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Oxidative stress-induced ocular degeneration: down-regulation by antioxidant agents

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INDICE

REDOX STRESS OR REDOX REGULATION?

The first published article on reactive oxygen species (ROS) dated since 1945. Since then the use of ROS as keyword in Pubmed search resulted in more than 117,000 English-written articles, almost 12,000 of which are review articles.
ROS are highly reactive molecules that originate mainly from the mitochondrial electron transport chain (ETC). Almost all cells and tissues continuously convert a small proportion of molecular oxygen into superoxide anion by the univalent reduction of molecular oxygen in the ETC. The ROS are produced by other pathways as well, including the respiratory burst taking place in activated phagocytes, ionizing radiation's damaging effect on components of cell membranes, and as byproducts of several cellular enzymes including NADPH oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase [Droge, 2002]. Cellular and tissue defenses against ROS include the enzymes superoxide dismutase (Mn-SOD, Cu/Zn-SOD, extracellular SOD), catalase, glutathione peroxidase, peroxiredoxins, and the nonenzymatic antioxidants, like glutathione (GSH), thioredoxin, ascorbate, α-tocopherol, and uric acid [Radi et al.,2001]. The oxidative damage potential is greater than antioxidant defence, and thus there is a constant small amount of toxic free radical formation, which escapes the cellular defenses. These may lead to a conclusion that endogenous levels of antioxidants are not high enough to fight the stress insults and that the addition of exogenous antioxidants could enhance the cellular antioxidant capacity. Human cells generate some hydrogen peroxide and other ROS molecules to use them as chemical signals to regulate everything from glucose metabolism to cellular growth and proliferation [Rhee, 1999]. ROSs induce various biological processes that include a transient elevation of intracellular Ca^{2+} concentration, phosphorylation of specific proteins, activation of specific transcription factors, modulation of eicosanoid metabolism, and stimulation of cell growth [Kaul and Forman, 2000]. Nitric oxide was identified as a signaling molecule as early as 1987 [Palmer et al., 1987] and is now a well-known regulator of some transcription factor activities and other determinants of gene expression. Hydrogen peroxide and superoxide have similar intracellular effects [Kamata and Hirata, 1999]. ROS can directly affect conformation and/or activities of all sulfhydryl-containing molecules, such as proteins or glutathione (GSH), by oxidation of their thiol moiety. Among many other enzymes and membrane receptors, this type of redox regulation affects many proteins important in signal transduction and carcinogenesis, such as protein kinase C, Ca2+-ATPase, collagenase, and tyrosine kinase [Dalton et al.,1999]. For several transcription factors, ROSs are physiological mediators of transcription control [Nordberg and Arner, 2001]. The well-known examples of redox-sensitive transcription factors are nuclear factor-κB (NF-κB) and activator Protein-1 (AP-1). Even the products oxidized by ROS (e.g., lipids, proteins, sugars, and nucleic acids) may act in the similar way. For example, lipid peroxidation

products may modulate signal transduction pathways and mediate biological processes through receptors or receptor-independent pathways. Low levels of ROS function as signalling molecules to induce adaptive responses. Overconsumption of exogenous antioxidants could thus lead to the state of "antioxidative" stress, where antioxidants might attenuate or block the adaptive stress responses. Additionally, reactive oxygen species in moderate concentrations are essential mediators of defence against microorganisms and unwanted cells [Salganik, 2001]. On the other hand, excessive oxidative stress for an extended period of time increases oxidative damage with negative influences on health and longevity. Oxidative or "antioxidative" stresses occur early in the process that may lead to pathology; however, being not associated with any specific clinical symptoms or signs at an early stage, often they are not recognized until the development of irreversible damage, that manifest itself as a symptom of a chronic disease. It is therefore important to recognise oxidative imbalance at an early stage in order to prevent the long-term oxidative stress[Poljsak and Milisav, 2012].

ROS production is increased by several environmental factors of stress, such as exposition to high levels of light, drought, heavy metals, salt concentrations, temperature extremes, air pollution, UV radiation, herbicides, and pathogen attacks. Whether ROS will act as damaging, protective, or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time [Gratao et al., 2005]. Now it is clear that although increased oxidative stress is not beneficial for the cell, the complete elimination of free radicals would disrupt, rather than extend, the normal functioning of the body: in order to maintain a state of homeostasis, living organisms are striving to keep those highly reactive molecules under tight control with the help of an intricate system of antioxidants.

Finally, due to the potential beneficial role of ROS demonstrated by several lines of research, ranging from their role as signaling molecules [Liu et al., 2003] to the more unexpected role in improvement of certain cancers [DeNicola et al., 2011], the term "redox regulation" might prove to be more accurate than "redox stress"; there have been even some situations where antioxidants are described to be "bad" [Perera ad Bardeesy, 2011]. However, the term "redox stress" is more commonly used.

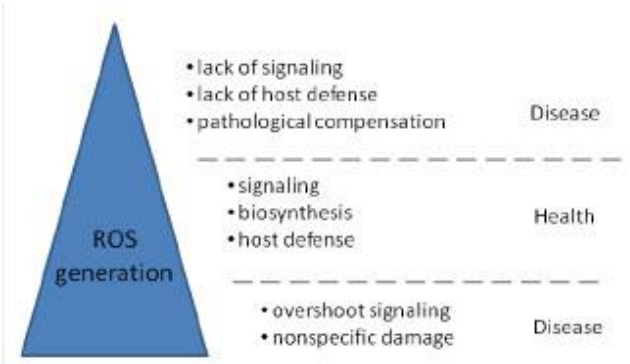


Fig. 1 Variations of ROS generation. At moderate levels, NOX have crucial roles in health through mechanisms including signaling, biosynthetic processes, and host defence. When ROS levels are too low, the condition may result in decreased antimicrobial defence. When levels are too high, there is increased damage.

FREE RADICALS ROLE IN PHYSIOLOGICAL PROCESSES

Role of Free Radicals (in specific ROS) in Normal Vascular Diameter Regulation.

Mitochondrial ROS, specifically superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), were demonstrated to play a role in normal vascular physiology in response to such factors as shear-stress [Liu et al., 2003]. In the vascular system, ROS were demonstrated to originate mainly from the mitochondria in a study performed on human coronary resistance arteries. The mitochondrial origin of ROS was confirmed using electrobiophysical methods that assessed the ROS generation and the response of vessel diameter to the presence of inhibitors of mitochondrial complexes and antioxidants [Liu et al., 2003].

Go and colleagues have studied in more detail the mitochondrial role in the signaling response to oxidized milieu that might be encountered in the vascular system. Their results provide a model whereby more oxidized environment in the plasma will lead to oxidation of cellular plasma membrane and cytoskeletal proteins. Oxidized proteins will then stimulate mitochondrial production of ROS that will initiate signaling pathways upregulating the cellular inflammatory response [Go et al., 2010]. The model described by Go *et al.* also provides explanation for the protective antioxidant role of small molecular weight-mitochondrial proteins such as thioredoxin 2 (Trx2). Earlier works have previously demonstrated Trx2 "regulatory" redox signaling pathway against mitochondrial ROS [Chen et al., 2002; Chen et al., 2006; Zhang et al., 2007]. The described model also provides partial explanation for the paradox that whereas moderate ROS levels contribute to regulation of vascular cell function [Liu et al., 2003], their excessive production is linked to pathological situations where redox damage and inflammation prevail in several chronic diseases. This kind of studies linking the cellular responses to alterations in redox potential on one hand and to the intracellular signaling pathways on the other hand is promising; since it can be translated into designing novel therapeutic agents that target relevant signaling pathways, as an alternative to the use of nonspecific antioxidant agents in clinical trials, or perhaps as a complementary tool to these agents.

Role of ROS in Oxygen Sensing.

Oxygen sensing is so critical to cellular health as it allows cells to initiate adaptive responses that will increase the likelihood of survival in anticipation for limited oxygen availability. Guzy and Schumacker have proposed that the ETC acts as an O_2 sensor by releasing ROS in response to hypoxia. The hypoxia-induced released ROS act as signaling molecules that trigger diverse functional responses, among which is the increased production and stabilization of the hypoxia-inducible factor-1 (HIF-1). This has been demonstrated at least in normal (non transformed) cells. As a matter of fact, a mutual regulation was reported for both HIF-1 and ROS. Under acute hypoxic

conditions, the mitochondrial ETC produces excess ROS. This is required for the induction of HIF-1 expression [Guzy and Schumacker, 2006], which in turn mediates adaptive metabolic responses culminating in a normalization of ROS levels and maintenance of redox homeostasis. Likewise, hypoxic induction of HIF-1 activity will end in normalization of the tissue O_2 levels by stimulating angiogenesis, which augments oxygen delivery to tissues and solves the problem of tissue hypoxia. However, in some cancer cells the picture is not the same, as cells transformation is expected to result in alterations in the above-described normal adaptations, hence even though angiogenesis might occur, it is less effective in maintaining oxygen homeostasis; this was extensively reviewed by Semenza [Semenza, 2008].

Role of ROS in the Immune System.

Essentially, ROS are deeply involved in both arms of the immunological defense system, the innate and the acquired responses. Upon exposure to environmental pathogens, exaggerated ROS production, as a part of the oxidative burst in activated phagocytes present in the local inflammatory milieu, represents one of the first lines of defense mounted against the invading pathogens. Although rapid, this innate immunity is usually only partially effective, since certain fraction of pathogens might escape and proliferate, thereby producing a larger number of pathogens. Acquired immunity will be initiated when pathogen-derived antigenic peptides that are the result of phagocytosis and digestion by activated phagocytes are presented to the T lymphocytes. As a result, the latter will proliferate and differentiate producing a large progeny of immunological effector cells, that are capable of mounting an efficient and antigen-specific immune response. ROS are involved in the acquired immune response because excess ROS continue to be locally produced by the activated phagocytes and consequently enhance the intracellular signal transduction cascades within the T lymphocytes and thereby decrease their activation threshold [Droge, 2002].

Role of ROS in Skeletal Muscle Physiology.

The skeletal muscle is a target organ for oxidative regulation and/or oxidative stress, since it requires a large supply of energy to ensure efficient contraction, and consequently it is liable to be exposed to excess mitochondrial ROS. The skeletal muscle production of ROS is promoted by multiple stimuli including muscle contraction, insulin, and hypoxia. Although under normal physiological conditions antioxidant systems control the level of ROS in skeletal muscle, oxidative stress can take place if ROS levels exceed the muscle antioxidant capabilities, and this can have damaging functional effects [Merry and McConell,2012]. Recent research has suggested that ROS can act as signaling intermediates in the regulation of skeletal muscle glucose uptake during contraction. However, results of such research have to be interpreted with caution, as they have been somewhat inconsistent depending on

the model studied and the experimental design [Sandstrom et al.,2006; Merry et al, 2010; Merry and McConell,2012].
Interestingly, muscle activity has been recently reported to affect the antioxidant defenses as well. Berzosa and colleague in healthy untrained male subjects have reported an augmented effect of acute exercise on the circulating total antioxidant status and antioxidant enzymes activities after both maximal and submaximal exercise periods [Bersoza et al., 2011]. Others [Hatao et al., 2006] have shown that the elevated levels of antioxidant enzymes activity was also detected in various body organs. Thus, it is thought that exercise, whether acute or chronic, helps in maintaining redox homeostasis since it increases the antioxidant defense mechanisms and due to the fact that long-term heavy exercise renders both animals and humans more resistant to oxidative damage [Bersoza et al., 2011]. Not only muscle contraction has drawn the attention of scientists interested in the field of muscle physiology, but also muscle immobilization, where ROS production was reported to increase in skeletal muscle tissue after immobilization: a finding that warrant further studies specially if we consider that immobilized subjects manifest great loss of their muscle mass [Droge, 2002].

Role of ROS in Genomic Stability, Regulation of Transcription, and Signal Transduction.

Cellular redox status is considered an emerging regulatory factor for genomic stability and transcription. In a recent review article by Rajendran and colleagues, the posttranslational enzymatic covalent modification of histone and nonhistone proteins, in the form of acetylation/deacetylation for finely regulating transcription, was discussed in relation to the cellular redox status. Various physiological processes such as cell cycle regulation, response to DNA damage, regulation of intermediary metabolism, programmed cell death, and autophagy, listing only few, are known to be regulated at the level of transcription of relevant genes. The authors have reviewed in detail various factors regulating transcription via modulation of chromatin dynamics. They have indicated that oxidative stress and cellular energy consumption are among the key transcription regulating factors, since the deacetylase activity of sirtuins, members of class III histone deacetylases, depends on the cellular redox status and NAD+ availability. In fact, the gene expression level of sirtuins has been shown to be under the control of the oxidative stress- and DNA damage-responsive transcription factor, E2F1, which regulates cell cycle and directly binds to the promoter of sirtuin 1, the most studied member of the sirtuins family. Moreover, exposure of cells to excess ROS such as H_2O_2 results in posttranslation modification of Sirt 1, in the form of desumoylation and hence inactivation of its deacetylation function, and consequently in acetylation and hence activation of pro-apoptotic Sirt 1 substrates, such as p53, and eventually in cell death. Under oxidative stress, the role played by ROS in transcription regulation is of critical importance and is able to affect vital processes such as glucose homeostasis, inflammation, cellular lifespan,

and multiple aging-related diseases including cancer [Rajendran et al., 2011]. Cells have an elaborate system to respond to redox status. This has been well studied in bacteria where the existence of a number of different ROS and redox status-responsive signaling pathways is well established [Wu and Bauer, 2008], as well as in the yeast *Saccharomyces cerevisiae* [Biteau et al., 2003]. In mammalian cells, similar yet incompletely understood protective redox-responsive signal cascades have been described. These cascades are critical for the survival of cells which happen to be in the midst of highly oxidizing environment, such as sites of infection and inflammation. While activated phagocytes utilize their capability of creating "oxidative burst" to kill invading pathogens, and this implies the overproduction of ROS, recruited lymphocytes on the other hand need to possess an armament of oxidative stress-induced signal transduction cascades to protect themselves against this same oxidative burst. The oxidizing milieu modulates lymphocytes signal transduction cascades and increases the activities of redox-responsive transcription factors, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). The latter will bind and activate the promoters of various genes. One of those genes is the gene for the protective protein thioredoxin (Trx). Trx is an oxidoreductase that works together with the glutathione system for establishing and maintaining a reducing intracellular redox state. Other set of genes whose products are protective antioxidants are peroxiredoxin I (i.e., a Trx peroxidase), heme oxygenase-1, the cystine transporter xc2, and manganese SOD (MnSOD) [Droge, 2002; Droge, 2006].

"Potential Beneficial" Role of ROS in Cancer.

Recently an interesting hypothesis arises that examines the following question: "Can antioxidants promote disease situations?" or as Perera and Bardeesy stated it: "When antioxidants are bad?" [Perera and Bardeesy, 2011]. This is a hot area of research and is finding increasing implications in cancer-related studies. Classically, ROS were demonstrated to promote various types of cancers. This was explained by different facts: the ROS ability to induce DNA damage and thus to enhance the rate of tumor-causing mutations and genetic instability, their pro-inflammatory effect, and their stabilizing influence on HIF essential for energy regulation. Accordingly, antioxidants were able to decrease tumorigenesis by neutralizing the deleterious effects of ROS [Reliene et al., 2004; Sablina et al., 2005; Aitio, 2006; Reliene et al., 2006]. Recently, a different face of the ROS coin has been revealed based on studying the effect of mutations activating the transcription nuclear factor, nuclear factor-erythroid 2-related factor 2 (Nrf2). Nrf2 is a redox stress-sensitive transcription factor that induces several antioxidant and detoxification genes. In the absence of redox stress states, Nrf2 is kept inactive by binding to another protein, Kelchlike ECH-associated protein 1 or KEAP1, ensuring effective Nrf2 repression. Somatic mutations in either Nrf2 or KEAP1 that prevent their binding will result in constitutive Nrf2 activation and transcription of Nrf2 target genes. Such mutations have been isolated from patients with lung cancer suggesting a protumorigenic role of

Nrf2. Furthermore, drug resistance in some antitumor therapy may take place as a result of such somatic mutations; this was reviewed by Hayes and McMahon [Hayes and McMahon, 2009]. More recently, De Nicola and colleagues have demonstrated that in mice several endogenous oncogenes, such as Kras, Braf, and Myc, actively induce Nrf2 expression, promoting a ROS detoxification program and hence creating a more "reduced" intracellular environment, a program that the authors suggest to be required for tumor initiation [DeNicola et al., 2011]. As Hansson and Libby elegantly described the immune response in atherosclerosis as "double edged sword" [Hansson and Libby, 2006], the description seems to perfectly fit the ROS. Therefore, the big picture reflecting the contributions of various mediators plus local environmental factors seems to be the actual determinant for ROS-induced consequences in both physiology and pathology, and hence it is essential to unravel the not-yet-well understood parts of this intricate picture for better understanding of the ROS induced alterations.

In conclusion: Although ROS have been classically known for their damaging effects, increasing evidence of their use in regulating and maintaining normal processes in living organisms has been accumulating. Therefore, the term redox regulation seems to better describe the redox status and its consequences. Both ROS and the protective antioxidant systems have to work in coordination to reach a state of redox homeostasis. Evidence of the roles played by ROS in several physiologic processes has been presented, such as maintaining vascular diameter and normal vascular cell function, participating with HIF in sensing the oxygen availability and initiating responses appropriate for cell survival, mounting effective immune response, acting as possible signaling molecules in regulating skeletal muscle glucose uptake, and regulating gene stability and transcription via affecting chromatin stability. Antioxidants are equally essential, and their genes expression is regulated by the ROS. In addition, muscle exercise is beneficial in rendering us more resistant to oxidative damage. Recent evidence points out to a potential link between the "reduced" cellular environment and tumor initiation.

FREE RADICALS CELLULAR ORGANELLES

THE ROLES OF THE ENDOPLASMIC RETICULUM AND MITOCHONDRIA IN OXIDATIVE STRESS/REGULATION

Both the endoplasmic reticulum (ER) and the mitochondrion have proven to be fascinating intracellular organelles that have stimulated a tremendous amount of research due to their unique characters. Their well-established roles in proper protein folding, posttranslational modifications, cellular trafficking, ions storage, energy production, cellular thermogenesis, and intermediary metabolism are just some examples. Both organelles have strong and interrelated ties to the redox cellular homeostasis, disturbance of which is implicated in many diseases. Increasing evidence accumulates that free radicals (in specific ROS) contribute to endothelial cell dysfunction, atherosclerosis, aging, diabetes mellitus (DM) and diabetic complications, and cardiovascular diseases (CVD) to name only few [Puddu et al., 2005; Doughan et al.,2008; Wenzel et al., 2008; Dai et al., 2011; McDermott-Roe et al., 2011].

Endoplasmic Reticulum and Endoplasmic Reticulum Stress.

Impaired biological processes within the cell, collectively defined as cellular stress, together with chronic inflammation have been causally associated to various metabolic diseases, such as DM, obesity and CVD [Puddu et al., 2005; Hotamisligil, 2006; Hansson and Libby, 2006; Doughan et al.,2008; Wenzel et al., 2008; Dai et al., 2011; McDermott-Roe et al., 2011]. The ER, ubiquitously present in eukaryotic cells, plays a key role in protein folding and modification as well as in dynamic storage of calcium. It is through its role in maintaining protein folding that the ER is intricately involved in the overall ROS production as will be discussed shortly. Although protein folding is a multistep process that is not yet fully understood, two factors are known to be essentially required for the formation of intra- and intermolecular disulphide bonds that are fundamental to the folding process; these are the availability of energy and an ER oxidizing environment. In addition, two ER enzymes, the protein disulphide isomerase (PDI) and ER oxidoreductin 1 (ERO1), are critical for the oxidative formation of disulphide bonds [Tu and Weissman, 2004]. The reactions they catalyze involve transfer of electrons and oxidation of cysteine residues in nascent proteins and utilize flavin adenine dinucleotide (FAD) and molecular oxygen. Electron transfer to molecular oxygen as a terminal electron receiver produces H_2O_2; hence excess load of protein folding can result in accumulated ROS. The latter will trigger cellular inflammatory response. The ER is thought to sense signals of altered cellular states triggered by a variety of stimuli, such as certain growth factors and hormones, limited availability of energy or nutrients, and the cellular redox state. The ER then acts accordingly aiming at restoring the normal cellular homeostasis. The ER itself might experience a state of ER stress, in which its capacity to correctly fold and

modify proteins is overwhelmed by an excessive demand for protein folding or by conditions accompanied by excessive unfolded or misfolded proteins. This will increase the amount of proteins of abnormal structure in the ER, triggering a defensive set of reactions collectively known as "unfolded protein response" or UPR, during which the cellular transcriptional and translational machineries are altered in order to restore the normal protein folding process. However, if the stress is extreme or prolonged, cellular homeostasis cannot be established and, alternatively, cellular pathways culminating in apoptosis will be activated [Schroder and Kaufman,2005; Ron ad Walter, 2007]. A less well-understood UPR system was recently described in the mitochondria (UPR mt) and its involvement in protecting cellular and specifically mitochondrial components against damaging consequences of metabolic stressors is increasingly acknowledged [Haynes and Ron, 2010]. At the molecular level, the relation between ER stress and oxidative stress can be explained by various routes. As mentioned earlier, during electron transfer to molecular oxygen as the terminal electron recipient in the ER protein folding process, some ROS will be generated. Furthermore, under ER stress conditions, manifested by excess accumulation of unfolded or misfolded proteins, the cell consumes extra reduced glutathione (GSH) to correctly fold these aberrantly folded proteins, adding more to the cellular stress. Consequently, the ER stress can result in oxidative stress which might trigger an inflammatory state. Thus, it seems that the ER is placed in a vicious cycle where ER stress can be caused by oxidative stress, and will also augment the perturbed oxidative redox state. Therefore, protective mechanisms essentially exist in the ER to limit the consequences of this damaging cycle. These include the protein kinase R-like ER Kinase (PERK) pathway-induced activation of an antioxidant program that utilizes the transcription factors: activating transcription factor-4 (ATF4) and Nrf2 [Harding et al., 2003; Cullinan et al., 2003; Cullinan ad Diehl, 2004]. As previously mentioned, activated Nrf2 will be translocated to the nucleus to increase the rate of expression of a group of antioxidant and oxidant detoxifying genes [Mathers et al., 2004; Zhang, 2006].

Role of Mitochondria in ROS Production.

The mitochondrial ETC represents the major source for cellular ROS production. The superoxide anion is non enzymatically formed by the ETC semiubiquinone compound and then enzymatically converted into hydrogen peroxide by superoxide dismutase (SOD). Superoxide anion can also be non enzymatically converted into hydrogen peroxide and singlet oxygen. Hydrogen peroxide can be converted into the highly reactive hydroxyl radical in the presence of reduced transition metals. Alternatively, hydrogen peroxide may be enzymatically converted into water by the enzymes catalase or glutathione peroxidase [Droge, 2002]. Mitochondria possess several unique characters among which are the presence of mitochondrial DNA (mtDNA), their mode of inheritance, the dynamic nature of their structure, their indispensable roles in fuel metabolism and energy production, and the established

links to various metabolic abnormalities. Therefore, it is expected that a defected mitochondrion is the underlying mechanism for a myriad of pathological conditions. The strong association between mitochondrial dysfunction, whether genetically determined or acquired, and chronic metabolic diseases such as type 2 DM and obesity was observed in many studies; yet a cause-effect relationship remained tentative for some time, till further studies demonstrated that impaired mitochondrial capacity and function are potential causes for insulin resistance and/or DM progression; this will be discussed below in more detail [Patti and Corvera, 2010]. The central regulatory role played by the mitochondria in whole body metabolism, energetics, and homeostasis necessitates that it will be under tight control. Its ultimate functional capacity in certain tissue and under certain physiological conditions is the result of a network of interfering parameters. These include the mitochondrial DNA copy number, the mitochondrial density, and levels and activity of specific mitochondrial proteins [Patti and Corvera, 2010]. Both transcriptional and posttranscriptional mechanisms exist to ensure tight control of the mitochondrial functional outcome. The nuclear DNA is deeply involved as well in implementing this control, and a strong link between nuclear and mitochondrial gene expression was demonstrated more than 15 years ago [Virbasius and Scarpulla, 1994]. As mentioned before, mitochondrial ETC is a potent source of ROS, and for obvious reasons such as the physical proximity to mtDNA, mitochondrial ROS generation is under tight control by various mechanisms, among which are the uncoupling proteins 1, 2, and 3 (UCP1, 2 and 3). UCPs are inner mitochondrial membrane proteins that are considered as natural regulators of mitochondrial ROS, responding to and controlling ROS production by diminishing the formation of a large proton gradient [Patti and Corvera, 2010]. It is thought that UCP1, which is present in the brown adipose tissue, evolved a thermogenic role in mammals as a side pathway of the original, more general function of protecting cells against the cold-induced production of ROS. On the other hand, UCP2 (ubiquitously expressed at low levels) and UCP3 (preferentially expressed in skeletal muscle) maintain their original function of decreasing ROS production through uncoupling and hence buffering ROS levels and do not appear to play a thermogenic role [Echtay et al., 2002; Andrews et al., 2008; Jastroch et al., 2010]. Other emerging roles of UCP have been suggested; UCP2, for example, is thought to exert a negative regulatory effect on pancreatic insulin secretion, as well as a ROS buffering effect on hypothalamic neurons controlling eating behavior [Chan et al., 2001; Zhang et al., 2001]. Several years ago, the dynamic nature of the mitochondrial structure was elucidated and was demonstrated to be attained by complex molecular machinery, several components of which have been well characterized [Shaw and Nunnari, 2002]. Abnormality in this machinery is linked to mitochondria—associated metabolic diseases. As an example, reduced expression of mitofusin 2 (Mfn2), one of the mitochondrial proteins responsible for its dynamic morphology, was demonstrated to be partly responsible for decrease glucose oxidation and cell respiration in obesity [Bach et al., 2003].

In conclusion: Both the ER and the mitochondria participate in maintaining normal cellular homeostasis. It is through the ER role in maintaining proper protein folding that this organelle is intricately involved in the overall ROS regulation. The ER senses signals of altered cellular redox states and then acts accordingly in order to restore and maintain normal homeostasis. During the UPR of the ER, ROS will be accumulated either due to actual production of ROS or due to consumption of the antioxidants such as GSH. Because the ER can be a part of a vicious cycle, where oxidative stress leads to ER stress, and the latter will further worsen the redox status, there are several protective mechanisms to limit the anticipated damage. A strong association and a potential cause-effect relationship exist between defective mitochondria and metabolic diseases. As in the ER case, several protective mechanisms exist to protect the mitochondria from oxidative damage. UCPs are natural regulators for mitochondrial ROS, responding to and controlling the ROS production by diminishing the mitochondrial large proton gradient. Recently, UCP2 has been linked to other functions as well [Alfadda ad Sallam, 2012].

ROLE OF FREE RADICALS IN DISEASES

Macromolecular "Toxicity".

In DM and obesity, the prevalent metabolic state is the one described by the term "glucolipotoxicity," in which excess extracellular glucose and fatty acids (FAs) exert various damaging effects. Excess glucose increases oxidative stress through several biochemical mechanisms, including glyceraldehydes autoxidation, protein kinase C activation, glycation, methyl glyoxal and sorbitol production, hexosamine pathway, and oxidative phosphorylation [Robertson, 2004]. Likewise, excess FA leads to peripheral insulin resistance and accumulation of lipid in non adipose tissue locations as the liver, heart, and pancreas, potentially resulting in failure of these organs. At the cellular organelles level, lipotoxicity has been recently linked to both oxidative and ER stress [Brookheart et al., 2009]. The link between excess glucose and lipid and inflammation was recently demonstrated in adipocyte where excess glucose and saturated FA, through free radicals generation (in specific ROS) and activation of the nuclear transcription factor NF-κB, induced inflammation as manifested by upregulation of active inflammatory mediators involved in monocyte adhesion and chemotaxis. The Toll-like receptor 4 (TLR4) was implicated in mediating the effect of excess saturated FA—but not excess glucose— on the expression of these inflammatory mediators [Han et al., 2010]. Moreover, and in contrast to excess saturated FAs, polyunsaturated FA were reported to exert on adipocytes anti-inflammatory effects that were linked to the nuclear receptor PPARγ [Han et al., 2010]. These observations were supported by *in vivo* studies on experimental animals [Shi et al., 2006; Saraswathi et al., 2007; Subramanian et al., 2008], but not yet in human.

Role of ROS in Insulin Resistance.

Insulin resistance (IR) is not only a key feature of T2DM, but is also a characteristic of a wide range of clinical conditions such as obesity, metabolic syndrome, and sepsis [Houstis et al., 2006]. IR can also occur, both *in vivo* and *in vitro*, as a consequence of certain experimental treatments with inflammatory cytokines such as tumor-necrosis factor-α (TNF-α), or with glucocorticoids such as dexamethasone. As a matter of fact, it is well established that elevated levels of TNF-α and/or glucocorticoids are detected in patients with the above-mentioned IR-associated clinical states [Stoner et al., 1983; Hotamisligil et al., 1995; Kirwan et al., 2002; Wang, 2005]. Several factors have been demonstrated to play a role in IR. ROS hold a unique position among these factors, based on studies conducted on cell lines or *in vivo*. When the murine adipocyte cell line 3T3-L1 was treated with the ROS H_2O_2 or with ROS inducers, it clearly developed resistance to insulin [Rudich et al., 1998; Lin et al., 2005]. Moreover, markers of oxidative stress have been significantly associated with obesity, IR, DM, and sepsis [Urakawa et al., 2003; Furukawa et al., 2004].

Similarly, conditions that increase ROS levels, for example, diseases with primary defects affecting ROS balance, such as familial amyotrophic lateral sclerosis, were found to be associated with IR [Hand and Rouleau, 2002]. Albeit strong, the association between ROS and IR in various pathologic settings did not initially imply a cause-effect relationship; it just elucidated a strong association state. Nevertheless, such a causal effect was demonstrated few years ago by Houstis and colleagues. Using two approaches, cell lines (3T3-L1) and animal model of genetic obesity (ob/ob mice), the authors have undoubtedly demonstrated that increased levels of ROS were indeed the cause for TNF-α- or dexamethasone-induced IR determined by the lowered glucose uptake rate. Experimental intervening by either pharmacological agents or in transgenic animals designed to decrease ROS levels was shown to substantially prevent the IR status [Houstis et al., 2006]. c-Jun NH2-terminal kinase (JNK) activation, which was detected upon stimulating the cell line with TNF-α or dexamethasone, was suggested to mediate the ROS-induced IR and was demonstrated to be linked to differential translocation of two important transcription factors; the pancreatic and duodenal homeobox-1 (PDX-1), which will be translocated from the nucleus to the cytosol thereby suppressing insulin biosynthesis, and the Forkhead transcription factor Foxo1, which will be translocated in the opposite direction, from the cytosol to the nucleus, thereby contributing to insulin resistance by enhancing gluconeogenesis [Kaneto et al., 2010]. Because the sphingolipid ceramide was reported to be increased in TNF-α- and dexamethasone-induced IR in 3T3-L1 cell line and in diabetic muscle, it was suggested as a potential ROS source in insulin resistance [Summers et al., 1998; Di Paola et al., 2000; Summers and Nelson, 2005].

Role of ROS in Mitochondrial Dysfunction and in Diabetes Mellitus.

Normally, the β-cells of the pancreas adapt their insulin secretion to the fluctuations in blood glucose concentration sensed by their glucose sensor, glucokinase. During hyperglycemia, the rate of insulin-dependent glucose utilization by glycolysis in the β-cells will increase. Compared to other cell types, the β-cells manifest an unusually high proportion of glucose-derived carbon skeleton entering the mitochondria in the form of pyruvate that will then enter the tricarboxylic acid (TCA) cycle. Mitochondrial ETC promotes ATP generation, which will then be exported to the cytosol. Under high ATP/ ADP ratio, the β-cells plasma membrane will be depolarized, and the potassium-ATP channels (KATP) will be closed, allowing the opening of voltage-sensitive Ca2+ channels. Increased intracellular Ca^{2+} is the key trigger for exocytosis and insulin release from the secretory granules [Rorsman, 1997; Maechler and Wollheim,2001]. This is referred to as stimulus-secretion coupling in the β-cells or glucose-stimulated insulin secretion (GSIS), as it was initiated by glucose utilization.

The pivotal role of normal mitochondrial ETC in the pancreatic β-cells glucose homeostasis has been established by a number of elegant studies over the past 30 years. Exposing the mitochondria to poisons or to restricted oxygen supply has

established the finding that blockade of the mitochondrial ETC inhibits GSIS from *β*-cells [Malaisse et al., 1979]. This was later confirmed in experiments using rho0 *β*-cells, where the mtDNA-encoded subunits of the ETC enzymes are suppressed, while insulin biosynthesis and cell viability are preserved. The mitochondrial dysfunction in these cells and the consequent loss of mitochondrial ATP production have resulted in loss of GSIS [Soejima et al.,1996; Kennedy et al., 1998; Tsuruzoe et al., 1998]. The lost response was restored by introducing normal mitochondria into the rho0 *β*-cells, confirming the mitochondrial origin of the defect [Soejima et al.,1996]. The stimulus-secretion coupling in the *β*-cells was further studied in transgenic animals with *β*-cells targeted deletion of the nuclear encoded mitochondrial transcription factor (TFAM), which is the major transcription factor controlling the mtDNA genes expression. The *β*- cells of this animal model manifest diabetic phenotype with both the ATP production and GSIS greatly diminished [Silva et al., 2000]. These animals represent a model for human mitochondrial diabetes, a rare form of DM, that is maternally inherited, caused by mutations in the mtDNA, and usually associated with other pathological findings as bilateral sensory-neural deafness [Wallace, 1999]. In patients with T2DM, the form of disease that affects almost 90% of all diabetic patients, some reports have demonstrated a decrease in the copy number of mtDNA in skeletal muscles and in peripheral blood cells [Antonetti et al., 1995; Lee et al., 1998]. As previously mentioned, accumulation of ROS in the mitochondria (due to excessive production and/or defective defense mechanisms) is accompanied by mitochondrial dysfunction; this was found to be an age-related process [Maechler et al., 1999]. Apparently, with advanced age the *β*-cells will be particularly susceptible to ROS damage, based on their low expression of the antioxidant protective enzymes, which will allow for the buildup of damaging effect of ROS [Tiedge et al., 1997; Acharya and Ghaskadbi, 2010]. The mitochondrial uncoupling protein UCP2 is considered as a negative regulator of insulin secretion. Overexpression of UCP2 in *β*-cells diminishes ATP production and GSIS [Chan et al., 2001]. Likewise, deletion of UCP2 in mice enhances pancreatic islet ATP generation and GSIS. Furthermore, increased UCP2 in obesity was suggested to be one of the links between obesity and *β*-cell dysfunction in obesity-induced T2DM [Zhang et al., 2001]. However, the role of UCPs is not fully understood, and specifically their response to the state of glucolipotoxicity that is highly manifested in uncontrolled DM and obesity requires further studies. It is increasingly acknowledged that diabetic complications are also strongly linked to a state of oxidative stress. Diabetic retinopathy, being a major cause of blindness among adults worldwide, has been the focus of intensive research, which demonstrated that oxidative stress plays a vital role in its pathogenesis. In a recent review article, Zhu and Zou have presented data concerning the pigment epithelium-derived factor (PEDF), which is a small secreted glycoprotein that was shown to exert protective effect on the retina, based on its antioxidant properties in addition to other functions as the neurotrophic, antiangiogenic, antivasopermeability, anti-inflammatory, and antifibrosis properties. Therefore, PEDF or its peptide

derivatives might represent a potential therapeutic approach in the prevention and/or treatment of diabetic retinopathy, an area that still needs further assessment [Zhu and Zou, 2012].

Role of ROS in Obesity and Obesity-Associated Comorbidities.

Obesity—defined as a body mass index of 30 Kg/m^2 or higher—is a chronic disease with serious adverse consequences and is currently a leading cause of preventable deaths

worldwide. It is an established independent risk factor for CVD. Obesity is also associated with a state of chronic inflammation in the adipose tissues as well in other organs, where tissue-infiltrating monocytes/macrophages increase in number and in activity. Several active mediators, chemotactic molecules, cytokines, and adipokines augment the chronic inflammatory state and result in the excessive production of ROS causing systemic oxidative stress. This is considered a potential mechanism linking obesity, vascular abnormalities, and the elevated risk of atherosclerosis and CVD. One of the main sources of ROS in those situations is believed to be the NADPH oxidase (Nox), a multiprotein complex that is expressed both in phagocytes and endothelial cells.

Feeding mice high-fat diet for 22 weeks to cause diet-induced obesity was associated with activation of Nox. The latter is believed to elevate the expression of TLR in the vascular tissues, and probably in adipocytes as well. TLR4, which is the receptor for endotoxin and lipid, and its intracellular signaling consequences induce overexpression of proinflammatory cytokines, as TNF-α and IL6, and of transcription factors such as NF-κB. Therefore, Nox-induced elevated TLR4 expression and signaling might be involved in the obesity-induced inflammation and insulin resistance. Such findings propose the components of Nox system as potential novel therapeutic targets for obesity-associated comorbidities [Chen et al., 2008]. In recent years, novel roles have been assigned to the ROS as they relate to the central nervous system control over our body weight. The site of these new roles for ROS is the hypothalamus, where there are neurons controlling our satiety and others controlling our hunger behavior. Such roles have been implicated as contributing factors underlying diverse findings such as the age-related decreased ability to lose weight and the caloric restriction-induced longevity. Interesting findings demonstrated that different hypothalamic neurons have distinct preference to fuel utilization, so that glucose is the preferred fuel for proopiomelanocortin (POMC) neurons that are responsible for satiety, while FAs are preferred fuel to neuropeptide Y/Agouti-related protein (NPY/AgRP) neurons responsible for feeding. Although ROS are produced in both types of neurons as a result of oxidation of glucose and FA, yet it was demonstrated that the ROS produced in the POMC neurons will be accumulating and hence impairing the POMC neurons over time and this is thought to be responsible, at

least in part, for our inability to lose weight as we get older. On the other hand, the ROS produced in the NPY/AgRP neurons that are active during negative energy balance will be buffered by UCP2 and this is thought to play a role in the mechanism of longevity induced by caloric restriction. This delicate neuronal system, although not completely well-understood, emphasizes the real need to be extra cautious with the use of any antiobesity pharmacological approach attempting to promote satiety or suppress hunger at the hypothalamic level [Chapman, 2008;Horvath et al., 2009].

Role of ROS in Inflammation and Infection

Role of ROS in Inflammation.

Recently, ROS were demonstrated to induce the assembly and activation of inflammasomes, which are multiprotein cytoplasmic complexes involved in mediating cellular inflammation in response to various damaging agents [Sorbara and Girardin, 2011; Tschopp, 2011; Escames et al., 2012; Salminen et al., 2012; Salminen et al, 2012]. The mitochondria are believed to be the main source of inflammasome-activating ROS, although other sources may exist. Excess ROS not only will result in the assembly and activation of the inflammasome but will also inhibit the process of mitophagy, which is a specialized type of autophagy responsible for removal of malfunctioning mitochondria. Therefore, the damaged mitochondria will persist, producing more ROS, and continuing the activation of inflammasome. Alternatively, the cells containing these damaged mitochondria might undergo apoptosis; which is, surprisingly, dependent on ROS as well. Likewise, the voltage-dependent anion channels (VDACs) in the outer mitochondrial membrane are also involved in both inflammation and apoptosis. Although it is so far uncertain what will direct the cell to either chronic inflammation or apoptosis, it is expected that this type of decision is under tight control [Zhou et al., 2011]. In line with the strong association between ROS and chronic inflammation, it was reported that ROS generation correlates with toxicity and pathogenicity of different types of pollutant, such as asbestos and silica particles. In a recent study, Dostert et al. have demonstrated the key role of ROS in mediating the injurious effects of these pollutants that may end in chronic inflammation or even tumor formation. Their findings indicate that upon particles phagocytosis by the immune cells, Nox will be assembled and activated, which will produce ROS in an iron-dependent process [Dostert et al., 2008]. Again, Nox might not be the only source of ROS; other ROS producers may be involved. In any case, the ROS will then activate the inflammasome complex formed of the protein NLRP3, the adaptor ASC, and the substrate procaspase-1. The described stress-related response will end in caspase-1 formation and processing followed by secretion of proinflammatory mediators, including IL-1β and IL-18 [Zhou et al., 2011].

Role of ROS in Infection.

ROS production has been used by human cells to fight infection, both bacterial and viral. Although the bactericidal effect of ROS is known since the 50s of the last century [Haase, 1950], active research in this area is still ongoing, especially with the aim to discover novel agents targeting bacterial strains with multiple antibiotic resistance, a serious clinical problem that is increasingly encountered. Recent experimental methodologies have been applied to this area; for instance, a genome-wide transcriptional profiling of the response of *Staphylococcus aureus* (*S. aureus)* to cryptotanshinone, a medicinal plant-isolated chemical agent exhibiting antimicrobial activity against a broad range of bacteria [Feng et al., 2009]. Cryptotanshinone (CT) demonstrated effective *in vitro* antibacterial activity against all *S. aureus* strains tested. Affymetrix GeneChips were used to determine the global transcriptional response of *S. aureus* to treatment with subinhibitory concentrations of CT. Both antibacterial and active oxygen radical generation functions of CT were positively correlated. Moreover, the *S. aureus* was found to undergo a defensive oxygen-limiting state upon exposure to the drug. Hence, the authors suggested that both actions of the drug, the antibacterial and the oxygen radical generation, may be responsible for its pharmacologic efficiency. This type of studies is promising since it sets the platform for developing and characterizing novel antibacterial agents with optimum activity against antibiotic resistant bacterial strains. The ROS involvement in viral infection has also been studied since quite a long time (late 1980s and early 1990s) [Schwarz, 1996]; more research is still being conducted and producing interesting results, especially in the field of human immunodeficiency virus-1 (HIV-1) infection and treatment. HIV-1 infection is known to be associated with a state of oxidative stress. Interestingly, HIV-1 treatment using highly active antiretroviral therapy (HAART) seems to worsen the oxidative stress status. This was recently published by Mandas and colleagues who have compared the HIV-1-infected patients treated with HAART with untreated patients and with normal control. Moreover, optimal adherence to the HIV-1 therapy further worsened the oxidative stress status as compared to poor adherence [Mandas et al., 2009]. More recently, higher oxidative stress status was demonstrated in patients coinfected with HIV-1 and HCV as manifested by higher oxidized glutathione level and more severe mitochondrial DNA damage as compared to patients who are monoinfected with HIV-1 [Shin et al., 2012].

In conclusion: Glucolipotoxicity is associated with both oxidative and ER stress. This is linked to activation of the transcription factor NF-κB and consequently of the proinflammatory gene expression. *In vitro* polyunsaturated FA anti-inflammatory effect is partly mediated by the nuclear receptor PPAR-γ. TNF-α or glucocorticoids-induced insulin resistance is mediated by excess ROS production, a potential source of which is the sphingolipid ceramide. Excess ROS in turn is thought to work through activation of the JNK signaling pathway, that results in differential translocation of

two transcription factors: PDX-1, and Foxo1. The biochemical consequences that will take place include suppression of insulin biosynthesis and activation of gluconeogenesis, which both enhance the progression of IR into diabetes. In normal β-cells, there is a glucose-stimulated insulin secretion that is dependent on the level of ATP production and is diminished by the mitochondrial UCP2. The age-dependent mitochondrial dysfunction is particularly important in the β-cells due to their relative deficiency of antioxidant protective enzymes. The redox state will not only affect the incidence of DM, but it is also involved in the incidence of diabetic complications. PEDF is believed to be protective against the occurrence of diabetic retinopathy and hence is suggested to be of therapeutic potential and must be further investigated. Activation of Nox enzyme is believed to elevate the expression of TLR4 in vascular tissues and is involved in the obesity-induced inflammation and associated vascular abnormalities. ROS exert different effects on the hypothalamic neurons involved in satiety or hunger behaviors; therefore, caution should be exerted with attempting to design anti-obesity approach working at the hypothalamic level. ROS induce the assembly and activation of inflammasomes and inhibit mitochondrial autophagy, both processes are related to aging and age-related diseases. Certain pollutants-induced chronic inflammation or tumor formation is induced by Nox-released ROS-induced activation of inflammasome complex. Both bacterial and viral infections have been related to ROS generation. Novel approaches are utilized to develop antibacterial agents with optimum activity [Alfadda and Sallam, 2012].

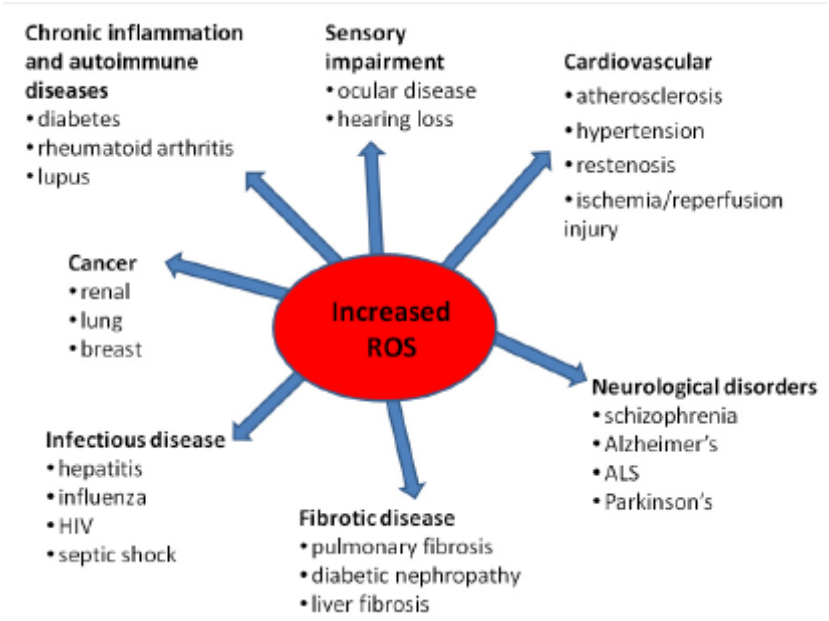


Fig 2. Overproduction of ROS and its contribution to various disease.

EVALUATION OF FREE RADICALS TOXICITY

To establish the mechanism of toxicity as free radicals (in specific ROS) mediated, there are direct and indirect methods. Direct methods relate to ROS measurement such as superoxide, H_2O_2, OH•. These species are very reactive, and their quantitation can be difficult. The only technique that can detect free radicals directly is the spectroscopic technique of electron spin resonance (ESR), sometimes called electron paramagnetic resonance (EPR). The indirect methods are used in order to overcome these problems. Indirect methods usually measure changes in endogenous antioxidant defense systems or measure the products of damage by ROS among the cellular components [Poljsak and Jamik, 2010]. Therefore, changes in endogenous antioxidant defense systems and damages on cellular components, caused by ROS, are measured by indirect methods. The principle behind fingerprinting methods is to measure products of damage by ROS, that is, to measure not the species themselves but the damage that they cause. Measuring the damage caused by ROS instead of direct measuring of ROS seems logical, since it is the damage caused by ROS that is important rather than the total amount of ROS generated. A good marker of oxidative damage must be increased in the presence of oxidative stress, and it must remain unchanged in its absence [Miwa et al., 2008]. The marker must measure a product that is endogenously present, not produced during the isolation procedure. Multiple methods of measurement are available today, each has their own benefits and limitations. Ascertaining the true importance of ROS has been hard, as these evanescent species are difficult to measure in vivo. It is generally accepted that two or more assays should be used whenever possible to enhance their validity, since different parameters are measured by different assays. There are inherent limitations in all methods, and no method can be said to measure accurately the amounts of ROS by itself [Halliwell and Gutteridge, 1985]. For example, in order to determine oxidative stress, both the ROS potential and the antioxidative defense potential should be measured. Besides, it is not possible for a reactive free radical produced in an extravascular space of living tissue, with a lifetime of microseconds, to diffuse into the blood to be detected at the distant site. Numerous in vitro methods are described for the antioxidation potential determination that are easy to perform and largely used in screening. However, the results of such tests are relevant only partially for humans as certain active compounds (e.g., those with large molecular masses) are poorly absorbed from the gastrointestinal tract and/or may undergo metabolic degradation. Therefore, new experimental models are required to provide information if protective effects take place in humans under realistic conditions. There are many challenges regarding the methodology for oxidative state detection on cellular level, for example, which method(s) to use, which antioxidant should be used for a standard, on the most appropriate positive control (e.g., an oxidising agent or catalysts of Fenton reaction), and so forth. There are no specific recommendations

on methods which evaluate best the oxidative stress in biological systems although there are several test systems with different end-points [Poljsak and Jamik, 2010]. Additionally, there are no reference values of what is the optimal antioxidative potential in urine, blood, or even intracellularly. It is not known how many antioxidants and in which combinations are needed for a beneficial antioxidant effect in vivo. Besides, typical oxidative stress status of an individual is not established yet because it is difficult to measure [Arguelles et al., 2007]. Determination of antioxidative potential per se is thus not enough, since it is difficult to establish how the individual antioxidants work: by preventing the formation of ROS, by scavenging free radicals, by inducing the signaling pathways, or by repairing the oxidative damage. The improvement in methodology will likely overcome at least some of these drawbacks.

ANTIOXIDANT THERAPEUTICS

Several natural antioxidants have been investigated *in vitro* or in animal models to assess their potential therapeutic effect in conditions linked to oxidative stress. Interestingly not all antioxidants are identical, results from recent studies emphasize that point, and some will be briefly summarized in the following section. In order to determine the protective role of vitamin E and/or dithiothreitol (DTT), Tsai and colleagues have studied rat hepatocytes that have been exposed to oxidative stress by treating them with Tert-butyl hydroperoxide and have assessed the cellular calcium homeostasis in these cells. Their results indicated that vitamin E not only blocks the elevation of intracellular ionic calcium ions but also prevents the loss of protein thiols from the cellular membranes, leading the authors to suggest that vitamin E conserves the
integrity of cell membranes and this might be important for the maintenance of intracellular calcium homeostasis [Tsai et al., 2010]. Another natural antioxidant, rottlerin, was studied by Maioli et al., in human breast cancer and human colon cancer cell lines, MCF-7 and HT-29, respectively [Maioli et al., 2009]. Rottlerin is a pigment that exerts a pleiotropic inhibitory effect on specific intracellular kinases and hence is thought to interfere with the NF-κB activation process. Similar polyphenolic phytochemical compounds as curcumin, resveratrol, and mangiferin were also reported to exert antioxidant activity that is mediated by NF-κB inhibition [Samuhasaneeto et al., 2009]. However, not all the antioxidant phytopolyphenols are identical in their mechanism of action; resveratrol and rottlerin, in fact, although acting as protein kinase C δ (PKC δ) inhibitors, inhibit NF-κB via different mechanisms. In addition, rottlerin exerts a free radical scavenging effect [Maioli et al., 2009].
Similar to rottlerin, curcumin, which is commonly used as food additive in many parts of the world, exerts antiinflammatory and antioxidant effect by scavenging free radicals and inhibiting NF-κB. Curcumin also inhibits lipid peroxidation as manifested by decreasing the hepatic malondialdehyde (MDA) level in a rat model of alcoholic liver disease. Samuhasaneeto and colleagues have induced liver injury in rats by feeding them ethanol and then assessed the protective effect of orally administered curcumin. On the other hand, and at least in this studied model, curcumin did not affect the SOD activity nor did it affect the PPARγ protein expression level. Curcumin seems to inhibit the early stages of alcohol liver disease in rats. As a matter of fact, early stages of the disease are mainly linked to oxidative stress that is induced by excessive accumulation of ROS. To a lesser extent, curcumin was found to decrease hepatocytes apoptosis that is caused by mitochondrial dysfunction and cytochrome C release [Samuhasaneeto et al., 2009]. While curcumin did not affect the level of SOD activity, another natural antioxidant and anti-inflammatory compound, the purple sweet potato color (PSPC), was recently reported to increase the activity of $Cu^{2+/}Zn^{2+}$ SOD, as well as of catalase. In a DGal-induced

mouse model for aging, oral administration of PSPC resulted in improvement in the mice behavior and cognitive performance, while in the isolated brain tissues, the increased activity of Cu2+/Zn2+ SOD and catalase, the low expression levels of induced NOS (iNOS) and of cyclooxygenase 2 (Cox2), the decreased nuclear translocation of NF-κB, and the lowered content of MDA led the authors to suggest that PSPC, through its antioxidant and anti-inflammatory capacity, ameliorates the cognition deficits and attenuates oxidative damage and inflammation in aging mouse brain [Shan et al., 2009]. Ginsenoside Rb1, a natural plant steroid belonging to the family of glycosides and triterpene saponins, was recently reported by Xia and colleagues to attenuate the myocardial oxidative stress and tissue histological damage in a model of streptozotocin-induced diabetes and myocardial ischemia/reperfusion injury. Since this protective effect was abolished by the eNOS inhibitor, L-NAME, it was suggested that ginsenoside Rb1 exerts its protective effect by enhancing the expression of eNOS and hence increasing the NO content, in addition to its antioxidant effect [Xia et al., 2011]. Interestingly, not all anti-inflammatory agents are antioxidants as well; diclofenac, a nonsteroidal anti-inflammatory drug (NSAID) that is usually prescribed to treat pain, fever, and inflammation is a clear example. It was recently reported that diclofenac resulted in apoptosis of neuroblastoma cell line. Diclofenac-induced apoptosis was related to its ability to cause mitochondrial dysfunction in the form of lowering the mitochondrial membrane potential and consequently releasing cytochrome C, and eventually causing cellular apoptosis. The diclofenac-induced mitochondrial dysfunction was related to its prooxidant activity since it was found to decrease the protein level and activity of mitochondrial SOD, though not its mRNA level. Furthermore, exogenous administration of the antioxidant Trx lowered the diclofenac-induced apoptosis and improved the mitochondrial SOD protein level. Such research has the potential to be of clinical significance as it can be applied in determining the optimum dosage and avoiding side effects and drug interactions caused by diclofenac [Cecere et al., 2010]. Epoetin δ is an erythropoietin that is prescribed to patients who are at increased risk of developing anemia. It is unique because, unlike other erythropoiesis-stimulating agents, epoetin δ is produced by gene-activation technology in a human cell line, and hence it has a human-type glycosylation profile. The antioxidant capacity of epoetin δ was recently assessed in primary human renal tubular cells, in which oxidative stress was induced by treatment with glucose oxidase enzyme; in these cells epoetin δ caused upregulation of several renoprotective genes, some of which, as carboxypeptidase M, dipeptide peptidase IV, and cytoglobin, were reported for the first time to be involved in the antioxidant renoprotection process [Beuf et al., 2010].

Potential Novel ROS Targeted Therapeutics

Taken together, the results of recently conducted research studying the mechanisms involved in mediating the ROS actions offer promising venues as they propose novel potential therapeutic agents for the ROS-linked diseases. Few examples were

presented in this review that should be further studied. The complexity and multifaceted nature of the process of redox regulation make it essential to better understand the key players in the process and then to design targeted means of controlling these players. An obvious example is the JNK signaling pathway, which is activated by various cell stressors including ROS, glucolipotoxicity, and ER stress [Urano et al., 2000; Ozcan et al., 2004; Wellen and Hotamisligil, 2005]. In the case of chronic ER stress, such as that seen in obesity [Chapman, 2008], the ER stress-induced metabolic disturbance would result in insulin resistance and, ultimately, T2DM. Could inhibitors for JNK signaling pathway be designed to specifically ameliorate the ER-stress associated activation of this pathway? Results published by Ozcan and colleagues have suggested that interventions that regulate the ER stress response offer new opportunities for preventing and treating T2DM [Ozcan et al., 2004].

In addition, the serine/threonine kinase, IkappaB kinase β (IKK) pathway is also activated by such stressors and is strongly involved in the development of β cell dysfunction, insulin resistance, and T2DM [Yuan et al., 2001; Wellen and Hotamisligil, 2005; Cai et al., 2005; Arkan et al., 2005]. Therefore, it is possible that such pathways could be targeted as an approach that is complementary to the classical antioxidants in the prevention and/or treatment of ROS-associated chronic diseases.

However, this approach is usually neither predictable nor straightforward; therefore *in vitro*, as well as experimental animal models studies have to be conducted first, and based on their results, carefully designed human intervention studies could be proposed. Even with such design, the hypothesis of targeting a specific signaling pathway with the

objective of ameliorating the redox stress-associated diseases remains subject to either approval or refutation. The recent work by Meijer and colleagues is a clear case for the inherent complexity of metabolic disorders. Based on results from animal studies implicating that the transcription factor activator protein-1 (AP-1) proinflammatory pathway is a promising target in the treatment of vascular diseases as atherosclerosis, this group has evaluated the profile of AP- 1 activation in human aortic wall samples and tested the potential benefit of AP-1 inhibition in a clinical trial involving patients with symptomatic peripheral arterial disease. Using doxycycline (an AP-1 inhibitor) or placebo in those patients did not affect any of the markers of inflammation and vascular dysfunction, except for the C-reactive protein which only revealed a borderline reduction in the group treated with doxycycline. This has led the authors to conclude that their findings did not corroborate the animal studies results and that AP-1 proved not to be a therapeutic target for progressive human vascular diseases [Meijer et al., 2012]. This review summarizes the key roles played by ROS, which are considered major redox species, although not the only ones; the thiol/disulfide redox system plays key roles as well in redox signaling and oxidative stress. In fact, the limited benefit of the classical antioxidant therapeutic

agents used so far in several clinical trials might be the result of the untargeted approach of these agents as mentioned above and importantly due to the fact that they are not affecting the cysteine-based redox regulators. Further research is indeed required for better clarifying the big picture of redox regulation both by ROS and non-ROS mediators.

In conclusion: In the field of antioxidant therapeutics, the key to future success of dietary antioxidant supplementation should be in the suppression of oxidative damage without the disruption of the well-integrated antioxidant defense network. To this purpose, several researches are currently conducted to better understand the mechanism of action of known antioxidant agents and to design and test novel therapeutic agents. As the case in any novel medication testing, systematic approach has to be undertaken utilizing *in vitro* and *in vivo* animal models and human trials; nevertheless, results might not be Predictable [Alfadda and Sallam, 2012].

REDOX STRESS AND AGING

AGING AND LONGEVITY THEORY

There is a growing interest in the topic of aging and in the search for a general theory that can explain what aging is and why and how it happens. To transform the enormous amount of now available diverse observations [Ekerdt, 2002] into a comprehensive body of knowledge, a theory of species aging and longevity is required. This theory may come in the future from a synthesis between systems theory (reliability theory) and specific biological knowledge. Reliability theory is a general theory about systems failure, which allows researchers to predict age-related failure kinetics for a system of given architecture (reliability structure) and given reliability of its components [Gavrilov and Gavrilova, 1991;Gavrilov ad Gavrilova, 2001]. As for specific biological knowledge, many researchers believe that it could be provided by evolutionary theories of aging based on the Darwinian theory of biological evolution by natural selection [Darwin, 1859; Weismann 1882/1889/1892 ; Fisher, 1930; Medawar, 1946/1952; Williams, 1957; Hamilton, 1966; Charleswoth, 1994/1997/2001; Kirkwood, 1977/1979/2000; Rose, 1991; Stearns, 1992/2000/2000; Reznick, 2001; Partiridge, 2001; Zwaan, 1999; Ricklefs, 19998]. In particular, in 1991 Rose provided a balanced scientific discussion of the evolutionary theories of aging, which evolutionary biologists suggest as "the intellectual core of gerontology"[Rose, 1991]. The evolutionary theories of aging are closely related to the genetics of aging, because biological evolution is possible only for heritable manifestations of aging.

Why did this theory take so long to be adopted?

The logical foundations for modern evolutionary theories of aging were completed only in the 1950s [Medawar, 1952; Williams, 1957], almost a century after Darwin suggested his theory of biological evolution [Darwin, 1859]. This happened because for many decades the evolution of aging was a puzzling phenomenon to the classic Darwinian theory of evolution by natural selection. Darwin's theory is based on the idea of random and heritable variation of biological traits between individuals (caused by mutations), with subsequent natural selection for preferential reproduction of those individuals who are particularly fit to a given environment. It is expected (and observed), therefore, that biological evolution acts to increase the fitness and performance of species evolving in successive generations. From this perspective it was difficult to understand why natural selection seemed to result in such bizarrely injurious features as senescence and late-life degenerative diseases, instead of eternal youth and immortality. How does it happen that, after having accomplished the maturity and productive adulthood, the developmental program formed by biological evolution fails even to maintain the accomplishments of its own work? Another

theoretical difficulty in understanding the evolution of aging was the timing problem. Many manifestations of aging happen after the reproductive period of evolving organisms, at ages which are beyond the reach of natural selection. It took almost a century to understand that it is exactly this theoretical difficulty of the timing problem that suggests an evolutionary explanation of aging. The problem of the biological evolution of aging was initially studied in a purely theoretical, non experimental way by August Weismann [Weismann 1882/1889/1892], Ronald Fisher [Fisher, 1930], Peter Medawar [Medawar, 1946/1952], George Williams [Williams, 1957], William Hamilton [Hamilton,1966], Brian Charlesworth [Charleswoth, 199; Charleswoth, 1974], and other researchers. The resulting evolutionary theories of aging were then partially tested by direct evolutionary experiments on laboratory fruit flies [Rose, 1991; Stearns et al., 2000]. Specifically, the researchers found that aging and lifespan do evolve in subsequent generations of biological species in a theoretically predicted direction depending on particular living conditions. For example, a selection for later reproduction (artificial selection of late-born progeny for further breeding) produced, as expected, longer-lived fruit flies [Rose, 1991], while placing animals in a more dangerous environment with high extrinsic mortality redirected evolution, as predicted, to a shorter lifespan in subsequent generations [Stearns et al., 2000].

Evolutionary Theory of Aging vs. Life History Theory

Current evolutionary explanations of aging and limited longevity of biological species are based on two major evolutionary theories: the mutation accumulation theory [Medawar, 1946; Medawar, 1952] and the antagonistic pleiotropy theory [Williams, 1957]. These two theories can be summarized as follows:

• Mutation accumulation theory: From the evolutionary perspective, aging is an inevitable result of the declining force of natural selection with age. For example, a mutant gene that kills young children will be strongly selected against (will not be passed to the next generation), while a lethal mutation with effects confined to people over the age of 80 will experience no selection, because people with this mutation will have already passed it to their offspring by that age. Over successive generations, late-acting deleterious mutations will accumulate, leading to an increase in mortality rates late in life.

• Antagonistic pleiotropy theory: Late-acting deleterious genes may even be favored by selection and be actively accumulated in populations if they have any beneficial effects early in life.

The main difference between the two theories is that in the mutation accumulation theory genes with negative effects at old age accumulate passively from one generation to the next, while in the antagonistic pleiotropy theory these genes are actively kept in the gene pool by selection [Le Bourg, 2001].

There were, however, attempts to find a better name for the antagonistic pleiotropy theory and to specify in more detail how one and the same gene could have both deleterious and beneficial effects. In particular, the disposable soma theory was

proposed [Kirkwood, 1977; Kirkwood and Holliday, 1979], which postulated a special class of gene mutations with the following antagonistic pleiotropic effects: these hypothetical mutations save energy for reproduction (positive effect) by partially disabling molecular proofreading and other accuracy promoting devices in somatic cells (negative effect).

While discussing the disposable soma theory, it is important to keep in mind that it was initially proposed to provide evolutionary justification for another theory of aging called Orgel's error catastrophe theory [Orgel, 1963; Orgel, 1970]. The error catastrophe theory, which ultimately failed at least in its original form, considered aging a result of a breakdown in the accuracy of protein synthesis within somatic cells [Johnson and McCaffrey, 1985; Dice ad Goff, 1987; Gallant and Kurlant, 1997; Gallant and Parker, 1997]. In any case, most researchers agree that the disposable soma theory is a special, more narrowly defined variant of the antagonistic pleiotropy theory of aging [Reznick et al., 2001; Le Bourg, 2001].

The Theory of Programmed Death

August Weismann (1834–1914) was one of the first biologists to use evolutionary arguments to explain aging. His initial idea was that there exists a specific death-mechanism designed by natural selection to eliminate the old, and therefore worn-out, members of a population. The purpose of this programmed death of the old is to clean up the living space and to free up resources for younger generations [Weismann, 1882].

Weismann's Evolutionary Theory and the Cell Division Limit

Suggesting the theory of programmed death, Weismann had to think about the exact biological mechanisms for this death program and came to an idea that there is a specific limitation on the number of divisions that somatic cells might undergo. Specifically, he suggested "that life span is connected with the number of somatic cell generations which follow after each other in the course of an individual life, and that this number, like the life span of individual generations of cells, is already determined in the embryonic cell" [Weismann, 1892]. Remarkably, his purely theoretical speculation over the existence of a cell division limit received a dramatic further development [Gavrilov and Gavrilova, 1991].

Testing the Theory of Programmed Death

One way of testing the programmed death hypothesis is based on a comparison of lifespan data for individuals of a single species in natural (wild) and protected (laboratory, domestic, civilized) environments. If the hypothesis is correct, there should not be very large differences in the lifetimes of adult individuals across compared environments. Indeed, for a self-destruction program to arise, take hold, and be maintained in the course of evolution, it must at least have some opportunity, however small, of expression in natural conditions. Consequently, the age at which such a program is "switched on" cannot be too high. Otherwise, because of the high mortality in the wild from predators, hunger, infections, and harsh natural conditions,

no one would live to the fateful age, and the self-destruction mechanism could not be expressed. It follows from this that lifespan in even the most favorable conditions cannot significantly exceed the ages reached by the most robust individuals in the wild, if, of course, the tested concept is correct. Analysis of the actual data reveals, however, a picture completely opposite to what would be expected from the programmed death theory: the lifespan of organisms in protected environments greatly exceeds the lifespan observed in natural conditions. Observations like this are common for many biological species [Comfort, 1964].

Mutation Accumulation Theory of Aging

According to this theory, persons loaded with a deleterious mutation have fewer chances to reproduce if the deleterious effect of this mutation is expressed earlier in life. For example, patients with progeria (a genetic disease with symptoms of premature aging) live for only about 12 years [Turker, 1996], and, therefore, cannot pass their mutant genes to subsequent generations. In such conditions, the progeria stems only from new mutations and not from the genes of parents. By contrast, people expressing a mutation at older ages can reproduce before the illness occurs, such as the case with familial Alzheimer's disease. As an outcome, progeria is less frequent than late diseases such as Alzheimer's disease because the mutant genes responsible for the Alzheimer's disease are not removed from the gene pool as readily as progeria genes and can thus accumulate in successive generations [Le Bourg, 2001]. In other words, the mutation accumulation theory predicts that the frequency of genetic diseases should increase at older ages. Mutation accumulation theory allows researchers to make several nontrivial testable predictions. In particular, this theory predicts that the dependence of progeny lifespan on parental lifespan should not be linear, as is observed for almost any other quantitative trait demonstrating familial resemblance (for example, body height). Instead, this dependence should have an unusual nonlinear shape with an increasing slope for the dependence of progeny lifespan on parental lifespan for longer-lived parents. This prediction follows directly from the key statement of this theory that the equilibrium gene frequency for deleterious mutations should increase with age at onset of mutation action because of weaker (postponed) selection against later-acting mutations [Charlesworth, 1994].

REDOX-STRESS AND AGING

In living cells, the oxidative damage potential is greater than antioxidant defense, and thus there is a constant free radicals formation in low amounts, which escapes the cell defenses. Estimates of how much oxygen is turned into free radicals vary; yet, even if we accept a conservative value of 0.15%, it still represents a substantial amount of free radicals formation [Zwaan, 1999]. High level of reactive oxygen species (ROS) compared to antioxidant defenses (the so called oxidative stress, or redox stress), is

considered to play a major role in a wide variety of degenerative processes, diseases, and syndromes.

In recent years, oxidative stress has been implicated in cancer, atherosclerosis/arteriosclerosis, heart attacks, strokes, ischemia/reperfusion injury, chronic inflammatory diseases (such as rheumatoid arthritis, lupus erythematosus, and psoriatic arthritis), acute inflammatory problems, photooxidative stresses to the eye (such as cataract), central nervous system disorders (such as certain forms of familial amyotrophic lateral sclerosis, certain glutathione peroxidase-linked adolescent seizures, Parkinson's disease and Alzheimer's dementia), as well as a wide variety of age-related disorders, perhaps even including factors underlying the aging process itself [Ekerdt, 2002].

The process of aging or senescence is complex; it may derive from a variety of different mechanisms and is caused by a variety of different factors. Harman already in the 1950s proposed the free radical theory of aging, which states that organisms age because their cells accumulate free radical damage over time. According to this theory [Darwin, 1859; Weismann, 1882/1889/1892], enhanced and unopposed metabolism-driven oxidative stress plays a major role in diverse chronic age-related disorders. Theory links oxygen consumption, metabolism, ATP, and ROS formation and holds that increases in ROS accompany aging and lead to functional alterations, pathological conditions, and even death [Medawar, 1946]. Furthermore, impairment of mitochondrial activity is assumed to be one of the main causes of the aging process [Medawar, 1952; Williams, 1957; Hamilton, 1966]. Mitochondria are the main site of intracellular oxygen consumption and the main source of ROS formation [Medawar, 1952; Charlesworth, 1994/2001]. Mitochondrial ROSs originate from the electron transport chain and the nitric oxide synthase reactions. Non mitochondrial sources of ROS include environmental pollutants, pollutants in food, radiation, or they are the by-products of other metabolic processes within organisms; however, the majority of free radicals are generated inside the cell rather than coming from the environment [Medawar, 1952; Kirkwood, 1977; Charlesworth and Partridge 1997].

In 2007 Halliwell and Gutteridge suggested to rename the free radical theory of aging as the "oxidative damage theory of aging" [Fisher, 1930], since aging and diseases are caused not only by free radicals but also by other reactive oxygen and nitrogen species. Recent studies, carried out in different species support the idea that oxidative stress is a significant marker of senescence [Gavrilov and Gavrilova, 1991]. Age-related mitochondrial deficits, as increases in oxidative damage and mutational load of mtDNA, were observed in multiple species and organ systems [Kirkwood and Austad, 2000], so supporting the mitochondrial damage theory [Kirkwood and Austad, 2000]. On the other hand, the "vicious cycle" theory, which states that free radical damage to mitochondrial DNA leads to mitochondria that produce more superoxide, has been questioned by some scientists, since the most damaged mitochondria are degraded by autophagy (mitophagy), whereas some defective

mitochondria (which produce less ATP as well as less superoxide) remain to reproduce themselves [Stearns, 2000].

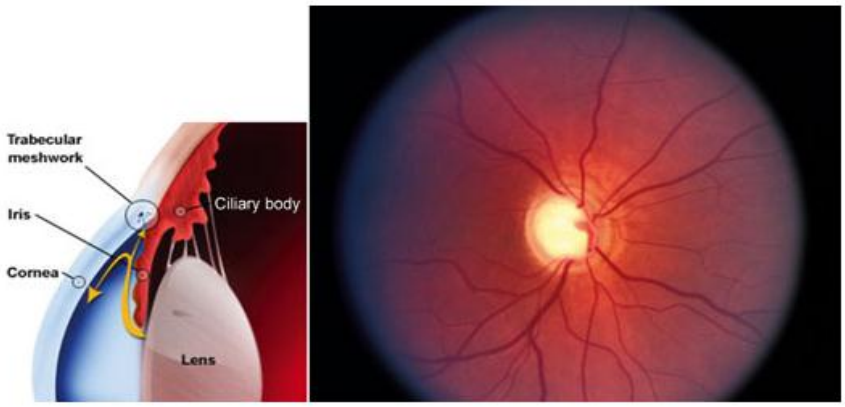
Oxidative stress being considered to play a major role in diverse chronic age-related diseases and aging, its reduction was supposed, and demonstrated, to be associated with prolongation of life expectancy; consequently, ROS-lowering interventions were proposed as antiaging strategies [Orgel, 1963; Orgel, 1970; Charlesworth, 1974; Carnes and Olshansky, 1993; Patnaik et al., 1994; Mueller and Rose, 1996; Ricklefs, 1998; Westendorp and Kirkwood, 1998; Wachter, 1999; Gavrilov and Gavrilova, 1999; Gavrilov and Gavrilova, 1999; Hendry and Berg, 1999; Hendry et al, 1999; Keeley and Bond, 1999; Le Bourg, 2001]. Since a biological antioxidant has been defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate [Fisher, 1930], it is worthwhile to investigate how treatments with diverse antioxidants might reduce oxidative stress in cell environment, how they would influence the health outcome and what adverse effects could they trigger.

REDOX STRESS AND GLAUCOMA

Humans, like other living organisms, are continuously exposed to reactive oxygen species (ROS) as a consequence of biochemical reactions as well as external factors. Oxidative stress is involved in many ocular diseases such as age-related macular degeneration, retinopathy of prematurity, retinal light damage, primary open-angle glaucoma (POAG), and cataract. The definition of oxidative stress implies increased oxidant production in mammalian cells characterized by the release of free radicals, resulting in cellular degeneration [Ohira et al, 2008; Bibizhayev, 2009]. In senile cataract and POAG, the oxidative stress reactions are particularly important.

PATHOPHYSIOLOGY DISTURBANCES IN GLAUCOMA

Glaucoma is a family of progressive ocular diseases that includes POAG, normal tension glaucoma (NTG), angle-closure glaucoma, secondary glaucoma, and glaucoma with onset in infancy; it is characterized by the progressive degeneration of retinal ganglion cells and visual field damage, with the ultimate loss of vision.



In normal eye, fluid flow in the anterior chamber maintains IOP and globe shape and supplies oxygen and nutrients to the non vascularized cornea, lens, and TM [Tian et al., 2000]; more than 80% of the AH is drained from the anterior chamber through the chamber angle tissue into the canal of Schlemm, from which it is drained by collector channels into the veins of the episclera and conjunctiva. There is general agreement that a rise in the resistance in the outflow routes via the trabecular meshwork (TM) and Schlemm's canal is the basis for most cases of open-angle glaucoma, which is traditionally diagnosed by either elevated IOP, optic nerve head atrophy with a defined clinical characteristic (cupping), and loss of visual field. The current treatment strategy involves medical, surgical, or parasurgical interventions, all aimed at reducing the IOP. This is now widely acknowledged to be insufficient, as many patients with glaucoma progress despite treatment, while many patients do not have

elevated IOP in the first place. Moreover, most current IOP-reducing agents either suppress AH production or increase outflow through the ciliary muscle, thus reducing AH flow through the TM, perhaps compromising an already compromised tissue and potentially stressing the cornea and lens. Patients with glaucoma usually receive several of these agents concurrently. Over the past three decades, much research has been devoted to discovering novel therapeutic strategies to improve the clinical outcome of the vast majority of patients with glaucoma. In particular, numerous studies have aimed at identifying pharmacological agents that can directly attenuate RGC death as occurs in glaucoma. Much has been learned from experimental studies on animal models of glaucoma and from information related to the treatment of other central nervous system degenerative diseases. However, till recently, the full comprehension of the pathogenesis of this disease were hampered by the fact that there was no complete agreement on the normal anatomy of the outflow routes and the mechanism for AH drainage [Bill, 1975]. The outflow pathway consists of trabecular lamellae covered with human trabecular meshwork (HTM) cells, in front of a resistor consisting of juxtacanalicular HTM cells and the inner wall of Schlemm's canal [Saccà et al., 2007]. The outermost juxtacanalicular or cribriform region has no collagenous beams, but rather several cell layers which some authors claim to be immersed in loose and increasingly accumulated extracellular material/matrix [Babizhayev et al, 1989; Tian et al, 2000]. The resistance to AH outflow, increased in POAG and considered its most critical risk factor, seems to be causatively linked to changes in the quality and amount of the extracellular matrix proteins (ECM) in the juxtacanalicular region of the TM [Tamm and Fuchshofer, 2007]. By means of immunocytochemistry and quantitative morphometry, increased deposit of fibronectin in trabecular tissues, mainly in the inner wall of Schlemm's canal and juxtacanalicular, or cribriform part of TM, was demonstrated along with aging and glaucoma disease progression [Babizhayev et al, 1989]. Aging is one major risk factor for the development of POAG [Tektas and Lutjen-Drecoll, 2009] and it is assumed that pre-existing age-related changes in the TM play a role in the development of increased outflow resistance and IOP in various types of glaucoma. The functional relationship between ciliary muscle (CM) and TM is also important. One main finding in aged eye is the increased thickness of the sheath of the elastic fibers and the presence of 'sheath-derived plaques' in anterior elastic tendons of the ciliary muscle (CM). The morphologic changes detected in POAG resemble those found in aging, but in POAG there is a significant increase in plaques compared to age-matched controls. This increase is attributable to fine fibrils and other components of the ECM that adhere to the sheaths of the elastic fibers and their connections to the inner wall endothelium. In POAG eyes, there is also a marked loss of TM cells, at places leading to fusion and thickening of trabecular lamellae [Tektas and Lutjen-Drecoll, 2009]. The main resistance to the AH outflow is located in the TM directly underneath the inner wall of Schlemm's canal [Maepea and Bill, 1992; Tian et al, 2000]. The bulk of AH outflow resistance is generated in or near the inner

wall endothelium of Schlemm's canal in normal eyes and probably also in glaucomatous eyes. Fluid flow through this region is controlled by the location of the giant vacuoles and pores found in the cells of the endothelium of Schlemm's canal, but the flow resistance itself is more likely generated either in the ECM of the juxtacanalicular connective tissue (JCT) or in the basement membrane of Schlemm's canal [Johnson, 2006]. Ultrastructural changes in glaucomatous TMs are similar to, but much more intense than, those observed in the normal trabecular meshwork in the elderly [Potau et al., 2000]. These changes include the thickening of basal membranes and trabecular beams, their enlargement or collapse, partial loss of endothelial cells, and the accumulation of materials such as pigment granules and calcium precipitates [Potau et al., 2000; Potau et al., 2000], central nucleus changes such as an increase in electrodense plaques and collagen, and the loss of endothelial cells [Lutjen-Drecoll, 2005]. Finally, recent studies indicate that the TM cytoskeleton may be also involved in the regulation of AH outflow; a cytoskeletal agent acting directly on the TM/ Schlemm's canal to reduce outflow resistance would be more consistent with normal physiologic function, and recent developments indicate that this approach is moving toward success [Tian et al., 2000].

OXIDATIVE DAMAGE IN GLAUCOMA

Oxidative stress may represent an important pathogenetic step in POAG because it could induce TM degeneration, favoring an IOP increase, thus priming the glaucoma pathogenetic cascade. The occurrence of oxidative DNA damage in TM has been demonstrated by measuring the increase in 8-hydroxy-2¢-deoxyguanosine, the most abundant DNA oxidative alteration, which is significantly increased in glaucoma-bearing subjects as compared with unaffected controls [Izzotti et al., 2006]. IOP elevation and visual field damage have been shown to be proportional to the DNA oxidative damage found in the human TM. [Saccà et al., 2005]. Patients with POAG appear to have a genetic predisposition rendering them susceptible to ROS-induced damage because of a more frequent deletion, as compared to controls, of the gene encoding for glutathione-S-transferase M1, a pivotal antioxidant activity. Furthermore, oxidative stress, occurring not only in TM but also in retinal cells, appears to be involved in the neuronal cell death that characterizes POAG. These findings provide a basis for the role of oxidative stress in the pathogenesis of glaucoma and provides new insight into the molecular mechanisms involved.

Mitochondrial DNA deletion was dramatically increased in the TM of patients with glaucoma vs. controls; this finding was paralleled by a decrease in the number of mitochondria/cell and by cell loss. Obtained results indicate that mitochondrion is targeted by the glaucomatous pathogenic processes and that a clinical relevant oxidative damage to the TM exerts a pathogenic role in glaucoma inducing mitochondrial damage and triggering apoptosis and cell loss [Izzotti et al., 2010].

Thus, the increase in oxidative DNA damage in the cellular component of the TM could directly affect the regulation of the ECM structure and the associated regulation

of IOP, leading to the clinical onset of glaucoma [Knepper et al., 1996; Lutjen-Drecoll, 1996]. Indeed, the permeability of the TM appears to be controlled by the endothelial cells lining the lumen of Schlemm's canal. These endothelial cells release vasoactive cytokines and other factors that able to increase the permeability of the endothelial barrier of the Schlemm's canal. Alterations in these cytokines may not allow sufficient flow through Schlemm's canal and, consequently, the IOP may rise to abnormal levels [Alvarado et al., 2005].

The hypothesis that ROS play a fundamental pathogenic role is supported by several lines of evidence, including the following: (i) outflow resistance in the anterior chamber increases in the presence of high levels of hydrogen peroxide; (ii) TM possesses abundant antioxidant activities; (iv) hydrogen peroxide compromises TM integrity.

This interpretation of glaucoma pathophysiology is in agreement with the view that increased IOP is secondary to a decline in TM cellularity [Alvarado et al., 1984; Alvarado et al., 1984]. A variety of statistical tests show that these changes in cellularity are highly significant and specific. These findings are compared to the loss of endothelial cells in the cornea, and they are discussed in relation to the important clinical characteristics of POAG [Alvarado et al., 1984]. The common denominator involved in the cellular alterations in the TM structure and optic nerve damage is, on the one hand, the oxidative stress and, on the other, the vascular damage described in both glaucoma and aging [Flammer, 1994]. Disk hemorrhages cannot only be a sign of damage; they can also provoke ischemia. Besides hypoxia, diseased vessel walls might play a direct role in the pathogenesis of optic nerve head cupping. Finally, a relationship between vascular dysregulation and AH dynamics is conceivable [Flammer, 1994]. Both IOP and vascular factors appear to play an important role in the pathogenesis of glaucomatous optic neuropathy (GON). A variation in ocular perfusion may lead to an increase in free oxygen radicals. This may finally lead to apoptosis [Flammer et al., 1999].

These considerations could bear relevance for POAG prevention and suggest that genetic analyses and the use of drugs or dietary measures attenuating the effects of ROS, if validated in future studies, could be useful tools contributing to the control of this disease [Izzotti et al., 2006].

OXIDATIVE DAMAGE OF TRABECULAR MESHWORK TISSUE AND GENERATION OF FREE RADICAL OXYGEN SPECIES IN PRIMARY OPEN-ANGLE GLAUCOMA

As described above, morphological and biochemical analyses of the TM of patients with POAG revealed loss of cells, increased accumulation of ECM, changes in the cytoskeleton, cellular senescence, and the process of subclinical inflammation. An important factor likely involved in the pathogenesis of POAG is oxidative stress. TM cells submitted to oxidative stress, in fact, showed all these alterations, together with the release of inflammatory markers. By pretreatment with antioxidants,

prostaglandin analogs, beta-blockers, or local carbonic anhydrase inhibitors, these effects were markedly reduced. It is tempting to speculate that the prevention of oxidative stress exposure to the TM may help to reduce the progression of POAG [Welge-Lussen and Birke, 2010].

Chronic changes in the composition of factors present in aqueous or vitreous humor may induce alterations both in trabecular cells and in the cells of the optic nerve head. Free radicals and ROS can affect the cellularity of the human TM. These findings suggest that IOP increase, which characterizes most glaucomas, is related to oxidative and degenerative processes affecting the human TM and, more specifically, its endothelial cells. This supports the theory that glaucomatous damage is the pathophysiologic consequence of oxidative stress. Glaucomatous subjects might have a genetic predisposition, rendering them more susceptible to ROS induced damage. It is likely that specific genetic factors contribute to both the elevation of IOP and susceptibility of the optic nerve/retinal ganglion cells (RGCs) to degeneration.

Oxidative stress, occurring not only in TM but also in retinal cells, appears to be involved in the neuronal cell death affecting the optic nerve in POAG [Izzotti et al., 2006]. The perturbation of the pro-oxidant/antioxidant balance can lead to increased oxidative damage, especially when the first line of antioxidant defense weakens with age. Thus, oxidative stress plays a fundamental role during the arising of glaucoma-associated lesions, first in the human TM and then, when the balance between nitric oxide and endothelins is broken, in neuronal cell. Vascular damage and hypoxia, often associated with glaucoma, lead to the apoptosis of RGCs and may also contribute to the induction of oxidative damage to the human TM [Saccà et al., 2008]. All these considerations could bear relevance for POAG prevention and suggest that genetic analyses and the use of drugs or dietary measures attenuating the effects of ROS, if validated in future studies, could be useful tools contributing to the control of this disease [Izzotti et al., 2006].

REDOX STRESS AND UV

Free radical reactions in the skin are one of the most interesting subjects of skin research because they are involved in various skin diseases, including tumors, as well as skin wrinkling and aging [Darr et al., 1994].
It is well known, in fact, that sun ultraviolet radiation is implicated in many types of skin damage, directly caused by its interaction with cell components, like membrane or DNA, as well as indirectly caused by free radicals production. Following UV-exposure, free radicals and reactive oxygen species (ROS) play a major role in producing lipid radicals (L•), that seem responsible for the destruction of the cell membrane and ultimately of the cell [Ananthaswamy and Pierceall, 1990]. Since the surface of human skin is affected by many surrounding environmental factors, such as solar light, recent increases in UVB (280–320 nm) and UVA (320–400 nm) radiation have been assumed to induce skin premature aging. Although being less implicated in acute skin damages (like UVB-induced erythema), UVA-rays penetrate deeper into the dermal matrix of skin tissues than UVB do, and are therefore the source of deleterious chronic skin effects [Urbach, 1992].
UVA–UVB-generated free radicals in skin should be measured by electron spin resonance (ESR) spectroscopy [Jurkiewicz et al.,1994] and imaging [Herrling et al., 1996]. The short lifetime of the generated free radicals, in fact, namely hydroxyl radical (•OH), superoxide anion radical (O2•−) and lipid radical (L•), demands the application of spin traps for scavenging and accumulating them, in order to get sufficient signal-to-noise ratio [Herrling et al., 2002].
The use of *in vivo* ESR to study processes in the skin appears to be an attractive and effective approach, because of the importance of this organ system and its accessibility, and during the last years this method has allowed the detection of UV-generated free radicals in skin cells and skin biopsies [Herrling et al., 2003]. Specifically, it has been possible to determine that in skin *in vivo* 20% of the free radical generation was due to UVB and these radicals were exclusively present in the epidermis, while UVA radiation was responsible for the remaining 80% of free radicals, which, coherently with UVA penetration rate in the skin, also showed their highest concentration in the epidermis. As a result we see the highest free radical density in the epidermis, which is correlated with the feasibility of generating a skin erythema.

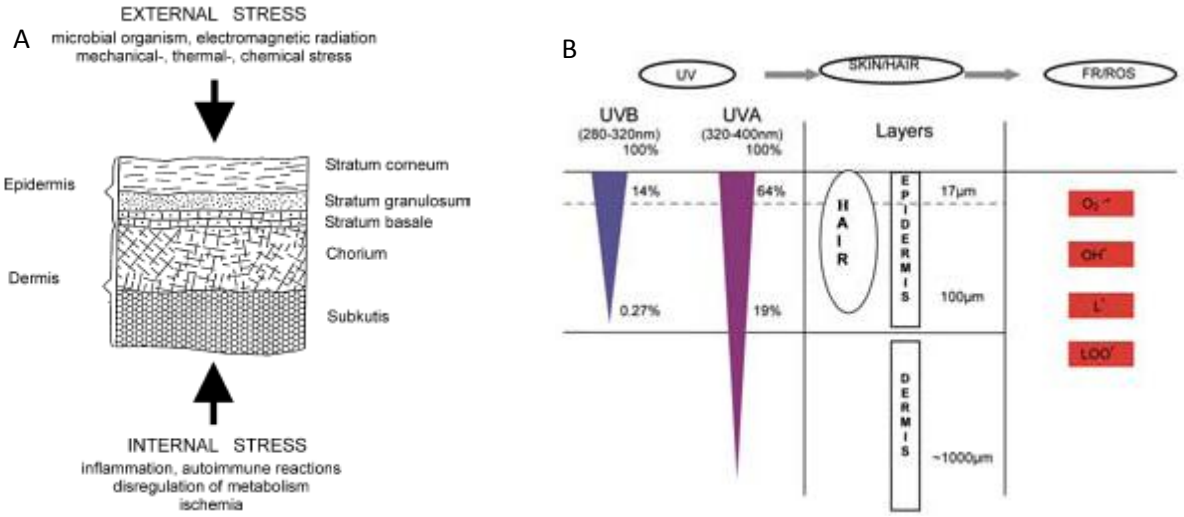


Fig. 3 A) Scheme of skin structure with representation of external and internal oxidative stress. B)Penetration depth of UVA and UVB wavelength in skin/hair and the FR/ROS consequently generated

Multiple lines of defense have evolved, aimed to protect skin from oxidative stress, including prevention, interception and repair. Primary defense mechanisms should prevent oxidative damage by protecting the skin with UV filters against aggressive solar radiation. On the other side, UVB filter protect only against free radicals and damages generated in the epidermis and have no influence on UVA generated free radicals in the dermis.

In skin, antioxidants are the second defense mechanism counteracting processes elicited by reactive oxygen species, such as lipid peroxidation; they in fact are responsible for scavenging of free radicals, like •OH and O2•−, and repair of secondary radical damages, like lipid radicals (Lip•) and lipid peroxy radicals (Lip OO•). Fig. 4 shows the protective effect of the different antioxidants.

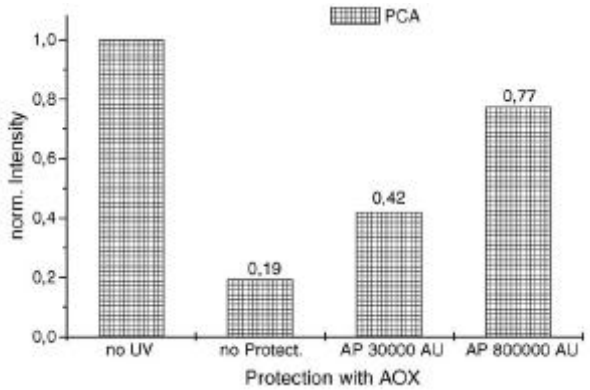


Fig. 4. Normalized signal intensity of the nitroxyl probe PCA in the skin protected by two different antioxidant formulations characterized by their antioxidative power (AP).

Antioxidants existing in the dermis and epidermis scavenge mainly the secondary radicals. Their assignment is avoiding cell damages caused by free radicals. Thus antioxidants give only an indirect protection, through a reduction of the secondary radicals and a repair of damages caused by the primary radicals. This radical protection caused by antioxidants is comparable with the radical protection caused by UVB and UVA/B filter in lower concentrations (see Fig. 4 and Table 1) using the RSF classification.

Protection formulation	SPF/PPD/AP	RSF
UVB filter (6%)	8/–/–	1.2
UVB filter (12%)	12/–/–	1.35
UVA/B filter (5%)	5/5/–	3
UVA/B filter (14%)	13/9/–	20
UVB filter (6%) + UVA/B filter (14%)	24/9/–	27
Antioxidant I	–/–/30000	1.4
Antioxidant II	–/–/800000	3.5

Table 1. Sun protection factor (SPF) and radical sun protection factor (RSF) of different UV filter and antioxidant formulations

Apart from using chemical and/or physical sunscreens to diminish the intensity of UV-radiation reaching the skin, supplementation of the skin with antioxidants, and thereby strengthening of its natural antioxidative potential, is an emerging approach to reduce ROS-induced skin damages caused by UV-radiation [Fuchs et al., 1989; Shindo et al., 1993].

ANTIOXIDANTS

RESVERATROL

Resveratrol was initially characterized as a phytoalexin (Langcake et al., 1976) , *i.e.* an antimicrobial substance synthesized by plants in response to infection. There were several pioneering reports on resveratrol, including a study of resveratrol as an inhibitor of arachidonate metabolism *via* interactions with 5-lipoxygenase and cyclooxygenase (COX) pathways in leukocytes (Kimura et al., 1985) . However, resveratrol attracted little interest until 1992, when it was postulated to explain some of the cardioprotective effects of red wine (Siemann and Creasy, 1992) . Since that time, many studies have shown that resveratrol can prevent or slow the progression of a variety of conditions, including cancers, cardiovascular diseases, and ischemic injuries (Jang et al., 1997; Wang et al., 2002; Sinha et al., 2002; Inohue et al., 2002; Bradamante et al., 2004) , as well as enhance stress resistance and extend lifespan. Attempts to demonstrate favorable effects *in vitro* have met with almost universal success and have led to the identification of multiple direct targets of resveratrol, such as COX, peroxisome proliferator-activated receptor (PPAR), endothelial nitric oxide synthase (eNOS) and silent mating type information regulation 2 homolog 1 (SIRT1) (De la Castra and Villega, 2005; Baur and Sinclair, 2006) .

From COX suppressor to PPAR activator

COX, a key enzyme in prostaglandin (PG) synthesis, has two isoforms, COX-1 and -2. COX-1 is constitutively expressed in most cells, whereas COX-2 is induced by inflammatory stimuli such as endotoxin lipopolysaccharide (LPS), suggesting that COX-2 plays a critical role in inflammation (Simmons, 2004; Smith, 2008) . However, growing evidence indicates that COX-2 expression is regulated differently among cell types, and that COX-2 also plays key roles in tumorigenesis (Oshima et al., 1996) , development (Morham et al., 1995; Dinchuk et al., 1995; Lim et al., 1997), and circulatory homeostasis (Dubois et al., 1998; Grosser et al., 2006) . Anti-inflammatory and cancer preventative properties of resveratrol have been demonstrated in a rat model of carrageenan-induced paw edema and in a mouse skin cancer model using dimethyl benzanthracene (DMBA) and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), respectively. In both models, the effects of resveratrol were attributed to the inhibition of PG synthesis *via* the inhibition of COX-1(Jang et al., 1997). Szewczuk *et al.(* Szewczuk *et al.*, 2004) showed that resveratrol could discriminate between COX-1 and COX-2, suggesting that resveratrol leads to the elimination of PG synthesis *via* COX-1. This is consistent with a report showing that COX-1 as well as COX-2 is involved in tumorigenesis (Tiano et al., 2002) .

PPARs are members of a nuclear receptor family of ligand dependent transcription factors (Mangelsdorf et al., 1995) . The PPAR subfamily comprises three isoforms, PPARα, β/δ, and γ, which play various roles in lipid and carbohydrate metabolism,

cell proliferation and differentiation, and inflammation; they are considered molecular targets against lifestyle-related diseases (Michalik et al., 2006; Sonoda et al., 2008) . The PGD2 metabolite 15-deoxy-Δ12,14 PGJ2 (15d-PGJ2) has been identified as a potent natural ligand of PPAR *γ (*Forman et al., 1995; Kliewer et al., 1995) . Inoue *et al*. reported that 15d-PGJ2 suppressed the LPS-induced expression of COX-2 in macrophage-like U937 cells, but not in vascular endothelial cells, and that the expression of COX-2 was regulated by a negative feedback loop mediated through PPAR*γ*, especially in macrophages (Inohue et al., 2000) . These findings indicate that PPARs participate in cell type-selective control of COX-2 expression and suggest that resveratrol may be an activator of PPARs. This concept was confirmed *in vitro* by cell-based reporter assays using human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs), as 5 *μ*M resveratrol activated PPAR*α*, *β*/*δ*, and *γ* in these cells (Inohue et al., 2003; Tsukamoto et al., 2010) . In a study using PPAR*α*- knockout mice, resveratrol treatment (20 mg/kg weight/day for 3 d) was shown to protect the brain against ischemic injury through a PPAR*α*-dependent mechanism, indicating that resveratrol activates PPAR*α* *in vivo* (Inohue et al., 2003).Concerning tissue selective expression of COX-2, we reported that physiological shear stress induced COX-2 expression for the production of prostacyclin (Inohue et al., 2002), as well as PGD2 (Taba et al., 2000), in vascular endothelial cells. Corticosteroids have been shown to induce COX-2 expression in cardiomyocytes (Sun et al., 2008; Yoshikawa et al., 2009) , and the production of PGD2 *via* COX-2 was involved in protection against ischemia/reperfusion injury in the heart (Tukudome et al., 2009) . Clinical investigations have demonstrated an association between the risk for cardiovascular disease and COX-2 selective inhibitors (Solomon et al., 2005; Bresalier et al., 2005) . These results indicate that because resveratrol seems to act as a tissue-selective suppressor of COX-2, it may be a more effective inhibitor than classical COX-2 selective inhibitors (Fig. 5).

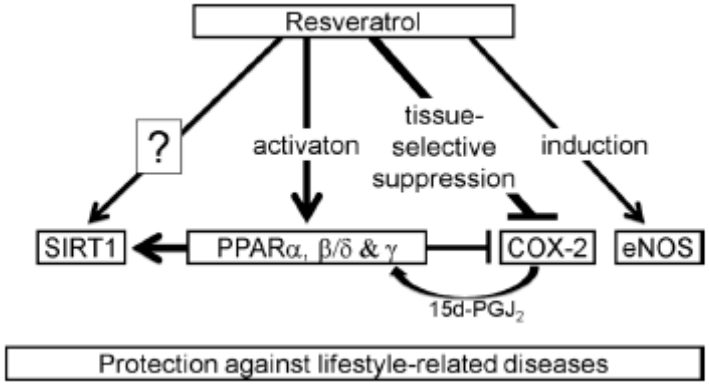


Fig. 5 Possible Molecular Targets for Protective Effects of Resveratrol

Tsukamoto *el al*. found that 5 *μ*M resveratrol also selectively activates PPAR*β*/*δ* (Tsukamoto et al., 2010) , that in Kupffer cells and adipose tissue macrophages

directs the expression of an alternative phenotype, which dampens inflammation and maintains homeostasis, avoiding metabolic problems such as insulin resistance (Kang et al., 2008; Odegaard et al., 2008) . Furthermore, prostacyclin analogues have been reported as ligands for PPARβ/δ (Hertz et al., 1996) . Vaticanol C, a resveratrol tetramer, activates PPARα and PPARβ/δ *in vitro* (5 μM) and *in vivo* (0.04% of diet for 8 weeks), but has no effect on SIRT1 activation (Tsukamoto et al., 2010). Polyphenolic compounds such as apigenin, chrysin (Liang et al., 1999; Woo et al., 2005) , and humulon (Yamamoto et al., 2000) (Fig. 6) suppress COX-2 expression and also activate PPARα and/or γ (Liang et al., 2001; Yajima et al., 2004) . Similarly, essential oil components such as carvacrol and citral activate PPARs and suppress COX-2 expression (Hotta et al., 2010; Katsukawa et al., 2010; Katsukawa et al., 2011) . These findings suggest that various food-derived components, including resveratrol, have similar effects on PPARs and COX-2.

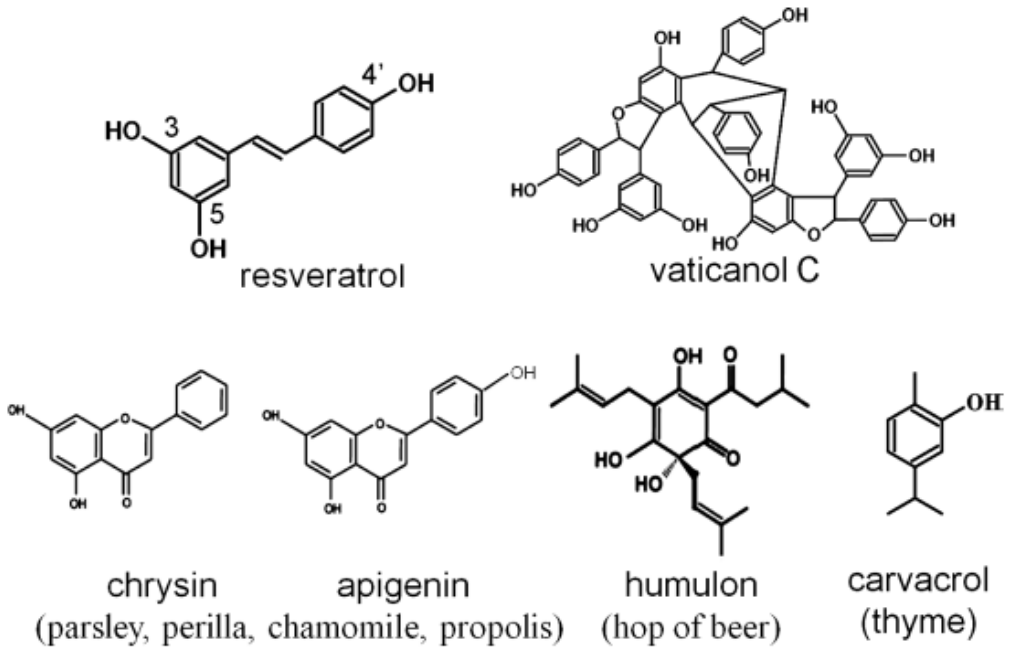


Fig 6 Chemical Structures of Resveratrol and Other Related Phenols

Link to eNOS

A growing body of evidence supports the theory that polyphenolic compounds have activities that maintain healthy cardiovasculature. Epidemiological studies have demonstrated that dietary polyphenol intake, especially red wine consumption, may improve endothelial function and reduce the risk between red wine consumption and incidence of coronary heart disease and atherosclerosis (French paradox) (Richard, 1987; St Leger et al., 1979). Resveratrol, a key compound implicated in the cardiovascular benefits associated with red wine consumption, exerts cardiovascular

protection by diverse mechanisms *in vivo* (Kopp, 1998; Frémont, 2000; Sato et al., 2002; Opiet and Lecoue, 2007).

At pharmacological doses, resveratrol increases vascular nitric oxide (NO) levels and improves NO bioavailability in animal models (Hattori, 2002; Zhang et al., 2003; Zou et al., 2003; Miatello et al., 2005; Rush et al., 2007; Xia et al., 2010) . NO in the vasculature is constitutively synthesized by endothelial NO synthase (eNOS) and plays a crucial role in maintaining cardiovascular homeostasis (Dudzinski et al., 2006; Dudzinski and Michel, 2007). NO relaxes vascular smooth muscle cells, thereby upregulating blood flow, and it prevents thrombogenic and atherogenic processes by vasodilatory and anti-aggregatory effects. Accordingly, these effects of NO may contribute to the cardioprotective effect of resveratrol. *In vitro* studies have been conducted to determine whether resveratrol acts directly on blood vessels or endothelial cells, facilitating NO production. Most reports focusing on short term effects (within 1 h) have demonstrated that resveratrol induces increased NO bioavailability or production. Chen and Pace-Asciak reported that 30 μM resveratrol inhibited the contractile response to phenylephrine in isolated rat aorta (Chen and Pace-Asciak, 1996). Similarly, resveratrol caused relaxation of isolated human saphenous vein and internal mammary artery rings (Rakici et al., 2005), as well as relaxed porcine arterial rings pre-contracted with KCl (Li et al., 2006). In these studies, the inhibitory effect of resveratrol was reversed by removal of the endothelium or inhibition of NOS. Orallo *et al.* reported that resveratrol (1—30 μM) relaxed the contractile response of rat aortic rings to phenylephrine and KCl in a NO dependent manner.

These results suggest that NO can mediate the biological activities of resveratrol, although it is unclear in which way it affects NO production in endothelial cells (Orallo et al., 2002). However, resveratrol did not affect eNOS activity, but instead inhibited NADH/NADPH oxidase and the subsequent decrease in superoxide generation, leading to an improved NO bioavailability. Wang *et al.* reported that 100 nM resveratrol caused the phosphorylation of Akt, extracellular signal-regulated kinase (ERK)1/2, and eNOS within 15 min, resulting in increased NO production in bovine aortic endothelial cells (Wang et al., 2011). Klinge *et al.* claimed that even at nanomolar concentrations, resveratrol acted as a phytoestrogen in endothelial cells, increasing NO production through membrane estrogen receptors (ERs) (Klinge et al., 2005; Klinge et al., 2008). Resveratrol induced the ER-mediated rapid activation of Src and mitogen-activated protein kinase ERK1/2, leading to eNOS activation in endothelial cells. However, in other studies, resveratrol-stimulated NO production was not prevented by pretreatment with an ER antagonist (Wallerath et al., 2002; Li et al., 2006; Takahashi et al., 2009). Compared with estradiol, resveratrol binds to ERα with a much lower affinity, having an IC50 of approximately 100 μM (Ashby et al., 1999), therefore it is not yet clear how ER mediates the effect of nanomolar resveratrol.

In contrast, other researchers have not detected any resveratrol-induced vasodilation. According to (Fitzpatrick et al., 1993) and (Taubert et al., 2002), in fact, resveratrol, even at 100 μM, failed to relax isolated rat aortic rings, and had no effect on the contractile response or NO production in isolated porcine coronary artery. Takahashi *et al*. (Takahashi et al., 2009) found that resveratrol at low concentrations (<20 μM) had no effect, but at high concentrations (>50 μM) increased NO production in endothelial F-2 cells. Moreover, resveratrol at low concentrations inhibited the increase in NO production in response to vascular endothelial growth factor. Overall, it remains unclear whether resveratrol rapidly increases NO production.

A major complication in the studies using resveratrol at high concentrations is that cell viability and damage were not examined. It is well known that resveratrol has cytotoxic actions toward cancer cells, and resveratrol at high concentrations can reduce cell viability and induce disruption of the plasma membrane, resulting in Ca2+ influx in endothelial F-2 cells (Takahashi et al., 2009). Furthermore, an injurious effect of resveratrol was reported in HUVECs (Yang et al., 2010). These observations suggest that an increased cytosolic Ca2+ concentration subsequent to cell damage may result in Ca2+-dependent eNOS activation in resveratrol-treated endothelial cells. In such a case, resveratrol at high concentrations may transiently increase NO production, but this effect would not be beneficial. Moreover, although high concentrations of resveratrol can have pharmacological effects, high concentrations are not produced by dietary intake; serum resveratrol concentrations are only 20—50 nM after oral administration of 25 mg of resveratrol in healthy volunteers (Goldberg et al., 2003; Walle et al., 2004). In general, routine consumption of red wine is required for cardioprotection, suggesting that genes specifically expressed in the vasculature play important roles in the beneficial effects of resveratrol. Consequently, the long-term effects of resveratrol have been examined. Wallerath *et al.* reported that the exposure of cultured endothelial cells to resveratrol for 24—72 h upregulated eNOS mRNA and protein expression levels, resulting in increased bioavailability of NO (Wallerath et al., 2002). However, their experiments were not conducted under dietary conditions, as the biological activity of resveratrol was observed between 10 and 100 μM. Räthel *et al.(*Rathel et al., 2007) and Appeldoorn *et al. (*Appeldoorn et al., 2009) confirmed that resveratrol at high concentrations significantly enhanced eNOS gene expression and enzyme activity, and NO production. In contrast, Nicholson *et al.(*Nicholson et al., 2010) reported that the exposure of HUVECs to nanomolar concentrations of resveratrol for 24 h increased the eNOS mRNA level, although neither eNOS protein nor NO production were determined. Nakata *et al*. also examined the long-term effect of nanomolar resveratrol on functional eNOS expression in HUVECs as a model of routine wine consumption (Takahashi and Nakashima, 2011). Resveratrol at 50 nM for 24 h did not alter eNOS protein levels or NO production, whereas daily treatment for 5 d significantly increased both eNOS protein and NO production without a loss of viability. Thus, eNOS induction may result from the cumulative effect of nanomolar concentrations of resveratrol. These

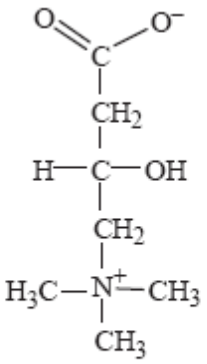
findings related to resveratrol account in part for the cardiovascular benefits of routine consumption of red wine. Further investigations are needed to resolve the effect of resveratrol on endothelial NO production.

Link to sirt1 and other targets

In lower organisms resveratrol has been considered a caloric restriction (CR) mimetic, primarily on the basis of its activation of sirtuin proteins and its capacity to extend lifespan (Horwitz et al., 2003; Wood et al., 2004). Corton *et al.* reported that PPARα mediates some CR effects and proposed that a pharmacological approach toward mimicking many of the beneficial effects of CR may be possible (Corton et al., 2004). Tanno *et al.* reported that manganese superoxide dismutase (Mn-SOD) was induced by 100 μM resveratrol *via* nuclear translocation and activation of SIRT1 in mouse myoblast C2C12 cells, and that the oral administration of resveratrol (0.4% in diet for 35 weeks) to TO-2 hamsters increased cardiomyocyte Mn-SOD levels, suppressed fibrosis, preserved cardiac function, and significantly improved survival (Tanno et al., 2010) . These results are consistent with the finding that hepatic expression of SIRT1 and Mn-SOD genes was induced by 0.02% resveratrol in the diet for 4 weeks in wild-type mice, but not in PPARα knockout mice (Tsukamoto et al., 2010).

However, according to Picard (Picard et al., 2004) 50 μM resveratrol promoted fat mobilization in white adipocytes by repressing PPARγ in 3T3-L1 cells, while Nakata *et al.* found that 5 μM resveratrol activates PPARα, β/δ, and γ in BAECs (Inohue et al., 2003; Tsukamoto et al., 2010). Other candidate targets of resveratrol such as quinone reductase 2 (QR2) (Buryanovskyy et al., 2004), AMP-activated protein kinase (AMPK) (Zang et al., 2006), and PPARγ coactivator (PGC)-1α (Lagouge et al., 2006) have also been reported. Resveratrol inhibits purified QR2, with a dissociation constant of 35 nM (Buryanovskyy et al., 2004); 10 μM resveratrol as well as apigenin stimulated AMPK in HepG2 hepatoma cells (Zang et al., 2006); and 50 μM resveratrol induced PGC-1α-responsive genes such as medium chain acyl-CoA dehydrogenase, cytochrome C, and estrogen receptorrelated receptor α in C2C12 cells infected with an adenovirus expressing PGC-1α. (Lagouge et al., 2006)

CARNITINE

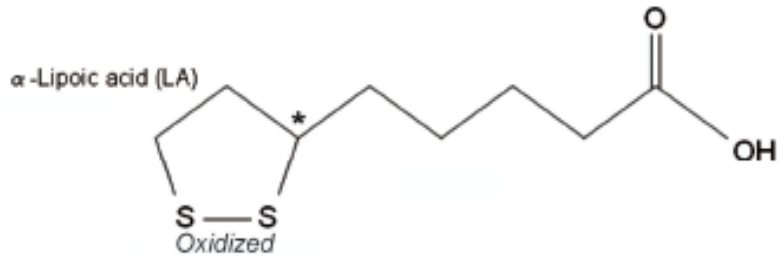


L-Carnitine

L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is derived from both dietary sources (75%) and endogenous biosynthesis (25%) in human body and plays an essential role in human intermediary metabolism (Bremer, 1983; De Vivo and Tein, 1990). L-carnitine's physiological roles are especially important in peripheral tissues where the oxygen tension is lower, because it shuttles the long-chain fatty acids across the inner mitochondrial membrane for oxidation and ATP production. Despite the low level of β-oxidation in brain, L-carnitine is actively transported through the blood–brain barrier and accumulates in neural cells (Shug et al., 1982; Mroczkowska et al., 1997). As hypothesized (Shug et al., 1982; Nalecz and Nalecz, 1996), a major modulatory role for l-carnitine in neural function may be played through l-carnitine-mediated transfer of acetyl groups for acetylcholine synthesis, as well as by influencing signal transduction pathways and gene expression (Binienda and Ali, 2001). Furthermore, l-carnitine is an important cofactor of peroxisomal oxidation especially for very long-chain fatty acids (C_n>C_{22}) (Ramsay, 1999). Obviously the major role played by l-carnitine as cofactor and in the transportation of free fatty acid (FFA) from cytosol to the mitochondria leads to decrease both in oxygen concentration and ROS formation (Mayes, 2000). Antioxidants can protect the human body from free radical effects and retard the progress of many chronic diseases as well as lipid peroxidation (Pryor, 1991; Kinsella et al., 1993; Lai et al., 2001; Gülcin et al., 2003). The most commonly used antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone. However, BHA and BHT were suspected of being responsible for liver damage and carcinogenesis (Wichi, 1988; Sherwin, 1990). Therefore, there is a growing interest on natural additives as potential antioxidants. (Grice, 1986; Moure et al., 2001; Gulcin et al., 2002; Oktay et al., 2003). Also, l-carnitine has a protective effect on the activity of mitochondrial enzyme succinate dehydrogenase, as well as the activity of the antioxidant enzymes catalase and superoxide dismutase against 3-NitroPropionicAcid-induced neurotoxicity (Binienda

and Ali, 2001). Moreover, it can be implemented to minimize age-associated disorders, for which free radicals are the major cause (Kalaiselvi and Panneerselvam, 1998). In addition, recent studies have shown that acetyl-l-carnitine, one of the short-chain acyl esters, enhances learning capacity in aging animals (Ando et al., 2001), improves the symptoms of nerve-degenerative disorders such as Alzheimer's disease (Pettegrew et al., 2000) and attenuates the neurological damage seen following brain ischemia and reperfusion (Calvani and Arrigoni-Martelli, 1999). In particular, Yasuia and co-workers demonstrated that the improvement in cognitive ability seen with acetyl-l-carnitine may occur through an amelioration of cellular dysfunction, via an inhibition of the increase in lipid hydroperoxidation observed in the brain tissue of untreated senescence-acceleration-prone mice (Yasuia et al., 2002).

α-LIPOIC ACID



α-Lipoic acid (LA) (IUPAC name: 5-(1,2-dithiolan-3-yl)pentanoic acid) was first isolated from bovine liver in 1951 [Reed et al., 1951]. Other names for LA include thioctic acid, 6,8-thioctic acid, 6,8-dithioctane acid and 1,2-dithiol-3-valeric acid. LA is an eight-carbon disulfide, highly reactive because of the tension of the S-S-C bond in the heterocyclic disulfide circle; it is relatively stable as a solid, but polymerizes when heated above its melting point (47.5°C) or under the influence of light when it is dissolved in a neutral solution. LA contains a single chiral center, thus resulting in two possible optical isomers: R-LA and S-LA. The R-isomer is synthesized endogenously and binds to proteins. For therapeutic purposes, *α*-Lipoic acid is usually administered as a racemic mixture of R-LA and S-LA [Singh and Jialal, 2008]. However, no difference between the plasma concentrations of R- and S-LA was observed following intravenous administration. LA is a small molecule that contains two oxidized or reduced thiol groups. Its oxidized form is usually defined as α-lipoic acid or just lipoic acid, and the reduced form of LA is known as dihydrolipoic acid (DHLA). LA and DHLA are amphipathic molecules that possess both hydrophilic and hydrophobic properties [Packer, 1998]. The biological effects of LA are primarily associated with its antioxidant properties and DHLA is the predominant form that interacts with reactive oxygen species (ROS), but the oxidized form of LA can also inactivate free radicals [Packer et al., 2001]. LA also exhibits antimutagenic and anticarcinogenic activities [Miadokova et al., 2000; Cremer et al., 2006; Ovotny et al., 2008]. Bilska et al. [Bilska et al., 2007] suggest that the biological action of LA may contribute to its influences on sulfane sulfur metabolism and the activity of the mitochondrial enzyme rodanase.

LA is naturally found in mitochondria, where it is bound to the subunit E2 and acts as the coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [Packer et al., 1995]. Humans can synthesize LA *de novo*, in liver and other tissues, from fatty acids and cysteine, but only in very small amounts; therefore, LA needs to

be absorbed from exogenous sources [Carreau, 1979). LA is primarily metabolized in the liver, *i.e.* the main detoxifying organ for toxic substances and drugs, through mitochondrial β-oxidation. Following the oral administration of a single dose of 1 g of R-LA to a male volunteer, 3-ketolipoic acid and the dimethylated products 2,4-bismethylmercapto-butanoic acid and 4,6-bismethylmercapto-hexanoic acid were detected in his plasma [Packer et al., 1995; Biewenga, 1997].

Bioavailability and safety of oral supplementation of LA

Dietary LA is obtained from both animal and plant sources. LA is found primarily in animal-derived foods, such as red meat and liver, heart and kidney. The most abundant plant sources of LA are spinach, broccoli, tomatoes, brussel sprouts, potatoes, garden peas and rice bran [Kataoka, 1998; Lachman et al., 2000]. LA administered as single-dose tablets (from 50 to 600 mg) was entirely absorbed after 30 min to 1 h [Breithaupt-Groglee, 1999]. LA absorption when prepared as an aqueous solution rather than in a gelatin has been shown to be more effective. The half-life of LA in plasma is 30 min, and the endogenous plasma levels of LA and DHLA are 1–25 × 10^9 and 30–140 × 10^9 g/ml, respectively [Teichert and Preiss, 1992].

LA exhibits low toxicity in low doses. Treatment of male or female Wistar rats with LA administered at the doses of 31.6 or 61.9 mg/kg/d for four weeks did not cause any adverse effects [Cremer et al., 2006]. In addition, the long-term (two-year) administration of up to 60 mg/ kg/day did not cause any adverse effects [Cremer et al., 2006]. Higher doses of LA (121 mg/kg) caused significant changes in liver enzyme activities. After four weeks of administration, the concentrations of alanine aminotransferase (ALAT) and glutamate dehydrogenase (GLDH) were increased, and some histopathological changes in the liver and mammary glands were observed [Cremer et al., 2006]. Following the oral administration of LA to rats, the measured LD_{50} value was 2,000 mg/kg of body weight, indicating that this antioxidant has very low acute toxicity. Doses of 2,000 mg/kg produced sedation, apathy, piloerection, hunched posture and/or eye closure in some rats [Cremer et al., 2006]. In clinical trials in which LA supplementation up to 2,400 mg/day was used, no adverse effects compared with the placebo were observed [Ziegler et al., 1992; Ghibu et al., 2009; Shay et al., 2009]. Similarly, LA administered intravenously at the doses of 600 mg/day for three weeks did not cause serious side effects [Borcea et al., 1999]. However, the intraperitoneal administration of racemic LA at a high chronic dose (100 mg/kg/day for 2 weeks) in aged rats caused an increase in plasma lipid hydroperoxide level and oxidative protein damage in the heart and brain [Cakatay et al., 2005; Kayali et al., 2006]. One possible explanation for these effects is that DHLA, a derivate of LA, is capable of removing the Fe^{2+} from ferritin and reducing ferric to ferrous increasing the possibility of oxidative damage [Cakatay et al., 2005].

LA in humans is readily absorbed from the diet and is converted to DHLA by reduced nicotinamide adenine dinucleotide or by reduced nicotinamide adenine. The

mitochondrial reduced form of nicotinamide adenine dinucleotide-dependent dihydrolipoamide dehydrogenase demonstrates a marked preference for R-LA, whereas the cytosolic reduced form of nicotinamide adenine dinucleotide-dependent glutathione reductase shows greater activity toward the (S)-(+)-LA stereoisomer. The activity of this reductase is particularly important in the heart, kidney and liver. The amount of LA available in dietary supplements (200–600 mg) is likely to be up to 1,000 times greater than the amount that could be obtained from diet alone [Singh and Jialal, 2008]. In other studies, it was also reported that the dietary supplementation of LA induced a decrease in oxidative stress while restoring reduced levels of other antioxidants. Supplementation of 600 mg/d "per os" LA for 2 months decreased urinary F2-isoprostane concentration (a biomarker of lipid peroxidation) and increased the lag time of LDL oxidation in healthy men [Marangon et al., 1999].

Antioxidant action of LA and DHLA

According to Packer et al. [Packer et al., 1995], a therapeutic antioxidant should be absorbed from the diet and easily converted by cells and tissues into a usable form. It should also possess a variety of antioxidant characteristics, including interactions with other antioxidants, in both the membrane and aqueous phase and low toxicity. LA fulfills all of these requirements, making it a potentially highly effective therapeutic antioxidant. This characteristic makes DHLA one of the most potent naturally occurring antioxidants. LA as an antioxidant is able to directly scavenge ROS, regenerate endogenous antioxidants, such as glutathione, and vitamins E and C, and possess metal chelating activity [Biewenga et al., 1997; Bast ad Haenen, 2003; Bilska and Wlodek, 2005]. LA and DHLA create a potent redox couple that has a standard reduction potential of 0.32 V, whereas the redox potential of reduced glutathione/glutathione disulfide (GSH/GSSG) is 0.24 V. Therefore, LA/DHLA is often called the "universal antioxidant", and it seems that LA/DHLA redox might regenerate several other antioxidants, such as vitamin C and vitamin E [Ghibu et al., 2009]. DHLA is not destroyed while quenching free radicals, but it can be recycled from LA [Shupke et al., 2001]. It has been shown that DHLA is an essential cofactor for mitochondrial bioenergetic enzymes, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Moreover, the beneficial action of LA may result in its ability to reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase/endothelial cell-mediated ROS generation, restore GSH/GSSG contents and enhance the mitochondrial expression of key antioxidant enzymes, including glutathione reductase [Bitar et al., 2010]. Furthermore, the beneficial action of LA was implicated during lipopolysaccharide (LPS)-induced oxidative stress [Skibska et al., 2006; Goraca et al., 2009; Goraca et al., 2009].

LA can scavenge a number of free radicals, and it can therefore be used in the prevention or treatment of several pathological conditions that are mediated by oxidative stress. LA has been shown to have a protective effect against the hepatotoxic effects of antitubercular drugs that produce many metabolic and

morphological aberrations in liver [Saad et al., 2010]. This antioxidant may be effective in preventing the development of hepatic steatosis and hepatic fibrosis [Park et al., 2008;Min et al., 2010]. Tabassum et al. [Tabassum et al., 2010] showed that LA reduced mitochondrial oxidative stress in liver tissue to which methotrexate (a folic acid antagonist used as a cytotoxic chemotherapeutic agent) had been administered. The mice that were exposed to methotrexate followed by LA showed a decrease in lipid peroxidation, and the detoxification of free radicals by antioxidants was enhanced.

Scavenging actions of LA and DHLA

LA is a unique endogenous and exogenous antioxidant because it is a direct ROS quencher in its oxidized and reduced form. Lipoic acid is both water and fat soluble and therefore elicits its antioxidant action in the cytosol as well as in the plasma membrane, serum and lipoproteins (the water and lipid components of blood), in contrast to vitamin C, which is hydrophilic, and vitamin E, which is hydrophobic. LA may scavenge hydroxyl radicals, hypochlorous acid and oxygen singlets [Suzuki et al., 1992; Yan et al., 1996]. DHLA scavenges superoxide radicals and peroxyl radicals, thereby preventing the free radical-mediated peroxidation of proteins [Packer et al., 1995]. DHLA has the salubrious property of neutralizing free radicals without becoming involved in the process.

Regeneration of other antioxidants

An antioxidant is a compound that scavenges free radicals. It becomes oxidized and is not able to scavenge additional ROS until it has been reduced. DHLA is a potent reducing agent that has more antioxidant activity than LA and that has the capacity to reduce the oxidized forms of several important antioxidants, including vitamin C and glutathione [Suh et al., 2004]. DHLA can regenerate vitamin C and vitamin E from their oxidized forms [Biewenga et al., 1997; Kozlov et al., 1999; Bast and Haenen, 2003]. DHLA may reduce the α-tocopheryl radical (the oxidized form of tocopherol) directly or indirectly by reducing the oxidized form of vitamin C (dehydroascorbate), which is able to reduce this radical. Moreover, DHLA can also reduce the oxidized forms of coenzyme Q10, which may additionally reduce the α-tocopheryl radical [Bast and Haenen, 2003]. Coenzyme Q10 is an important component of the mitochondrial electron transport chain that has antioxidant activity as well. LA directly recycles and prolongs the metabolic lifespan of vitamin C, glutathione and coenzyme Q10, and it indirectly renews vitamin E.

Therapeutic usage of lipoic acid LA in aging

Mitochondria provide energy for basic metabolic processes, and their decay with age impairs cellular metabolism and leads to cellular decline. Mitochondrial mass is reduced with age, which leads to a defective energy homeostasis. Oxidative mitochondrial decay and the production of damaging free radicals is a major

contributor to aging [Zhu et al., 2007]. Aged rats given LA supplements showed a decrease in lipid peroxidation and an increase in the activities of mitochondrial enzymes, such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, NADPH dehydrogenase and cytochrome C oxidase. Moreover, no significant changes in mitochondrial enzyme activity were found in young rats treated with LA [Arivazhagan et al., 2001]. The authors conclude that LA reverses the age-associated decline in mitochondrial enzymes and, therefore, may lower the increased risk of oxidative damage that occurs during the aging process [Arivazhagan et al., 2001; Arivazhagan et al., 2003]. It was also shown that a decline in the mitochondrial oxidative capacity of skeletal muscle may contribute to the whole-body aging process [Short et al., 2005]. Animal studies have found that LA protects the heart mitochondria against aging effects [Janson, 2006]. LA corrected the age-related increase in oxidative stress and the age- related decline in mitochondrial enzyme activity of the citric acid cycle and respiratory chain in the liver and kidney of aging rats [Arivazhagan et al., 2001; Mc Chaty et al., 2009]. Some of this decay can be reversed in old rats by feeding them normal mitochondrial metabolites, such as acetylcarnitine, and high doses of LA. In aged rodents and dogs reducing oxidative damage by providing an antioxidant either in supplements or in fruit and vegetables can improve learning, memory and motor functions [Bickford et al., 2000; Roudebush et al., 2005]. In particular, LA was reported to be effective in protecting the rat brain from oxidative damage and memory loss [Stoll et al., 1993] and, in dogs, treatment with LA improved cognitive function through increased mitochondrial function [Head et al., 2009]. This work suggested a link between oxidative damage to mitochondrial dysfunction and cognitive decline [Zhu et al., 2007]. Furthermore, recently, it has been shown that the anti-inflammatory and antiapoptotic actions of LA in aged diabetic rats occur through a PI3K/Akt signaling pathway [Bitar et al., 2010].

MnSOD

Superoxide dismutases, or SODs, are enzymes that play a pivotal role in metabolizing $O2^{\bullet -}$; in fact they convert two molecules of superoxide to one each of dioxygen and hydrogen peroxide, with consumption of two equivalents of H^+.
Aerobic metabolism makes some 18 times more energy available per glucose than does glycolysis, making possible large and complex organisms. Thus, ability to employ O_2 constitutes a decisive evolutionary advantage. Ability to survive O_2 is such a stringent selection criterion that very few organisms lacking SOD survived the transition from reducing to oxidizing environment brought about by the evolution of oxygenic photosynthesis some 2.4 billion years ago [Blankenship, 2010]. The evolutionary pressure to develop protection against superoxide was sufficiently intense that SODs evolved on at least three separate occasions. One of these was sufficiently ancient and important that this enzyme is found in all kingdoms of life, indicating that it evolved even before differentiation of eubacteria from archaea.
The most primitive versions of SOD employ Fe, consistent with the prevalence of Fe and its ready availability in the reducing environment in which life is believed to have begun. However more modern organisms employ a version of this enzyme that requires Mn for activity, consistent with diminished bioavailability of Fe and increased Fe toxicity as O_2 levels rose. Thus, changes in the inorganic chemical makeup of the environment appear to have driven evolution of SOD[Blankenship, 2010].
In mammals three distinct isoforms of SOD have been identified and characterized: copper-zinc superoxide dismutase (Cu/ZnSOD, encoded by the *sod1* gene), manganese superoxide dismutase (MnSOD, encoded by the *sod2* gene) and extracellular superoxide dismutase (ECSOD, encoded by the *sod3* gene). These forms of SODs elicit similar functions, but characteristics of their protein structures, chromosome localizations, metal cofactor requirements, gene distributions and cellular compartmentalization are distinctly different from one another (reviewed in [Parge et al., 1992]). Among the three SOD isoforms, MnSOD is the only SOD that has proved to be essential for the survival of aerobic organisms [Carlioz and Touati, 1986].

Transcription of sod2 gene

The human sod2 is located on chromosome 6q25.3. Two forms of human sod2 transcripts exist, due to molecular structure and organization of the gene (five exons interrupted by 4 introns). [Wan et al., 1994]. The induction of sod2 in response to oxidative stress has been well established in organisms, tissues and cells growing under various stress conditions. Stimuli such as ionizing radiation [Eastgate et al, 1993; Akashi et al., 1995], 12-O-tetradecanoylphorbol-13-acetate (TPA) [Fujii and Taniguchi, 1991], interferon-gamma (IFN-γ) [Harris et al., 1991] and proinflammatory cytokines, such as tumor necrosis factor α (TNF α) [Wong et al.,

1988; Wong et al., 1988], interleukin-1beta (IL-1 β) [Visner et al., 1990], interleukin-4 (IL-4) and interleukin-6 (IL-6), can rapidly modulate sod2 gene transcription [Dougall and Nick, 1991]. In the proximal promoter regions of the sod2 gene there are many transcriptional regulatory elements, that act as binding sites for several common transcription factor, i.e NF-κB, Sp1, AP-1and AP-2, as well as C/EBP, that play important roles in regulating the constitutive or inductive expression levels of MnSOD.

The redox-sensitive transcriptional factor NF-κB acts as a regulator of genes by serving as an "immediate responder" to harmful cellular stimuli [Xu et al., 1999; Fattman et al., 2003; Hoffman et al., 2006]. The stimulus-dependent MnSOD mRNA level elevation was demonstrated to be controlled at the transcription level [St Claire, 2002] and NF-κB was identified as the most crucial transcriptional factor regulating its induction [Eastgate, 1993]. Interestingly, functional studies have demonstrated that while the NF-κB is necessary for cytokine-mediated induction of MnSOD expression [Xu et al., 1999; Rogers et al., 2000;Kiningham et al., 2001; Guo et al., 2003], p50, a member of the NF -κB family, elicits a negative role in sod2 expression [Dhar et al., 2007].

Specificity Protein 1 (Sp1) is a zinc-finger protein that acts as a transcription factor by binding directly to DNA through three consecutive zinc-finger domains in the C-terminus and enhances gene transcription with one of the two glutamine-rich domains [Dynan and Tjian, 1983; Dynan and Tjian, 1983;Briggs et al., 1986]. Thus, the GC-rich motif contained within the three sod gene promoters suggests a common regulatory role of Sp1 in the expression of SODs [Yeh et al., 1998;Xu et al. 2002]. In the 5′-flanking region of sod2, transcription factor Sp1 is essential for not only the constitutive but also the inducible expression of MnSOD [Tanaka et al., 2000;Xu et al. 2002]. Multiple Sp1 binding elements are needed to induce sod2 expression via the proximal promoter and the intronic enhancer element. A unique DNA looping structure in the 5′-flanking region formed by direct interactions between distant and local Sp1 is able to synergistically activate transcription in vivo [Mastrangelo et al., 1991].

Activator Protein 1 (AP-1) is a homo- or hetero-dimeric protein composed of proteins belonging to the c-Fos, c-Jun and Fra families. AP-1 acts as a transcriptional regulator to modulate signal transduction processes involved in cell proliferation and transformation [Angel et al., 1991]. The expression of c-fos and c-jun genes is responsive to a variety of stimuli, including cytokines, growth factors and oxidative stress.The activity of AP-1 is also subject to redox regulation. Thus, alteration in sod genes expression also modulates AP-1 activity [Zhou et al., 1991;Huang et al., 2001].Therefore, AP-1 could activate the expression of sod genes and could be activated by the expression of SODs, as demostrated in different models [Qadri et al., 2004; Zhao et al., 2002;Baldelli et al., 2008]

Activating Protein 2 (AP-2) is a family of five transcription members (AP-2alpha, to AP-2epsilon) [Eckert et al., 2005]. AP-2 plays a negative role in the constitutively low expression of MnSOD by suppressing Sp1-dependent transcription [Zhu et al., 2001; Zhu et al., 2001; Xu et al., 2002] or if AP-2 binding site in the sod2 promoter is methylated[Huang et al., 1997]. Consistent with this possibility, a low Sp1/AP-2 ratio plays a role in dysregulating the promoter activity of the sod2 gene [Miao e St Claire, 2009].

CCAAT-Enhancer-Binding Proteins (C/EBP) consist of six members, C/EBP α to C/EBP ζ, which can interact with the CCAAT box motif present in many gene promoters [Ramji and Foka, 2002]. The C/EBP binding site located in the sod2 intronic enhancer region elicits the supportive role of MnSOD induction in response to cytokine stimulation. [Kiningham et al., 2001; Qiu et al., 2008].

MnSOD in pathology

Abnormal cellular redox status has been associated with many types of diseases. The physiological role of MnSOD as a cytoprotective enzyme has been clearly confirmed by the extremely short life-span of MnSOD knockout mice, which died shortly after birth with dilated cardiomyopathy and neurodegeneration [Li et al., 1995; Lebovitz et al., 1996]. The importance of MnSOD beyond the need for survival in the aerobic environment has also been well-established. For example, in the development of cancer, which involves either the activation of oncogenes or the inactivation of the tumor suppressor gene, the levels of ROS and superoxide dismutase are regulated reciprocally. It has been demonstrated that, at an early stage of cancer development, oxidative stress and relatively low levels of antioxidant enzymes result in DNA damage and cell injury. Because MnSOD plays a critical role in the defense against oxidant-induced injury and apoptosis of rapidly growing cancer cells, it is considered a unique tumor suppressor protein [Kinnula and Krapo, 2004]. The tumor suppressive effect of MnSOD has been demonstrated in numerous cell types with malignant phenotype via modulating redox-related transcriptional factors (reviewed in [Oberley, 2005]). However, after cancer has progressed, the expression of MnSOD can be higher in aggressive cancer compared to benign counterparts. These findings have been reported for advanced cancer tissues and blood samples of leukemia. Significantly higher MnSOD levels in malignant ovarian cancer tissue compared to normal ovarian epithelium and benign lesions have also been identified in a large number of samples using comparative tissue microarray analysis [Devi et al., 2000; Hu et al., 2005]. ROS partially renders cancer cells more dependent on the function of superoxide dismutase to protect itself from damage caused by increased amounts of superoxide radicals. The necessity to alleviate ROS stress, coupled to the loss of MnSOD suppressors as the cancer progresses, provides a mechanistic explanation for a high MnSOD level in some cancer types at advanced stages. Due to the pivotal role of mitochondria and MnSOD in regulating cell death and cancer development,

alteration of MnSOD activity and levels could be a potential target for therapeutic intervention. A number of studies have identified the association of *sod2* genetic polymorphisms with various diseases including type II diabetes and hypertension [Nakanishi et al., 2008; Hirooka, 2008; Arsova et al., 2008; Mikhak et al., 2008]. Mutations detected in the *sod2* promoter region reveal the possibility for decreased expression of MnSOD in several human cancer cells [Xu et al., 1999]. Among these mutations, C to T transition at -102 and an A insertion at -93 interrupting the single-strand loop structure have been suggested as reasons for reduced levels of MnSOD activity in some tumor cell lines. These mutations create an extra DNA binding site for the transcription factor AP-2 and alter sequence-specific interaction between DNA-protein and protein-protein in the transcription initiation complex [Xu et al., 1999;Zu et al., 2007]. C to G transversion at -38 modulates an AP-2 dependent dysregulation of *sod2* gene expression [Xu et al., 2008]. The newly synthesized polypeptide for MnSOD requires that it be transported across two mitochondrial membranes into the mitochondrial matrix where the enzyme is converted to an active form. This transport activity is mediated by the presence of a signal sequence within the N-terminal of the polypeptide. An extensively investigated cytosine to thymine (C to T) single nucleotide polymorphism in the *sod2* mitochondrial targeting sequence, which causes the substitution of alanine (GCT) with valine (GTT) at codon 16, can disrupt the secondary α-helix structure of MnSOD and affect the localization and efficiency of mitochondrial transport of MnSOD enzyme [Rosenblum et al., 1996]. Sutton *et al.* have reported that the MnSOD Ala variant generates 30-40% more active MnSOD enzyme and allows more efficient MnSOD import into the mitochondrial matrix than its Val counterpart, suggesting that the homozygous AA genotype may have higher MnSOD activity than its VV counterpart [Sutton et al., 2003]. This target sequence polymorphism of human MnSOD gene and its association with cancer risk have been recently reviewed [Bag and Bag, 2008]. With respect to the AA genotype having a beneficial role in higher MnSOD activity, indeed, the *sod2* homozygous variant genotype (Val/Val) has been associated with a greater risk of pancreatic cancer compared with the Ala allele. The occurrence of the VV variant also enhances the risk of non-small cell lung carcinoma in the presence of p53 and XRCC1 polymorphism [Liu et al., 2004;Wheatley et al., 2008]. However, there is significant variability in results regarding the association of Ala16Val polymorphism with increased risk of disease. Little overall association has been found between MnSOD polymorphism and other diseases, such as asthma, Parkinson's disease, lung cancer and prostate cancer [Li et al., 2005; Ho et al., 2006; Holla et al., 2006; Singh et al., 2008; Mikhak et al., 2008]. Another potentially important polymorphic substitution for isoleucine or threonine is found at amino acid 58 in the mature human MnSOD protein. The Thr variant is less stable and more susceptible to inactivation by S-thiolation reaction than the Ile counterpart due to its stability at the tetrameric interface [Borgsthal, et al., 1996]. Consistent with the tumor suppressive role of MnSOD, human malignant breast cancer cells overexpressing

Ile58 MnSOD have 3-fold higher MnSOD activity and a greater tumor-suppressive effect than cells overexpressing the Thr58 counterpart at an equal MnSOD protein level [Zhang et al., 1999]. Further direct testing of this possibility would be to replace cellular MnSOD with either version and then to study the effect on tumorigenesis, e.g., in a transgenic knockin model. Another relatively unique point mutation, C5782T in exon3 of *sod2* producing a L60F mutation in the mature enzyme, has been found in Jurkat T lymphocytes. The L60F mutation led to a deficiency in MnSOD activity and correlated with the malignant phenotype. This finding also explains the paradoxical effects of thiol reagents on antioxidants in some leukemia cells [Hernandez et al., 2003]. The different roles that MnSOD plays at different cancer stages suggest that specific strategies will be needed for specific situations. Thus, understanding how the expression of MnSOD can be selectively regulated at each stage of cancer development is likely to have important implications for the prevention and treatment of cancer.

Due to the key role played by MnSOD in counteracting free radical-derived cell damages, it appears extremely interesting to evaluate the feasibility and efficacy of a MnSOD oral administration. In scientific literature, in my knowledge, there is only one paper (Milesi *et al*., 2009), reporting the effects of an oral supplementation with a melon juice concentrate (at a dosage of 140 UI SOD/day) to healthy people. After 4 weeks of this diet (compared to placebo) sensible improvements were described for several stress parameters, like pain, sleep troubles, concentration, weariness, and irritability.

AIM OF WORK

Although free radicals are known to play a positive role in the organism, the etiology of many diseases seems to be due to these molecules. This evidence allows to hypothesize that the main cause of toxicity of these molecules can be rather their disbalance in cells. In the present study we considered:

- the presence of alterations presumably caused by an excess of free radicals (morphology, apoptosis markers, LPO marker, etc),
- the effectiveness of some antiradical substances in counteracting these damages.

in three experimental conditions:

- aging, studied both *in vivo* and *in vitro*,
- animal model of glaucoma, studied only *in vivo*,
- irradiation with UV rays, studied only *in vitro*.

The antiradical substances utilised are:

- resveratrol,
- L-carnitine,
- alfa lipoic acid,
- Mn superoxide-dismutase (MnSOD).

Concerning aging, it has been studied *in vivo* in a physiological condition, i.e. in cornea of 24 months-old rats (comparable to centenary humans), and *in vitro* in a experimental condition, i.e. in cultured mouse fibroblasts, submitted to serum deprivation (aging cell model, according to literature).

The cornea was studied (hematoxylin-eosin staining, NADPH-diaphorase cytochemistry, TUNEL, caspase 3 and iNOS immunocytochemistry, and Western blot) both in control old animals and in old animals subjected to a long oral treatment with L-carnitine, utilized at a dosage of 0.1 g/Kg, comparable to those available in a human diet. Results were compared to those obtained in 4 months-old (young adult) rats.

In serum-deprived mouse fibroblasts cell viability was analyzed through MTT test after incubation with L-carnitine at concentrations ranging from 0.3 to 1.2 mM and for times comprised between 24 and 48 h. Results were compared to those obtained in untreated serum-deprived and not serum-deprived cells.

Concerning glaucoma, an animal model of this pathology was obtained by a procedure, previously developed by the research team that supported me during PhD period, consisting in methylcellulose injection in the anterior eye chamber of adult rats (Pescosolido et al., 1998; Calandrella et al., 2007). In this experimental model the insurgence of ocular hypertension had been monitorised by a tonometer and the increased concentration of free radicals had been demonstrated by the malonyldialdehyde assay. In my research I examined both the optic nerve and retina obtained from 6 months old rats in the following conditions:

- No treatment (control animals),
- methylcellulose-injection in normally fed animals (glaucoma model),
- methylcellulose-injection in alfa lipoic acid-fed animals,
- methylcellulose-injection in MnSOD-fed animals,
- methylcellulose-injection in alfa lipoic acid+MnSOD-fed animals
- injection of methylcellulose + L-carnitine in normally fed animals.

These above drugs were administered at dosages comparable to those available in a human diet. In both tissues, in all the above experimental conditions, alterations were examined by hematoxylin-eosin staining, TUNEL, iNOS and caspase3 immunocytochemistry, and Western blot.

Concerning UV irradiation, I used a line of human keratinocytes (HaCat) and primary cultures of human keratinocytes. Both cell types were subjected to an UV dose known for causing large amounts of free radicals in the epidermis without causing thymine dimerization (Al-Adhami et al., 2006) Resveratrol was added to the incubation media for 30 min before and 24 h after UV irradiation.

The cell damage and free radicals formation were studied by means of MTT test, TUNEL, caspase3 and iNOS immunocytochemistry, and NADPH diaphorase cytochemistry. Results obtained in UV-treated, resveratrol-treated and resveratrol + UV-treated cells were compared to those obtained in untreated cultures.

MATERIALS AND METHODS

AGING EXPERIMENTAL CONDITION

Cornea of old rats

Animals and treatment

Corneas were taken from 9 male Wistar rats sacrificed at 6 months of age (young adult animals, body weight comprised between 300 and 350 grams) and from 9 male Wistar rats sacrificed at 24 months of age (extremely old animals, comparable to centenary men; body weight comprised between 400 and 450 grams).

4 young and 4 old animals were fed with normal pellet diet, while 5 young and 5 old animals were fed (for the 4 months preceding the sacrifice) with an enriched containing L-carnitine.

The enriched diet contained the following components:

- 500 grams of peeled and washed carrots,
- 500 grams of peeled and washed potatoes,
- 200 grams of salmon,
- 10 grams of extra virgin olive oil,
- bread crumbs or pellet powder to give consistency,
- 6. 5 g of L-carnitine.

The above mixture, stored in freezer, was utilized for 50 daily portions, which were administered every day in the morning. Each daily portion contained 100 mg L-carnitine, corresponding to a dosage of 0,25gr/kg of body weight. The animals submitted to the enriched diet were housed one per cage, to ensure that each rat assumed the right amount of nutrients. Due the exceptional palatability of the food, the daily portions were always entirely assumed. All the animals were systemically anesthetized and sacrificed by carotid hemorrhage.

Sectioning

Eyes were enucleated, corneas were removed and underwent the following treatments:

- fixation in 4% paraformaldehyde for 6 hours,
- washes with PBS pH 7.4,
- immersion in 30% sucrose in PBS pH 7.4 overnight at 4°C,
- inclusion in OCT (Killiks, Bio-optica),
- cryostat sectioning.

The sections, collected on microscope slides, were stored al -20°C.

Hematoxylin-eosin staining

10 µm thick cryostat sections, stored at -20°C and allowed to dry at room temperature for 30 minutes, were subjected to the following treatments:
• rehydration with decreasing concentration alcohols ;
• 5 minutes distilled water;
• 1 minute hematoxylin;
• 10 minutes running water;
• 2 minutes distilled water;
• 45 seconds eosin;
• 2 minutes distilled water;
• dehydration with alcohols;
• closure with glycerol-PBS 1:3.
Sections were observed with a light microscope.

Immunohistochemical localization of inducible nitric oxide synthase (iNOS) and caspase-3.

10 µm thick cryostat sections, stored at -20°C and allowed to dry at room temperature for 30 minutes, were subjected to the following treatments:

• 5 minutes washes in PBS;
• 2 minutes post-fixation with increasing concentration alcohols;

• 5 minutes washes in PBS;
• block with NRS serum 10% in PBS, 1 hour in a humid chamber;

• incubation with rabbit polyclonal antibody anti-iNOS (Santa Cruz), diluted 1:50, or with rabbit polyclonal antibody anti-caspase-3 (Santa Cruz), diluted 1:100, in PBS + 1% BSA, for 1 hour (or overnight) at room temperature;
• washes in PBS;
• incubation with FITC-conjugated goat anti-rabbit IgG antibody Alexa Fluor (Molecolar Probes),
diluted 1:200 in PBS, for 30 minutes;
• 5 minutes washes in 1X PBS;
• closure with Eukitt.
Specimens were observed in a fluorescence microscopy.

TUNEL assay

10 µm thick cryostat sections, stored at -20°C and allowed to dry at room temperature for 30 minutes, were subjected to the following treatments:

1. rehydration with decreasing concentration alcohols

2. incubation with 30µg / ml Triton X-100 at 37°C for 20 min;
3. washes with PBS;
4. incubation with the TUNEL reagent (In Situ Cell Death Detection Kit, AP / ROCHE), containing 1/10 in volume of terminal-desossinucleotidil transferase and the remaining fraction of nucleotides labeled with a fluorescent chromophore, 10 µl per section at 37 ° C for 1 hour;
5. washes with PBS;
6. closure with Eukitt.
7. Specimens were observed by fluorescence microscopy.

Western blot and immunodetection of iNOS and caspase-3

Proteins were extracted from rat corneas by the lysis buffer 20 mM TrisHCl, pH 7.6, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 0.1 M DMSF; 1 mM,1 M DTT.

Specimens were kept for 10 minutes on ice, then centrifuged at 13,000 rpm for 5 seconds, transferred again on ice for 10 minutes and finally centrifuged for 10 minutes at 13,000 rpm. Then supernatants were collected and submitted to quantification with Bradford assay.

After quantification, proteins were subjected to electrophoretic separation on SDS-PAGE (SDS-Polyacrylamide-Gel-Electrophoresis). Electrophoretic upper gel was 4x Tris HCl, pH 6.8, containing 0.5 M SDS 0.4%, while running buffer was 4x Tris HCl, pH 8.8, containing 1.5 M SDS 0.4%.

Western blotting was performed by standard procedures, using rabbit policlonal anti iNOS and anti caspase-3 antibodies (Santa Cruz), diluted 1:500 in skim milk and goat secondary antibodies anti-rabbit IgG (Jackson) diluted 1:5000 in skim milk. Results were normalized by the immunolocalization of actin, constitutively expressed in cells, carried out utilising goat policlonal anti actin antibody (Santa Cruz) diluted 1:1000.

All the experiments were repeated three times and t he statistic analysis was done using the ANOVA test. Results were considered significant when P was < 0.05.

Proteins were transferred by pressing the gel against a nitrocellulose filter under in 300 mA constant electric field. After this, the filter underwent to washing with T-TBS 1X 0.1% and blocking with T-TBS 1X + 5% milk. Detection was performed by chemiluminescence (ECL).

Cultured fibroblasts

Murine fibroblast 3T6 cell line was maintained in DMEM/high glucose medium, supplemented with 10% newborn calf serum, 2 mM glutamine and Penicillin–Streptomicin. All the experiments were done plating discrete amounts of cells in 30, 50 and 100 mm Petri dishes: $7x10^4$ cells (30 mm dishes); $5\ x10^5$ cells (50 mm dishes); 10^6 cells (100 mm dishes). Isolation of DNA was performed using the ApopLadder Ex kit (Takara).

Acetyl-L-carnitine (0.3, 0.6 and 1.2 mM) was dissolved in fresh serum-free medium and administered to cells, 24 h after plating, for a period of 5 h (for evaluating the cytochrome C release) or 24 h (for all the other evaluations). Viable cells were counted in a Burker chamber after erythrosin staining (f.c. 0.1% in PBS).

Both untreated and L-carnitine-treated fibroblasts were submitted to the below described procedures.

MTT assay

Cells were plated on 24 well chambers (10^4 cells/well) and grown for 24 h; they were then washed with PBS and incubated for 4 h in 0.3 ml of serum-free medium containing 0,5mg/mL methylthiazoletetrazolium (MTT). The reaction was stopped by the addition of 0.6 ml of a 0.08N HCl solution in isopropanol and optical density was measured at 570 nm.

Chromatin staining

Cells were fixed in 4% paraformaldehyde for 1 h at room temperature, washed in PBS and incubated with 0.1% hematoxylin QS for 1 min; cells were then washed again and incubated with 1% eosin for 2 min (for cytoplasm counterstaining) and observed in a light microscope.

TUNEL assay

Cells were fixed in 4% paraformaldehyde, washed in PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, for 2 min on ice. Cells were then washed, air dried and incubated with 50 ml TUNEL mix (terminal transferase was omitted from the mix to be used with the negative controls) for 1 h at 37°C in the dark. Finally, cells were washed for 30 min at 4°C in the dark, the dishes sealed and observed in a fluorescence microscope (Zeiss Axiophot).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, washed in PBS and incubated with goat policlonal anti procaspase3 and anti total procaspase3-caspase3 antibodies (Santa Cruz), diluted 1:500 in BSA for 1h at room temperature. Positivity was revealed using the Vectastain ABC kit (Vector Laboratories), and counterstained with hematoxylin QS (X1575) following the manufacturer's instructions. Specimens were observed in a light microscope.

Western blotting and immunodetection of caspase 3

Proteins were extracted by the lysis buffer 20 mM TrisHCl, pH 7.6, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 0.1 M DMSF; 1 mM,1 M DTT.

Cells were collected in tubes and specimens were kept for 10 minutes on ice, then centrifuged at 13,000 rpm for 5 seconds, transferred again on ice for 10 minutes and finally centrifuged for 10 minutes at 13,000 rpm. Supernatants were collected and submitted to quantification by Bradford assay.

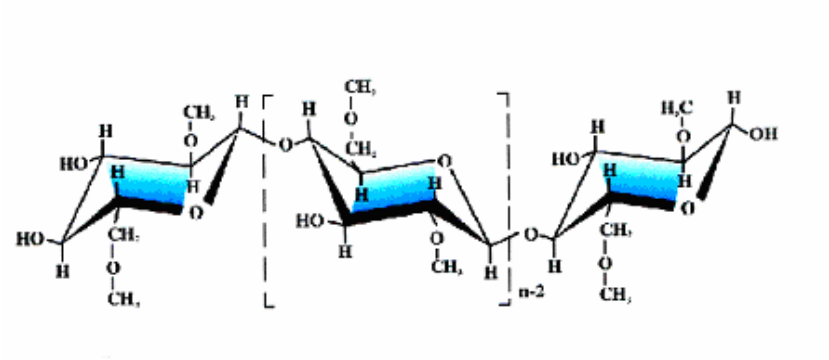
Western blotting was performed by standard procedures, using goat polyclonal anti caspase3 antibody (Santa Cruz), diluted 1:1000 in skim milk. Results were normalized by the immunolocalization of tubulin, constitutively expressed in cells, carried out using rabbit polyclonal anti tubulin antibody (Sigma) diluted 1:1000.

All the experiments were repeated three times and the statistic analysis was done using the ANOVA test. Results were considered significant when P was < 0.05.

GLAUCOMA EXPERIMENTAL CONDITION

Hypertone induction

The experimental model of human glaucoma used in the present research derives from a method developed in rabbits (Zhu and Cai, 1992) and adapted for rats in the laboratory where I worked for my thesis (Pescosolido et al., 1998; Calandrella et al., 2007). In this model, 15 µl of 2% methylcellulose (Aldrich Chem. Co.), diluted in saline, were injected in the anterior eye chamber, creating a barrier to aqueous humor outflow, with consequent onset of ocular hypertension.



Methylcellulose structure

Animals and treatments

32 male 4 months-old Wistar rats (young adult animals; body weight comprised between 300 and 350 grams) were utilised.

14 animals were fed with normal pellet diet, while 18 animals were fed since weaning and till the sacrifice with three different enriched diets (6 animals for each diet).

The enriched diets contained the following components:

- 500 grams of peeled and washed carrots,
- 500 grams of peeled and washed potatoes,
- 200 grams of salmon,
- 10 grams of extra virgin olive oil,
- bread crumbs or pellet powder to give consistency,
- 7mg SOD, or 150 mg ALA, or 7mg SOD + 150mg ALA.

The above mixture, stored in freezer, was utilized for 50 daily portions, which were administered every day in the morning. Each daily portion contained 0,14mg SOD, corresponding to a dosage of 2 UI/kg of body weight, or 3mg ALA, corresponding to a dosage of 8.57 mg/kg of body weight, or 0,14mg SOD and 3mg ALA. The animals submitted to an enriched diet were housed one per cage, to ensure that each rat assumed the right amount of nutrients. Due the exceptional palatability of the food, daily portions were always entirely assumed.

For inducing ocular hypertension, rats were systemically and locally anesthetized and were inoculated with 15µl of MTC 2% (or MTC + L-carnitine) in saline, in the anterior chamber of an eye, by a hypodermic needle connected to a cannula (PE 60) (Zhu et al., 1992). General anesthesia was performed by intraperitoneal injection of thiopental sodium (Farmotal, Pharmacia & Upjohn) 2.5% in saline, by intraperitoneal injection of thiopental sodium (Farmotal, Pharmacia & Upjohn) at 2.5% in saline, about 1 ml, while oxybuprocaine (0.4% in saline) eye drops to 0.4% (Novesina, Novartis Farma SpA) was utilised as the local anesthetic.

In all the experiments one eye/animal was treated and the controlateral eye was utilised as control.

For L-carnitine intraocular administration, the injected solution contained: 7,5 ml of MTC 4% in saline + 3 ml of 50 mM L-carnitine in saline + 4,5 ml of H_2O. In this solution, the L-carnitine final concentration was 0.6 mM, corresponding to a concentration previously tested in *in vitro* experiments (Calandrella et al., 2007).

More precisely:

- 6 normally fed rats, 6 SOD-fed, 6 ALA-fed, 6 ALA+SOD-fed rats were injected with 2% methylcellulose (MTC).
- 6 normally fed rats were injected with 2% MTC + L-carnitine.
- 2 normally fed rats were injected with a saline solution (NaCl 0.9%) (shame operated control).

In MTC-inoculated eyes ocular hypertension (monitorised by a tonometer) reached a maximum (55-60 mmHg) within 3-6 hours and then stabilized within 24-48 hours on values (25-30 mmHg), intermediate between physiological (0-12 mmHg) and peak pressure values. In shame operated eyes intraocular pressure values were comparable to those found in untreated eyes.

24 hours after the hypertone induction, animals were systemically anesthetized, carried out as above described, and sacrificed by carotid hemorrhage. The suffering degree of treated animals was assessed by the behavioral test of Irwin and the recovery of body weight after anesthesia. The ocular inflammation was evaluated

with the test Drize (Drize et al., 1944), adapted for rat. All experiments are in accordance with Association for Research in Vision and Ophthalmology (ARVO) and Italian and European standards for the treatment of experimental animals.

Sectioning

After eyes enucleation, retina and optic nerve were removed and underwent the following treatments:

- fixation in 4% paraformaldehyde for 6 hours,
- washes with PBS pH 7.4,
- immersion in 30% sucrose in PBS pH 7.4 overnight at 4°C,
- inclusion in OCT (Killik, Bio-optica),
- cryostat sectioning.

Hematoxylin-eosin staining, immunohistochemical localization of inducible nitric oxide synthase (iNOS) and caspase-3, TUNEL assay

Retina and optic nerve cryostat sections were processed as described in previous paragraphs.

Reaction of histochemistry NADPH – diaphorase

Retina and optic nerve cryostat sections sections, left to dry at room temperature for 30 minutes, were subjected to the following treatments:

• 3 washes in Tris buffer pH 7.6 for 10 minutes;
• incubation for one hour at 37 ° C in 0.25 mg / mL nitro-blue tetrazolium, 1mg/mL NADPH and 0.5% Triton X-100 in 0.1 M Tris Buffer pH 7,6;
• washing with PBS for 2 minutes;
• closure with glycerol-PBS 1:3
Sections were observed with a light microscope.

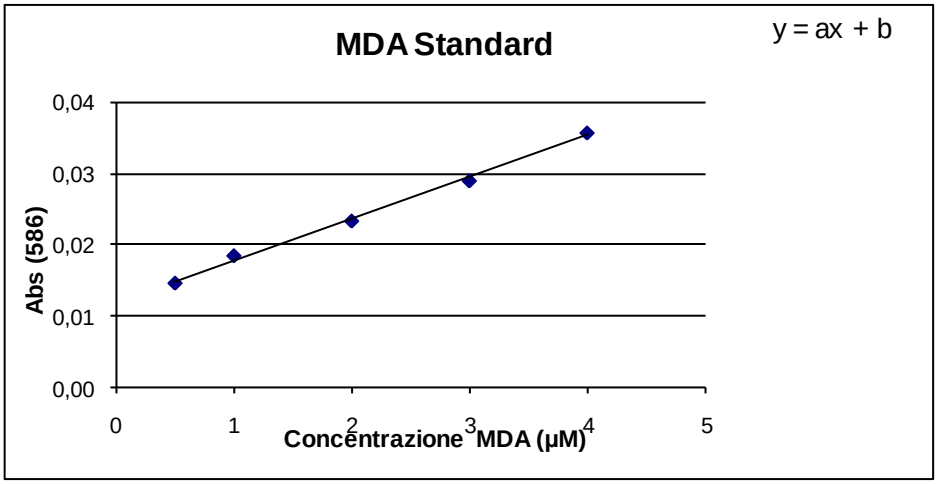
Western blot and immunodetection of iNOS and caspase-3

Rat retinas were processed as described in previous paragraphs.

Lipid peroxidation assay (LPO-586)

The assay LPO-586 is used to investigate oxidative stress in cellular membranes, and consists in the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with malondialdehyde (MDA) at 45°C: one MDA molecule reacts with two R1molecules to form a stable chromophore, with absorbance at 586 nm.

For preparation of the standard curve of MDA, the malondialdehyde was supplied as acetal, due the aldehyde instability. At 45°C acetal (TMOP) is hydrolyzed during the incubation with acid, regenerating MDA. To quantify MDA in samples, after spectrophotometer readings, we referred to a standard curve with increasing concentration of TMOP.



For LPO assay, retinas were homogenated in a glass-glass potter with 230 µl of lysis buffer in three freeze / thaw cycles. 650 µl of reagent R1 and, later, 150 µl of HCl 12 N were added to samples and specimens were centrifuged at 15,000 g for 10 minutes after incubation for 60 minutes at 45° C. Supernatants were collected and the absorbance measured at 586 nm.

In each sample MDA concentration can be calculated from absorbance at 586 nm, after subtracting blank, by using a linear regression analysis,where X is the value of the concentration of MDA, while Y represents the absorbance value.

Y = AX + B A = regression coefficient B = intercept
X = (Y-B) / X

UV IRRADIATION CONDITION

Cell cultures

Primary cultures of normal human epidermal keratinocytes (HEK) were obtained from skin biopsies of healthy volunteers (n = 6) after their informed consent (32). Primary cultures were grown in a 5% O_2 humidified atmosphere in keratinocyte growth medium: DMEM and Ham's F12 (both from Lonza, Walkersville, MD), media (2:1 mixture) , containing 10% fetal bovine serum (Invitrogen), 0.18 mM adenine and 0.4 µg /ml hydrocortisone (both from Calbiochem), 5 µg /ml insulin, 0.1 nM cholera toxin, 2 nM triiodothyronine, 1% penicillin–streptomycin, 4 mM glutamine (all from Sigma-Aldrich) and 10 ng /ml epidermal growth factor (Austral Biologicals). When HEK cultures became sub-confluent (60–80%), cells were transferred to keratinocyte growth medium containing growth factors (KGM-Gold; Lonza, Walkersville, MD).

The spontaneously immortalized human keratinocyte cell line HaCaT was from Lonza (Milan, Italy). HaCaT cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS, PAA, Coelbe, Germany) and 1% penicillin–streptomycin (Biochrom KG, Cerlin, Germany) at 37°C in a 5% CO_2 humidified atmosphere.

UV and resveratrol treatments

Both HEK and HaCaT cultures were used for experiments at 60– 80% of confluence and before all measurements/experiments, they were transferred into 96-well plates at the density of 0.5×10^4 cells per well and were starved for 24 h in a medium deprived of all supplements.

For UV treatment, cell monolayers were exposed to low-dose UVA+UVB irradiation, produced by Solar Simulator (Dermalight Vario with filter A2, Dr. Hoehnle AG, UV Technology, Planegg, Germany), with emission spectrum from 280 nm and emission peak at 375 nm. The light effluence rate on the cell monolayer was 4 mJ/ cm^2.

For resveratrol (RV) treatment, cell monolayers were processed as follows:

a) Incubation in 10 and 50 µM RV (Biomol Research Lab, Plymouth, MA), previously diluted in DMSO (DMSO final concentration 0,10%), for 30 min,

b) washing in PBS,

c) UV irradiation,

d) incubation in 10 and 50 μM RV for 24 h.

Some cell monolayers were incubated in 0.10% DMSO.

Some cell monolayers were submitted to a and d steps only.

Washing in PBS was introduced in order to avoid UV-induced resveratrol oxidation.

MTTassay

To assess both HEK and HaCaT proliferation, 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide (MTT) colorimetric assay was applied. All cell monolayers (untreated, UV-treated, UV+resveratrol-treated, UV+DMSO treated and resveratrol alone-treated) were washed in PBS and 200 μl of MTT solution (5 mg/ml) were added into each well. After 4 h at 37°C the medium was removed, 100 μl of DMSO were added to dissolve tetrazolium granules and the absorbance at 540 nm was measured. All measurements were done in triplicate and data of at least three independent experiments were statistically evaluated.

RESULTS

AGING EXPERIMENTAL CONDITION

Cornea of old rats

After the diet was enriched with carnitine, behavioral differences were observed in treated rats compared to pellet-fed ones. In fact, 24 months old-rats have, usually, both reduced motility and responsivity to external stimuli; carnitine-treated animals, instead, were more reactive and had better motility.

In cornea I studied:

1. Morphological features (hematoxylin-eosin staining)
2. Oxidative stress onset and progress (iNOS expression and NADPH diaphorase positivity)
3. DNA damage (TUNEL)
4. Apoptotic death induction (caspase-3 expression)

Morphological analysis

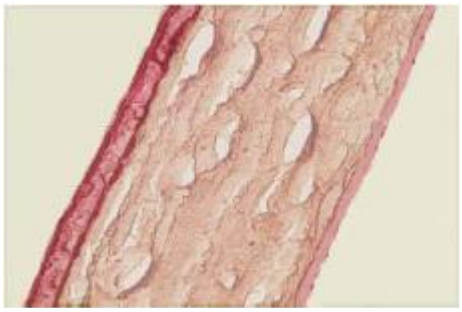


Fig. 7a Section of cornea from 24 months old-rats (50x)

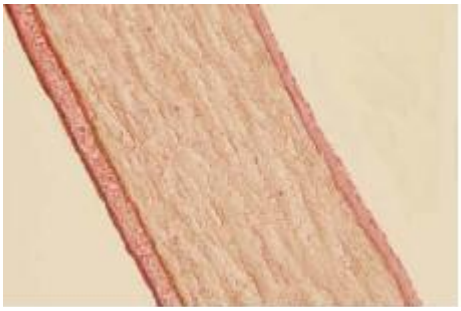


Fig 7b Section of cornea from 6 moths old-rats (50x)

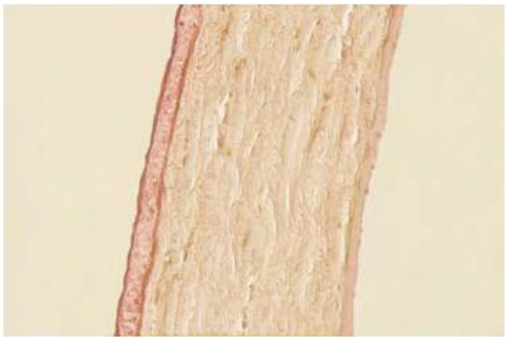


Fig 7c Section of cornea from 24 moths old-rats carnitine-treated (50x)

The stroma appears deteriorated in old animals, compared to young rats; moreover in corneas from old rats a reduction both in keratinocytes and endothelial cells can be observed (data not shown).

iNOS immunolocalization

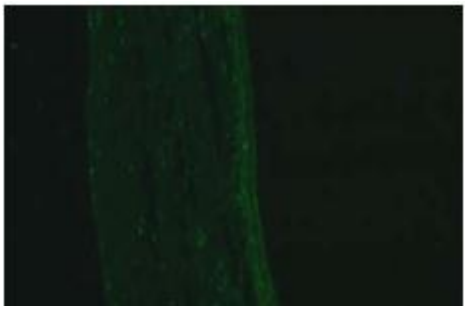


Fig 8a Section of cornea from 24 months old-rats (50x)

Fig 8b Section of cornea from 6 months old-rats (50x)

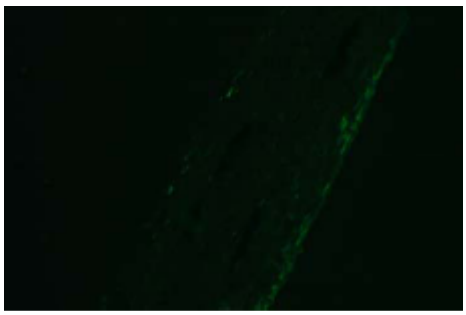


Fig 8c Section of cornea from 24 months old-rats (50x) carnitine-treated

After iNOS immunolocalization in specimens from old rats a marked positivity was found, especially located in epithelium and endothelium, allowing to hypothesize an iNOS strong induction. Positivity appeared reduced in specimens from carnitine-treated old animals and totally absent in sections from young rats.
After NADPH-diaphorase cytochemistry, the staining, that marks the presence of oxidative stress, was found in relevant amount in all layers of specimens from old

rats, while was lower in specimens from carnitine-treated old animals and totally absent in sections from young rats. This cytochemical method being the almost only approach able to reveal the *in vivo* presence of free radicals in extracellular matrix, our observation of a significant staining in stroma of old animals appears particular relevant.

NADPH-diaphorase staining

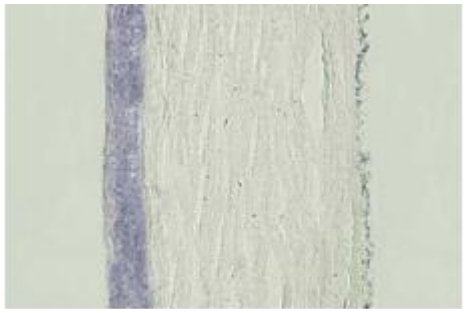


Fig. 9a Section of cornea from 24 months old-rats (50x)

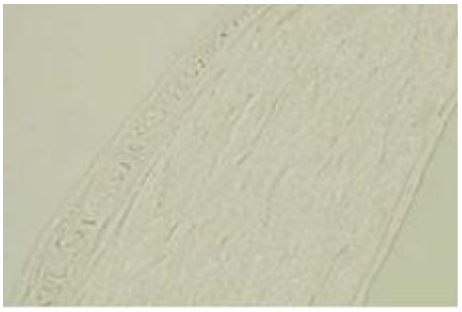


Fig.9b Section of cornea from 6 months old-rats (50x)

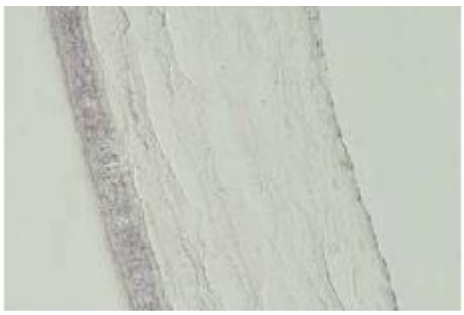
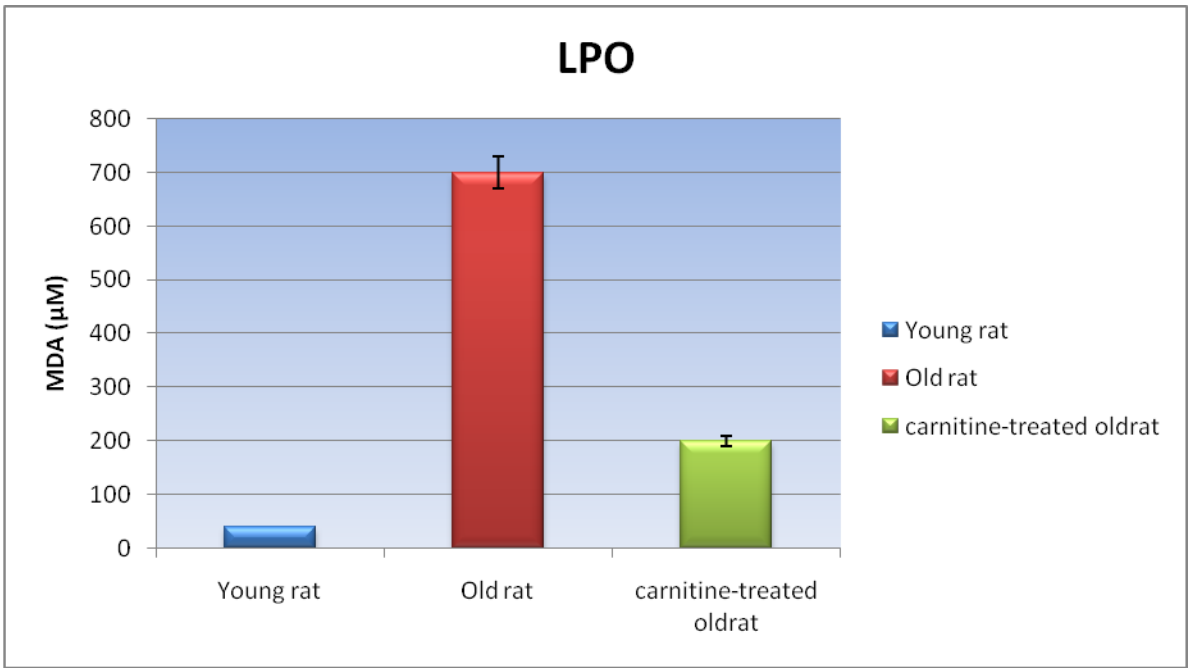


Fig 9c Section of cornea from 24 months old-rats (50x) carnitine-treated

Lipoperoxidation

The age activates diverse cellular and molecular signals of stress such as DNA damage. This results in the establishment of an oxidative stress. This stress cause damage of the cell membrane, which was assessed by measuring the production of MDA, a widely recognized marker of lipoperoxidation, whose production occurs in cells directly from a damage to the membrane structure and function. Samples taken from old rat eyes showed extensive lipoperoxidative damage, as indicated by the very high cytoplasmic MDA concentration. The treatment with carnitine reduces the level of lipoperoxidation to half.



Bars report the level of malondialdehyde (MDA). The values represent Mean ± S.E:M.

DNA fragmentation

In this study TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick end Labeling) was used to evaluate DNA degradation in corneal cells. TUNEL, in facts, marks nuclear damage due to the endonuclease-caused DNA fragmentation, both at early and advanced death stages.

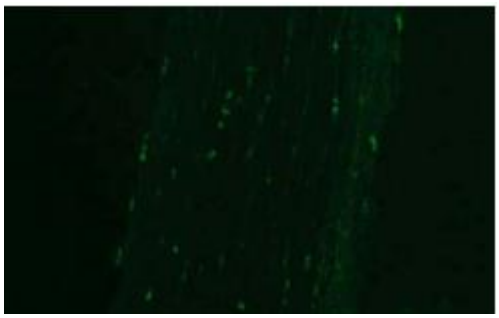


Fig 10a Section of cornea from 24 months old-rats (50x)

Fig 10b Section of cornea from 6 months old-rats (50x)

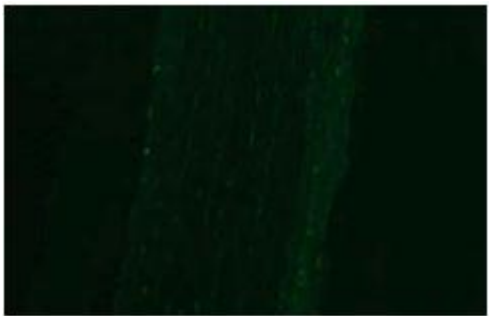


Fig 10c Section of cornea from 24 months old-rats (50x) carnitine-treated

In the section from control old animal a generalized fluorescence can be observed, due to DNA fragmentation. Positivity is reduced in specimens from carnitine-treated old animals, suggesting a DNA lower degradation rate. Positivity is totally absent in specimens from young rats.

Caspase-3 immunolocalisation

The DNA fragmentation revealed by TUNEL (see above) indicates the presence of apoptosis or necrosis. Carnitine being known to stabilize mitochondrial membrane, the reduction of DNA fragmentation that I found in carnitine-treated old rats argued in favor of the idea of apoptosis key role. On this basis, and in order to investigate if apoptotic program was progressed to an irreversible step, I performed the immunolocalization of caspase-3, which is known to be involved in final stages of cellular suicide-program.

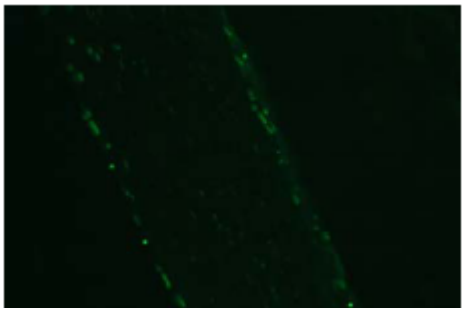


Fig 11a Section of cornea from 24 months old-rats (50x)

Fig 11b Section of cornea from 6 months old-rats (50x)

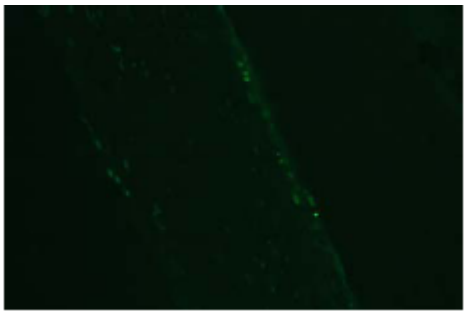


Fig 11c Section of cornea from 24 months old-rats (50x) carnitine-treated

In specimens from old animals a strong expression of activated caspase-3 was observed both in epithelial and endothelial cells; this expression decreases in cornea from carnitine-treated rats. As known, membranes submitted to oxidative stress undergo to lipoperoxidation phenomena, destabilization and release of pro-apoptotic factors, with consequent increase of procaspase-3 that proceeds to caspase-3.

On this basis I can suppose that in rat cornea carnitine supplementation reduces the naturally occurring apoptosis, *via* stabilization of the mitochondrial membrane, involved in fatty acids transport.

Cultured fibroblasts

Serum depletion is probably one of the best known ways to induce apoptosis in several cell culture systems [Araki et al., 1990; Galli et al., 1993; Kulkami and McCulloch, 1994] and it has also been reported to induce oxidative stress [Pandey et al., 2003].

Morphological analysis

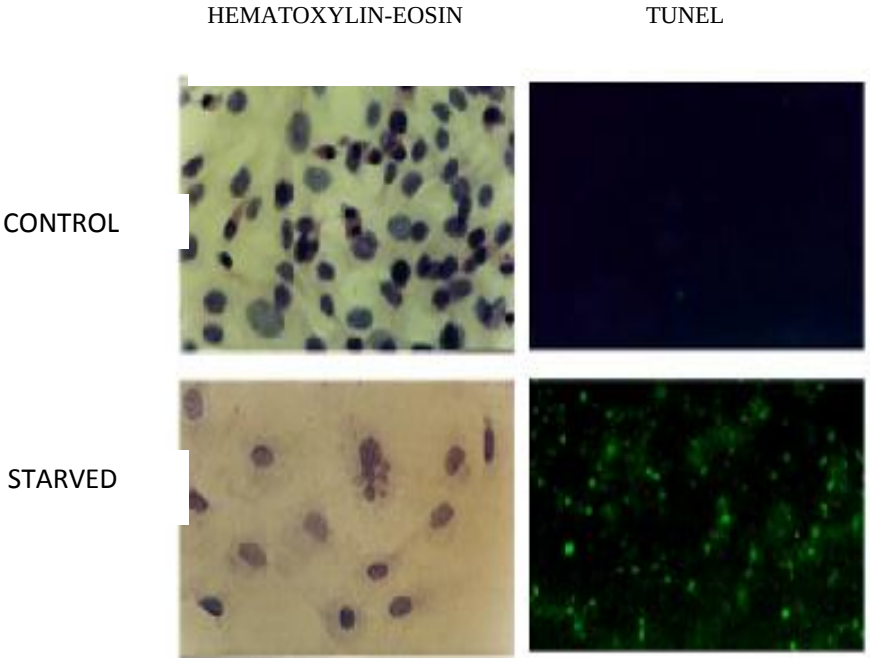


Fig. 12

Staining of serum-deprived 3T6 cells with hematoxylin–eosin evidenced loss of the typical fibroblast morphology and presence of apoptotic bodies. Apoptosis was unequivocally assessed also by TUNEL staining.

MTT test

The colorimetric Mosmann assay (MTT test) is a method used to quantify loss of mitochondrial function [Mossman, 1983; Gutierrez-Canas et al., 2003]. In our experimental condition the test showed a survival rate of 20% after 24 h of serum deprivation (Table 2) testifying a severe activation of apoptotic mitochondrial pathway.

Table 2
Effect of serum deprivation on cell survival

	0 h	6 h	18 h	24 h	48 h
Control	24653 ± 1300	33487 ± 1570	59331 ± 1650	74964 ± 3800	118283 ± 4789
Serum starved	24653 ± 1400	22473 ± 2000	21666 ± 20983	16851 ± 1600	13012 ± 1500

Serum deprivation severely reduces fibroblast survival rate: after 24 h from serum withdrawal, cell viability is reduced to 22% compared with control cells.

Carnitine treatment

Hematoxylin eosin staining and TUNEL

As already mentioned, carnitine is known to interact with the mitochondrial membrane and to play an active role in the overall metabolism of this organelle. As above described, the possible cytoxicity of carnitine was assayed using three different drug concentrations: 0.3, 0.6 and 1.2 mM. Only the highest one seemed to have some cytotoxic effect, so I assumed that 1.2 mM carnitine was the toxicity border limit concentration. To evaluate the cyto-protective action of carnitine, fibroblasts were starved for 24 h in the absence or presence of 0.3, 0.6 and 1.2 mM carnitine. Serum-depleted cells showed an abnormal morphology (Fig. 13, upper panel) while those treated with carnitine maintained the typical fibroblast shape and displayed a normal chromatin condition.

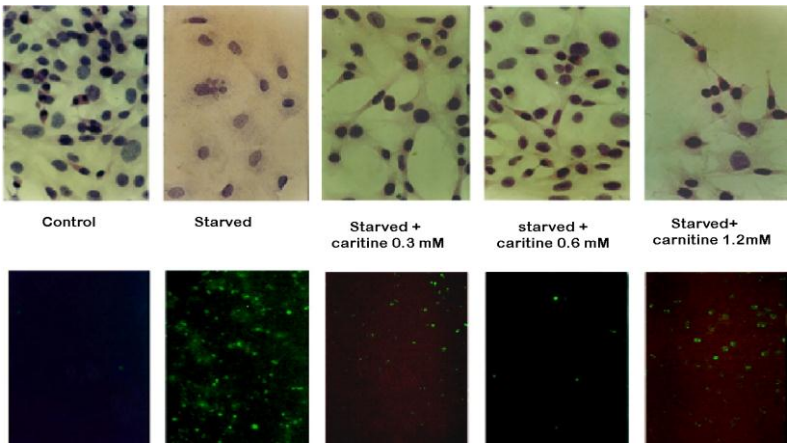


Fig. 13 Effect of carnitine on cell morphology and chromatin condensation. Upper panel: cells treated with 0.3 mM and 0.6 mM carnitine show no differences in cell morphology and chromatin status with respect to control cells. Cells treated with 1.2 mM carnitine show some cytoplasmic alteration and their number is strongly reduced compared with control. Lower panel: TUNEL reaction shows a strong signal in serum-depleted cells while it is very low in carnitine treated cells; control cells show no positivity to the reaction.

Cells treated with 0.6 mM carnitine showed, in particular, a comparable phenotype to one of the control cells even if a minor reduction of the total number of cells was observed (see TUNEL assay results (Fig. 13, lower panel) also showed that carnitine treatment protects fibroblasts from DNA fragmentation. No signal was detected, on the other hand, in control cells that had been neither depleted nor treated with carnitine. It should be pointed out that pre-treatment with carnitine (16 h) and subsequent starvation in the presence of the drug produced identical results to those reported above (data not shown), indicating that carnitine protects cells and does not rescue them from apoptosis. The concentration of the drug plays an important role since its protective effect is most evident at 0.6 mM carnitine while some fluorescent signal was observed at 1.2 mM. Since the cells treated with 0.6 mM carnitine showed

the lowest amount of fragmented DNA, this concentration was used throughout the subsequent experiments.
The most significant increase was observed after 24 h of treatment which indicates that this is the longest time span of protecting action by the drug.

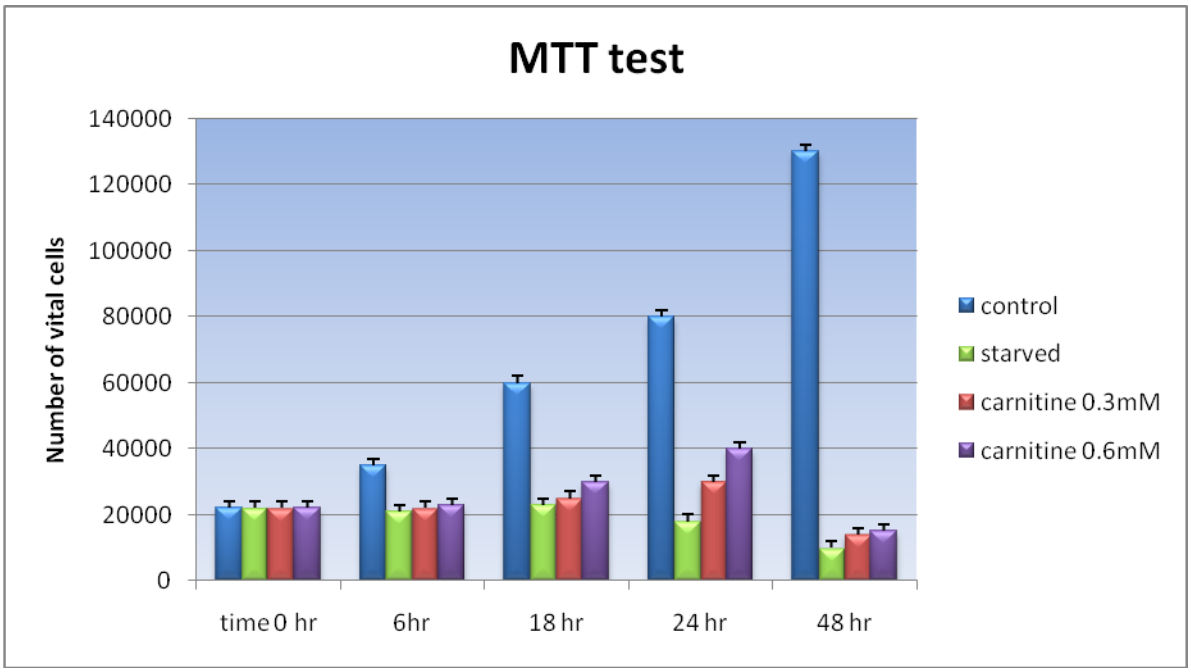


Fig. 14.
Administration of carnitine enhances cell proliferation compared with untreated serum-depleted cells, but this effect decreases at 48 h of treatment. Time T0 corresponds to serum depletion or serum depletion and carnitine administration performed 24 h after plating. Data represent the average values and standard errors obtained from three different experiments. Results were considered significant when P b 0.05 (**P b 0.02 vs. Starved; *P b 0.05 vs. Starved).

Caspase3

To determine the involvement of the mitochondrial pathway, caspase 3 was investigated. This protease is normally found in its inactive form (procaspase 3), but it is activated during apoptosis upon the release of mitochondrial factors into the cytosol.
For caspase 3, two different antibodies were used, the first recognizing the C-terminal end and the second binding to the N-terminal portion of the inactive precursor. As expected, procaspase 3 (N-terminal) was observed in control cells but not in the serum-deprived ones (Fig. 15).

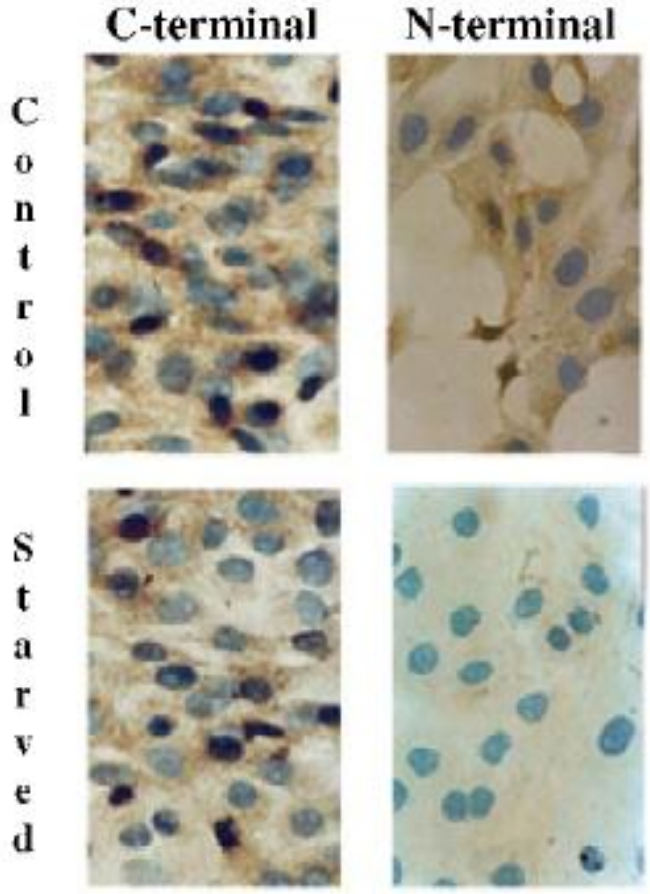


Fig. 15. Apoptosis through the mitochondrial pathway. Left panel: control and serum-depleted cells were probed with two different antibodies specific for caspase 3: one recognizes the C-terminal domain of the protein, while the other is specific for the N-terminal domain that is present only in the procaspase 3 inactive precursor. As expected, procaspase 3 (N-terminal) can be detected only in control cells, confirming that serum-depleted cells have activated the reactions responsible for zymogen maturation. For the conditions used in the experiment, control cells also give positive reaction also when probed with the Cterminal specific antibody. Cells were counter-stained with hematoxylin QS (X1575).

GLAUCOMA EXPERIMENTAL CONDITION

Intraocular pressure (IOP) after hypertension induction

After injection of 15µl of 2% methylcellulose (MTC) in the anterior eye chamber, the intraocular pressure increased until 60 mmHg in 5 minutes, returning to the basal pressure in the following 60 minutes. The following events are sequentially involved in pressure changes in the first hour:

1. MTC occludes the trabecular meshwork (TM)
2. Compensative mechanism re-establishes the basal pressure.

In the time comprised between 60 and 180 minutes the pressure gradually increases until second maximum peak (approximately 54 mmHg), due to the additional occlusion of Schlemm's canal.

After 6 hours after MTC injection IOP stabilizes around 25mmHg(fig 16A). After 72 hours IOP decreases until 9-10 mmHg (fig 16B), due to the ocular damage.

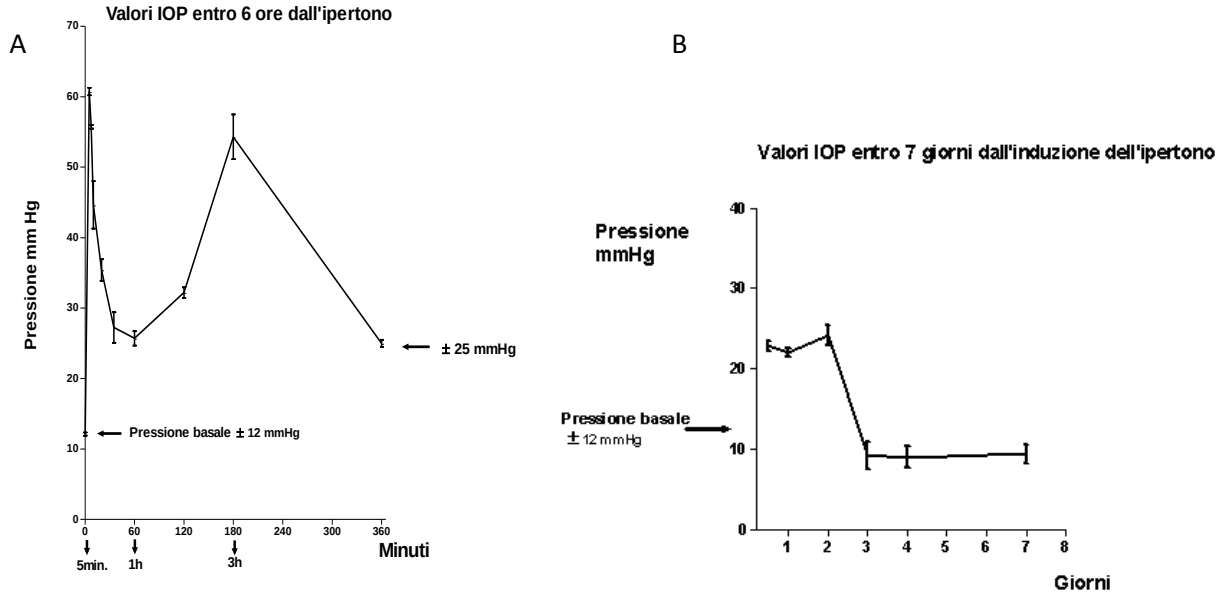


Fig. 16: IOP in rat after injection of 15µl of 2% methylcellulose. Values are the mean ± ES.

Morphological analysis

In eye sections taken from normal rats, optic nerve fibers are tightly arranged (fig17A) and surrounded by quiescent astrocytes (small and round nuclei), disposed in rows (fig 17A'); retina also shows its characteristic morphology (see fig 17C).

In eye sections taken from treated rats, 24 h after MTC injection, the following alterations are detected:

- in optic nerve, fibers do not run longitudinally, the papilla shows an excavation or "cupping" (fig 17B) and the majority of astrocytes shows drumstick-shaped

and expanded nuclei (fig 17B'); both nuclear hypertrophy and chromatin fragmentation are considered as cell activation signs.

- in retina, picnotic and/or vacuolated nuclei and fragmented chromatin are found in glial cells of the nerve fiber layer (fig 17C', arrows), in Muller cells (Fig 17C", arrows) and in neurons of ganglionic cell layer (fig 17C', RGC); these alterations argue in favor to the hypothesis that retinal cells are undergoing to cell death.

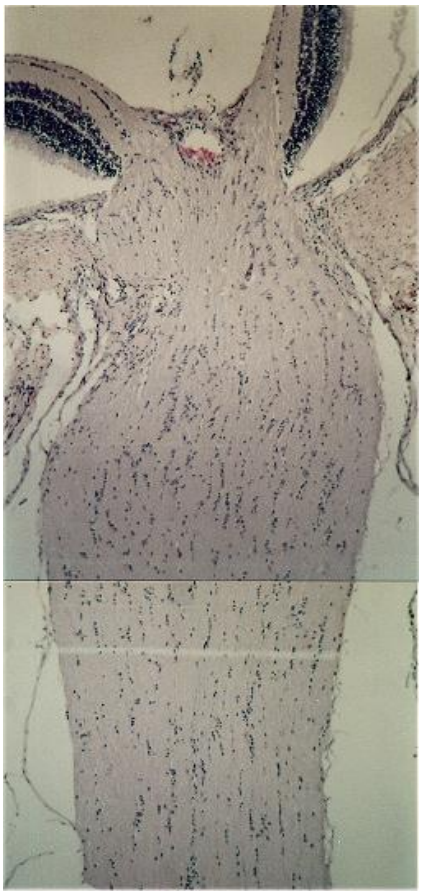


Fig 17A

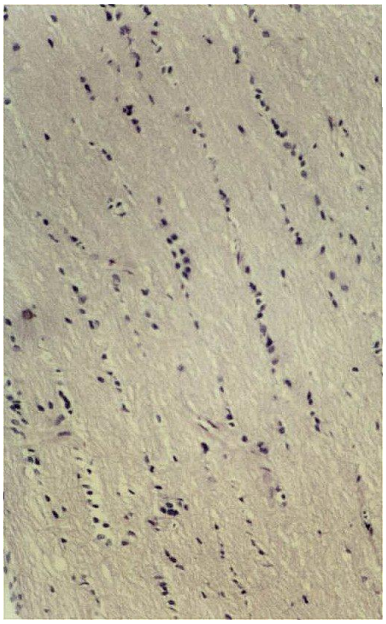


Fig. 17A'

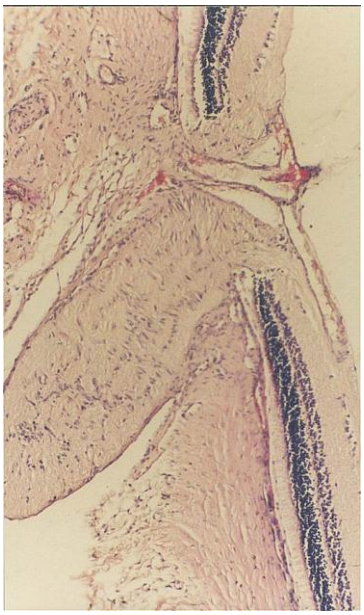


Fig. 17B

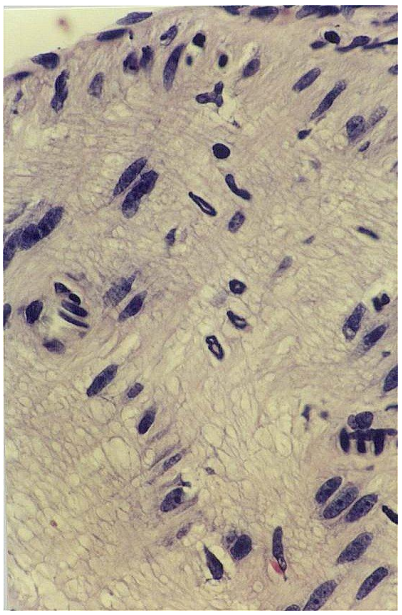


Fig. 17B'

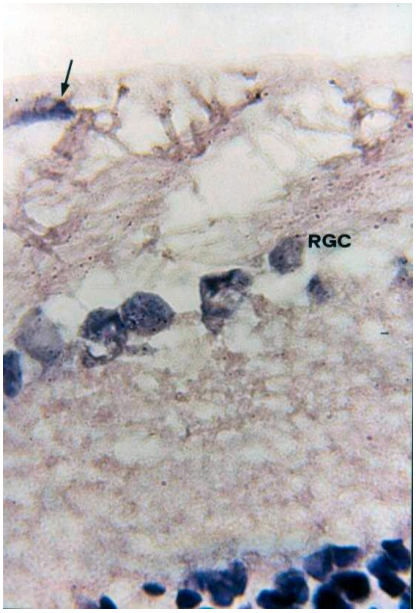


Fig. 17C

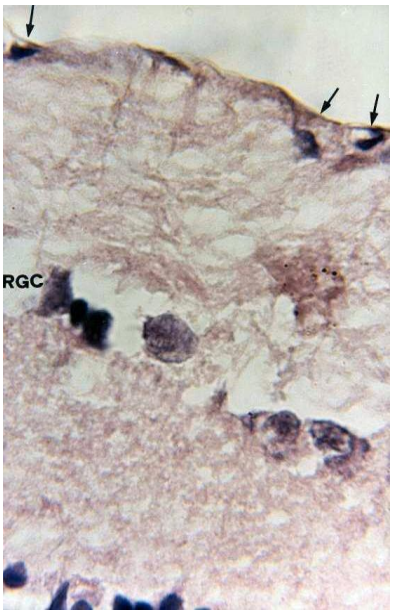


Fig. 17C'

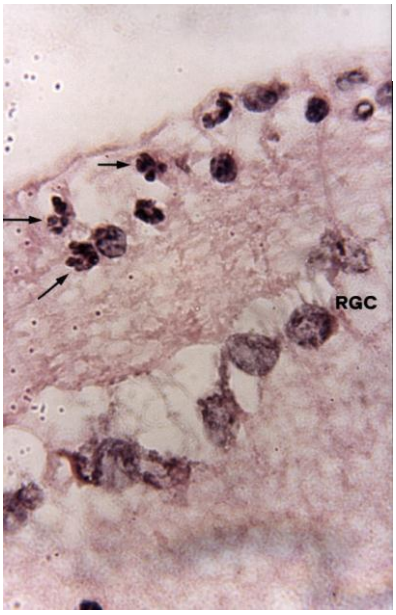


Fig 17C"

Fig. 17A and 17A') optic nerve from control rat.

Fig.17B and 17B') Optic nerve from MTC-injected rat, sacrificed 24 hours after hypertension induction.

Fig. 17C) Retina from control rat.

Fig. 17C') Retina from MTC-injected rat, sacrificed 24 hours after hypertension induction.

Fig. 17C") Retina from MTC-injected rat, sacrificed 24 hours after hypertension induction

Carnitine-injection treatment

Eyes taken from MTC-injected rats (24 h after hypertension induction) by the research team that supported me during PhD period, had been previously submitted to iNOS immunolocalization, (Pescosolido et al.,2009).

These results allowed to hypothesize that the observed cell damages could be due to an hypoxia/hyperoxia stress, leading to free radicals production, lipoperoxidation and cellular membranes destabilization. This hypothesis is in good agreement with literature: Goldstein and Geyer reported this damage for retina, including iNOS activation and NO increased levels (Goldstein et al., 1995; Geyer et al., 1995). As known, high levels of NO are cytotoxic because it reacts with radical molecules (Crow et al., 1996; Ullrich et al., 2000) causing lipid peroxidation. It has also been demonstrated that in retina the expression of iNOS causes neuronal death in vitro, while in vivo it has been showed that iNOS induction causes apoptosis in the inner nuclear layer (Sennlaub et al., 2002).

On the basis of these above considerations, in the present study I wanted to analyze the possible effectiveness of carnitine , well-known as able to protect cell membranes, in counteracting the effects of oxidative stress in this experimental model. Therefore I administered carnitine together with MTC in the anterior eye chamber (see material and methods).

iNOS immunolocalization and Western blot

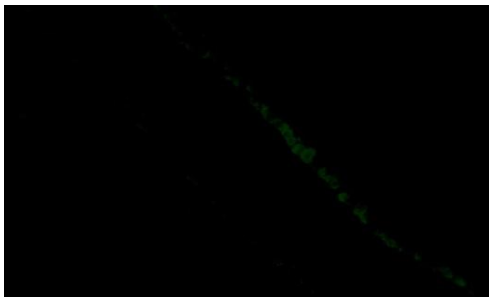


Fig 18a Section of retina from control rat (x50)

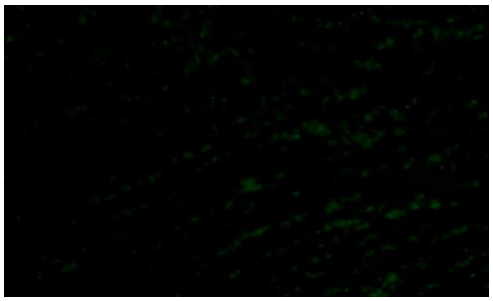


Fig 18b Section of optic nerve from control rat (x50)

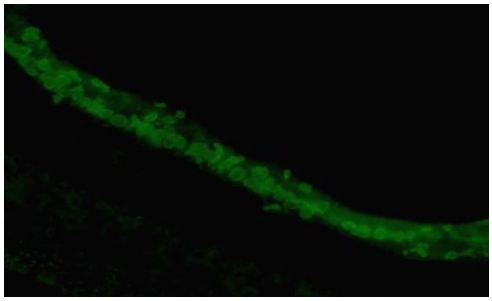


Fig 18c Section of retina from 2%MTC injected rat (x50)

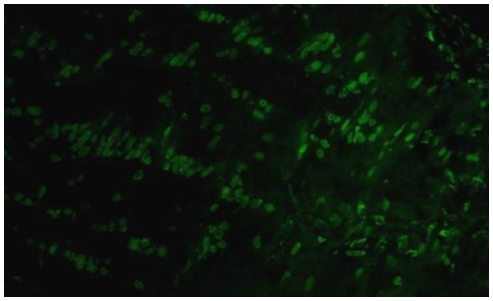


Fig 18d Section of optic nerve from 2%MTC injected rat (x50)

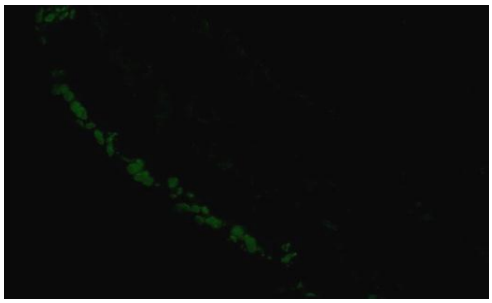


Fig 18e Section of retina from 2%MTC+0,6mM carnitine-injected rat (x50)

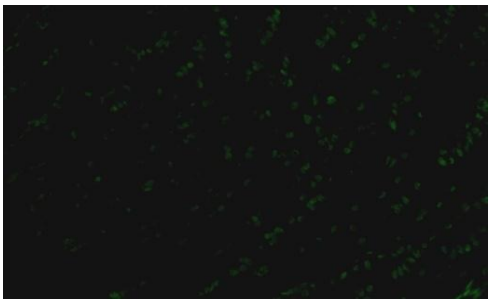


Fig 18f Section of optic nerve from 2%MTC+carnitine 0,6 mM-injected rat (x50)

24 hours after hypertension induction with methylcellulose, high levels of iNOS are expressed both in glial retinal cells ad astrocytes of optic nerve, suggesting the presence of ischemic events. An important role is played by the reperfusion, that avoids cell necrosis, but on the other hand generates high levels of free radicals after the short hypoxic period.

In specimens from 2% MTC + 0,6mM carnitine-treated rats, levels of iNOS significantly decreased. This result, suggesting a cytoprotective effect for carnitine, is in agreement to previous our data (Pillich et al., 2005) and literature (Mutomba et al., 2000; DiMarzio et al., 1997).

Western blots were performed in order to quantify iNOS expression (130 kDa) and results were normalized by actin localization (50 kDa). Since NO is known to be early produced after pressure and ischemic stress, I decided to assay iNOS expression also 6 hours after hypertension induction.

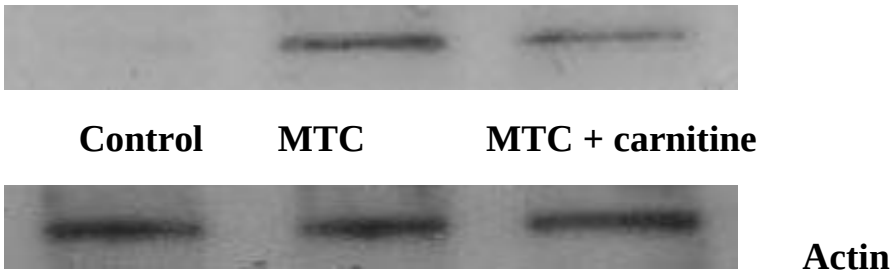


Fig.19a Rat retina homogenates, 6h after treatment

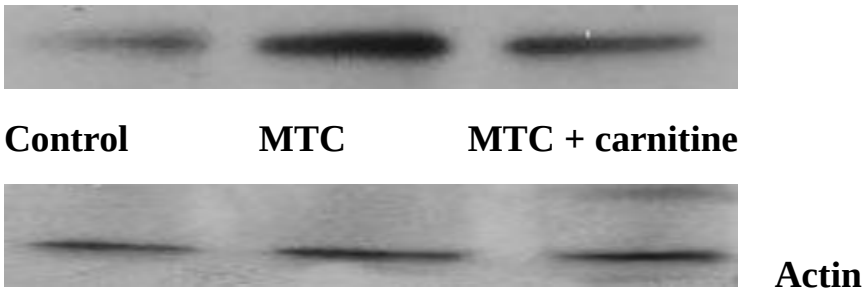


Fig.19b Rat retina homogenates, 24h after treatment

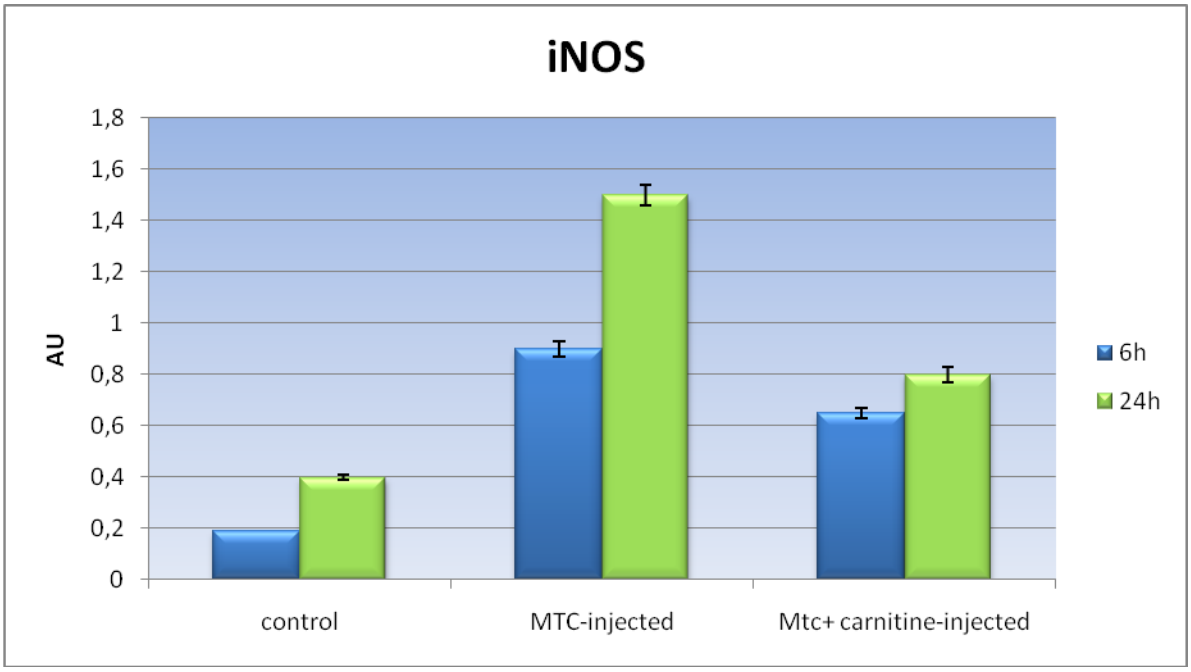


Fig.20 Bars report the level of malondialdehyde (MDA). The values represent Mean ± S.E:M.

Concerning the 24 h treatment Western blots results confirm immunolocalisation data (Fig.19b and Fig.20).

Concerning the 6 h treatment iNOS (130kDa) was already detectable as supposed (Fig.19a and Fig.20).

Summarizing, the co-administration of both methylcellulose and carnitine in rats decreases iNOS levels, suggesting an improved response to ischemic damage.

Lipoperoxidation

Lipid peroxidation is well-known to damage cell membranes and therefore can be used as oxidative stress marker (Petit et al., 1995; Chancerelle et al., 1995). Specifically, lipid peroxidation rate (LPO586) can be evaluated on the basis of concentration of malonyl-dialdheyde (MDA), one of the main products resulting from polyunsatured fatty acids decomposition. Lipid peroxidation reaction, that occurs in ocular tissues after pressure damages, is increased during reperfusion, therefore it is advisable to perform LPO586 test at least three hours after injection.

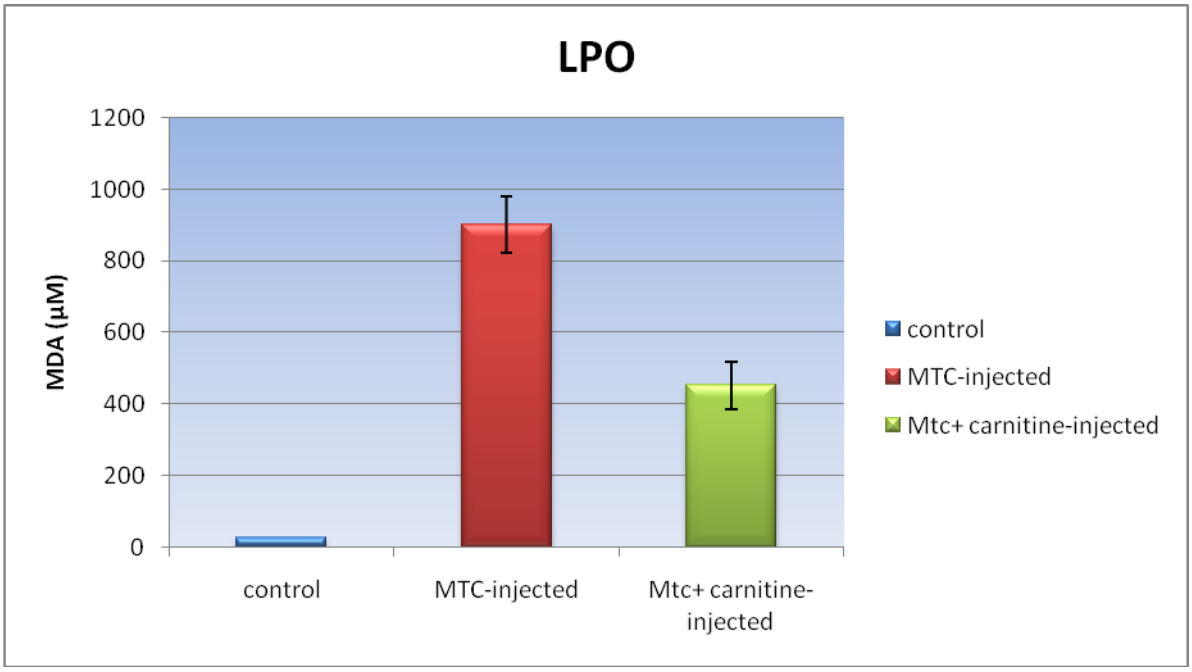


Fig. 21 Bars report the level of malondialdehyde (MDA). The values represent Mean ± S.E:M.

In the above described eye hypertension model, lipoperoxidation quantitative assay, carried out 3 hours after MTC injection, showed lower MDA levels in specimes from 2% MTC+0,6mM carnitine-treated animals compared to 2% MTC-treated ones, confirming the protective effect exerted by carnitine (Fig.21).

Therefore carnitine is effective in protecting the lipid compound of cell membranes. Concerning mitochondria this protection is addictive with the key role played by carnitine in long chain fatty acids transportation.

Caspase-3 immunolocalisation and Western blot

Since retinal cells undergo apoptosis as result of hypertension damage (Mutomba *et al.*, 2000; Calandrella *et al.* 2007), I wanted to investigate if carnitine is able to prevent cell death and downregulate caspase activity.

Fig.22a Section of retina from control rat (x50)

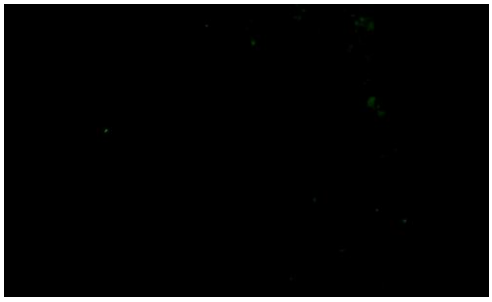


Fig.22b Section of optic nerve from control rat (x50)

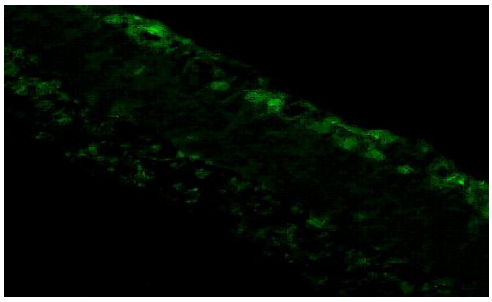


Fig.22c Section of retina from 2%MTC injected rat (x50)

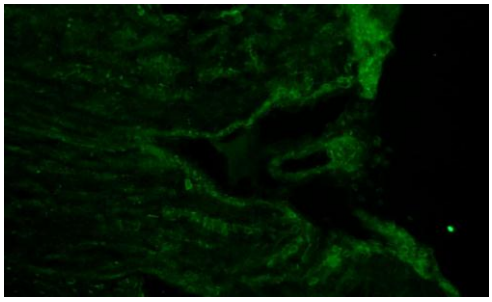


Fig.22d Section of optic nerve from 2%MTC injected rat (x50)

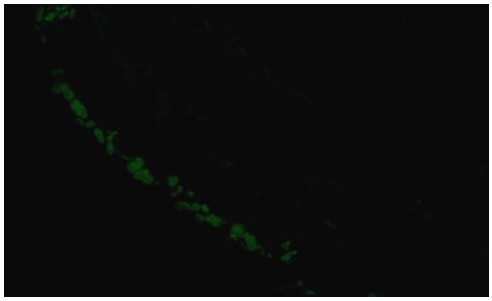


Fig.22e Section of retina from 2%MTC + 0,6 mM carnitine injected rat (x50)

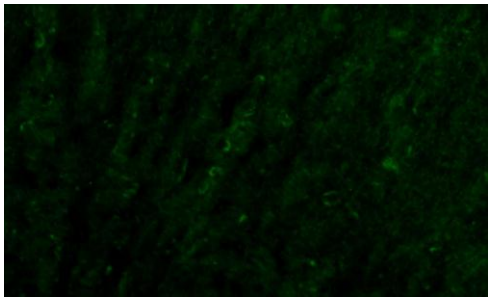


Fig.22f Section of optic nerve from 2%MTC + 0,6 mM carnitine injected rat (x50)

24 hours after hypertension induction with methylcellulose, high levels of caspase-3 are expressed both in glial retinal cells ad astrocytes of optic nerve (Fig.22c and Fig.22d) due to the triggered cell death process, suggesting a reduced stability of mitochondrial membranes: the increased lipid peroxidation reaction, that occurs in

tissues due to the oxidative stress, leads to the pro-apoptotic factors release such as caspase-3.

In specimens from 2%MTC + 0,6mM carnitine-treated rats, levels of caspase-3 significantly decreased (Fig.22e and Fig.22f). This result, suggesting a cytoprotective effect for carnitine, is in agreement to previous our data (Pillich et al., 2005) and literature (Mutomba et al., 2000; DiMarzio et al., 1997).

Western blots were performed in order to quantify caspase-3 expression (30 kDa) and results were normalized by actin localization (50 kDa).

Since caspase-3 is known to be late produced after pressure and ischemic stress, I decided to assay caspase-3 expression both at 6 and 24 hours after hypertension induction.

Concerning the 24 h treatment Western blots results confirm immunolocalisation data (Fig.23b and Fig.24).

Concerning the 6 h treatment caspase-3 (30kDa) was not detectable as supposed(Fig.23a and Fig.24).

Summarizing, the co-administration of both methylcellulose and carnitine in rats decreases caspase-3 levels, suggesting an improved response to ischemic damage.

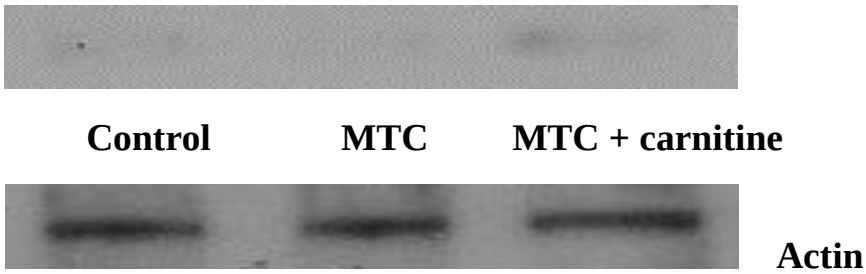


Fig.23a Rat retina homogenates, 6h after treatment

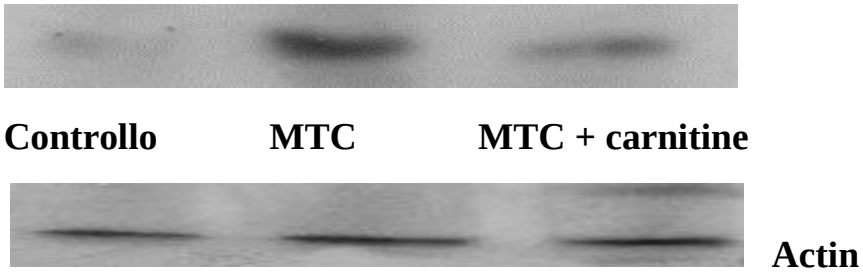


Fig.23a Rat retina homogenates, 6h after treatment

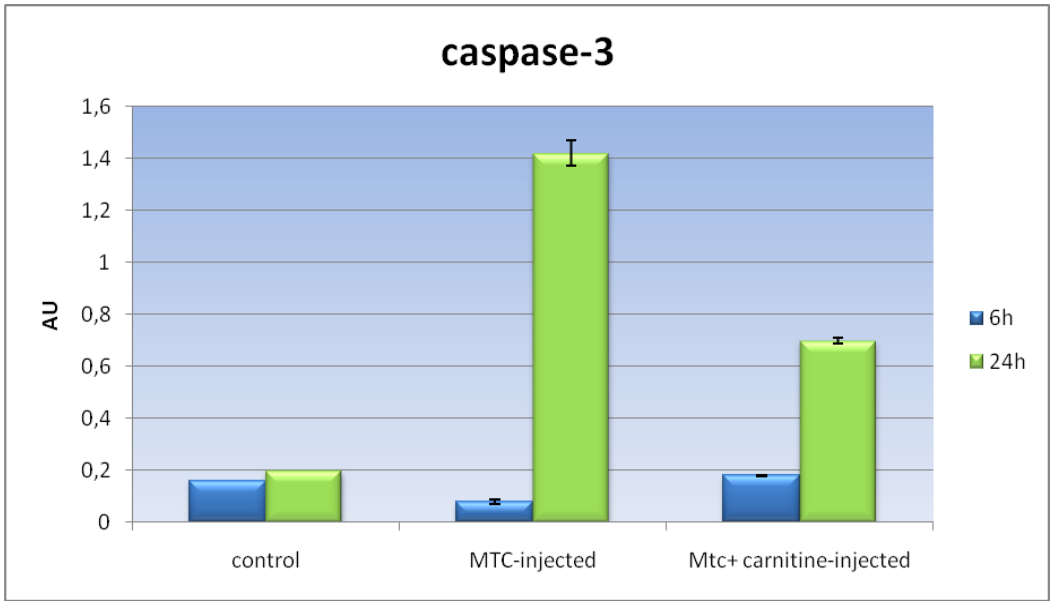


Fig 24 Bars report the level of caspase-3. The values represent Mean ± S.E:M.

Western blots for caspase-3 confirm previous immunolocalisation data.

30kDa protein is detectable 24 hours after hypertension induction with methylcellulose, but not after 6 hours, so an early activation of apoptotic program in retinal cells can not be confirmed. The co-administration of both methylcellulose and carnitine in rats decreases caspase-3 levels, suggesting a down-regulation of apoptotic program.

α-lipoic acid and SOD treatment

After the study of damage markers produced by ocular hypertension and above described, I decided to investigate if two well-known free radical scavengers, *i.e.* α-lipoic acid and SOD, play a role in cell protection. Specifically, I studied the effectiveness of a dietary supplementation in mitigating hypertone ocular damages. The treatment was performed with α-lipoic acid, MnSOD, or α-lipoic acid+ MnSOD, administered for 8 weeks before MTC injection.

The enriched diets contained:

- 0,14 mg/day SOD
- 3 mg/day α-lipoic acid
- 0,14 mg SOD+3 mg α-lipoic acid/day

Control rats were fed with a normal pellet diet.

Hematoxylin/eosin staining

Optic nerve and retina of MTC-injected animals fed with α-lipoic acid and SOD showed milder alterations (Fig 25a, 25d) with respect to those previously described in MTC-injected normally fed ones (Figs, 17B,17B', 17C', 17C"). In optic nerve the α-lipoic acid + SOD- treatment seems especially effective in preventing the hypertone-induced disarrangement of columnar pattern of astrocytes.

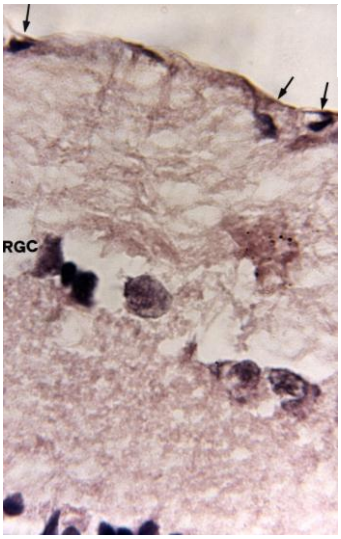


Fig.25a

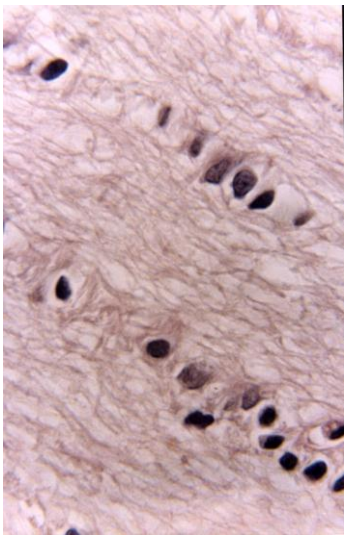


Fig.25b

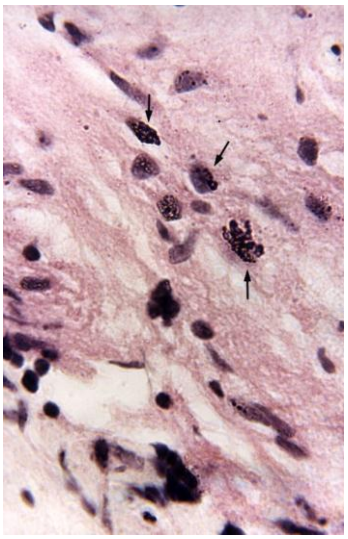


Fig.25c

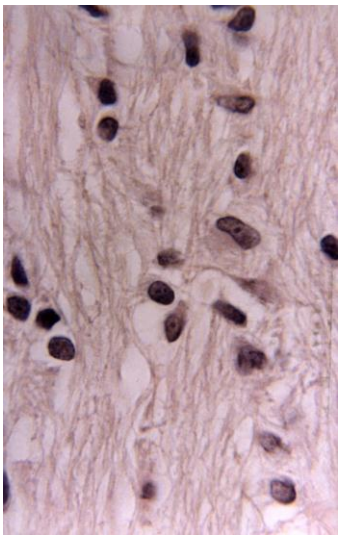


Fig.25d

Fig. 25a) Retina from MTC-injected rat, fed with α-lipoic acid+ SOD. RGC=retinal ganglionic cells. Arrows=Muller cells.

Fig. 25b) Optic nerve from control rat.

Fig. 25c) Optic nerve from MTC-injected rat. Arrows= vacuolated nuclei.

Fig. 25d) Optic nerve from MTC-injected rat, fed with α-lipoic acid+ SOD.

DNA fragmentation

TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick end Labeling) was used to evaluate DNA degradation in retina and optic nerve. TUNEL, in facts, marks nuclear damage due to the endonuclease-caused DNA fragmentation, both at early and advanced death stages.

Fig.26a Optic nerve head and retina from control rat (x50)

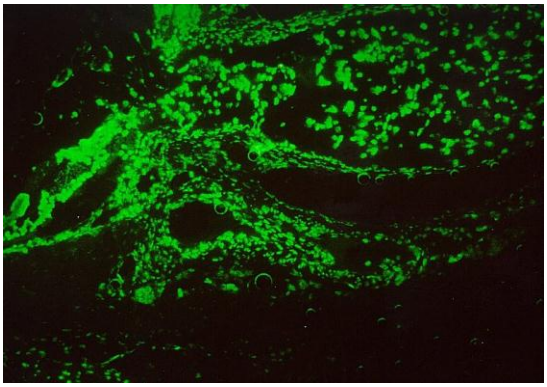


Fig.26b Optic nerve head and retina from MTC-injected rat (x50)

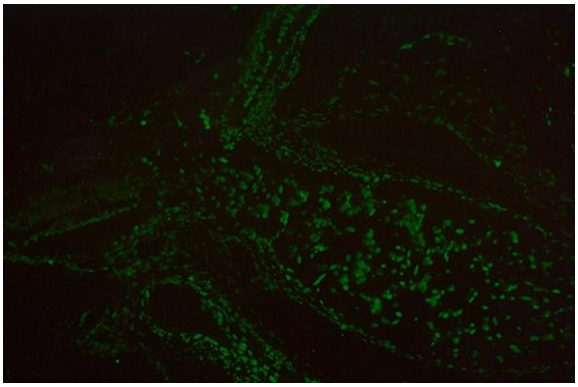


Fig.26c Optic nerve head and retina from MTC-injected rat α-lipoic treated

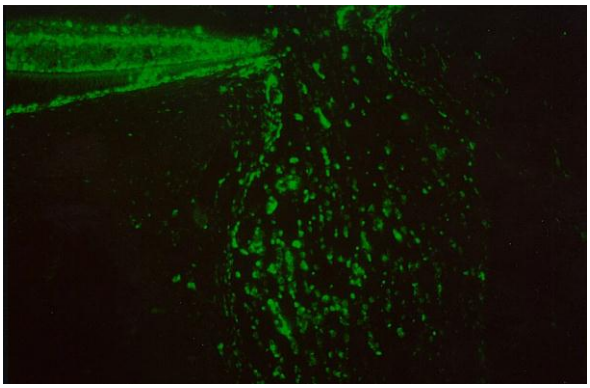


Fig.26d Optic nerve head and retina from MTC-injected rat SOD treated

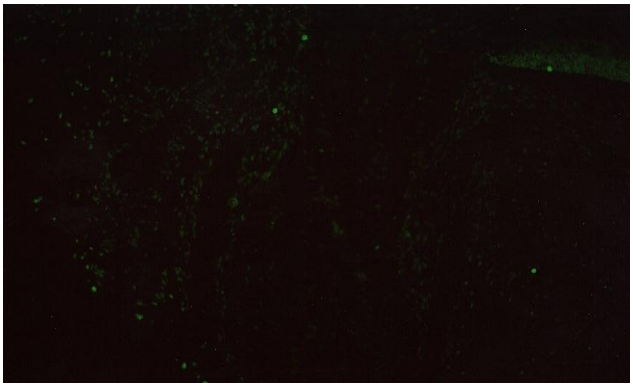


Fig.26e Optic nerve head and retina from MTC-injected rat α-lipoic acid+SOD treated

Results can be summarised as follows:

- Specimens from normally fed MTC-injected animals showed an extremely high fluorescence level, due to DNA damage (Fig.26b);
- specimens from SOD-fed rats showed a mild decrease of fluorescence (fig.26d);
- in specimens from α-lipoic acid-treated animals the fluorescence decrease was sensibly high; this is probably due to the great solubility of α-lipoic acid in membranes and in blood-retina barrier (Fig.26c);
- in specimens from α-lipoic acid + SOD-treated animals the staining pattern was quite similar to that found in not injected rats. Therefore the two drugs seem to act in additive manner in preventing DNA degradation (Fig.26e).

iNOS immunolocalization

Fig. 27a Optic nerve head from control rats

Fig. 27a' Retina from control rats

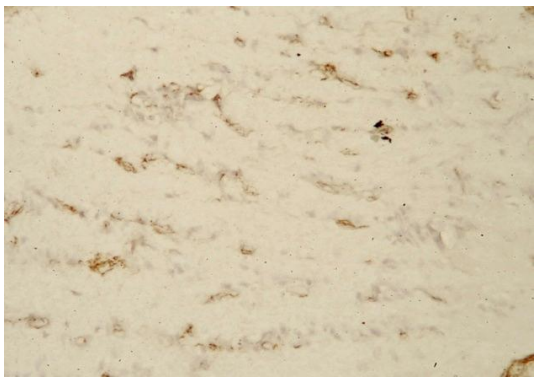


Fig. 27b Optic nerve head from MTC-injected rat

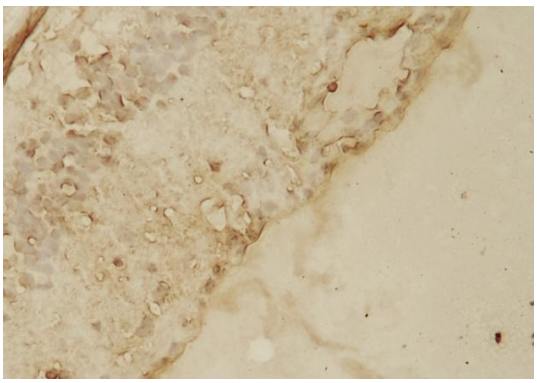


Fig. 27b' Retina from MTC-injected rat

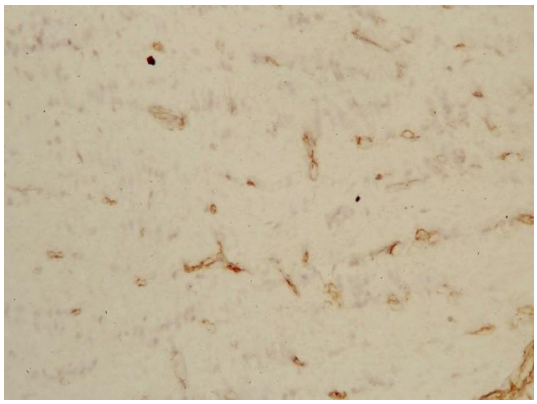


Fig. 27c Optic nerve head from MTC-injected rat+ SOD

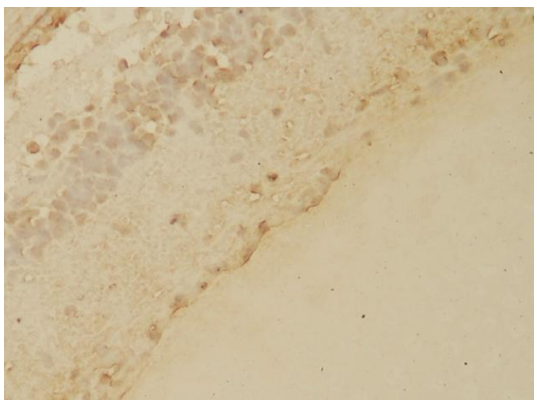


Fig. 27c' Section of retina from MTC-injected rat+ SOD

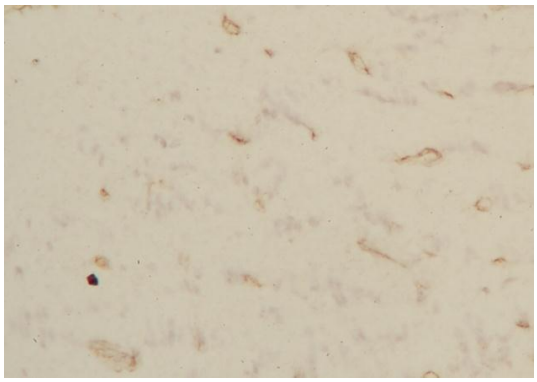


Fig. 27d Optic nerve head from MTC-injected rat+ α-lipoic acid

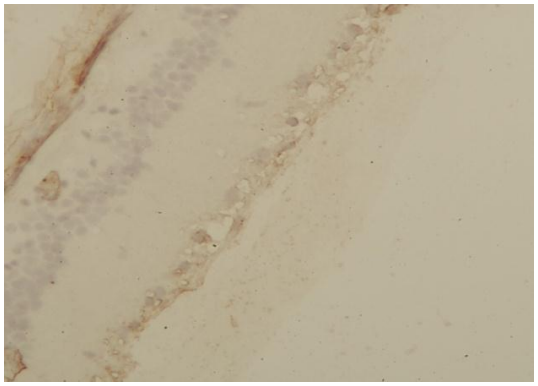


Fig. 27d' Retina from MTC-injected rat+ α-lipoic acid

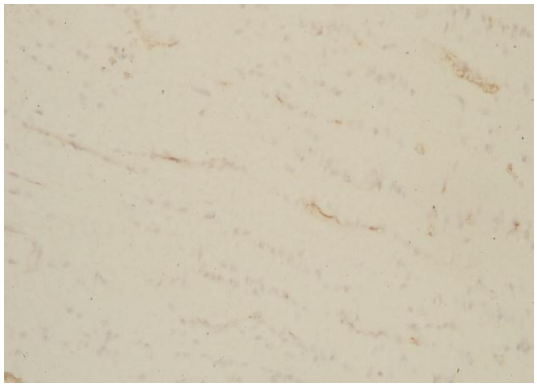


Fig. 27e Optic nerve head from MTC-injected rat+ α-lipoic acid+SOD

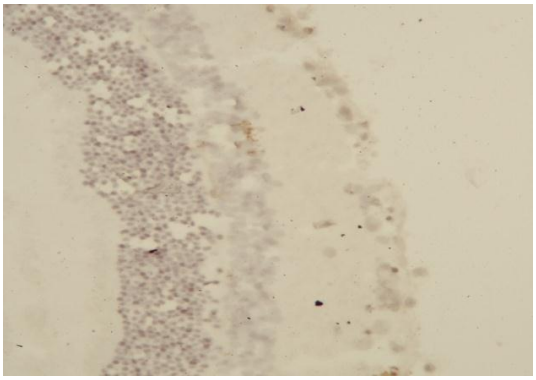


Fig. 27e' Retina from MTC-injected rat+ α-lipoic acid+SOD

24 hours after hypertension induction with methylcellulose, high levels of iNOS are expressed both in glial retinal cells ad astrocytes of optic nerve, suggesting the presence of ischemic events. An important role is played by the reperfusion, that avoids cell necrosis, but on the other hand generates high levels of free radicals after the short hypoxic period.

Results can be summarized as follows:

- Specimens from normally fed MTC-injected animals showed a high positivity, due to DNA damage (fig 27b, 27b');
- specimens from SOD-fed rats showed a mild decrease of positivity (fig 27c, 27c'); this low SOD effectiveness is probably due to its difficulty in entering into cells (probably it acts on free radicals iso-hydric balance);

- in specimens from α-lipoic acid-treated animals the staining decrease was sensibly high (fig 27d, 27d'); this is probably due to the great solubility of α-lipoic acid in membranes and in blood-retina barrier;
- in specimens from α-lipoic acid + SOD-treated animals the staining pattern was quite similar to that found in not injected rats (fig 27e, 27e'). Therefore the two drugs seem to act in additive manner in preventing iNOS over-expression.

Western blots were performed in order to quantify iNOS (130 kDa) and results were normalized by tubulin localization (55 kDa).

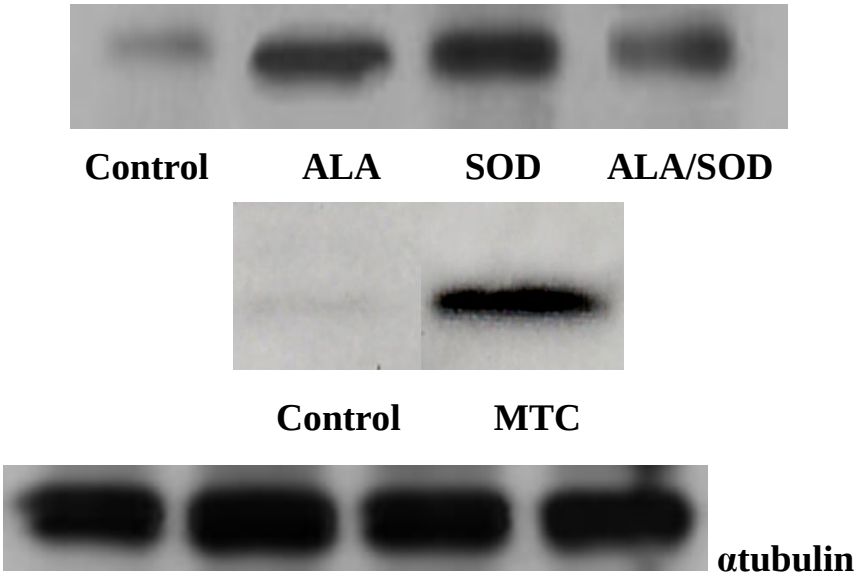


Fig.28 Rat retina homogenates

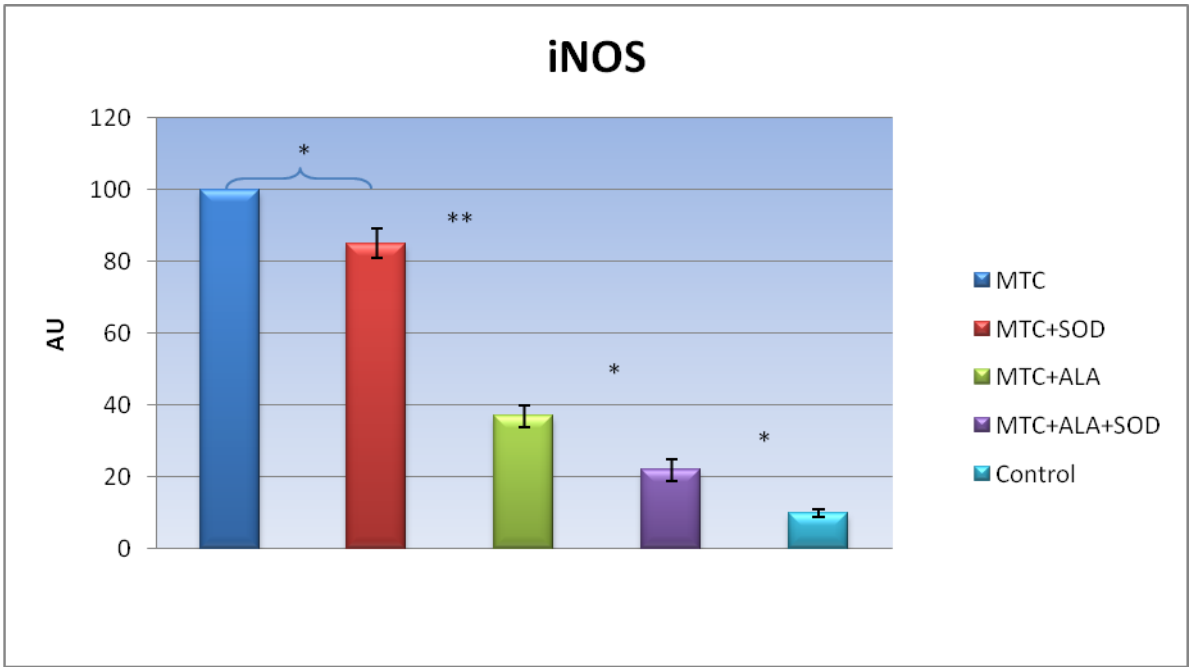


Fig 29 Bars report the level of iNOS. The values represent Mean ± S.E:M.

Western blots for iNOS confirm previous immunolocalisation data.

Lipoperoxidation

The extent of membrane damage was assessed by measuring the production of MDA, a widely recognized marker of lipoperoxidation, whose production occurs in cells directly from a damage to the membrane structure and function. Samples taken from MTC-injected eyes showed extensive lipoperoxidative damage, as indicated by the very high cytoplasmic MDA concentration. The pre-treatment with ALA + SOD reduces the level of lipoperoxidation to 40%.

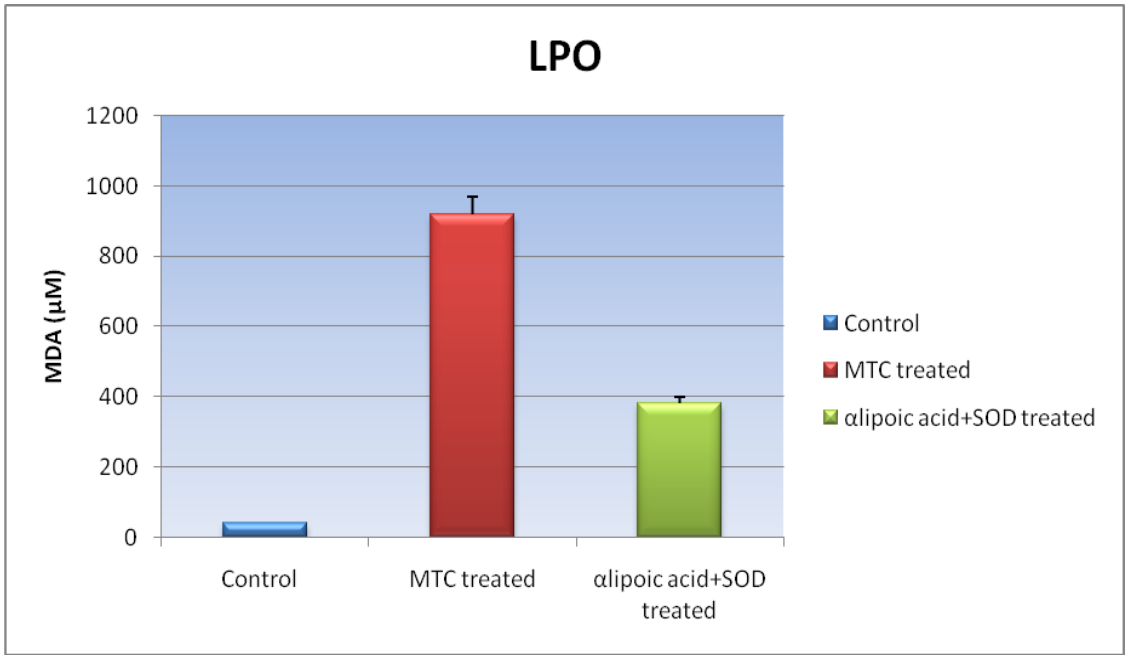


Fig 30 Bars report the level of malondialdehyde (MDA). The values represent Mean ± S.E:M.

Caspase-3

Since retinal cells undergo apoptosis as result of hypertension damage (Mutomba *et al.*, 2000, Calandrella *et al.* 2007), I wanted to investigate if α-lipoic acid and SOD are able to prevent cell death and down-regulate caspase activity.
Results can be summarized as follows:

- Specimens from normally fed MTC-injected animals showed a high positivity, due to DNA damage (fig31b, 31b');
- specimens from SOD-fed rats showed a mild decrease of positivity (fig 31c, 31c'); this low SOD effectiveness is probably due to its difficulty in entering into cells (probably it acts on free radicals iso-hydric balance);
- in specimens from α-lipoic acid-treated animals the staining decrease was sensibly high(fid 31d, 31d'); this is probably due to the great solubility of α-lipoic acid in membranes and in blood-retina barrier;
- in specimens from α-lipoic acid + SOD-treated animals the staining pattern was quite similar to that found in not injected rats (fig. 31e, 31e'). Therefore the two drugs seem to act in additive manner in preventing caspase-3 over-expression.

Western blots were performed in order to quantify caspase-3 (30 kDa) and results were normalized by tubulin localization (55 kDa).

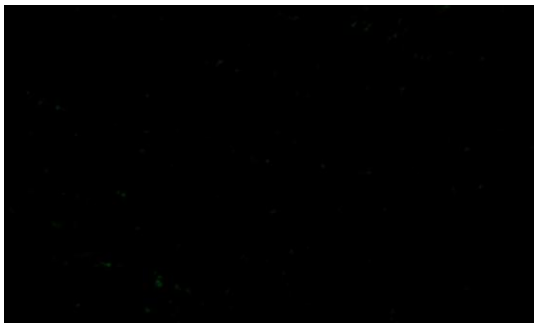


Fig.31a Optic nerve head from control rats

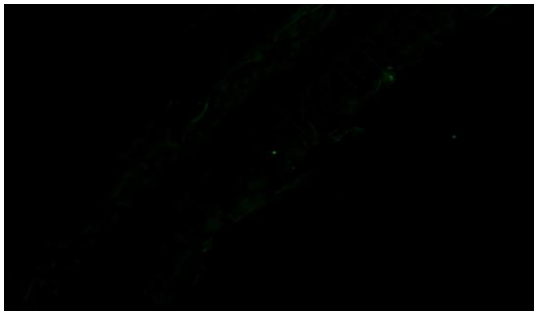


Fig.31a' Retina from control rats

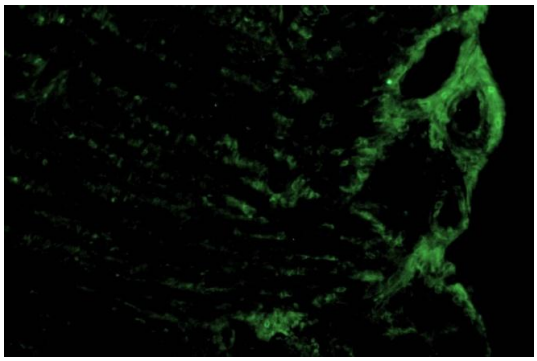


Fig. 31b Optic nerve head MTC-injected from rat

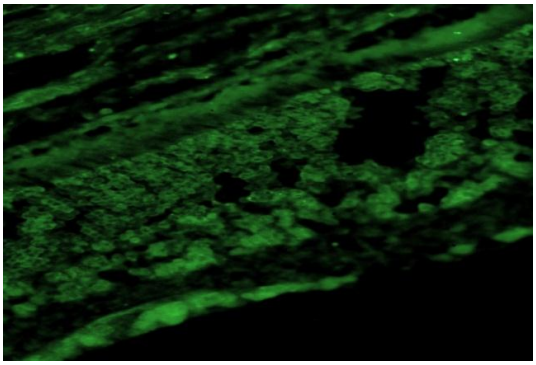


Fig. 31b' Retina from MTC-injected rat

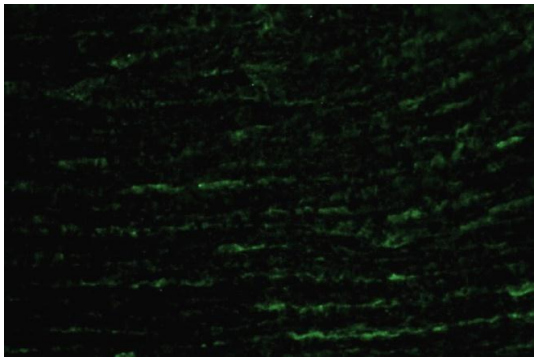


Fig.31c Optic nerve head from MTC-injected rat+SOD

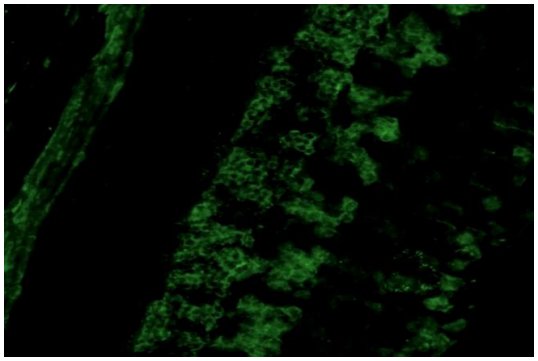


Fig.31c' Section of retina from MTC-injected rat+SOD

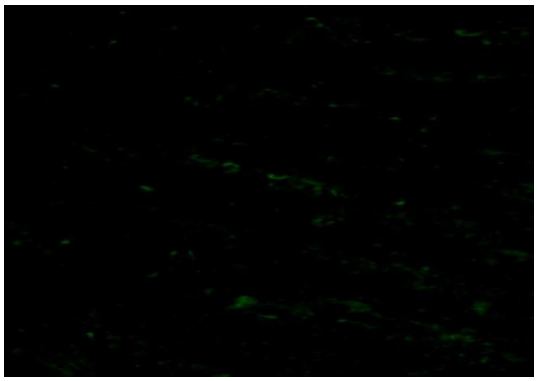


Fig. 31d Optic nerve head from MTC-injected rat+ α-lipoic acid

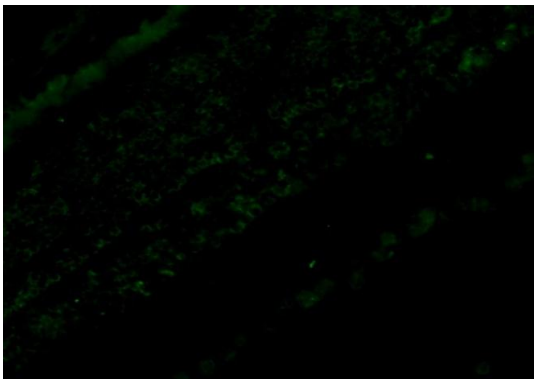


Fig. 31d' Retina from MTC-injected rat+ α-lipoic acid

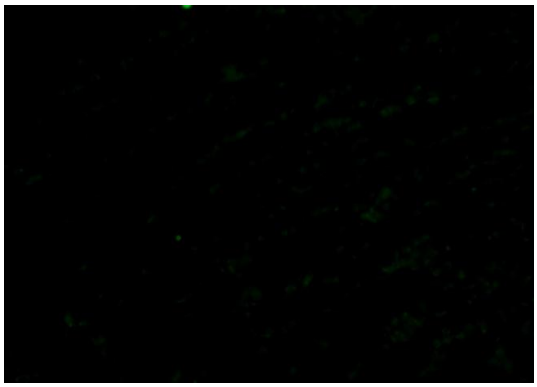


Fig. 31e Optic nerve head from MTC-injected rat+ α-lipoic acid+SOD

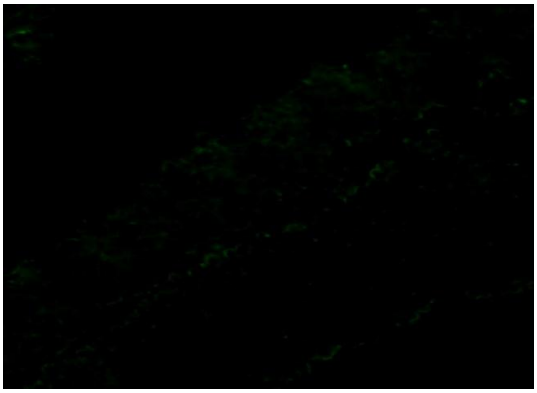
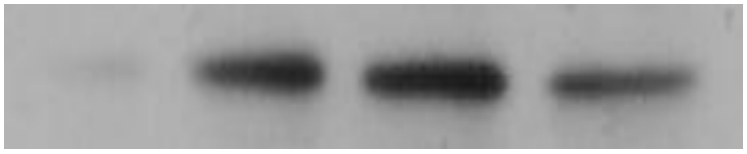
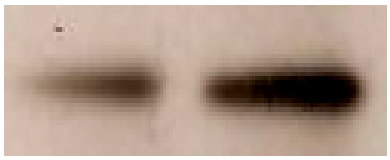


Fig. 31e' Retina from MTC-injected rat+ α-lipoic acid+SOD



Control ALA SOD ALA/SOD



Control MTC

αtubulin

Fig.32 Rat retina homogenates

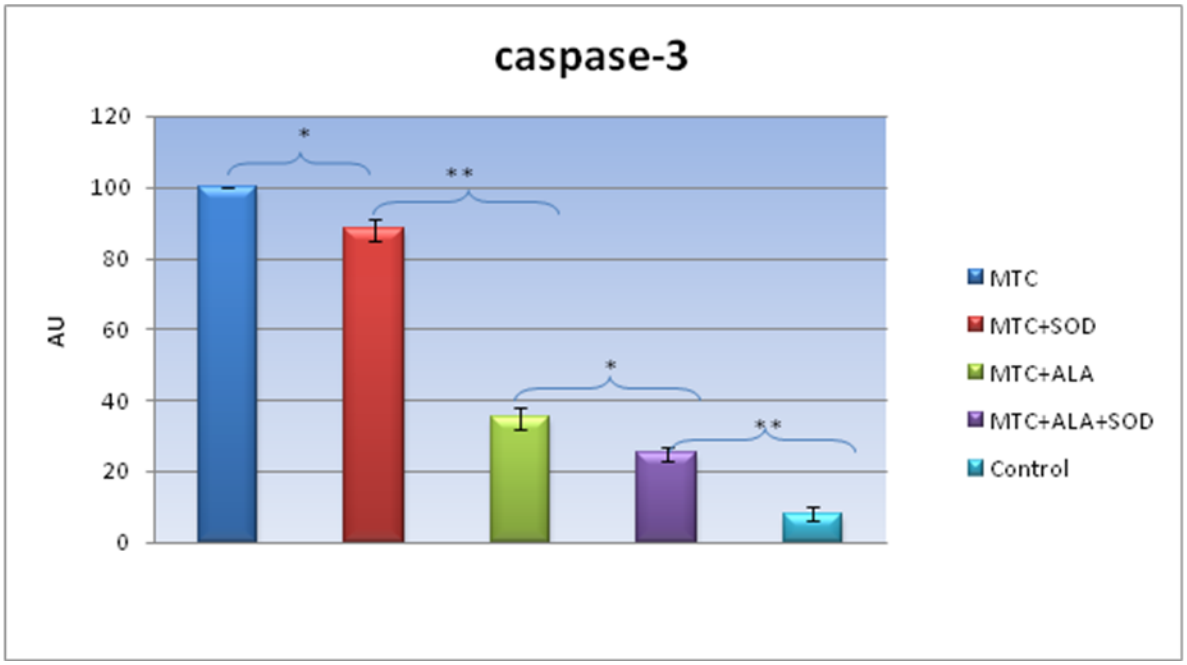


Fig 33 Bars report the level of caspase-3. The values represent Mean ± S.E:M.

Western blots for caspase-3 confirm previous immunolocalisation data.

UV IRRADIATION EXPERIMENTAL CONDITION

Both HaCat cells (immortalized keratinocyte line) and primary keratinocytes (HEK) are widely accepted models for the human epithelia pathologies.

HaCat cells

Irradiation of HaCat cultures with 4mJ/cm^2 of UVA/B resulted in decreasing numbers of viable cells as compared to non-irradiated ones. In previous experiments, where cells were incubated with resveratrol at concentrations ranging from 10 to 50 µM, 50 µM resveratrol was the most effective and non-toxic concentration (data not shown). Incubation of UV-irradiated keratinocytes with resveratrol at concentration 50 µM for 24 hours significantly increased the number of cells in culture as compared both to irradiated and also control cells (fig 34). Moreover I observed that resveratrol was effective in improving cell viability also in non-irradiated cells (data not shown).

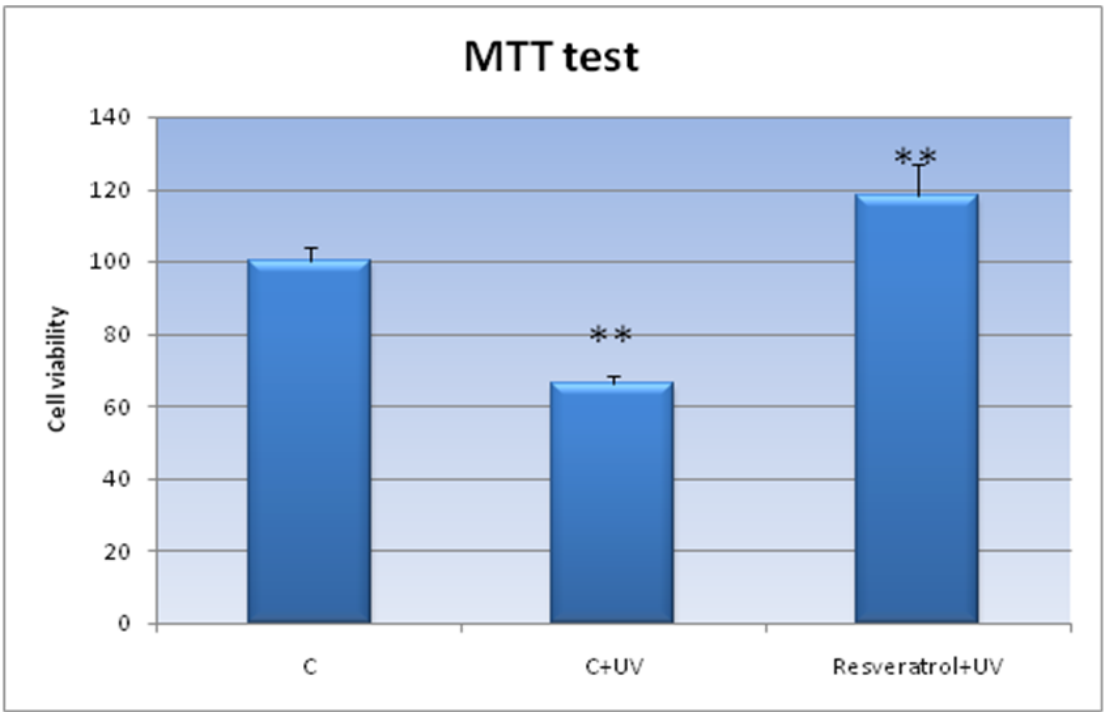


Fig 34 Bars report viability percentage. The values represent Mean ± S.E:M

Primary keratinocytes (HEK)

In HEK cultures resveratrol addition gave effects similar to those found in HaCat, even if the extent of variations was reduced (fig. 35).

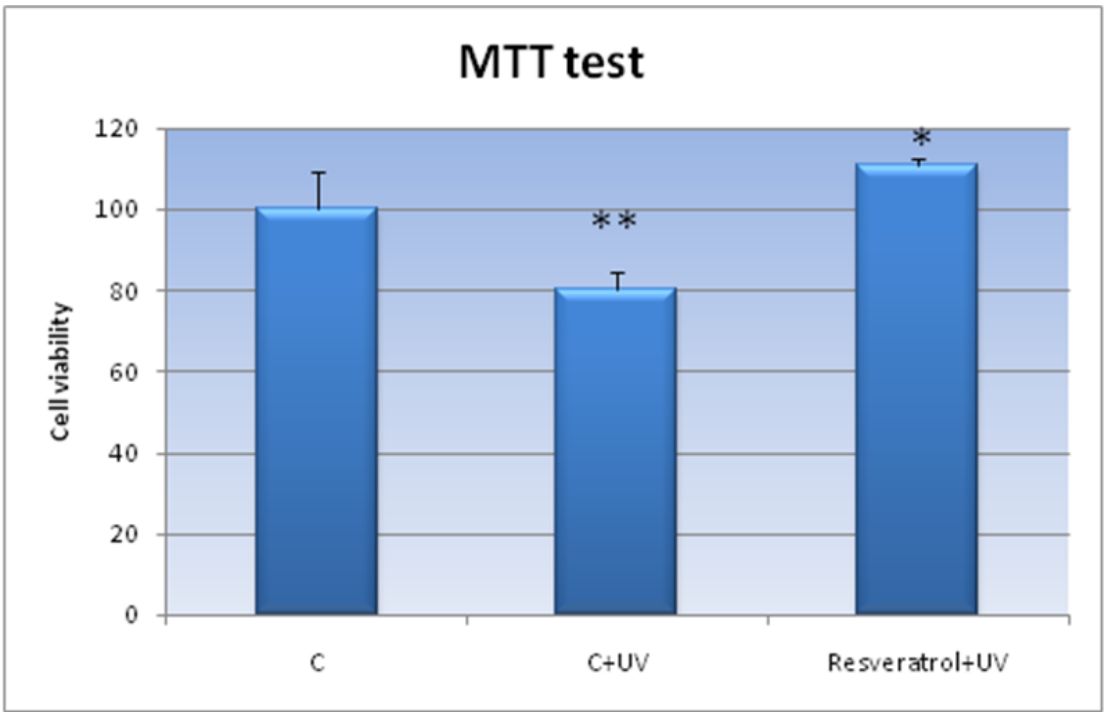


Fig 35 Bars report viability percentage. The values represent Mean ± S.E:M

DISCUSSION

AGING

During human aging, corneal keratinocytes and endothelial cells undergo numerical decrease, probably in response to oxidative stress or to a decreased protection exerted by the antioxidant systems (Patel et al., 2001; Cejkova et al., 2004); moreover tissue becomes more sensitive to infections, due to decreased ability in counteract physiological stresses. In this research, 4 months-prolonged dietary administration of carnitine to aged rats produces cytoprotective and anti-apoptotic effects in corneas undergoing physiological aging process.

In particular, carnitine causes:

- MDA level reduction;
- lower DNA degradation;
- iNOS down-regulation and free radical concentration reduction;
- caspase-3 down-regulation, due to increased stabilization of mitochodrial membranes.

Concerning aging experimental condition obtained with serum deprivation in fibroblasts, the carnitine-supplemented medium stimulates the mitochondrial metabolism, in this way protecting the whole cell from apoptosis induced by serum withdrawal.

OCULAR HYPERTENSION

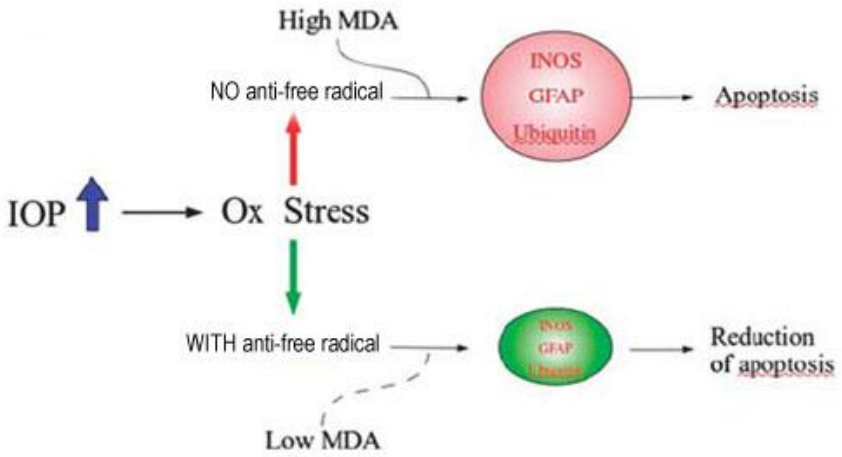
The injection of MTC into rat eye anterior chamber causes an acute hypertensive insult in retina and optic nerve, mimicking the structural, functional, and molecular alterations described for human acute glaucoma.
In ocular hypertension-affected animals, the iNOS over-expression, together with the very high cytoplasmic MDA concentration, argue in favor to an extensive lipoperoxidative damage of mitochondria and, more generally, of cell membranes. Moreover, the severe alterations of normal columnar arrangement of astrocytes and the appearance of cupping in optic nerve, as well as the chromatin condensation in retinal ganglionic neurons, are clear indications of cell sufferance, due to a mechanical and hypoxi-regenerative stress and consistent with an extensive death of astrocytes and RCGs'. Although the TUNEL reaction, that occurs at double strand break points and/or single strand nicks in DNA, is not sufficiently specific to discriminate between apoptotic and necrotic cell death, the significant increase of expression of caspase-3 supports the idea that cell death in these eye districts occurs via apoptosis.

Carnitine, that has a key role in the stabilization and function of the cell membrane, when injected together with MTC, is able to protect from apoptotic death due to oxidative stress. As matter of fact, carnitine reduces the production of MDA but does not completely restore the cell vitality, suggesting that apoptosis is not entirely due to mitochondrial damage. In conclusion, by enhancing the mitochondrial performance, carnitine also improves the overall homeostatic response to the hypertensive insult and limits apoptotic phenomena.

α-lipoic acid is known to directly inhibit membrane phospholipase A_2 (PLA_2), which is in turn activated by mitochondria-derived free radicals (Neufeld et al., 1997; Neufeld, 1999; Garthwaite et al., 2002; Dalkara et al., 1998;Ullrich and Bachschmid, 2000; Lee and Peter, 2003;Risuleo et al., 2003;Ishii et al., 2000; Cifone et al., 1997; Mutomba et al., 2000; Hengartner, 2000; Moretti et al., 2002; Pillich et al., 2005; Alagoz et al., 2002); MnSOD plays a key role in protecting aerobic life from the deleterious effects of oxygen. Methods for increasing the bioavailability of these two drugs might be valuable strategies for the treatment of various diseases involving ROS production, including neurological conditions, cardiovascular disease, and cancer.

In hypertension-affected eyes, dietary administered α-lipoic acid and MnSOD causes:

- MDA level reduction;
- lower DNA degradation;
- iNOS down-regulation and free radical concentration reduction;
- caspase-3 down-regulation, due to increased stabilization of mitochodrial membranes.



The increase of the intraocular pressure (IOP) results in oxidative stress as shown by the overexpression of inducible nitric oxide synthase (iNOS). Mitochondrial lipid peroxidation causes the accumulation of intracellular MDA: a hallmark of lipoperoxidation. The activation of the ubiquitin (Ub)-mediated proteasome pathway is directly related to the execution of the apoptotic death as also shown by the stimulation of caspase 3 expression. Treatment with methylcellulose in the presence of free radicals scavengers reduces the level of all markers of apoptosis, and therefore anti- free radicals also improve the overall homeostatic response and limit apoptotic phenomena.

UV IRRADIATION

UVA- and ⁄ or UVB are known to produce several dose-dependent damages, in different tissues; in this research I focused on the effects exerted in keratinocyte cultures by UV dosages known for causing only free radical production, and not thymine dimerization.
Resveratrol, a small molecule produced (Crozier et al., 2009) by higher plants to combat bacterial infections and withstand stresses and injuries (Korkina et al., 2009; Langcake ad Pryce, 1977; Korkina et al., 2009), has proved to exert tumor chemopreventive, anti-inflammatory, cardioprotective, anti-neurodegenerative, anti-diabetic, and antiaging effects (Reagan-Shaw et al., 2008; Korkina et al., 2009; Athar et al., 2009; Baxter, 2008); recently it was shown to interfere with UVA- and ⁄ or UVB-induced redox stress in keratinocytes.
In my research I carried on Mossman assay (MTT test) to evaluate cell viability of an immortalized cell line (HaCat) and of primary human keratinocytes; it is known in fact that primary cultures and cell lines differently react to treatments.

I quantified the damage produced by UVA/B and tried to identify the higher non-toxic resveratrol dosage effective in counteracting these damages. In both cell populations, RV was able to mitigate the cell death with similar trends but different percentages.

Although preliminary, these results argue in favor to the possible therapeutic role of resveratrol in preventing or ameliorating UV damages in solar light-exposed tissues.

CONCLUSIONS

At low, regulated levels, free radicals are involved in many vital physiological processes. They play a role in various signaling cascades, such as response to growth factors and control of inflammatory responses [Finkel, 2011]. They participate in regulation of many cellular processes, including differentiation, proliferation, growth, apoptosis, cytoskeletal regulation, migration, and contraction [Krause, 2007].

On the other hand free radicals contribute to a wide range of pathologies and many of the implicated diseases are leading causes of death. Cancers, cardiovascular diseases, and neurological diseases all show robust evidence for free radicals involvement. Therefore recent decades have seen a surge of interest in the role of free radicals in health and disease. From basic science research to clinical trials, the biomedical community has rapidly advanced toward a better understanding of free radicals-metabolizing systems and their contribution to specific conditions.

In the present study I focused on "pathological" free radicals role in different experimental conditions and on effectiveness of the free radicals-scavengers carnitine, α-lipoic acid, MnSOD and resveratrol in restoring cell homeostasis.

Despite the different ways of administration (diet, eye injection, or culture medium supplementation) **carnitine** effects in corneas of aged animals, in methylcellulose-injected eyes and in serum-deprived fibroblasts were substantially similar, consisting in free radicals decrease, iNOS and caspase-3 down-regulation and DNA damage mitigation.

Concerning **α-lipoic acid** and **SOD**, their dietary administration to rats submitted to methylcellulose injection was effective in preventing redox stress-induced damages; in fact free radicals concentration decreased, iNOS and caspase-3 were down-regulated, and DNA damage was mitigated.

In cultured keratinocytes, finally, medium added **resveratrol** was able to mitigate UV damages and, moreover, to increase cell viability also in untreated cells.

In conclusion, dietary administration of exogenous free radical scavengers might be a promising strategy for preventing and/or treating human pathologies characterized by redox unbalance.

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