

Neurodegenerative and Morpho-functional Alterations During Senescence: Positive Modulation by α -Lipoic Acid and Superoxide Dismutase

Ocular Senescence in a Rat Model

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Abstract- We have evaluated the modulation of degenerative events at the retinal and optic nerve level in a rat model system. Degeneration was ameliorated in aging rats by the combined oral administration of α -lipoic acid and superoxide dismutase. Twenty four month-old rats were fed *ad libitum* with a diet supplemented by these two compounds. Positive control rats received a compound-free diet, while the negative control group consisted of untreated young animals. The cyto-protective effects of α -lipoic acid and superoxide dismutase were investigated by morphological analysis of retinal and optic nerve head sections. The level of nuclear DNA damage was assessed by TUNEL fluorescent reaction. Lipo-peroxidation assays evaluated the oxidative stress and consequent alteration of the cell membrane function. Apoptotic phenomena were investigated by immuno-localization and Western blotting to measure the expression of caspase-3 and inducible nitric oxide synthase. The results reported in this work provide evidence that α -lipoic acid and superoxide dismutase counteract the degenerative damage in aging animals, reducing the effects of oxidative stress.

Keywords- α -lipoic Acid; Apoptosis; Oxidative Stress; Rat Model

I. INTRODUCTION

The concept of oxidative stress and its consequences at the physio-molecular level were introduced and reviewed in detail by Sies, who defined it as an imbalance between oxidants and antioxidants in favor of the former: the prevailing of the latter may lead to cell damage [1-5]. In a physiologically normal situation, the cell maintains an internal reducing environment due to an ensemble of molecules and enzymes counterbalancing the formation of oxidizing compounds. Cells living in aerobic conditions produce most of the energy required for the maintenance of their vital functions from mitochondrial respiratory activity. In these organelles, the largest amount of oxygen consumption takes place; hence, they are the principal site of the synthesis of Reactive Oxygen Species (ROS) [6, 7]. Nitrogen-derived compounds, as also called Nitrogen Reactive Species (NRS), represent an additional family of free reactive radicals. Nitric oxide is one of the main active nitrogen reactive radicals and the enzyme involved in its production, Nitrogen Oxide Synthase (NOS), is found in three different isoforms. They catalyze the conversion of L-arginine to L-citrulline with the release of NO; two of the NOS isoforms are located at the brain at the endothelium level, where they are constitutively expressed, generating moderate amounts of NO. Their action depends upon the intracellular concentration of Ca^{2+} and calmodulin. The third isoform, also known as inducible NOS (iNOS), does not depend upon calcium and is not found in "healthy" tissues. This enzyme, unlike the others, produces high concentrations of NO, and therefore, is significantly toxic. The expression of iNOS has been monitored in the astrocyte cells of the optic nerve in glaucomatous patients. This pathological condition causes, among other phenomena, an increase in Intra-Ocular Pressure (IOP) and ischemia at the optic nerve level [8, 9]. As senescence advances, oxidants are no longer produced in a sufficient amount to counteract the accumulation of free radicals [10]. This may cause irreversible molecular damages that accumulate with age [11]. The role of the production of free radicals at the mitochondrial level has been discussed in different experimental models. In particular, Nohl and Hegner reported that in aging rats, there was a higher production of superoxide radicals as compared to younger animals. Furthermore, the super-expression of SOD could counterbalance the effects of aging in heterogeneous systems [12-14]. As far as the retinal tissue is concerned, studies have demonstrated that it is particularly susceptible to oxidative damage. This is plausibly due its metabolic activity, since it contains a very high number of mitochondria and is subjected, by definition, to frequent if not continuous photo-chemical stress [15-18]. Therefore ocular nervous cells produce very high levels of radical species which, if not adequately neutralized, may cause severe damage to the hydrocarbon chains of unsaturated fatty acids, the aminoacid-residues, the nitrogen bases of nucleic acids, and carbohydrates [19]. The end of this molecular catastrophe may be followed by the alteration and jeopardy of all structures with consequential cell death. Death of a significant number of retinal ganglion cells also brings about a reduction of the number of axons which form the optic nerve, resulting in a decrease of optic nerve function, which eventually determines a neuro-sensorial vision deficit [20]. To counteract the oxidative insult, the cell has a series of antioxidant agents that can delay or hinder the oxidation of the substrate molecules [11].

One of the most important enzyme systems involved in these defense mechanisms is SOD, a ubiquitary enzyme playing a key role against the accumulation of ROS and NRS produced by cell metabolic processes. This enzyme catalyzes the dismutation of the superoxide anion in hydrogen peroxide and molecular oxygen. In mammals, it exists in three different isoforms coded by distinctive genes, and each form has its own cellular localization; however the reaction they mediate is the same in all cases [21]. Studies conducted on “knock-out” mice demonstrated that mitochondrial SOD is required for survival. As a matter of fact, animals deprived of this isoform have been known to show cardiac and liver conditions, abnormal lipid peroxidation, mitochondrial mal function, and early death. On the contrary, animals lacking the cytosolic isoform show a normal phenotype, even if they seem to be less resistant to traumatic episodes [22]. Furthermore, the super-expression of SOD in mutant or transgenic strains of *C. elegans* and *D. melanogaster* results in an increased tolerance for oxidative stress, which manifests in an overall extension of the duration of life [23, 24]. This does not correspond to published data obtained on mice with premature aging phenomena or on subjects affected by Down syndrome. However, this discrepancy may be apparent only since results published on mice are controversial [25, 26]. On the other hand, Down syndrome shows such a complex symptomatology that can be simply ascribed to a redox defect. Also, the mitochondrial-driven pathologies are multi-faceted, therefore linking them to a unique mechanism may become unlikely [27].

A second line of defense is represented by endogenous antioxidants, among which is α -lipoic acid (ALA). In higher primates, small amounts of ALA is synthesized from fatty acids and cysteine [28]. This compound is considered an efficient scavenger of free radicals and is capable of increasing the levels of intracellular glutathione and ascorbate in the liver. This scavenging activity tends to diminish as age advances [29, 30].

The research developed in our laboratory in past years focused on the biological action of natural substances and the study of their actions, especially with respect to the evaluation of cell survival and cell death [31-35]. Therefore, in light of the data discussed above, we investigated the action and the role of ALA and SOD in the molecular and physiological phenomena underlying ocular degeneration. In particular, we concentrated on the evaluation of the degenerative effects associated with senescence, paying particular attention to detrimental aspects due to the accumulation of free radicals.

II. MATERIALS AND METHODS

A. Animals, Treatments and Sample Preparation for Morphological Analysis

Experimental procedures were conducted within the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (European Communities Council Directive of 24 November 1986 (86/609/EEC), Italian Health Ministry guidelines, and EU Directive 2010/63/EU).

Twenty-four Wistar-Harlem male rats (20 to 24 months old, with average weight of 300 and 350g) were divided into two groups. The first group was pre-treated for 8 weeks with a diet supplemented by ALA and SOD (generously provided by Alfa Wassermann, Bologna, Italy); the second was the control group, not subjected to any dietary supplement. The dosage was 857 mg/day for ALA and 2 IU/day for SOD. Negative control was represented by a group of 12 young males (age 4 to 6 months, average weight 200 e 250 g) fed *ad libitum*. Animals were sacrificed by carotid hemorrhage, and ocular bulbs were enucleated. The corneas were rapidly cut vertically, crystalline lens and vitreous humour were removed. Tissues were fixed in paraformaldehyde 4% for 6 hours at 4 °C, followed by a rapid wash with saline solution with a pH of 7.4. They were then immersed in a 30% sucrose solution in 1X PBS with a pH of 7.4 at 4 °C overnight; embedding was done in a Killik medium (Bio-Optica, Milan, Italy) to perform a cryostat cut (10 μ m sections). The tissue was sliced horizontally for the longitudinal observation of the optic nerve. Sections were mounted on microscope slides and stored at -20 °C. Morphological analyses were carried out according to standard procedures. In all cases, the magnification was 500x. For further details see [36, 37].

B. Molecular Evaluation of the Cell Damage

TUNEL, lipoperoxidation, fluorescence immunolocalization and Western blotting immunodetection assays were carried out according to standard protocol. For further details about the experimental data treatment, see published work from our laboratory [35, 38-40].

C. TUNEL Assay and Analysis of the DNA Fragmentation

One consequence of cell death is the activation of DNases. This causes the formation of single strand nicks and/or the fragmentation of DNA in monomers of 180-200 base pairs, or multiples. DNA rupture may be evidenced by in-situ labelling. Cell nuclei are permeabilized; fluorescent dUTP and terminal-deoxynucleotide-transferase conjugates the nucleotide at the level of the ruptures in the sugar-phosphate backbone. The fluorescence intensity provides data about DNA damage.

The fluorescent signal was analyzed by CytoVision (LAS x) dedicated to Zeiss/Leica microscopes (version 2014).

D. Membrane Lipoperoxidation

Oxidative stress at the membrane level was evaluated by a commercial kit (LPO-586; Oxis Health Research Products Portland, Or. USA). The assay allows for quantitative analysis of the intra-cellular formation of malonyl-dialdehyde (MDA)

deriving from the decomposition of poly-unsaturated fatty acids. This molecule reacts with a chromogenic compound (N-methyl-2-phenylindole) after the incubation of the cell extracts at 45 °C, thus forming a stable chromophore. The absorbance at 586 nm is directly transformed in the intracellular concentration of MDA.

E. Western Blotting

The enzyme Poly-ADP-Ribose-Polymerase (PARP) is activated in response to DNA fragmentation, which occurs during apoptosis. In this situation, PARP is inactivated by the caspases, cleaving it into two fragments of 116 kDa and 85 kDa. The proteolytic fragments are evidenced by immuno-electrophoresis utilizing a polyclonal antibody (PARP H-250 Santa Cruz Biotechnology, Inc.) directed against the protein and revealed by a secondary anti-rabbit antibody. Electrophoretic bands of Western blotting immuno-detection were quantified by the Optiquant software (Perkin- Elmer).

F. Statistical Analysis

The reported data are a summary of at least four separate experiments. They are presented as \pm SEM. Statistical analyses were performed using a two-way ANOVA test followed by Bonferroni's post-test to determine the statistical differences between the experimental cells and the control cells (* $p < 0.05$). All p values < 0.05 were considered significant.

III. RESULTS

A. Morphological Analysis of the Retina and Optic Nerve Head

Sections of the rat eye were stained according to the eosin-hematoxylin (HE) protocol to assess possible alterations of astrocytes cells and retinal layers. This also allowed monitoring modifications of the chromatin to be made at the nuclear level [35, 36]. In untreated young rats, no alterations were found, while in the control untreated aging rats, a reduction of the neural tissue was seen. This reduction was possibly due to apoptotic loss of the retinal ganglion cells (RGC), of the optic nerve axons and astrocyte cells. The reduction of the diameter of the optic nerve was estimated by light microscopy and is attributable to the aging process. In the aging rats that received a dietary supplement of ALA and SOD, on the contrary, a minor papillary loss and the typical columnar arrangement of the RGCs was observed (Fig. 1, panels A, C and D, respectively). In the following sections, we address the mode of ocular cell loss.

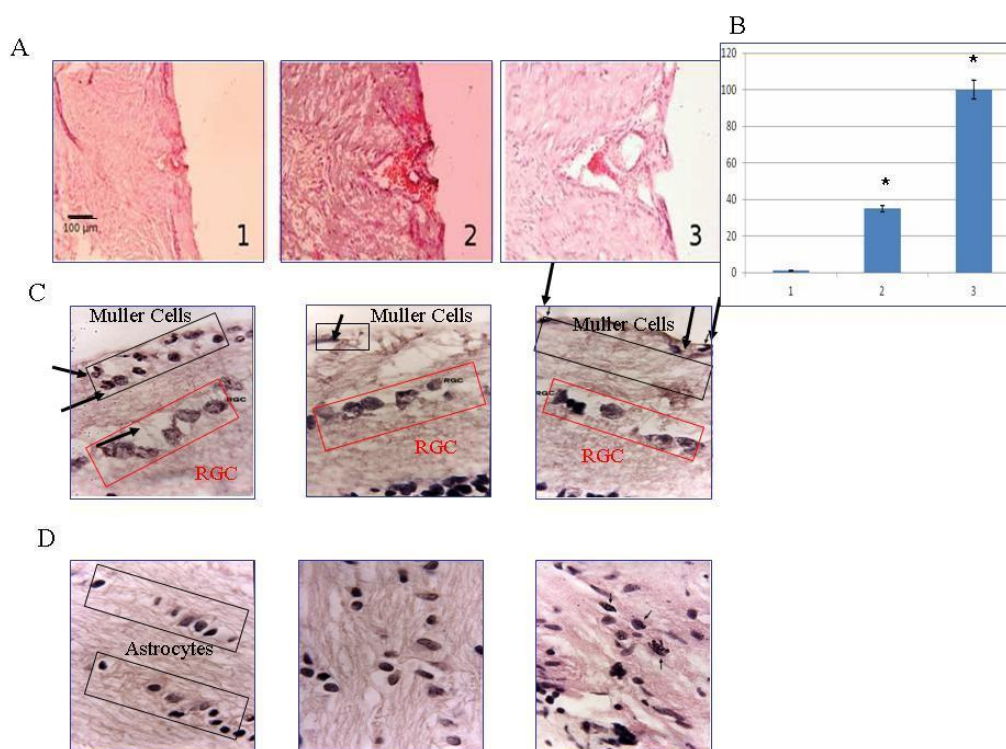


Fig. 1 Morphology of the retina and optic nerve head. A) 1: Optic nerve head of a six-month young rat (negative control); 2: Old rats fed with ALA and SOD supplements; 3: Old rats subjected to a normal diet. B) Quantitative estimation, expressed in μ^2 , of the macular degeneration, evaluated in percentage of the whole macular area (100% old rats and 0% control). Data report the mean values of 24 sections obtained from 6 different optic nerve heads (4 for each animal, data are reported \pm SEM) C) Retinal samples obtained from animals treated as in A. D) Glial cell sample (astrocytes) obtained as in B). In general, a decrease of the papillary excavation and an improved columnar arrangement improvement of chromatin structure in astrocytes (arrows) are evidenced after administration of ALA and SOD. In the center and bottom panels, from left to right, samples were obtained from animals treated as in 1, 2, and 3 shown in the top panel. Sections were eosin-hematoxylin stained. RGCs, Muller cells, and astrocytes appear in red and black boxes, respectively

B. Treatment with ALA and SOD Decrease DNA Degradation as Evidenced by TUNEL Assay

The fluorescent technique, first developed by Gavrieli and co-workers in 1992 [41], has become one of the main methods for detecting advanced apoptotic cell death. The increased fluorescence in apoptotic cells is due to the specific incorporation of fluorescent nucleotides at the free 3'-OH ends, both at the single strand or double helix level, of fragmented DNA. The reaction is mediated by the terminal transferase. In apoptotic cells, the endogenous nuclei cause typical DNA fragmentation, electrophoretically visualized as the DNA inter-nucleosomal ladder, a hallmark of apoptosis [42]. In the young control animals, the fluorescent reaction was hardly evident. On the contrary, in old untreated animals, a greater fluorescence was seen. The fluorescent labeling decreased in nuclei obtained from animals fed with ALA and SOD, where the endonucleolytic attack occurred at a lesser extent (Fig. 2, panels A and B). The eye sections of rats treated with ALA and SOD therefore corroborate the idea regarding the cytoprotective action of the two antioxidant drugs, which is exerted both on astrocyte cells and RGCs (Fig. 2, panel C). In the following section, data about the apoptotic cell death consequent to the age related oxidative stress are presented.

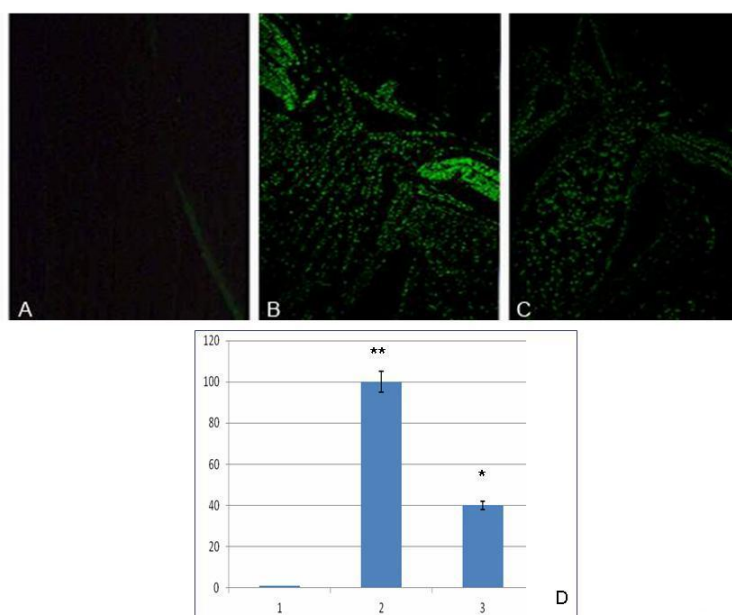


Fig. 2 Nuclear DNA degradation. DNA samples were obtained from animals treated as Fig. 1 and subjected to the TUNEL fluorescent reaction: A) Young rats (negative controls); B) Untreated aging rats; C) ALA+SOD treated aging rat; D) Fluorescence level is quantitatively expressed as percent emission. Controls represent emission 0% (1=A), and 24 month old rats represent emission 100% (2=B). The fluorescence level in histogram (3=C) shows the reduction the DNA degradation induced by ALA + SOD administration. Fluorescence emission is evaluated after Compton $E = K \times L(\text{emis.}) \times C$; C represent concentration of fluorescent emitter (in TUNEL reaction due to DNA degradation). The overall reduction of fluorescence positivity in animals fed with supplements indicates cytoprotection by ALA and SOD

C. Membrane Lipoperoxidation is Reduced after Treatment with ALA and SOD

Oxidative stress induces an increased lipoperoxidation of the cell membrane with resulting damage and malfunction; this phenomenon is frequently observed due to aging [43]. The extent of the membrane damaged was assessed by measuring the production of malondialdehyde (MDA) [44], a widely recognized marker of lipoperoxidation. This compound is not typically present in "healthy" cells but derives directly from damage to the membrane. The samples obtained from old rats that not fed with the antioxidant dietary supplements show a very high cytoplasmic MDA concentration. On the contrary, the treatment with ALA and SOD reduces the intra-cellular level. Therefore, it is plausible to infer that these antioxidants improve the overall homeostatic response, thus limiting the apoptotic phenomena (Fig. 3).

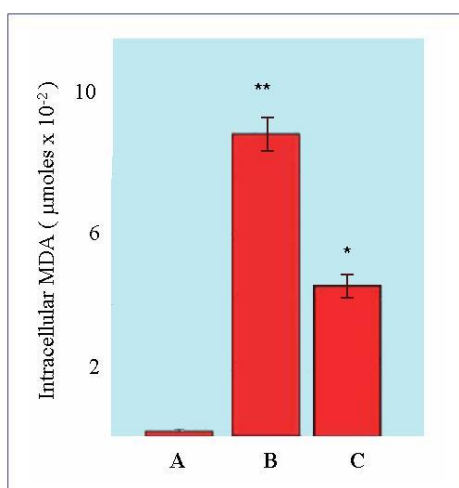
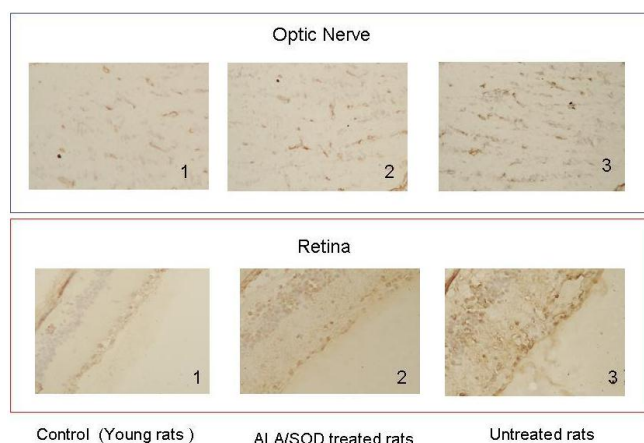


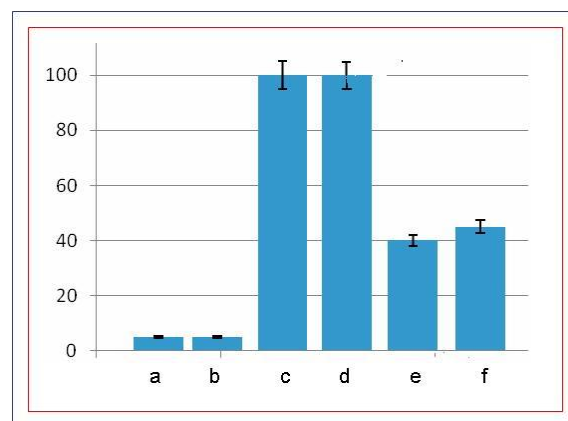
Fig. 3 Role of ALA and SOD in the modulation of membrane lipoperoxidation in retina samples. Bars report the level of malondialdehyde (MDA) evaluated in cell extracts obtained from: A) Young negative controls; B) Untreated aging rats; C) ALA+SOD treated aging rat. The values represent Mean \pm S.E.M. Controls = young rats untreated; positive rats = old rats untreated; positive rats ALA+SOD = old rats after treatment with ALA and SOD. Data were obtained from four different experiments and \pm SEM is reported

D. Immuno-localization and Western Blot Analysis of iNOS and Caspase-3

The inducible NO synthase and the executioner caspase-3 are two additional diagnostics of apoptosis [45-47]. The first enzyme was absent in the eyes of young rats, whereas positivity to iNOS was very evident in the normally fed adults, suggesting that the production of high and cytotoxic levels of NO. In the aging rats fed on a diet supplemented with ALA and SOD, the immuno-fluorescent signal was lower, indicating a reduced expression of iNOS. This supports the idea that the enzyme is activated when an age-dependent damage occurs (Fig. 4).



Immuno-localization of iNOS in optic nerve and retina



Quantification of iNOS in optic nerve and retina

Fig. 4 Immunolocalization of iNOS in sections of the optic nerve head and retina. (Left Panel) 1, 2, and 3 indicate, respectively: Young negative control rats; Aging rats fed with ALA + SOD supplements; Normally fed aging rats. (Left Panel) Quantification of the absorbance at 620 nm (brown color) which is proportional to level of iNOS in retina and/or optic nerve. Violet spots are due to unspecific (non-reacted) color deposition and are therefore not evaluated. Values represent mean \pm SEM of 20 tests; a, b: Control young rats; c, d: Old normally fed rats (100%); e, f: Old rats retina or nerve treated with ALA+ SOD. Retina and nerve respectively. The high level of iNOS evidenced in normally fed old rats is significantly reduced in animals fed with supplements, indicating a lower level of free radicals in these latter ones

The activation of caspase-3 was also monitored only in the case of the old rats, but the expression of this enzyme was reduced in the animals fed with ALA and SOD (Fig. 5).

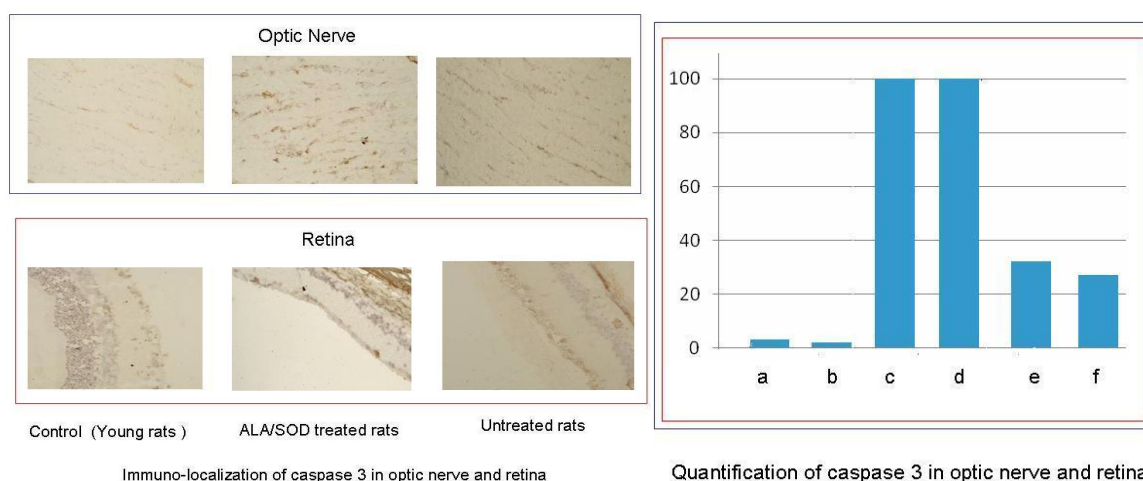
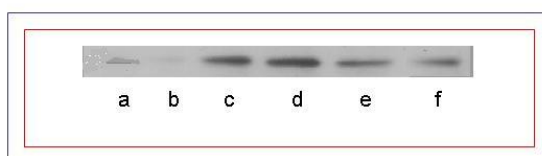


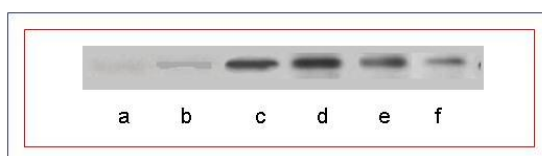
Fig. 5 Immunolocalization of caspase-3 in sections of the optic nerve head and retina (Left Panel). Samples were obtained from animals treated as in Fig. 4. Quantification as in Fig. 4B (Right Panel). For legends and details, refer to previous figure. In animals treated with ALA/SOD there is an evident reduction of positivity to caspase-3 which is diagnostic of an overall reduction of apoptotic phenomena induced by these food supplements

Analysis of iNOS levels by Western blot confirms that the aging rats fed with ALA/SOD show a reduced level of this enzyme, thus validating the data obtained by immuno-localization. As expected, a reduction of caspase-3 was also observed in the retinal extracts obtained from rats subjected to the anti-oxidants, indicating that the apoptotic program is not activated at a comparable level. The data obtained by Western blotting are shown in Fig. 6 and quantitatively summarized in Table 1.

Immuno-blotting of caspase 3 in retina and optic nerve



Immuno-blotting of INOS in retina and optic nerve



Reference standard: α -tubulin

Fig. 6 Western blotting on samples of retina and optic nerve. Samples were obtained from animals treated as in Fig. 4. For legends and details, refer to previous Figs. 4 and 5. A, B, and, C report the level of the standard α -tubulin marker and refer to untreated, ALA/SOD-fed and normally fed animals, respectively. Only the standard reference for the caspase 3 is shown since the one monitored for iNOS is virtually the same. Primary data of immuno-blotting experiments are quantitatively summarized in Table 1 where also the statistical significance is reported

TABLE 1 SYNOPSIS OF PRIMARY WESTERN BLOTGING DATA SHOWN IN FIG. 6

Antigen	Young Rats	Untreated Aging Rats	ALA+SOD Treated Aging Rats	$p(1)_{1-2}$	$p(2)_{2-3}$
iNOS	6 ± 2	100%	26 ± 4	≤ 0.01	≤ 0.1
Caspase-3	8 ± 2	100%	23 ± 3	≤ 0.01	≤ 0.1

The bands of iNOS and Caspase-3 in the different samples were quantified by chemi-luminescence. Values are expressed as percent with respect to untreated aging rats and the mean values \pm SD are reported.

IV. DISCUSSION

One of the main aims of this work was to investigate the histological and bio-molecular phenomena underlying cell senescence; this was pursued using an animal model previously developed in our laboratory for the experimental study of glaucoma. We also attempted to modulate the neuro-degenerative alterations using the anti-oxidant molecules ALA and SOD.

These molecules were administered to the experimental animals as dietary supplements. The idea was to abrogate the damage caused by the accumulation of free radicals during the normal lifespan. With respect to this, we would like to point out that the old rats used in the experimental work can be compared to ultra-centenarian humans. The data presented in this work are consistent with the idea that the combined oral administration of ALA and SOD as food supplements counteract degenerative events linked to aging in old rats of over twenty weeks of age. As a matter of fact, in aging and supposedly damaged tissues where a higher accumulation of free radicals occurs, an enhancement of the expression of iNOS is found. It is commonly accepted that the accumulation of NO is irreversibly detrimental for cells and tissues. Toxicity induced by the over-expression of iNOS was demonstrated both in inflammatory and degenerative pathologies, such as multiple sclerosis and Parkinson's and Alzheimer's diseases. Furthermore, the increased iNOS expression in rats is the basis of the apoptotic death of the retinal photoreceptors; it should also be pointed out that apoptosis has been ascribed to high intracellular concentration of NO. The results of the work discussed here demonstrate the reduction of nitric oxide mediated by the administration of antioxidants. This may improve retinal degeneration derived by an oxidative stress due the aging of the organism. It is plausible to assume that this amelioration may also occur in the case of pathological cell degeneration due to a phlogistic, traumatic, or dys-metabolic origin. The higher stability of the mitochondrial membrane, as evidenced by the reduction of the LPO reaction induced by free radicals, may hinder the release of pro-apoptotic factors indispensable for the trigger and execution of the apoptotic program. It is known that an increased permeability of the mitochondrial membrane could result in the release of cytochrome c into the cytoplasm matrix, one of the most potent signals for the activation of the intrinsic apoptotic pathway. When cytochrome c is actually released, it interacts with Apaf-1 to form the apoptosome complex that promotes the activation of caspases and ending in the apoptotic death cascade.

The results presented here demonstrate, finally, that during the aging process the RGCs, the optic nerve axons and astrocyte cells undergo a series of degenerative phenomena. These are essentially caused by oxidative stress and the subsequent accumulation of oxygen and nitrogen free radicals. These noxious compounds are not efficiently removed or neutralized by the resident enzyme systems, the efficiency of which tends to decline with age. The final effects of the administration of ALA and SOD consist in the defense from the ocular degeneration as proven by morphological and bio-molecular results. In the aging rats that received a dietary supplement of ALA and SOD, in fact, minor papillary loss and restoration of the RGCs was observed. The decrease of DNA fragmentation and iNOS expression were also seen. Lastly, the diminished LPO reaction strongly suggests the stabilization and consequent conservation of the cell membrane integrity. The overall interpretation is that these antioxidants control the accumulation of free radicals, thus slowing the age-related tissue degeneration. These studies were conducted in a rat model of ocular degeneration, and the result is that ALA and SOD play an important role in the protection of the retinal cells and optic nerve fibers from. However, it should be considered that this protective role might also apply in human cells. Moreover, it is worth noting that analogous protective benefits may be attained in other areas of the body. This would occur not only during aging, but also in the treatment of pathological phenomena such as neo-plastic proliferation, inflammation, trauma, and dystrophy.

V. CONCLUSIONS

The aim of this work was to evaluate the modulation of degenerative events induced by the accumulation of free radicals at the retinal and optic nerve level. The negative effects were positively modulated by the administration of α -lipoic acid and super-oxide dismutase as dietary supplements. These compounds improved the condition of ocular nerve tissues in aged rats. In control untreated rats, no amelioration was observed. These data were obtained by morphological and bio-molecular tools. The administration of combined ALA and SOD to aged rats helps to maintain proper ocular tissue organization, and membrane integrity, while decreasing DNA fragmentation and the expression of caspase-3. In conclusion, these powerful antioxidants can hinder and/or prevent neural degeneration at the ocular level, possibly acting as scavengers of free radicals that normally accumulate during the aging processes. The possibility that similar protective mechanisms may take place also in other parts of the organism, and in humans as well, remains to be examined, but the idea represents a great potential for research regarding age-dependent degenerative phenomena.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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