since the population over 55 is most susceptible to lens opacification and is expected to increase 4 fold worldwide and significantly in the United

States [4], cataract is a major disease both in terms of numbers of people involved and in economic impact.

Of the 17 million cases of blindness in the world, half are in the developing countries of Africa and Asia. Published data estimate that 1.2% of the entire population of Africa is blind, with cataract causing 36% of this blindness [5]. In developing countries, there is simply not a sufficient number of surgeons to perform cataract operations. Therefore, a significant number of people developing cataract become permanently blind. The disease is becoming more frequent with increase in the life span of this population. While the majority of cases occur in older age groups, young subjects are not exempt and, in them, the rate of maturation is faster. Cataract is far more prevalent in India and developing countries than in Western countries [6], and it was reasonable to hypothesize that there could be a nutritional cause of cataract, at least to explain this difference. Early studies showed that those people who ate poorer qualities of food were indeed more at risk of cataract [7,8], an index of poverty consistently identified as a risk factor of cataract in many different populations [6].

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## COMPLICATIONS OF CATARACT SURGERY

While cataract surgery is generally recognized as being one of the safest operations, there is a significant complication rate. At least 5 to 10 million new visually disabling cataracts occur yearly, with modern surgical techniques resulting in 100,000 to 200,000 irreversibly blind eyes. In the United States, 300,000 to 400,000 new visually disabling cataracts occur annually, with complications of modern surgical techniques resulting in at least 7000 irreversibly blind eyes. From 30 to 50% of all patients in the United States having cataract extraction develop opacification of the posterior lens capsule within two years and require laser treatment with its own significant risk of complications [9]. Since the number of cataract operations is so large, even a small percentage of complications represents a significant number of people. Of the patients having cataract surgery, 0.8% develop retinal detachments [10], from 0.6% to 1.3% were rehospitalized for corneal edema or required corneal transplantation [11] and about 0.1% presented with endophthalmitis [10]. Thus, aside from secondary cataract, about 2% of 1.3 million people, or 26,000 individuals in the United States annually develop serious complications as a result of cataract surgery.

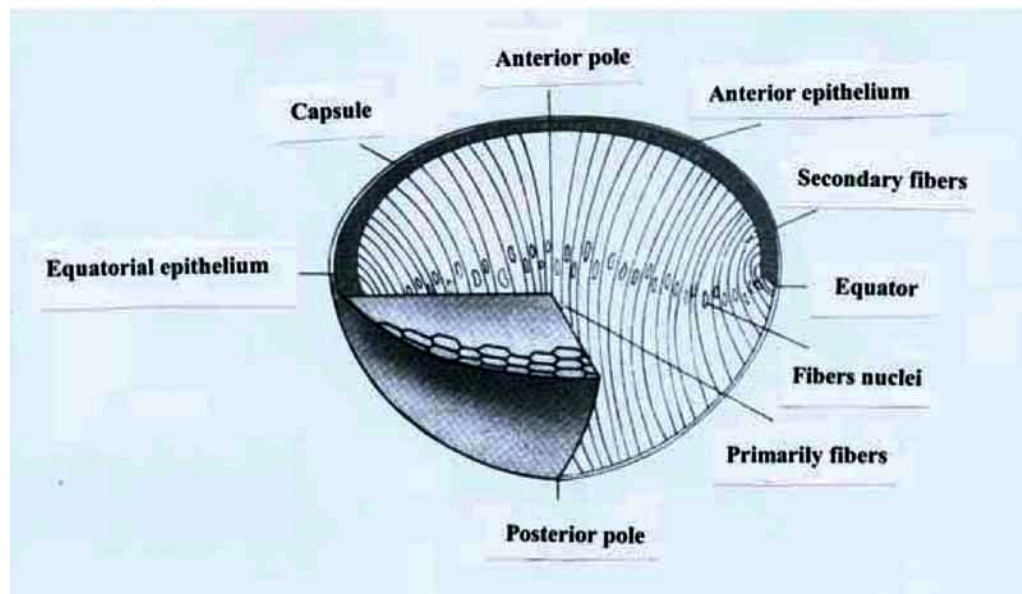
Most morbidity associated with senile cataracts occurs postoperatively. While the risk of death as a result of cataract extraction is almost negligible, studies have shown an increased risk of mortality in patients who underwent surgery. In a comparison of 167 patients aged 50 years or more who underwent cataract extraction at the New England Medical Center over a period of 1 year with 824 patients who elected one of six other surgical procedures, it was found that the former had almost twice the mortality of the latter. Further analysis showed no significant correlation between diabetes and increased mortality [12]. Age-related cataract was reported to be associated with increased risk of death. After adjustment for age, sex, and other mortality risk factors,

mixed cataracts with a nuclear/posterior subcapsular component were significantly associated with higher risk of death by Cox proportional hazards regression analyses. These findings are compatible with the hypothesis that mixed types of cataract with a nuclear/posterior subcapsular component are indicators of accelerated aging.

The large and growing number of people blind with cataract and the significant complication rate should be sufficient reason to consider the search for a medicinal cure of cataracts. The considerable discomfort experienced by patients as their vision diminishes and the complete loss of accommodation resulting from the removal of the lens should also be recognized. Besides possible complications, an artificial lens just does not have the overall optical qualities of a normal lens. Identification of the risk factor(s) and unravelling the mechanism(s) through which the human eye loses its transparency and turns opaque would help to develop an effective preventative approach to cataract blindness.

## MORPHOLOGICAL AND MOLECULAR ASPECTS OF THE LENS TRANSPARENCY

The normal human eye lens is a transparent, pale yellow resilient biconvex body enclosed in an elastic capsule. It is suspended behind the pupil of the eye by strands which fuse with the capsule. The lens is a relatively simple tissue that has but a single layer of epithelial cells on the anterior side of the tissue (Fig. 1) [13,14]. The lens grows throughout life from the epithelium that lines the inner surface of its transparent capsule. The disciform eye lens continues to grow throughout life. The fresh fibers, developing by elongation from cubic anterior epithelial cells, are continually laid down in the equatorial zone. The older cells in the anterior and posterior cortex and supra-nuclear cortex gradually lose their nuclei and become more and more compressed in the center



**Fig. (1).** Scheme of the human lens structure. In the present article Figs. 1, 2, 3 are presented as the original drawings. Around the minute hand at 30 minutes are shown: posterior lens pole, equatorial epithelium, capsule, anterior pole, anterior epithelium, secondary fibers, equator, lens fiber nuclei, primarily lens fibers.

or nucleus of the lens. There is a growing awareness that the interaction of tissues of the eye influences the overall viability of the lens organ (Fig. 2). Both development and cataract disease are affected by the environment of the respective tissues of the eye. The water content in the nucleus is the lowest and highest in the cortex of the lens [15]. The whole lens consists of about 65% water and 35% proteins. These structural proteins are called: "crystallins", and form most of the dry weight of the lens [6]. Transparency of the normal eye lens depends on the short range order that exists in the supramolecular organization which results in interference effects [16-18]. The conformational change during cataractogenesis leads to the formation of aggregates which scatter light producing opacification [6, 19]. For that reason, an aggregation process was proposed as the main contributor to cataractogenesis [20]. Traditionally, the lens is considered as a "sac filled with proteins". That is why until now the majority of investigators have been searching for the cause of cataract high molecular weight protein aggregate formation, in the crystalline physico-chemical properties alteration, in the reduction of the protein solubility, in the change of their amino acid content [21-23]. Biochemical characteristics of cataract manifestation are the following: (1) formation of large high-molecular weight aggregates of low solubility in the lens tissue; (2) appearance of "blue" fluorescence of non-tryptophan nature; and (3) disintegration of the lens fiber plasma membranes [23,24]. At the same time, it is well known that the protein aggregate formation in the lens is accompanied by an intrusion of lenticular fiber membrane fragments into the cytoplasmic fraction (Fig. 3) [24,25]. The content of the above-mentioned aggregates was revealed to contain phospholipids which can be found in the lens cytoplasmic protein fraction using phosphorus registration [26]. However, the reports of

membranes and phospholipid components found within cytoplasmic protein isolates does not necessarily mean that membrane fragmentation occurs in the native lens. Morphological and biophysical techniques have shown that membrane derangement occurs in human cataractous lenses as the primary light scattering centers which cause the observed lens opacity (Fig. 3). Spector in the 1980's tried to illustrate his hypothesis that protein aggregation begins at the membrane surface through S-S bonds resulting in membrane disintegration [23]. Phospholipid molecules modified with oxygen, accumulating in the lipid bilayer, change its geometry and impair lipid-lipid and protein-lipid interactions in lenticular fiber membranes. Electron microscopy data of human lenses at various stages of age-related cataract document that these disruptions were globules, vacuoles, multilamellar membranes, and clusters of highly undulating membranes. Other potential scattering centers found throughout the mature cataract nucleus included variations in staining density between adjacent cells, enlarged extracellular spaces between undulating membrane pairs, and protein-like deposits in the extracellular space. Lipid peroxidation (LPO) is a pathogenetic and causative factor of cataract. Since in the transparent lens most lipids localize in the membranes, we have suggested that processes inducing the damage of the lenticular fiber plasma membrane lipid bilayer participate in the aggregation of crystallins.

#### LIPID PEROXIDATION AND CATARACTS

Among endogenous processes which can cause injury to membranous structures of cells and tissues one of the most important is lipid peroxidation (LPO). Reactive oxygen species (ROS) is the term usually used to indicate components that can cause oxidative stress. It includes oxygen radicals

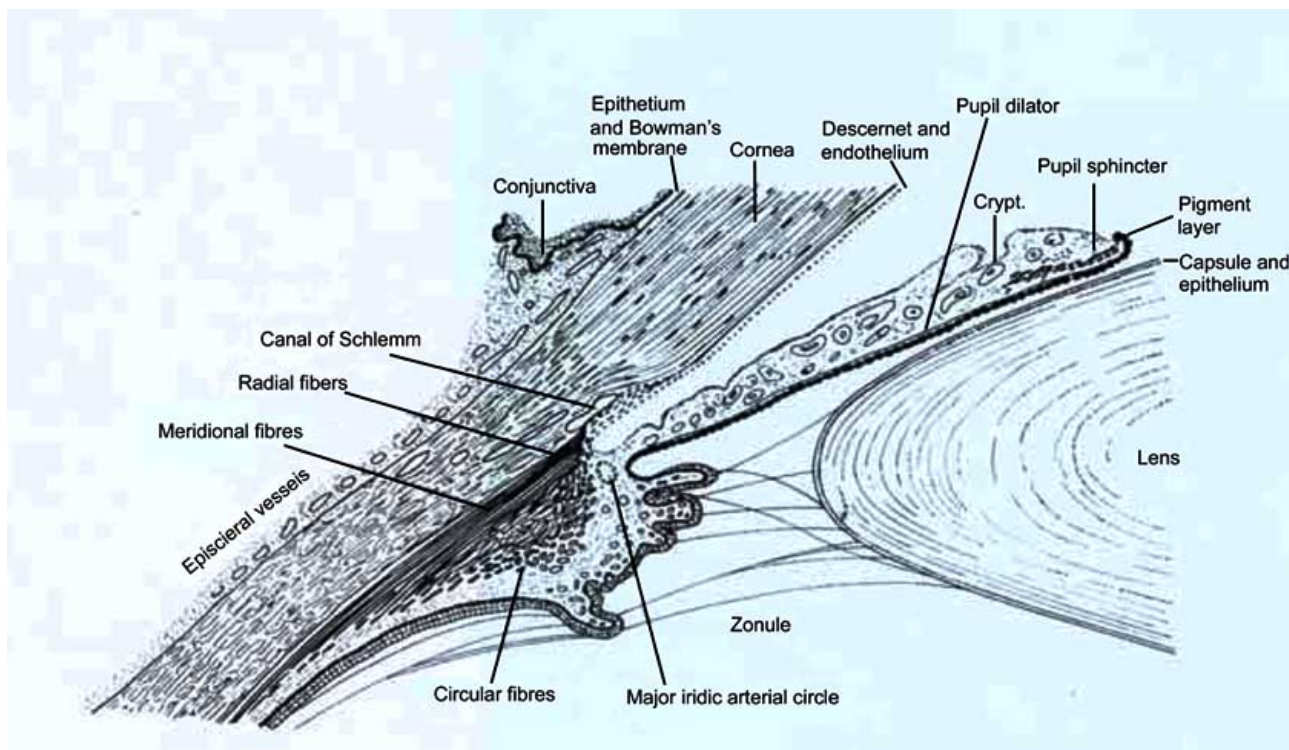
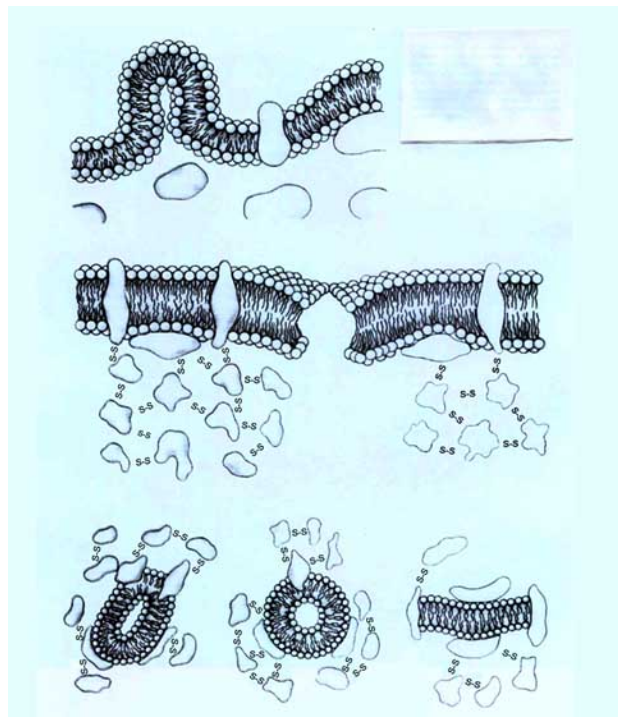


Fig. (2). Scheme of the lens in the anterior chamber of the eye.



**Fig. (3).** Scheme of the lipid peroxidative damage to the lens fiber cell plasma membranes.

such as superoxide,  $O_2^{\cdot-}$ , and hydroxyl radical,  $OH^{\cdot}$ , as well as oxidants like hydrogen peroxide,  $H_2O_2$ , and singlet oxygen,  $O^1_2$  ( $^1\Delta g$ ) [27]. During aging and, in particular, during the development of senile cataract, activity of enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic (ascorbate, cysteine, glutathione) antioxidant systems in the lens and aqueous humor is reduced [28-30].

Accumulation of LPO products in the lens may be facilitated by the presence of compounds in the lens which are photosensitizers of free-radical oxidation reactions of the 3-hydroxykynurenine or N-formylkynurenine type, which absorb light in the near UV-region of the spectrum (360-400 nm) [31]. Two major types of processes can occur with proteins. The first of these involves direct photo-oxidation arising from the absorption of UV radiation by the protein, or bound chromophore groups, thereby generating excited states (singlet or triplets) or radicals *via* photo-ionization. The second major process involves indirect oxidation of the protein *via* the formation and subsequent reactions of singlet oxygen generated by the transfer of energy to ground state (triplet) molecular oxygen by either protein-bound, or other, chromophores [31]. The photo-oxidation products of tryptophan under the influence of light can generate active forms of oxygen and products of its successive single-electron reduction (singlet oxygen, superoxide anion-radicals, hydrogen peroxide, hydroxyl radicals), which can

be found in the lens tissue and also in the aqueous humor [32]. Several varieties of cataract have been described in the literature, whose mechanisms of development have been linked with the generation of active forms of oxygen. They include psoralene cataract, cataract induced by the action of hyperbaric oxygenation, and cataract arising in animals fed with the catalase inhibitor 3-amino-1H,1,2,4-triazole [33-35]. Free radical enhancers, diquat injected intravitreally as a single dose (300 nmole in 30  $\mu$ l of isotonic saline) in the right eyes of 5-wk-old Dutch belted rabbits, induced early cataract after 24-72 h [36]. The lens of the contralateral control eyes injected with isotonic saline had no change.

On the basis of these facts it has been postulated that LPO may play a role in the etiology and pathogenesis of cataract. The direct proof of LPO activation has been obtained in cataracts [37-40]. The results of determination of the lipid peroxidation different molecular products revealed in the lipid extracts from the lens, are shown in Fig. 4. In UV absorption spectra of lipid extracts from lenses with a cataract, there are two additional maxima at 230 and 274 nm (Fig. 4). The first of these corresponds to absorption of diene conjugated structures. The maximum at 274 nm corresponds to triene conjugates- secondary molecular lipid peroxidation products. The results of the determination of diene conjugates in lipid extracts from the lenses at different stages of cataract maturation are presented in Fig. 4, Table 1. It is evident that the content of the hydroperoxides having conjugated double bonds and determined by a characteristic maximum in UV spectrum at 230 nm, increases at the initial stages of the opacification up to almost mature stage of cataract (55-64% opacification degree) assessed by quantitative morphometric analysis technique (Fig. 5). However, at further stages of mature and hypermature cataract the lipid peroxidation primary molecular product level drops a little. At the same time, determination of the content of fluorescent end products of the lipid peroxidation- Schiff bases determined by lipid extract fluorescence intensity at 430 nm (fluorescence excitation, 365 nm) revealed an increase monotonously by cataract development (Fig. 6, Table 1). The fluorescent end lipid peroxidation product concentration in the lens correlates strongly with the opacity degree ( $r=+0.956$ ,  $P<0.01$ ) (Fig. 7) [40].

An important regularity was revealed in our studies: accumulation of the lipid peroxidation products depends on the cataract development stage, but does not depend on its kind (age-related, complicated, diabetic), suggesting a universal role of lipid peroxidation in the lens opacification process. Determination of total thiols and the content of lipid peroxidation products for the same lenses show that with the development of cataract, the level of the total thiols of the lens fibres rapidly falls with intensification of the free radical oxidation reactions of lipids (Fig. 7) [40]. The new research phenomenon revealed by the authors is that the total thiols of the lens fibres are rapidly inactivated with increase in the intensity of the free radical induced reactions of the lipids through drop in the level of glutathione. These processes promote the formation of high molecular weight protein aggregates in the lens and progression of the cataract [40]. The presence of high correlations between the degree of































**Table 6. Cumulative Changes in Clinical Characteristics of Cataracts in Patients at 6 and 24 Months in Relation to Baseline Study**

Clinical group	ADVANCE No. of Eyes (%)								
	Visual acuity			Glare test			Image analysis		
	Improvement range (%)	Stationary	Deterioration range (%)	Improvement range (%)	Stationary	Deterioration range (%)	Improvement range (%)	Stationary	Deterioration range (%)
Group I <u>Control</u>	2 (5.7) (14-60)	21 (60.0)	12 (34.3) (17-40)	-	7(43.8)	9(56.3) (11-170)	-	31(88.6)	4 (11.4) (11-39)
Overall Mean ±SEM (no. of cases)	0.93 ± 0.03 (n=35)			1.37± 0.08 (n=27)			1.04± 0.01 (n=70)		
Group II (Treated with instillations of NAC, 1% sol)	37 (90) (7-100)	4 (9.8)	-	16 (88.9) (27-100)	1 (5.6)	1 (5.6) (100)	17 (41.5) (12-50)	24 (58.5)	-
Overall Mean ±SEM (no. of cases)	1.43± 0.04 * (n=41) (p<0.001)			0.50± 0.05 * (n=33) (p<0.001)			0.86± 0.01 * (n=82) (p<0.001)		

a. Trial 1/ Trial 2 (follow-up period over 6 months).

acetylcarnosine in eye drops are displayed correspondingly, on the figures **18a-c** using the retro-illumination photography analysis technique. The most striking results have been obtained using a 1% NAC instillation in canines with age-related cataracts. We have determined the efficacy of cataract treatment, and we have revealed a new phenomenon of melting snow upon the instillation of NAC, within only several weeks of chronic treatment (see photos, Figures **18a,b** and **19a,b**). The cortical appearance of cataract reversal starts from the periphery and then the lens becomes more transparent. This is then accompanied by the improved

visual behavior of the animal. During treatment of canines, there is indication of improvement (melting of snow) but the return to a clear lens or sufficient retreat of the opacity to return normal functional vision strongly depends on the initial status of the lens opacities. Those animals that have immature and ripe cataracts show a significant visual response, however in total cataracts, the data suggest improvements only in the cortical layers. These results are evidence that N-acetyl-carnosine manufactured by Innovative Vision Products, Inc. is one of the most important natural-like antioxidants for cataract prevention and cure.

**Table 7. Mean ± SD of Changes (IMPROVEMENT) in Visual Functions. The Measure of Visual Acuity Readings After 4 Months of Treatment was Divided by the Clinical Baseline Measure of Visual Acuity for Each Eye Individually to Get Ratios, and then the Average of Those Ratios Through Each Clinical Group of Eyes was Calculated. Similarly with glare, the Calculating of the Ratio of Glare Sensitivity at Red and Green Target After 4 Months of Treatment to the Baseline Reading of Glare Sensitivity for Each Eye was Undertaken and then the Ratios were Averaged Through the Whole Groups of Eyes**

Treatment group	Visual acuity improvement	Glare test improvement
4-month follow-up of older drivers with cataract		
Control group	0.92 ± 0.04 (n=30)	1.42 ± 0.07 (n=30)
NAC-treated group	1.61 ± 0.03* + (n=35)	0.42 ± 0.04* (n=35)
4-month follow-up of older adult no-cataract drivers		
Control group	0.97 ± 0.03 (n=34)	1.30 ± 0.06 (n=34)
NAC-treated group	1.21 ± 0.03* (n=38)	0.37 ± 0.06* (n=38)

NAC= N-acetylcarnosine (Can-C™) \* p< 0.001 compared to Control group who received placebo eye drops or did not receive eye drops ; + p< 0.001 : an improvement of visual acuity is statistically significantly better in the group of older drivers with cataract than an improvement of visual acuity in the group of older adult non-cataract drivers.



ascorbate) and iron-sequestering proteins (apoferritin and apolactoferrin). A secondary protection involves enzymatic removal of lipid-derived hydroperoxide intermediates typically catalysed by glutathione-requiring enzymes (Se-dependent GSH peroxidases and certain Se-independent enzymes, such as GSH-S transferase B). Carnosine has been proposed to act as anti-oxidant *in vivo* and its activity in the crystalline lens can be related to the prevention of the free-radical-induced inactivation of activity of the proprietary antioxidant enzymes in the crystalline lens, such as SOD. The protective effects of carnosine and related compounds against the oxidative damage of human Cu,Zn-superoxide dismutase (SOD) by peroxy radicals generated from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were established [60]. A unique property of carnosine is its water solubility combined with its ability to trap LPO products. Carnosine exhibits an ability to inhibit LPO catalysts besides inhibiting free metals, scavenging OH· and lipid peroxy (RO<sub>2</sub>·) radicals or donating hydrogen ions. In addition to inhibiting the generation of lipid peroxy radicals, carnosine catabolizes lipid hydroperoxides to their alcohols both in aqueous medium and in a phospholipid system [45]. This differs from the native peroxidase which uses GSH as a cofactor and prior hydrolysis by Ca<sup>2+</sup>/ phospholipase A<sub>2</sub> to eliminate the phospholipid-derived hydroperoxides [45]. A possibility exists from our studies that carnosine is reacting directly with MDA and other aldehydes/ketones. Indeed carnosine has been shown to protect against MDA-induced crosslinking and toxicity, and a hydroxynonenal-carnosine adduct has recently been characterized, providing further evidence for carnosine's potential as an aldehyde scavenger [61]. The presented results can be explained in part by the adduction of the various LPO products directly by carnosine following de-acetylation of N-acetylcarnosine.

The experimental and clinical results reported here provide a substantial basis for the application of N-acetylcarnosine as an ophthalmic prodrug of L-carnosine in the treatment and prophylaxis of age-related cataracts and other ophthalmic disorders which have a component of oxidative stress in their genesis. The ability of L-carnosine to inhibit LPO reactions as well as to diminish the content of LPO products makes N-acetylcarnosine applied with a lubricant carboxymethylcellulose a prominent tool in the therapy especially, of the posterior subcapsular and cortical cataracts, whose mechanism can be related with the toxic effects of LPO products [42]. In the cataractous lens, cross-linking of proteins by any means increases their effective molecular weight and produces light scattering and consequent lenticular opacity. The production of such high molecular weight protein complexes by disulphide bridges and covalent links with dialdehydes has been implicated in the formation of senile and other cataracts in humans (see section "Lipid Peroxidation and Cataract"). Both types of cross-linking may be caused by depletion of the lens' reduced glutathione and accumulation of LPO products in the lens tissue. The results of our studies strongly suggest that L-carnosine released from its ophthalmic prodrug N-acetylcarnosine during its application to the eyes with cataracts is able to prevent the loss of reduced glutathione and to remove the secondary LPO products in biological

systems. This, in turn, may lead to dissociation of the intermolecular protein cross-links due to glutathione-protein thiol-disulphide exchange mechanism and utilization of lipid peroxides and dialdehydes derived from LPO process, anchoring protein-lipid complexes in the lens [43]. There is a rising evidence that carnosine prevents oxidation and glycation, both of which contribute to the crosslinking of proteins [62]. The results suggest that histidine is the representative structure of L-carnosine for an anti-cross-linking agent, containing the necessary functional groups for optimal protection against crosslinking agents. It has been proposed that the imidazolium group of histidine of carnosine may stabilize adducts formed at the primary amino group [62]. Cellular aging is often associated with an increase in protein carbonyl groups arising from oxidation- and glycation-related phenomena and suppressed proteasome activity. These "aged" polypeptides may either be degraded by 20S proteasomes or cross-link to form structures intractable to proteolysis and inhibitory to proteasome activity. For example, effects of L-carnosine on proteasome activity in the lens might explain the apparent decline in cataract, as proteasome activity is known to decline during lenticular ageing [63, 64]. Esterase activity in the lens is decreased in senile cataract and diabetes. Glycation and a steroid inactivate esterase. Harding JJ *et al.* [65] tested the inactivation of esterase with fructose, fructose 6-phosphate (F6P) and ribose as model glycation reactions and prednisolone-21-hemisuccinate (P-21-H) as a model steroid and investigated the ability of carnosine to protect esterase against inactivation. The activity of esterase was measured by a spectrophotometric assay using p-nitrophenyl acetate as the substrate. The modified esterase was examined electrophoretically. The esterase was progressively inactivated by F6P, fructose, ribose and P-21-H. P-21-H was more effective than the sugars. Carnosine significantly inhibited the inactivation of esterase induced by all four compounds. Carnosine decreased the extent of the cross-linking. These results provide further evidence for carnosine's role as an anti-glycation compound. It is also proposed that carnosine may be an anti-steroid agent.

Carnosine when present at surprisingly high levels (about 20 mM or over) can delay senescence of cells and reverse the senescent phenotype, restoring a more juvenile appearance. As better antioxidants/ free-radical scavengers than carnosine do not demonstrate these antisenescent effects [66], additional properties of carnosine must contribute to its antisenescent activity. Having shown that carnosine can react with protein carbonyls, thereby generating "carnosinylated" polypeptides using model systems [66], it might be generally proposed that similar adducts can be generated in senescent cataractous lens fiber cells exposed to carnosine during the therapeutic treatments with 1% N-acetylcarnosine lubricant eye drops *in vivo*. However, polypeptide (crystalline)-carnosine adducts are yet to be detected with lens individual crystalline fractions or in the lens protein extracts that might occur relatively rich in carnosine, and carnosine's reaction with carbonyl functions generated during amino acid deamidation is needed to be revealed with the lens crystallins to support a general proof of this concept during N-acetylcarnosine ophthalmic treatments of age-related cataracts.

Protein glycation, which promotes aggregation, involves the unwanted reaction of carbohydrate oxidation products with proteins. Glycation of lens alpha-crystallin occurs *in vivo* and may contribute to cataractogenesis. Anti-glycation compounds such as carnosine may be preventive, but interestingly carnosine reverses lens opacity in human trials. The mechanism for this observation may involve carnosine's ability to disaggregate glycated protein. Seidler *et al.* [67] recently investigated this hypothesis using glycated alpha-crystallin as the *in vitro* model. Methylglyoxal-induced glycation of alpha-crystallin caused aggregation as evidenced by increased 90 degrees light scattering. After addition of carnosine, light scattering returned to baseline levels suggesting that the size of the glycation-induced aggregates decreased. Additionally, carnosine decreased tryptophan fluorescence polarization of glycated alpha-crystallin, suggesting that carnosine increased peptide chain mobility, which may contribute to the controlled unfolding of glycated protein. These data support the hypothesis that carnosine disaggregates glycated alpha-crystallin. However, the used concentrations of L-carnosine demonstrating as efficacious to disaggregate glycated alpha-crystallin were excessively high (50 mM) in the provided experiments. To distinguish between the carbonyl trapping and antioxidant activity of the advanced glycation end product (AGE) inhibitors, Baynes *et al.* [68] measured the chelating activity of carnosine by determining the concentration required for 50% inhibition of the rate of copper-catalyzed autoxidation of ascorbic acid. In their studies L-carnosine exhibited the anti-glycating activity with the estimated IC<sub>50</sub> 4 μM [68] for inhibition of copper-catalyzed oxidation of ascorbic acid that proposes this natural dipeptide as a potent inhibitor of glycation reactions in the lens proteins mediated by metal-catalyzed oxidation of ascorbate present in the aqueous humor.

Further attention should be directed mainly to the application of N-acetylcarnosine when combined with a lubricant carboxymethylcellulose in the ophthalmic formulations. This therapeutic modality gives the most efficacious uptake of L-carnosine in the aqueous humor protecting carnosine from the short-term hydrolysis and this dipeptide is known to be an endogenous component in the mammalian and lens tissues [43]. LPO reactions are widely involved in the genesis of ophthalmic disorders, such as cataract, glaucoma, inflammatory, corneal, retinal disorders, systemic disorders having a component of oxidative stress in their genesis [44]. The present study presents an evidence suggesting that the developed by Innovative Vision Products, Inc. N-acetylcarnosine/ lubricant carboxymethylcellulose ophthalmic formulation reverses lens opacity in humans and canines.

## CONCLUSION

Senile cataract is a very common disorder. The high costs of the surgical procedure, the limited numbers of trained surgeons, the potential risk of surgical complications, and the simple fact that an artificial lens does not have the optical qualities of a natural lens, makes a non-surgical procedure such as N-acetylcarnosine eye-drops a very attractive proposition. N-acetylcarnosine eye drops (Can-C<sup>TM</sup>Nu-Eyes<sup>TM</sup>) approved for use in USA as a lubricant formulation appear to be a safe, effective means to prevent cataracts, and to

possibly even treat cataracts that are forming. In years to come, this type of therapeutical approach may give another option versus cataract surgery and benefit the course of cataract reversing and prevention of this age-related eye-disease. The importance of lipid oxidation in the formation of age-related cataracts is emphasized in this review article. The primary goal is to stress the worldwide cataract problem and to offer a non-surgical treatment employing an ingenious formulation of N-acetylcarnosine releasing an antioxidant L-carnosine in the aqueous humor. This study has contributed to the demonstration that lipid peroxidation is a key factor in human age-related cataracts.

## ACKNOWLEDGEMENTS

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## APPENDIX

### Experimental Details, Materials and Methods

The test material consisted of opaque human lenses at different cataract stages obtained during operation by intracapsular cryoextraction. Transparent human lenses were removed from the donor eyes provided by a bank. Before the operation, all the lenses were examined on a slit lamp and were divided into groups according to clinical characteristics of opacity. The average age of the cataract patients and donors was 65 ± 9 years.

### Morphometric Evaluation of the Lens Opacities

To evaluate objectively the lens opacity by means of biomicroscopy and further photoregistration, an image of the lens was obtained on "Leitz" TV-analyser. The values of optical density in different lens zones were determined. The image was subsequently divided into zones with pre-set values of optical density. Their areas were measured, the redistribution of the zones over the lens surface was determined and the part of every zone in the whole opacity area of the lens was established [69].

### Lipid Extraction from the Lens

Immediately after the lens material had been obtained, lipids were extracted from the lens [37]. Phospholipid content was assessed by the results of organic phosphorus evaluation [37]. Total lipid amount in the extract was determined gravimetrically, as well as by characteristic absorption in 206-210 nm area of the lipid sample after dissolution in 4 ml of methanol/heptane mixture (5:1 by vol.).

### Determination of Lipid Peroxidation Products

Accumulation of the lipid peroxidation (LPO) primary products was estimated spectrophotometrically from characteristic absorbents of diene conjugates in the UV-region at 232 nm characterizing the level of hydroperoxides of polyunsaturated fatty acids, as well as by LPO secondary products absorbance at 274 nm, corresponding to the

concentration of conjugated trienes and cetodienes on HITACHI-557 spectrophotometer (Japan). The content of end fluorescent LPO products was determined from the lipid extract fluorescence intensity at 365 nm excitation and 420-440 nm emission wavelengths [37], measured on a HITACHI-MPF-4 spectrofluorometer (Japan).

### **Gas Chromatography of Halogen-Substituted Derivatives of the Fatty Acids**

Content of the polyunsaturated fatty acids in the lens is rather moderate, hence direct registration of their decrease in the course of LPO is quite difficult [37]. However, evidently, to register directly an increase of oxiproducs in unsaturated fatty acids is of rather greater importance than to reveal a decrease in the acid content. In the applied gas-chromatography method we used not methyl esters of fatty acids but their halogen-substituted derivatives. By fluorine atom introduction into fatty acid molecule we succeeded in selective labeling of its functional groups. Using this property, it is possible to determine the change of the number of oxigroups which gain in content in fatty acids in the LPO course both under *in vitro* and *in vivo* conditions. Electron-capture detector used in gas-chromatography of fluorine-substituted compounds is found to be more sensible than flame-ionization one: minimum detectable sample flow in substances with high affinity of the electron, such as fluorine-substituted compounds, for electron-capture detector, is of  $10^{-13}$  g/s – that is why this method is the optimum one to measure even lower levels of oxidervative fatty acids in tissue lipid extracts. For selective determination in the lens lipid fraction of the substances containing oxigroups, fluorine-substituted derivatives of the fatty acids were obtained. For this purpose, after evaporation in the solvent nitrogen current, 100  $\mu$ l of hexafluorine/isopropanol-benzol mixture in proportion (1:4) and 40  $\mu$ l of penta-fluorine-propine acid anhydride were added to every sample of the lens lipids, the mixture was then sealed off and kept at 60 °C for 30 min. After this, the ampoule content was evaporated in nitrogen current, the lipid residue was diluted in 150  $\mu$ l of isoocane and introduced into “Tracor-560” gas chromatograph (USA) equipped with “Tracor-770” autosampler (USA), “FSOT-I” capillary column (USA) of 50 m length with 0.25 mm inside diameter and also with an electron-capture detector. Hydrogen was used as carrier-gas, with 1:30 flow split. The following temperature conditions were maintained: initial temperature of 140 °C (2 min), final temperature of 280 °C (15 min), temperature gradient of 3°C/min. Gas-chromatographic determination of halogen-substituted fatty acid derivatives was carried out by utilizing the method of inner standard by retention time comparison with the standards. “Sigma” fatty acid standards were used in the work.

### **Determination of Reduced Thiols**

The content of reduced thiols in the lenses was determined from the reaction with 5,5'-dithio-bis-2-nitrobenzoic acid [40, 70].

### **Cataract Induction with Phospholipid Peroxidation Products**

Cataract modeling with lipid peroxides was carried out in rabbits of chinchilla race of 4 months of age. The experi-

ments were conducted in accordance with the ARVO Resolution on the Use of Animals in Research. After inducing anaesthesia under aseptic conditions a microsection of the conjunctiva in 4 mm of the limbus was performed. Exposed sclera was pierced with a stilet-needle of 0.17 mm diameter, and, by means of microsyringe, 0.05 ml of liposomal suspension containing 0.4 mg of phospholipids (dilinoleoyllecithin or dipalmytoyllecithin (Serva)) was injected into the posterior vitreous of rabbit eyes under indirect binocular ophthalmoscope control. In the case of highly oxidized phospholipids, malonyldialdehyde (MDA) content in the liposomes was 22.2 nmol MDA/ $\mu$ mol of phosphorus, and in low oxidized phospholipids it was 2.0 nmol MDA/ $\mu$ mol of phosphorus.

### **Peroxidation Reaction System**

The techniques for phospholipid extraction, purification, preparation of liposomes (reverse-phase evaporation technique), peroxidation of phosphatidylcholine and measurements of TBA-reactive substances were described earlier [43,53].

### **Enzyme Assays**

The ability of transparent and cataractous human, rabbit and mice lenses to metabolize hydrogen peroxide in the surrounding medium was evaluated as previously described [30]. For the assays of enzyme activities, the lenses were weighted and homogenized in double glass distilled water unless otherwise mentioned. Glutathione peroxidase activity was assayed as described [29,30]. Glutathione reductase activity was measured as described [30]. Superoxide dismutase activity was tested by the method described earlier [30]. Xanthine oxidase (grade I from buttermilk, Sigma, St. Louis, MO, oxidoreductase, EC 1.1.3.22) (0.06 unit/ml) and xanthine (Sigma)  $5 \cdot 10^{-5}$  M were used to generate superoxide anions in 0.02 M  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$  (pH 8.3) buffer with addition of Triton X-100 (10 mg/ml). The presence of superoxide radicals was established by the nitro blue tetrazolium ( $5 \cdot 10^{-5}$  M) assay. Catalase activity was measured spectrophotometrically at 250 nm as described by Babizhayev et al. [30].

### **Pharmacokinetic Studies**

Rabbit eyes were treated with 1% N-acetylcarnosine, L-carnosine or placebo and extracts of the aqueous humor from the anterior eye chamber were analyzed for imidazole content by reverse phase analytical high performance liquid chromatography (HPLC), thin-layer (TLC) and ion-exchange chromatographic techniques. The techniques were described in details [53].

### **Clinical Studies**

All patients were evaluated at entry and followed up every 2 months for a 6-month period, or at 6-month intervals for a 2-year period, for best-corrected visual acuity and glare testing [55]. In addition, cataract was measured using stereocinematographic slit-images and retro-illumination examination of the lens. Digital analysis of lens images displayed light scattering and absorbing centers in two- and three-dimensional scales [55].

## Cataract Studies in Canines

The eyes of animals in each group were examined at regular intervals using a Zeiss SL-10 slit lamp. The appearance and progression of opacity was different in animals so the staging varied. Posterior subcapsular opacity (PSC) and cortical opacities occurred during the early stages of cataract formation.

## Statistical Analyses

Statistical analysis was performed using Student's t-test;  $p=0.05$  was taken as the upper limit of significance. Correlation and linear regression analysis were used to assess associations.

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