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BIOGENESI MITOCONDRIALE, MISFOLDING PROTEICO E RISPOSTA CELLULARE ALLO STRESS NELL'AGING E NEI DISORDINI NEURODEGENERATIVI: APPROCCIO PROTEOMICO

MITOCHONDRIAL BIOGENESIS, PROTEIN MISFOLDING AND CELLULAR STRESS RESPONSE IN AGING AND IN NEURODEGENERATIVE DISORDERS: PROTEOMIC APPROACH

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INTRODUCTION

Life is the interplay between structure and energy, yet the role of energy deficiency in human disease has been poorly explored by modern medicine (1).

Numerous experimental evidence supporting the idea that the reduction of the expression cell, the activity of antioxidant proteins and the consequent increase in oxidative stress are the fundamental cause of the aging process and neurodegenerative disorders (2).

Several studies suggest that the accumulation of oxidative molecules damaged is one of the factors that could cause senescence. The predominant molecular cause of aging is the accumulation of protein mutated.

Moreover, several conditions including protein, lipid or glucose oxidation disrupt redox homeostasis and lead to accumulation of unfolded or misfolded proteins in the aging brain. Alzheimer's and Parkinson's diseases or Friedreich ataxia are neurological diseases sharing, as a common denominator, production of abnormal proteins, mitochondrial dysfunction and oxidative stress, which contribute to the pathogenesis of these so called ''protein conformational diseases''(3).

The central nervous system has evolved the conserved mechanism of unfolded protein response to cope with the accumulation of misfolded proteins (3).

Harman in 1972 first proposed that mitochondria may have a central role in the process of aging. According to this theory, free radicals generated through mitochondrial metabolism can act as causative factor of abnormal function and cell death (4).

The mitochondrial genome plays a key role in aging and neurodegenerative disorders. Mitochondrial dysfunction is indeed characteristic of various disorders, and damage lead to altered mitochondrial respiratory chain activity as found in Parkinson's disease, in Alzheimer's disease and in Huntington's disease.

These dysfunctions in the activity of respiratory complex, possibly associated with alterations of the oxidant/antioxidant balance, are thought to underlie defects in energy metabolism and induce cellular degeneration (5).

The mitochondrial DNA mutations, both inherited and acquired, cause a malfunction of the electron transport chain (ETC), which leads to the decrease of ATP production, increased formation of toxic free radicals and altered homeostasis of Ca2+. These determine the toxic consequences of ETC dysfunction that promote mitochondrial

damage, including oxidation of mitochondrial DNA, proteins, lipids and altered mitochondrial permeability, an event associated with the degeneration and cell death (6).

Mitochondria are the main significant source of oxidants, and in vitro studies have indicated that approximately 1–2% of electron flow through the ETC results in the univalent generation of superoxide (4).

Moreover, various toxins in the environment can injure mitochondrial enzymes, leading to increased generation of free radical that over the life span would eventually play a major role in aging.

During the last few years, cellular oxidant/antioxidant balance has become the subject of intense study, especially by those interested in brain aging and in neurodegenerative mechanisms (7).

As one of the main intracellular redox systems involved in neuroprotection, , the *vitagene system* is emerging as a neurohormetic potential target for novel cytoprotective interventions.

Vitagenes encode for cytoprotective heat shock proteins (Hsp) Hsp70 and heme oxygenase-1, as well as thioredoxin reductase and sirtuins.

Nutritional studies show that ageing in animals can be significantly influenced by dietary restriction. Thus, the impact of dietary factors on health and longevity is an increasingly appreciated area of research.

Reducing energy intake by controlled caloric restriction or intermittent fasting increases lifespan and protects various tissues against disease.

Genetics has revealed that ageing may be controlled by changes in intracellular NAD/NADH ratio regulating sirtuin, a group of proteins linked to aging, metabolism and stress tolerance in several organisms (3).

Consistently, the neuroprotective roles of dietary antioxidants including curcumin, acetyl-L-carnitine and carnosine have been demonstrated through the activation of these redox-sensitive intracellular pathways.

Although the notion that stress proteins are neuroprotective is broadly accepted, still much work needs to be done in order to associate neuroprotection with specific pattern of stress responses (3).

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REDOX BALANCE AND ANTIOXIDANT DEFENSE SYSTEMS

Oxidative stress

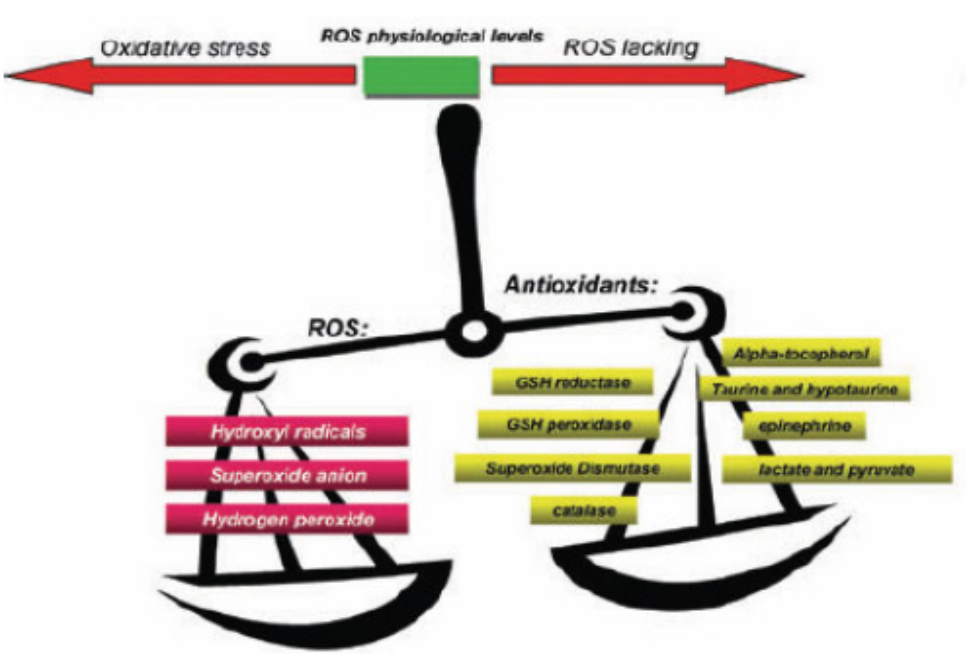
The overproduction of free radicals, molecular species that are continuously produced in small quantities in our bodies, is an important cause of cellular aging and plays a role in the pathogenesis of a wide variety of pathological states (8).

Under normal conditions, cells have efficient antioxidant defense systems. When the rate of generation of free radicals exceeds the capacity of antioxidant defenses, a state of "oxidative stress" is established which is the “primum movens" for the consequences that can lead to irreversible cell damage (9).

Numerous experimental data shows the involvement of oxidative stress in the mechanism of aging and neurodegeneration (10).

Oxidative stress is therefore characterized by an imbalance of the redox state of oxidants/antioxidants that may lead to altered cellular function and oxidative damage of fundamental biological macromolecules like protein (protein carbonyls, nitration of tyrosine), lipids (products of lipid peroxidation) and nucleic acids (11,12).

Oxidative stress is induced by both exogenous and endogenous sources (13). The first include drugs and toxic chemicals that change the balance of oxidants / antioxidants on behalf of oxidation; the latter includes overproduction of reactive oxygen intermediates by the mitochondrial electron transport chain. One of the main causes of oxidative stress is therefore the excessive release of reactive oxygen species (ROS).

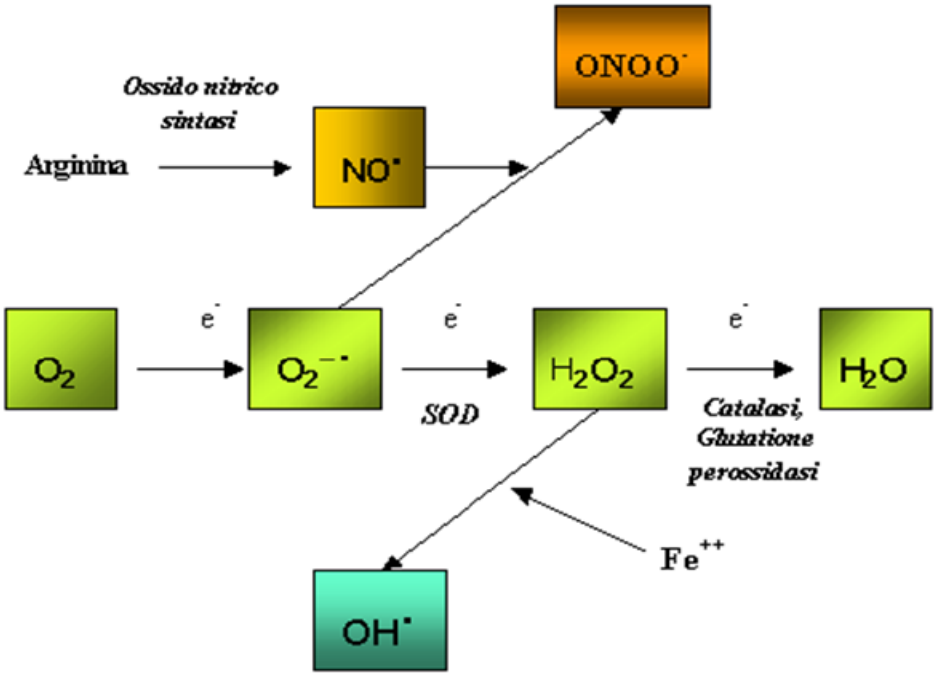


Modification of the normal balance between ROS and antioxidants (from Nutrition and oxidative stress.

Reactive oxygen species (ROS)

ROS are defined as molecular entities that react with cellular components, causing harmful effects on their functions. ROS include both free radicals (containing highly reactive unpaired electrons) such as superoxide anion (O2-•), nitric oxide (NO•) and hydroxyl radical (OH•) and other molecular species, such as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-).

The majority of cellular ROS are generated during the incomplete metabolic reduction of oxygen to water (see figure below).



Reactive oxygen species (ROS)

Free radicals are highly reactive molecules, have a very short half-life and can have a provenance endogenous and exogenous.

Reactive oxygen intermediates are produced continuously for a process of "leakage or short circuiting" of electrons from the normal locations of biological origin, which are rapresented mainly by the mitochondrial respiration, the tissue content of xanthine oxidase and, to a lesser extent, by the metabolism of arachidonic acid and by the processes of auto-oxidation of catecholamines or heme proteins (14).

The oxygen molecule is a biological paradox that on the one hand is a molecule essential for aerobic life, the other a biological hazard due to its high toxicity. In fact, the oxygen that is taken from the external environment is required in the mitochondrial respiration for the production of energy in the form of ATP according to a complex process that takes the name of "oxidative phosphorylation"; oxygen acting as a final acceptor of electrons removed from the molecules and combining with the protons subtracted under the same, allows the complete oxidation to water and carbon dioxide molecules of various nature (glycidol, fatty acids, amino acids, etc.), with release of all the energy contained in them (14).

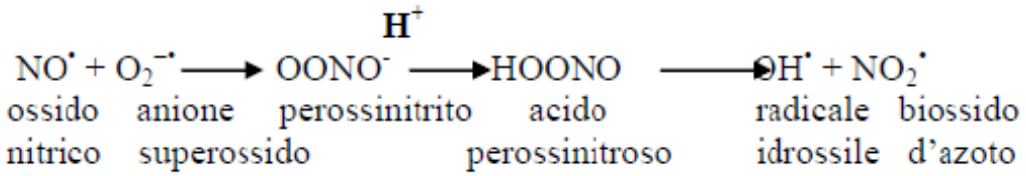
The activation of molecular oxygen can usually be done in two ways: one by using electrons and energy (8). The free radicals which can be formed during the sequential reduction mono electronics O_2 are the superoxide anion (O_2^-), hydrogen peroxide (HOOH) hydroxyl radical (OH^-).

During activation energy, it takes 22 kcal of energy because you have electronic transitions in molecular orbital of oxygen leading to the formation of singlet oxygen (O_2) which is not a radical, as there is an unpaired electron, but has a strong oxidizing ability, and, for degradation, generates superoxide anion (O_2^-).

Ionizing radiation, the photosensitizing substances, heat, death of a cell, are all phenomena entropic thermodynamically favoring the release of the amount of energy and, therefore, sufficient because these electronic transitions take place (14).

Even the excessive production of NO, a molecule physiologically important for the regulation of vascular tone and processes immunomodulators, can generate radical forms if associated with a concomitant overproduction of superoxide anion (15).

The endothelium appears to continuously produce small amounts of superoxide can react with nitric oxide (both are free radicals) to form nitrate ions, a product not radical. For this reason, variations in the production of nitric oxide and superoxide by the endothelium may represent a mechanism for the regulation of vascular tone. The peroxynitrite anion, degrading, form a hydroxyl radical (15,16).



If two radicals react with each other, cancel each other, and when a radical species reacts with a molecule not radical, it produces a new free radical and triggers a chain of reactions until it will form a stable compound. It then passes from the stage of "generation" to that of "propagation" of the free radical (14).
The formation of reactive oxygen species is therefore an eventuality cannot be eliminated in the cellular environment.

ROS Toxicity

Certain clinical situations or the intensification of external factors such as environmental pollution, smoking, a diet rich in fats, alcohol abuse, solar radiation, the use of certain medications, physical and mental stress, are all conditions generally associated with the overproduction of free radicals.
When the generation of free radicals exceeds the capacity of detoxifying antioxidant defenses, it establishes a condition of "oxidative stress". This represents a risk to the structural and functional integrity of important molecules such as DNA, proteins and lipids (13).
Free radicals and in particular the $OH^{\cdot}$, which is the most reactive free radical, can react with various molecules, dramatically changing both the chemical state and profoundly altering the function. The proteins can be oxidized at the level of sulfhydryl groups through a process that involves the deactivation of channel proteins, receptor or important enzyme activities (16,17); for example, enzymes such as phosphofructokinase, complex I and complex IV of mitochondrial respiratory chain are inactivated with severe deterioration of the cell's ability to supply energy. The calcium pump is inactivated with a consequent tendency to maintain high levels of calcium citosolubile.
Nucleic acids are sensitive to free radical attack at both of the bases of the pentose resulting in rupture of the propellers with the formation of modified bases such as 8-hydroxy-guanine and alteration of the genetic code (14).

The best known harmful effect of ROS is lipid peroxidation a chain reaction that leads to the formation of lipid peroxides and hydroperoxides from the oxidation of a methylene bridge at the level of a polyunsaturated fatty acid of membrane lipids such as arachidonic acid and linolenic acid. The lipohydroperoxides tend to move from the hydrophobic membrane to the surface leading to a disorganization of the structure of the membrane itself.

Consequently it causes irreversible damage to the morpfofuctionality of intracellular and cellular membranes or lipoproteins (18).

The continued against oxidative damage important molecules like DNA, proteins and lipids, elicited by activated oxygen species and of NO, is considered, in light of current experimental and clinical evidence, the most important cause of physiopathogenetics and biochemical changes observed during aging of the CNS including neurodegenerative disorders (15).

Several lines of evidence suggest that accumulation of oxidative molecular damage is a causal factor in senescence.

Among the correlative evidence supporting the involvement of oxidative stress are the following: (a) oxidative molecular damage to DNA and proteins increases exponentially with age, and concomitantly, the rates of mitochondrial $O2^{-}\cdot$ and H_2O_2 generation as well as the susceptibility of tissues to experimentally induced oxide.

Among the correlative evidence supporting the involvement of oxidative stress are the following: (b) experimental regimens that extend the lifespan, such as caloric restriction in mammals and reduction of metabolic rate in insects, decrease the accumulation rates of oxidative damage; (c) mitochondria make two rather contradictory contributions to cell survival. The classically recognized function is the synthesis of ATP for energizing endergonic reactions, the other is generation of reactive oxygen species which may compromise the long-term survival of cells and constitute a major underlying cause of the aging process. Indeed, these two rather conflicting functions are part of the same process, namely mitochondrial respiration.

The alteration in the redox and mitochondrial dysfunction that follows, is involved in so many disease states neuropathogenesis including neurodegenerative disorders such as multiple sclerosis (MS), Parkinson's disease (PD) the Alzheimer's disease, (AD) and the aging (19).

CNS has a large potential oxidative capacity (20) due to the high level of tissue oxygen consumption. However, the ability of the brain to withstand oxidative stress is limited

because of: (a) a high content of easily oxidizable substrates, such as polyunsaturated fatty acids and catecholamines; (b) relatively low levels of antioxidants such as glutathione and vitamin E and antioxidant enzymes (such as glutathione peroxidase, catalase and superoxide dismutase); (c) the endogenous generation of reactive oxygen free radicals via several specific reactions; (d) the elevated content of iron in specific areas of the human brain, such as globus pallidus and substantia nigra (SN), while cerebrospinal fluid has very little iron-binding capacity owing to its low content of transferrin; (e) CNS contains non-replicating neuronal cells which, once damaged, may be permanently dysfunctional or committed to programmed cell death (apoptosis).

Numerous experimental evidence lead to the conclusion that the dysfunction at the level of cellular energy metabolism is an important factor in the neurotoxicity mediated by NO and that the cellular content of thiols is crucial in determining the sensitivity of cells to oxidative stress and nitrosative (21).

Mechanisms of Antioxidant Defence

In the cell, in the normal conditions, there is a steady-state balance between pro-oxidants and antioxidants which is necessary to ensure optimal efficiency of antioxidant defenses, that, during the normal cellular metabolism, can adequately cope with all the free radicals generated (6).

Furthermore, recent studies show that a minimum amount of free radicals, which until now have been considered only due to aging, is the necessary condition for the functionality of the cells, because it leads to a greater efficiency of defense systems and increased cell survival (21).

In the cell, as regards oxidative insult, at the level of the cytoplasm, in mitochondria and also in the extracellular fluid, there are efficient antioxidant defense mechanisms of enzymatic and non-enzymatic nature. In the first case it is cytoprotective enzymes (superoxide dismutase, catalase, glutathione peroxidase), said "scavengers", because they function as "scavengers" in respect of free radicals, tending to remove them as formats or to prevent their formation. In the second case it is of antioxidants can react with free radicals, abducting or neutralizing them, and therefore capable of blocking the series of reactions of lipid peroxidation, and then to prevent the detrimental action

is that the propagation of free radicals; between these molecules are able to bind the O_2 singlet (β-carotenes, retinoids); xanthine oxidase inhibitors (allopurinol); molecules of low molecular weight, is water-soluble such as ascorbic acid (vitamin C) and glutathione, which operate in blood plasma and in the cytosol, both fat-soluble such as α-tocopherol (vitamin E), bilirubin, uric acid and estrogen that instead exert their action in the heart hydrophobic cell membranes or of plasma lipoproteins (14) .

In recent years, since oxidative stress has been felt at the base of some if not all aspects of neurodegeneration, numerous experimental studies were conducted in order to reduce the effects of oxidative stress through the use of scavengers of free radicals. Among these are some nutritional compounds.

There are two classes of antioxidants, endogenous and exogenous. Among the former are the tripeptide glutathione (GSH), various vitamins, and products of reactions catalyzed by enzymes that are subjected to upregulation in response to oxidative stress, such as bilirubin dall'emeossigenasi, and products ARE (antioxidant responsive element) (22). Among the latter, the nutritional antioxidants, there are several classes of molecules: those that increase the endogenous levels of GSH or otherwise in functionality reactive-SH; vitamins, phenolic and polyphenolic compounds (23,24).

The ***tripeptide glutathione*** (γ-glutamyl-cysteinyl-glycine) is an endogenous antioxidant of great importance. Glutathione (GSH) is required for the maintenance of the thiol redox state of the cell, for the protection from oxidative damage, for the detoxification of electrophilic and reactive metals endogenous and exogenous, for the storage and transport of cysteine, as well as for the synthesis of proteins and DNA, for the regulation of the cell cycle, and for cell differentiation (25). The glutathione and glutathione-dependent enzymes play a key role in protecting the cell from the effects of reactive oxygen species. The key element of the functional cysteine glutathione is the part that provides the thiol reactive group. Glutathione is the main defense against reactive oxygen species (ROS), which are reduced by glutathione in the presence of glutathione peroxidase.

As a consequence of the GSH is oxidized to GSSG, which, in turn, is rapidly reduced back to GSH by glutathione reductase at the expense of NADPH. The thiol-disulfide redox cycle contributes also to maintain the reduced protein thiols and enzymatic. In the absence of a process of reduction of protein disulfides, vulnerable cysteine residues essential enzymes may remain oxidized, with consequent alteration of the catalytic activity.

Glutathione contributes, also, for the storage and transfer of cysteine also. The cysteine to cystine autoossida quickly producing toxic oxygen radicals. To escape the toxicity of cystine, most of the protein is collected in the non-cysteine glutathione. Moreover, exerting a protective action by reactive oxygen species, glutathione is an excellent scavenger of the products of lipid peroxidation, such as HNE and acrolein, which bind proteins inhibiting its activity. Glutathione also reacts with the carbon atoms, saturated, unsaturated and aromatic. This detoxification involves the nucleophilic attack on an electrophilic carbon atom by GSH.

This reaction may start spontaneously, but very often is catalyzed by glutathione S-transferase. Glutathione also forms metal complexes through non-enzymatic reactions. The GSH intervenes to storage, mobilization and release of metal ions between ligands, in the transport of metals across cell membranes, serves as a source of cysteine to bind the metals, and by reductant for redox reactions involving metals (25). The sulfhydryl group of the cysteine portion of GSH has a high affinity for metal ions such as mercury, silver, cadmium, arsenic, lead, gold, zinc and copper, forming a complex thermodynamically stable which can be eliminated by the body. Glutathione reacts with free radical molecules. Thus, the possibility of increasing glutathione levels could prove useful against oxidative stress.

Vitamin E, a phenolic compound, acts as an antioxidant by removing the free radicals by the atom-H phenolic. The reactions of vitamin E, vitamin C, and glutathione may be connected with different recycling processes, contributing, thus, increasing the efficiency of these parts against oxidative stress.

Polyphenols are natural substances ubiquitously present in fruits and vegetables, as well as, beverages obtained from plants such as tea, red wine and olive oil. Flavonoids compose the largest group of polyphenols. Their skeletal structure consists of an aromatic ring condensed to a heterocyclic ring, attached to a second aromatic ring. Flavonoids are mainly divided into:

anthocyanins, glycosylated derivative of anthocyanidin, present in colorful flowers and fruits, and anthoxantins, colorless compounds further divided in several categories including flavones, flavans, flavonols, flavanols, and isoflavones. The remarkable antioxidant activity of these compounds is conferred by the numerous phenolic hydroxyl groups on the aromatic ring.

The rapid donation of a hydrogen atom to lipid peroxyl radical results in the formation of the polyphenol phenoxyl radical (PP•) according to the reaction ROO• +

PPH→ROOH → PP• that can be stabilized by further donation of another hydrogen or by reacting with another radical. In addition, flavonoids present efficient iron chelating activity, for which the 3-OH is important (26). The physiological effects of flavonoids are particularly significant in those pathologies where
the oxidative stress hypothesis is accepted and supported by experimental data, such as AD. In vitro, flavonoids are capable of scavenging superoxide anions (27) and hydroxyl radicals (28). Once ingested, these compounds are capable of elevating the redox and antioxidant level (29). In red blood cells, polyphenols enhance cell resistance to oxidative insult (30), as well as inhibit LDL oxidation in plasma (31). The importance of these molecules in protecting cells from oxidative stress goes beyond the simple radical oxygen species (ROS) scavenging properties. In a recent study on neuronal Cells (32), three different mechanisms of protection have been identified: Flavonoids can prevent cell death after glutamate injury by scavenging radicals, maintaining the correct glutathione levels and inhibiting Ca2+ influx, which represents the last step in the cell death cascade. These properties, together with anti-inflammatory properties attributed to some polyphenols (33), renders this class of compounds suitable for application where oxidative stress, together with inflammation and antioxidant defense depletion take place, such as AD.

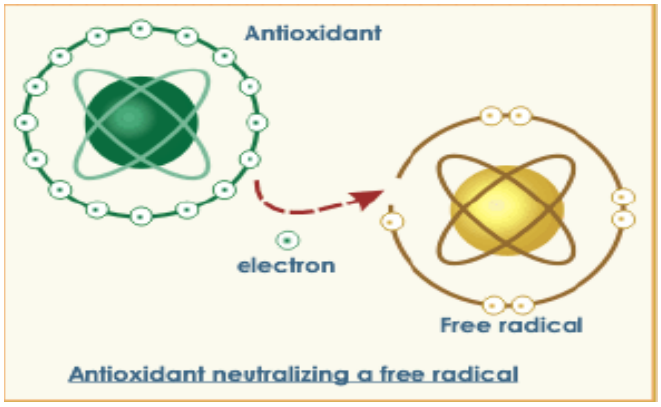
Spices and herbs often contain phenolic substances with potent antioxidative and chemopreventive properties (34). Spices form an important class of food adjuncts and are used to enhance the sensory quality of food.

Recent studies show that some of the biochemical effects of spices are due to their active principles. Turmeric (Curcuma longa Linn, family: Zingiberacee) has been used as a coloring agent and food additive in Indian culinary preparations from time immemorial (35).

The active antioxidant principle in Curcuma longa has been identified as curcumin (diferuloyl methane). It is generally assumed that the phenol moiety is responsible for antioxidant properties of any plant phenolic compound. Consequently, the free radical chemistry of curcumin (an omethoxyphenol derivative) has focused on its phenol ring (36). The possible involvement of the _-diketone moiety in the antioxidant action of curcumin has been considered and, as recently shown (37), the H-atom donation from the β-diketone moiety to a lipid alkyl or a lipid peroxyl radical has been reported as the potentially more important mechanism underlying its antioxidant action.

Similar to the well-known synergism between lipid soluble antioxidant action (38) might position itself at the border of the cell membrane adjacent to the acqueous milieu, in short “pops out” of the membrane to be repaired by water soluble antioxidant, such as catechins or ascorbate. Curcumin, as a powerful lipid soluble antioxidant, positions itself within the cell membrane, where it intercepts lipid radicals and becomes a phenoxyl radical. Being more polar than curcumin, phenoxyl radical travels to the membrane surface. Owing to the high reduction potential of phenoxyl curcumin radical (0.8 V, at physiological pH 7), this allows the curcumin intermediate to be easily repaired by electron donors with favorable oxidation potential, such as epigallocatechin gallate (0.43 V), catechin (0.55 V), or vitamin C (0.28 V). Such electron and associated proton transfer reaction will maintain optimal concentrations of curcumin at expense of water soluble antioxidants, in spite of its fast turnover and low physiological uptake. Moreover, curcumin free radicals can also react with each other or with other free radicals forming, either stable products such as curcumin dimers, vanilin and ferulic acid (39) or, through a peroxyl linkage at the 3’ position of the curcumin phenolic ring, coupling products which generate, via intramolecular Diels-Alder reaction, not radical stable compounds (40). Curcumin contains two electrophilic α,β-unsaturated carbonyl groups, which can react with nucleophiles such as glutathione (41). By virtue of its Michael reaction acceptor function and its electrophilic characteristics, curcumin and several other polyphenolic compounds have been recently demonstrated to induce the activities of Phase I and Phase II detox system (42,43), e.g., inhibition of COX-1 and COX-2 enzymes (44) and stimulation of glutathione-S-transferase (45). In addition to its ability to scavenge carcinogenic free radicals (46), curcumin also interferes with cell growth through inhibition of protein kinases. Although the exact mechanisms by which curcumin promotes these effects remains to be elucidated, the antioxidant properties of this yellow pigment appear to be an essential component underlying its pleiotropic biological activities. Of particular interest is the ability of curcumin to inhibit lipid peroxidation and effectively to intercept and neutralize ROS (superoxide, peroxyl, hydroxyl radicals) (47) and NO-based free radicals (nitric oxide and peroxynitrite) (48). In this regard, curcumin has been demonstrated to be several times more potent than vitamin E (49).

It should be noted cheanche though most of the cytoprotective enzymes is localized in the intracellular antioxidant agents are located within both intra-and extracellular (6).



Mechanism by which an antioxidant neutralizes a free radical,
(from www.gmvirtual.com/acaibasics.html)

Defense mechanism "Heat Shock Response"

Oxidative stress has been shown that alters the expression of antioxidant enzymes in mammals (50) and also stimulates the expression of several transcription factors that bind to DNA including AP-1, fos, jun, myc, erg-1, NFkB and the SAPK (51).

Moreover, it is well known that brain cells are continually challenged by conditions which may cause acute or chronic stress.

In order to adapt to different environmental conditions and to the different types of insults eukaryotic cells have developed a system with different responses, which reveal and control various forms of stress (52). One of these answers, known as "heat shock response", has attracted considerable interest as a fundamental mechanism for cell survival under different conditions.

In mammalian cells, the induction of the *"heat shock response"* requires the activation and translocation into the nucleus of one or more dell'heat shock transcription factors that control the expression of a series of specific genes, denominated vitagenes (14), which encode for cytoprotective HSPs.

Recent studies have demonstrated that the heat shock response contributes to establishing a condition cytoprotective in a great variety of pathological conditions, such as ischemia and reperfusion injury, inflammation, cancer, as well as metabolic and neurodegenerative disorders. The "stress response" determines the gene expression

in response to environmental changes such as high temperature, radiation, exposure to excitotoxins and other stressful conditions that lead to protein denaturation. Thus, the induction of Hsps is not only an index of physiological stress, but is used by cells to repair processes but is used by cells to repair processes after the insults to prevent the damage resulting to the accumulation of these proteins denatured (53).

The HSPs can be either inducible and constitutive. The constitutive form performs basic physiological functions. However, some of these are up-regulated by stress. The inducible form prevents the denaturing of proteins and assembly of abnormal polypeptides during exposure to conditions stressogene. Denatured proteins induce stress proteins. The family of HSPs of 70-kDa is one of the most studied. This family includes the form constitutively expressed Hsc-70 and the inducible form of Hsp-70 also called Hsp-72.

Another important family of HSPs is represented by the Hsp-32 or heme oxygenase (HO). There are three isoforms of heme oxygenase: HO-1, or inducible isoform; HO-2, or constitutive isoform; and the recently discovered HO-3.

Heme oxygenase-1 exerts protective role, by degrading the intracellular levels of prooxidant heme and by producing biliverdin, the precursor of bilirubin, this latter being an endogenous molecule with potent antioxidant and antinitrosative features and also produces carbon monoxide, a molecule involved in regulating vessel active pathway of NO (54).

Increasing evidence suggests that the HO-1 gene is redox regulated and contains in its promoter region the antioxidant responsive element (ARE), similarly to other antioxidants that bind specific transcription factors Nrf2 as sensitive to the alteration or NFkB redox balance; in fact, nitrosative stress and depletion of GSH up-regulate the protein. It was seen that the cells overexpressing the Hsps are resistant to several types of oxidizing agents and to the heat shock; the Hsps play a protective role against oxidative damage to DNA (55).

Previous studies suggest a correlation between the mechanisms of oxidative stress and the induction of HSPs as: (i) inhibition of antioxidant defense increases susceptibility to killing by heat shock (Mitchell and Russo, 1983); (ii) hsp confers resistance to oxidative stress (56) (iii) induction of HSP70 protein is inhibited by antioxidant compounds, such as flavonoids, furthermore,a role for NO in inducing hsp70 proteins HSP70 induction (16).

The thioredoxin system, originally identified in Escherichia coli, in 1964, as a hydrogen donor for ribonucleotide reductase required for DNA synthesis, plays a key role in cell function by limiting oxidative stress directly via antioxidant effects and indirectly by protein–protein interactions (57).

The thioredoxin system, comprising primarily by the enzyme thioredoxin reductase (TrxR) and its related protein thioredoxin (Trx), has aroused a keen interest by different researchers, and in the light of new experimental evidence, is a leading multifunctional systems redox ubiquitous deputy to the redox regulation of the cell (58).

Thioredoxin (Trx) is one of the members of a family of proteins redox-active evolutionarily conserved feature of a catalytic center disulfidrilic / dithiol within the sequence of the active site (-Cys-Gly-Pro-Cys-), which is subjected to reversible reactions of oxidation at the level of cysteine residues following the reduction of flight disulfidrilic of a wide variety of target proteins oxidized (59). The thioredoxin (Trx) has evolved similar to a protein chaperone by ensuring the maintenance of the structure dithiol / disulfidrilic protein biological function. Indeed, several studies show that the scientific Trx binds to specific proteins, modulating its structural conformation.

Several experimental evidences show that in mammals the redox regulation of many cellular processes is regulated by the cooperation between the system of the thioredoxin and the glutathione (60). In fact, both systems are involved in a variety of redox-dependent pathway as the supply of reducing equivalents for ribonucleotide reductase (an enzyme involved in the first phase of the synthesis of deoxyribonucleotides for DNA repair), and for methionine sulfoxide reductase (an enzyme involved in antioxidant defense) and end-in the regulation of cellular redox balance (58).

The TRX system is considered one of the key systems Members of the redox homeostasis of the cellular microenvironment in synergistic cooperation with the redox system of GSH. Taken together, both systems, constitute a powerful antioxidant mechanism deputy to redox control of gene expression, signal transduction, cell proliferation, the cellular protection against oxidative stress, anti-apoptotic function, of the effects mediated by factors of growth and by cytokines, as well as the adjustment of the redox state of the extracellular environment (61).

The Trx gene promoter contains a number of elements of the stress response, several transcription factors that bind sites like: SP1, AP-1, NF-kB and antioxidant response elements (ARE) (3). A difference of reduced glutathione (GSH), which is largely responsible for the low power and redox of the global content of thiol groups free of organelles within the cell and because of its high intracellular concentration (1-10 mM), the system of thioredoxin may play a critical role in the regulation of protein thiol redox target, mainly involved in signal transduction and in the regulation of gene expression (62).

In addition, the thioredoxin (Trx), which behaves essentially as a soluble protein after disintegration of the cells, exists in an isoform predominant cytoplasmic (Trx-1) and a mitochondrial (Trx-2) (63). Molecular studies show that both the cytoplasmic isoform of the mitochondrial Trx protect against oxidative stress and both are essential for the viability of mammalian cells (64).

Given the huge amount of functions carried out by the redox Trx, it is plausible to say that it is a critical molecule essential for cell viability. The overexpression of the system of Trx / TrxR is generally associated with the activation of cellular mechanisms of tolerance to stress and, in general, to a resistance to oxidative damage and / or nitrosative mediated by a wide variety of stress agents, including compounds such as doxorubicin and etoposide (65,67). The Trx plays a cytoprotective role against different forms of stress in a variety of biological systems.

It has been characterized as a fundamentally stress-inducible protein with a typical cytosolic intracellular localization (63). Many chemical and physical stimuli, such as UV irradiation and hydrogen peroxide (H2O2), induce the expression and secretion of Trx as redox-sensitive molecule with cytokine-like activity and chemokine-like which prevents cell damage by oxidative stress. Furthermore treatment of cells in culture with phorbol esters, hydrogen peroxide (H2O2), hypoxia, chemotherapeutic agents such as cisplatin and hemin, causing the translocation of TRX from the cytoplasm to the nucleus where it regulates the activation and the redox activity of DNA binding of transcription factors (Jun, Fos, p53, CREB, PEBP2/CBF, Myb) critics who are involved in fundamental processes such as gene expression, cell growth and apoptosis.

The Trx-1, the most extensively studied isoform, is primarily a cytosolic soluble protein without a specific localization signal (63). Several studies indicate that the Trx is expressed constitutively associated with protein sulfhydryl on the surface of the plasma membrane of different cell types (63).

Thioredoxin plasma levels in normal individuals vary between 20 and 30 ng/ml (68,69) and increase in certain human diseases including HIV infection and cancer (3). Elevated Trx levels may contribute to increased cancer cell proliferation and resistance to chemotherapy by several mechanisms as the stimulation of DNA synthesis and the activation of redox-modulated transcription factors.

Recent work suggests that Trx-1 is involved in nerve growth factor (NGF) signaling pathways. NGF, a neurotrophic factor regulating development, maintenance and function of the CNS, has been shown to activate Trx-1 expression via cyclic AMP (cAMP)-response elements (CREs) present in the Trx-1 gene promoter, and also to induce nuclear translocation of Trx1 (3). Recent data suggest that, beyond its ability to regulate the function of proteins through thiol-disulfide exchange reactions, Trx and its substrates may also have beneficial effects during oxidative stress by upregulating HO-1(70), with important cytoprotective pleiotropic effects deriving from heme degradation and bilirubin formation (71). Besides the role as a source of reducing equivalents, Trx per se acts as antioxidant or ROS scavenger. In fact, Trx eliminates singlet oxygen, hydroxyl radical and hydrogen peroxide. Finally, the NO-dependent expression of Trx has been shown to be involved in the neuroprotection against oxidative stress mediated by estrogens (3).

The *sirtuins* are a group of proteins linked to aging, metabolism and stress tolerance in several organisms. In mammalian cells seven sirtuins have been identified. SIRT1, 2, 3, 6 and possibly 5 are NAD-dependent deacetylases, SIRT4 and 6 are ADP-ribosyltransferases, and the activity of SIRT7 has not been defined (72).

The sirtuin family of histone deacetylases (HDACs) was named after their homology to the Saccharomyces cerevisiae gene silent information regulator 2 (Sir2). In the yeast, Sir2 has been shown to mediate the effects of caloric restriction on the extension of life span, with high levels of Sir2 activity promoting longevity (73). Like their yeast homologs, the mammalian sirtuins (SIRT1-7) are class III HDACs and require NAD+ as a cofactor to deacetylate substrates ranging from histones to transcriptional regulators. Through this activity, sirtuins are shown to regulate important biological processes, such as apoptosis, cell differentiation, energy transduction or glucose homeostasis (74).

Sirtuin-mediated deacetylation and ADP ribosylation are related in that both cleave NAD as the initial chemical step of the reaction cycle.

In deacetylation, the ADP-ribosyl transfer directly participates in the removal of the acetyl group from the protein substrate to generate 2,3-O-acetyl-ADP-ribose, whereas in ribosylation, the ADP ribosyl moiety is transferred to the protein substrate.

Deacetylation of sirtuin substrates can inhibit or induce their activities, whereas ADP-ribosylation has only been shown to be inhibitory.

Several studies have determined a role for the human SIRT1 protein in cell survival. SIRT1 specifically associates with the p53 tumor suppressor protein and deacetylates it, resulting in negative regulation of p53-mediated transcriptional activation.

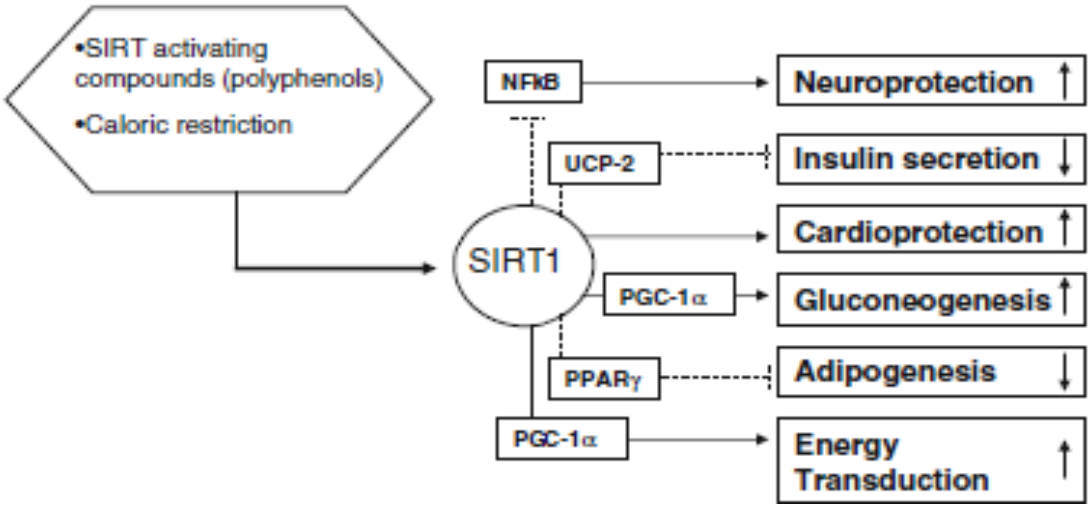
Importantly, p53 deacetylation by SIRT1 also prevents cellular senescence and apoptosis induced by DNA damage and stress.

SIRT1 regulates important aspects of mitochondrial biology, e.g. it deacetylates the essential cofactor PGC-1a (PPAR-c coactivator-1a) in mitochondrial biogenesis.

An up regulation of the mitochondrial activity might be of therapeutic benefit for various diseases related to aging such as metabolic disorders (e.g. diabetes type 2) or mitochondrial disorders.

These studies provide a powerful indication that SIRT1 activation offers a promising approach for treating metabolic disorders.

In addition, SIRT1 activation significantly decreases neuronal cell death induced by amyloid-beta (Ab) peptides through inhibition of NF-jB signalling (74).



Carnosine is a natural dipeptide (β-alanyl-L-histidine), present in long-lived mammalian tissues (75) to high concentrations (2-20 mM) (76), in the brain, has been found in glial cells and in some type of neurons. At physiological concentration, this dipeptide also has antitumoral properties, acting as an antioxidant and antiglycating (77).

Since β-alanine is non-proteinogenic aminoacid, it is obvious that carnosine is not product of protein catabolism: Instead it is synthesized enzymatically by carnosine synthetase, an enzyme present in brain and muscle that shows broad substrate specificity (78,79).

The hydrolysis of carnosine is catalyzed by two enzymes recently cloned and characterized; Both enzymes belong to the M20 metalloprotease family.

The enzyme named CN1 exhibits narrow specificity and the characteristics of the enzyme previously designated XHis dipeptidase or carnosinase (3).

The enzyme named CN2 displays broad substrate specificity and is ubiquitously expressed like the enzyme previously designated cytosol non-specific dipeptidase (3).

It has been shown that carnosine prevents neural toxicity in vitro (80,81) and protects neuronal cells from ischemic injury (82) and aggregated forms of the peptide β-Amyloid (83).

Moreover, carnosine protects neurons isolated from reactive oxygen species overprodotte by excitotoxic insults N-methyl-D-aspartate (84), prevents the in vivo inactivation of Cu-Zn-SOD in "senescence accelerated mouse-prone - (SAMP) - mice, an effect associated with a prolonged average "life-span" (85).

These data suggest that carnosine may retard the aging process, as demonstrated in vitro, in cultured human cells senescent (86,87).

This dipeptide, reacting non-enzymatically with protein carbonyl groups, can prevent the subsequent cross-linking and degradation of glycosylated proteins (84). This process called "carnosinylation'" might be relevant during aging, where it was observed a significant accumulation of carbonyl groups at the protein level, resulting from phenomena of glycation (88).

Remarkably, increased formation of protein carbonylated, which occurs during aging, suppresses the activity of proteasomal (86,89,90). The polypeptides of the aged, in fact, may also be degraded by the 20S proteasome, or cross-linked to form aggregates for proteolysis and inhibit the proteasome activity. In these conditions, the carnosinylation and glycated proteins / oxidized, through inhibition of the cross-

linking with the normal macromolecules, may influence the fate and the biological significance of important proteins during the conditions of radical attack and oxidative stress, such as aging processes. Furthermore, it was recently reported that carnosine can act as a selective inhibitor of the activation of guanylate cyclase, NO-dependent, suggesting its use for the treatment of several disorders (cancer, sepsis, etc.). Associated with aberrant activity of the system intracellular signal: NO-cGMP soluble guanylate cyclase (91). More recently, it has been demonstrated that carnosine and some of its synthetic analogues neutralize the damage peroxynitrite-dependent, as the nitration of free thyroxine and dell 'α1AP, and the modification of LDL (92,93). The peroxynitrite by acting as oxidizing and nitrating highly toxic, rapidly decomposes, generating reactive species capable of reacting with different biological molecules which, as thiols, lipids, amino acids, antioxidants and nucleic acids, and is implicated in many human disease states (94,95).

Carnosine has been shown to delay ageing in cultured human fibroblasts, male Drosophila (96) and senescence-accelerated mice (97). Therapeutic potential has also been invoked in cataractogenesis and diabetes (98,99). The occurrence of carnosine and its analogue homocarnosine (c-aminobutyryl-histidine) in brain, and homocarnosine in CSF, and their age-related alterations suggested a role for these peptides with respect to suppression of onset or progression of AD and other neurodegenerative diseases (3).

In brain cells, carnosine has been shown to be neuroprotective because of its ability to counteract both oxidative and nitrosative stress related to several pathological conditions including ischemia ,methamphetamine neurotoxicity and neurodegenerative disorders (100).

Importantly, carnosine may indirectly influence neuronal excitability by modulating the effect of zinc and copper (101). Furthermore, carnosine has been shown prevent β-amyloid aggregation and toxicity and this effect can be due to the known ability of this peptide to inhibit protein misfolding and avoid the formation of advancedglycation end-products .

Antioxidant and Neuroblastoma cells

Recent evidences show that a minimum amount of free radicals, which until now have been considered only due to aging is a necessary condition for optimal cell function because it leads to a more efficient defense systems and increased cell survival (16).

Bis(2-hydroxybenzylidene)acetone is a potent inducer of the phase 2 response through the Keap1-Nrf2-ARE pathway to study the protective effects of antioxidant molecules, including the HBB-2.

This double Michael reaction acceptor reacts directly with Keap1, the sensor protein for inducers, leading to enhanced transcription of phase 2 genes and protection against oxidant and electrophile toxicities.

Experimental evidence show that Bis(2-hydroxybenzylidene) is a potent chemoprotective agents, infact low concentrations (in the submicromolar range) of bis(2-hydroxybenzylidene)acetone markedly increased the activities of NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) and glutathione reductase, and the levels of total glutathione, three markers of the phase 2 response. In contrast, at high concentrations (in the micromolar range) the same compound caused G2/M cell cycle arrest and apoptosis (102).

It was assumed the role of an antioxidant compound synthesis as the bis(2-hydroxy-benzilidene) acetone or HBB-2 as a possible modulator of vitagenes its use in vivo.

It was also assumed its neuroprotective effect in human neuroblastoma cells exposed to nitrosative stress induced by SIN-1 to support the hypothesis that HBB may have anti-aging in the central nervous system.

The human neuroblastoma cells SH-SY5Y subclones neuroblasts are an extensively characterized cell line SK-N-SH (103,104) obtained in 1970 by a fabric of a metastatic neuroblastoma of a little girl of four years (105).

Neuroblastomas are tumors in childhood-onset originating from immature neuroblasts during the development of the peripheral nervous system (106).

The cell line SH-SY5Y cells are a cell population from neuroblast-like morphology, with a teardrop-shaped cell body (107) and small neurites, which grow to form small aggregates or clusters (105, 108) while a smaller part is in suspension, and represents the cells in mitosis. These cells are a well characterized model for the study of in vitro differentiation into a neuronallike phenotype inducible by various exogenous differentiating agents such as retinoic acid, phorbol esters, nerve growth factor (NGF),

and the membrane permeable analogue of cAMP, the cAMP-dibutiril (104, 109, 110). The differentiation of these cells is associated with the extension of long neuritis and can be quantified by morphological analysis of neuritogenesis (104,107).

The line SH-SY5Y is also used as a model for the study of neuronal cell death induced by oxidative stress associated with several chronic neurodegenerative diseases such as Alzheimer's disease, Alzheimer's and Huntington's (111). They are also used as a model for the dopaminergic neurotransmission and sympathetic study of Parkinson's disease (112).

Express adenosine A1 and A2A receptors and, in the presence of inhibitors of phosphodiesterase, increase levels of cAMP even in the absence of a receptor-mediated stimulation of adenylate cyclase evidence to suggest that the basal levels of cyclic nucleotides are subject to a high turnover (113). Other features of the cell line include the ability to convert glutamate into GABA, choline into acetylcholine, and the presence of dopamine-β-hydroxylase activity. Express μ opioid receptors and δ, in particular δ2 and the α2 adrenergic receptors (114, 110).

They are also considered an appropriate model for the study of σ receptors (113).

The cells also express the calcium channels, and N-type and L-type muscarinic receptors (M3) and nicotinic receptors for neuropeptide Y (Y2 receptors) (112,115).

Oxidative stress and type II Diabetes

Oxidative stress is caused by an unbalance between a relative overload of oxidants and a depletion of antioxidants, and it is implicated in the pathogenesis of several chronic diseases, including atherosclerosis, ischaemia/reperfusion injury, chronic inflammatory diseases, renal failure and diabetes mellitus (116,117).

Several mechanisms seem to be involved in the genesis of oxidative stress in diabetic patients. Among these, glucose autoxidation, not enzymatic protein glycation as well as the formation of advanced-glycation end-products (AGEs) have been demonstrated in patients with diabetes and a direct relationship with the circulating blood glucose levels and glucose variability has been repeatedly demonstrated (116,118,119).

In particular, it has been shown that two subclasses of AGEs, such as N-ε(carboxymethyl)lysine and pentosidine, accumulate in various tissues of poorly controlled diabetic patients (120).

The interplay between oxidative stress and AGEs is very complex. In fact, reactive oxygen species (ROS) accelerate the formation of AGEs, which in turn, as glycated proteins, are also able to produce ROS via complex biochemical mechanisms (121,122).

In addition to the above mentioned AGEs and protein carbonyls, various other products derived from lipid peroxidation accumulate in biological fluids and tissues of diabetic patients. 4-hydroxy-2-nonenal (HNE) is considered an important marker of lipid peroxidation, but at the same time it is directly involved in both cytotoxicity and mutagenic activity. In fact, 4-HNE further reacts with protein residues, such as histidine, to generate stable Michael adducts (123,124).

Interestingly, HNE-modified proteins have been identified in the serum and in renal tissues of type 2 diabetic patients (123,125).

The free radical-based oxidation of arachidonic acid (126,127), is one of the most relevant biochemical pathways that generate isoprostanes. Increased levels of F2-isoprostanes can be found in the plasma or urine of patients affected by several chronic inflammatory or degenerative diseases, including diabetes, and are currently used as in vivo indicators of lipid peroxidation (116,128,129).

It is of interest that in addition to their well known role as in indicator of the oxidative stress status, several lines of evidences demonstrate that F2-isoprostanes are directly involved in vascular function and endothelial mediated platelet aggregation (130,131).

It is hypothesized, in *vitro*, the role of oxidative stress in the pathogenesis and in clinical history of mellitus diabetes type 2, to evaluate the presence of systemic oxidative stress, glutathione status and cellular stress response in plasma and lymphocytes of patients with type 2 diabetes.

Eukaryotic cells, have developed various pathways to counteract oxidative stress-related damage. Among these stress, induced proteins, chaperones are essential to help the correct *folding* and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses (11). Therefore, normal chaperone function plays a pivotal role in the endogenous response of several tissues to an increased cellular stress, whereas altered chaperone function has been associated with the development of several diseases (132).

Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Diabetic nephropathy (DN) is one of the most severe complications of type 1 and type 2 diabetes and is the foremost cause for dialysis in the Western world (133).

Prime risk factors for developing DN are poor glycemic control and high blood pressure; yet, appropriate treatment of individual patients to minimize these risk factors can only delay the onset, but does not eliminate susceptibility to develop DN (134,135).

Recent experimental evidences have demonstrated that susceptibility to DN is strongly associated with a polymorphism in the CNDP1 gene (136). This gene encodes the serum carnosinase (CN-1) protein which degrades carnosine into β-alanine and histidine.

The (CTG)n polymorphism in the serum carnosinase (CN-1) gene affects CN-1 secretion (137). Since CN-1 is heavily glycosylated and glycosylation might influence protein secretion.

It is tested the role of N-glycosylation for CN-1 secretion and enzyme activity.

Furthermore has also been hypothesized whether CN-1 secretion is changed under hyperglycemic conditions.

Recently, is identified an allelic variant of human carnosinase 1 (CN1) that results in increased enzyme activity and is associated with susceptibility for diabetic nephropathy in humans. Investigations in diabetic (db/db) mice showed that carnosine ameliorates glucose metabolism effectively (138).

It is hypothesized renal carnosinase metabolism in db/db mice. Kidney CN1 activity increased with age and was significantly higher in diabetic mice compared to controls.

Carnosine treatment largely prevents the alterations of renal carnosine metabolism.

Susceptibility to diabetic nephropathy (DN) is strongly associated with a polymorphism in the CNDP1 gene, encoding the serum carnosinase (CN1) protein (139). The shortest allelic form (homozygous for the five-leucine allele, the so-called CNDP1 Mannheim) is more common in the absence of nephropathy and associated with lower serum carnosinase activities (140). CN1 is a dipeptidase which catalyses the hydrolysis of the dipeptides carnosine, anserine and homocarnosine (141). CN1 is present in different allosteric conformations (142). In mammals, two types of L-

carnosine-hydrolyzing enzymes (CN1 and CN2) have been identified. In humans, CN1 is expressed in various tissues and secreted from the liver into the blood, in rats CN1 expression is confined to the kidney (141).

Carnosine, anserine and homocarnosine are the most abundant histidine-containing dipeptides. They are widely distributed in mammals and tissue distributions and concentrations are species specific (143). All three peptides have antioxidant activity (144). Carnosine increases ischemia tolerance of neurons and hepatic cells by scavenging of reactive oxygen (145) species and acts as a natural inhibitor of the angiotensin converting enzyme (146). It restores erythrocyte deformability, inhibits protein glycation and cellular senescence and reduces the synthesis of matrix proteins such as fibronectin and collagen type VI of podocytes and mesangial cells (140). In the central nervous system, carnosine meets many criteria for a neurotransmitter (147).

Recent studies, showed that carnosine influences glucose metabolism, however, mesangial expansion, as a sign of nephropathy, was not affected by carnosine treatment in diabetic mice (148).

In a previous study, we showed that the human serum histidine dipeptide concentrations are not correlated to CN1 activity (149). Since the absence of diabetic nephropathy is associated with low CN1 activity, these findings suggest that local effects in the kidney might be of importance. We therefore investigated renal histidine dipeptide metabolism and the impact of exogenous carnosine in db/db mice, as well as established model of diabetic nephropathy. Db/db mice are characterized by defective hypothalamic leptin signaling, persistent hyperphagia and obesity, high plasma leptin, glucose and insulin levels and progressive diabetic nephropathy.

Vitagenes and UCP proteins in Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and represents the most common cause of dementia in the elderly, accounting for 50-60% of all cases in Western world (150,151). The prevalence rates for AD rise exponentially with age, increasing markedly after 65 years.

AD is characterized by cognitive decline beginning usually with impairment of episodic memory, involving progressively all cognitive functions in the late stage

(152). Although some cases are familiar, sporadic AD is more common, affecting more than 15 million people worldwide (153).

The pathological hallmarks of AD are amyloid plaques, containing amyloid-β peptide, derived from the transmebrane amyloid precursor protein, and neurofibrillary tangles, composed of hyperphosforylated tau protein, in the medial temporal lobe structures and cortical areas of the brain together with neuronal death and synapses loss (154,155). Many approaches have been undertaken to understand AD, including Aβ aggregation, but the heterogeneity of the etiologic factors makes it difficult to define the clinically most important factors determining the onset and progression of the disease (156). Accumulation of Aβ characterizes AD as a protein misfolding disease, suggesting a pathogenic role for oxidative stress in protein clearance defect by the ubiquitin-proteasome system (157,158). In particular, misfolded Aβ is considered to be the key mediator of cellular oxidative stress in AD (159), and different evidences exist which indicate that oxidative stress is central to neurodegeneration in AD (160,161).

Consistently, increasing evidence indicates that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis (162).

It is well known that living cells are continually challenged by conditions which cause acute or chronic stress.

Eukaryotic cells to adapt to environmental changes and survive different types of injuries, have evolved networks of different responses which detect and control diverse forms of stress (163). One of these responses, known as the *heat shock response*, has attracted a great interest as a universal fundamental mechanism necessary for cell survival under a wide variety of toxic conditions (8,3). Consistent with this, integrated survival responses exist in the brain, which are under control of redox regulated genes, called *vitagenes*, including heat shock proteins (Hsps), Sirtuins and Thioredoxin, that actively operate in detecting and controlling diverse forms of stress and neuronal injuries (3,164).

Sirtuins are a family of histone deacetylases that, in humans, includes at least seven members (silent information regulator two: SIRT 1-7) that exhibit different cellular and subcellular localizations and substrate specificities (165). The best studied sirtuin is SIRT-1, an NAD^+ dependent enzyme that deacetylates several different protein

substrates involved in the regulation of cellular energy metabolism and redox state, thereby influencing cell survival and plasticity (15,166).

Thioredoxin (Trx), is a major redox control system, consisting of a 12 kD a redox active protein Trx, and a homodimeric selenoprotein called thioredoxin reductase (TrxR1). TrxR1 is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized thioredoxin protein. It is usually located in the cytosol, but it translocates into the nucleus in response to various stimuli associated with oxidative stress. Trx, thus, plays a central role in protecting against oxidative stress (167,168).

Uncoupling proteins (UCPs) are members of the super family of anion carrier proteins located in the inner membrane of mitochondria. These proteins have several hypothesized functions including thermogenesis in certain tissues, protection from reactive oxygen species (ROS), neuroprotection and export of fatty acids. UCPs influence the production of mitochondrial reactive oxygen species. In general, the available data indicate that UCP activity results in decreased superoxide and hydrogen peroxide production (169,170).

Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

Glaucoma is a progressive optic neuropathy characterized by degeneration of neuronal tissue due to loss of retinal ganglion cells (RGCs), with accompanying compromission of visual field over time (171).

Research studies have demonstrated that RGC damage in glaucoma is not limited to the primary insulted neurons, but also involves neighboring neurons.

The increase in the prevalence of glaucoma with age is not accounted for only by the increase in ocular hypertension alone, being accompanied by an increase in the vulnerability of the optic nerve to the effects of glaucoma risk factors which increase as function of age. In particular, factors such as tissue hypoxia, disturbed protein metabolism and oxidative stress have been identified to interact in a vicious cycle underlying the pathogenesis of glaucoma (172,173), ultimately leading to apoptotic retina ganglion cell death (174,175, 176). In view of these considerations glaucoma can be viewed as a neurodegenerative disease which, similarly to other neurodegenerative pathologies, i.e., Alzheimer's and Parkinson's disease, where

irreversible functional deficit ensues as consequence of neuronal dysfunction and death. There is now a growing body of evidence demonstrating a link between AD and glaucoma.

Amyloid deposits, consisting of Aβ, which area characteristic feature of several neurodegenerative diseases such as Alzheimer's (AD), mild cognitive impairment, and Parkinson's disease (177) have been recently implicated in the pathogenesis of retinal damage, macular degeneration, and glaucoma (178). Accordingly, drugs designated to target β-amyloid (Aβ) has been found to reduce apoptotic degeneration of RGCs, asobserved in vitro and in vivo. Furthermore, the presence of increased levels of Aβ characterizes glaucoma as a protein misfolding disease, also suggesting a role for oxidative stress in the pathogenesis of retinal degenerative damage associated to glaucome.

Although oxidative stress has been recognized to play a critical role in the development and progression of glaucoma, yet, the exact mechanisms remain elusive. Oxidative stress can cause oxidative attack to DNA, proteins, and lipids, leading to DNA and protein modification, thus sustaining the pathophysiology of degenerative damage of RGCs (171). Relevant to protein misfolding, of emerging interest are heat shock proteins (HSPs), specialized molecular chaperones which mediate various cellular functions. HSPs are up regulated in response to conditions of stress in order to restore normal cell integrity (175). The heat shock response, an important component of vitagene family, contributes to establishing a cytoprotective state in a wide variety of human diseases. Vitagenes include, besides HSPs 70 and 32, the latter also called heme oxygenase-1 (HO-1), thioredoxin and sirtuins (179,180).

Several families of HSP have been implicated in neurodegenerative diseases and glaucomatous RGC apoptosis with increased levels of circulating autoantibodies to alpha-crystallins and HSP-27 and increased immunostaining of HSP-60, HSP-27 in RGCs and the retinal blood vessels in glaucoma patients (181,182).

In a rat glaucoma model, treatment with geranylgeranylacetone increases HSP-72 synthesis while reducing markedly RGC loss, possibly through interactions with different protein kinases, such as Akt kinase, and the inhibition of NF-kB. In this study we tested the hypothesis that there may be a causal relationship between AD and glaucoma that may be explained by systemic oxidative stress and dysregulation of cellular stress response.

Present work elucidated cellular stress response in peripheral cells in patients with glaucoma as com- pared to healthy volunteers, as control, in order to gain insight into the pathogenic mechanisms operating in the neurodegenerative damage associated with this disease and exploit the possible role of vitagenes in opening up new therapeutic targets for limiting the oxidative damage associated to degeneration occurring in glaucoma**.**

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AIM OF THE RESEARCH

Under normal conditions, the cell antioxidant defense systems operate very efficient there is a balance between pro-oxidant and antioxidant necessary for the maintenance of antioxidant defense system that, during normal cellular metabolism, is able to eliminate all free radicals that are generated.

In recent years, since oxidative stress has been considered the basis for some aspects of neurodegeneration, numerous experimental investigations have been conducted in order to reduce the effects of oxidative stress through the use of scavengers of free radicals (22). The cells are able to fight oxidative stress with many resources including bioactive molecules (glutathione, thioredoxin, flavonoids), lipoic acid, enzymes (heat shock proteins, superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase, etc.) and redox sensitive transcription factor protein.

The heat shock proteins (Hsp) is one of the most studied active defense systems against oxidative damage. The heat shock response to contribute took office a cytoprotective state in a wide variety of human diseases such as inflammation, cancer, aging and neurodegenerative disorders.

Vitagenes encode for heat shock proteins (Hsp) Hsp70 (53), and heme oxygenase-1, the thioredoxin and the sirtuin protein systems, open new prospetive in medicine and pharmacology that molecules capable of activating these defense mechanisms are a potential target for novel cytoprotective strategies (183,184).

The aim of the present study are as follows:

1. **Antioxidants and neuroblastoma cells**
2. **Oxidative stress and type II Diabetes**
3. **Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells**
4. **Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes**
5. **Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease**

1) Antioxidants and neuroblastoma cells

To understand the possible mechanisms underlying the protective molecules with antioxidant activity including the HBB-2 in the Central Nervous System will be studied in vitro, the role dell'HBB-2 cytoprotective against inflammatory and oxidative insults mediated by SIN-1, generating peroxynitrite (NO) and in human neuroblastoma cells (SH-SY5Y).

To assess the cytoprotective effects dell'HBB-2 will be studied in vitro in human neuroblastoma cells (SH-SY5Y) to:

- examine the neuroprotective effect of L-acetylcarnitine in human neuroblastoma cells SH-SY5Y cells exposed to oxidative stress induced dall' H_2O_2.
- evaluate the expression levels of HSP-70 and HO-1, as markers of oxidative stress and cell viability to investigate the cytoprotective effects of L-acetyl-carnitine in human neuroblastoma cells SH-SY5Y cells exposed to oxidative stress in the absence and presence of L-acetyl-carnitine.
- assess levels of stress protein Hsp-70 in human neuroblastoma subjected to nitrosative stress after treatment with HBB-2.

2) Oxidative stress and type II Diabetes

Diabetes is a chronic inflammatory disease and this study was designed to evaluate systemic oxidative stress and cellular stress response in patients suffering from type 2 diabetes and in healthy subjects.

Furthermore, one measure of oxidative marker is lipid oxidation, indexed by HNE, which can also occur in brain and peripheral tissues under oxidative stress.

Reactive carbonyl compounds, which are known precursors of carbonyl stress, can be generated during the AGEs-mediated free radical formation.

As previously reported, AGEs, such as pentosidine, are reliable markers of oxidative stress in diabetes and diabetic nephropathy, but they can trigger per se further inflammation thus creating a selfsustained vicious circle responsible for tissue damage.

Isoprostanes, in contrast to lipid hydroperoxides, are chemically stable end products of lipid peroxidation, and the measurement of their levels in plasma or urine may permit a sensitive and specific method for detection of lipid oxidative damage in *vivo*.

The heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders.

In the present study have examined, *in vitro,* the role of oxidative stress in the pathogenesis and clinical history of diabetes mellitus type 2

To assess the presence of systemic oxidative stress, will be studied, in *vitro*, the role of systemic oxidative stress and cellular stress response in the pathogenesis and clinical history of diabetes mellitus type 2 to:

.-examine the content of reduced and oxidized GSH in plasma of type 2 diabetic patients as a measure of the antioxidant status.

-evaluate protein oxidation by measuring the amount of protein carbonyls (DNPH).

-assess expression levels of pentosidine in plasma of patients with type 2 diabetes.

-examine expression levels of HNE in plasma from type 2 diabetic patients.

- assess the effects of observed systemic pro-oxidant conditions on lipids-derived circulating F2 isoprostanes in plasma of patients with type 2 diabetes.

-dose expression levels of stress proteins by Western blot analysis using specific antibodies (Hsp70, Hsp32, Trx , TrxR-1, Sirt-1, Sirt-2) in plasma and lymphocytes of patients with type 2 diabetes compared to controls.

3) Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Diabetic nephropathy (DN) is the foremost cause for dialysis in the Western world (133).

The vitagenes may therefore represent important targets for new therapeutic strategies. Modulation of cellular stress pathways and the search for neuroprotective strategies, using pharmacological interventions and food could play a key role in the treatment of diseases in general.

Carnosine is an endogenously synthesized dipeptide, it acts as a free radical scavenger and possesses antioxidant properties.

The objective is to determine the levels of expression of markers of oxidative stress in murine podocytes.

The aims of the present study are as follows:

-to examine the effect of glucose, carnosine and anserine in murine podocytes cells exposed to oxidative stress;

-to measure levels of expression of stress proteins by Western blot analysis, in murine podocytes, using antibodies specific: Hsp-70, HO-1, Trx, Sirt-1, HNE and DPNH.

-to investigate the oxidation of specific stress proteins by measuring of protein carbonyl levels in murine podocytes.

4) Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes

Alzheimer's disease (AD) is a progressive disorder characterized usually by early memory loss, however affecting all intellectual functions in the late stage and leadind to complete dependence for basic functions of life. The pathological lesions in AD include neurofibrillary tangles, neurite, plaques, the central core of which is amyloid-β peptide, derived from the transmembrane amyloid precursor protein (APP), amyloid angiopathy (156,162).

It has been hypothesized that oxidative stress may play an important role in the pathogenesis and progression of AD.

Uncoupling proteins (UCPs) are members of the super family of anion carrier proteins located in the inner membrane of mitochondria. These proteins have several hypothesized functions including thermogenesis in certain tissues, protection from reactive oxygen species (ROS), neuroprotection and export of fatty acids.

The objective of present study was determine the expression levels of stress responsive proteins such as sirtuin, thioredoxin and UCP protein in the blood of AD patients as compared to age-matched normal subjects to understand the potential role of these protective mechanism in the pathogenesis of AD pathology.

The aims of the research are as follows:

-to assess expression levels of stress proteins by Western blot analysis using specific antibodies (Trx, Sirt-1 e Sirt-2) in plasma and lymphocytes of patients with AD respect to control subjects.

-to evaluate levels of expression of UCP proteins by Western blot analysis in plasma of AD patients compared to controls.

5) Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

The objective is to determine cellular stress response in peripheral cells in patients with glaucoma as compared to healthy volunteers, as control, in order to gain insight into the pathogenic mechanisms operating in the neurodegenerative damage associated with this disease and exploit the possible role of vitagenes in opening up new therapeutic targets for limiting the oxidative damage associated to degeneration occurring in glaucoma.

The aims of the present study are as follows:

-to measure levels of expression of stress proteins by Western blot analysis in lymphocytes and plasma of patients with glaucoma, using specific antibodies: Hsp-70, HO-1, Trx, Sirt-1.

-to evaluate systemic pro-oxidant conditions by measuring the F2-isoprostanes levels in plasma of patients with glaucoma.

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MATERIALS AND METHODS

1. Antioxidant and neuroblastoma cells

Human neuroblastoma SHSY5Y cells (CRL-2266, American Type Culture Collection Rockville, MD, USA) is a well established cell line, corresponding to a third successive subclone of the SK-N-SH line originally established from a bone marrow biopsy of a neuroblastoma patient. The parental cell line comprises at least two morphologically different phenotypes, neuroblastic (N-type) and substrate adherent (S-type), which can undergo trans-differentiation (185). SHSY5Y cells can differentiate into cells with neuronal morphology, the phenotype of which varies depending on the inducing agent (186,187). SHSY5Y cells cultured in 50%MEM medium supplemented with 10% fetal bovine serum and 2 mM glutamine streptomycin (0.1 mg/ml) and 50% Nutrient F12 HAM. Cells were maintained at 37°C in CO_2 incubator in a saturated humidity atmosphere containing 95% air and 5% CO_2. The confluent cells were then exposed to treatment as shown in Table 1 and 2. After the various treatments, the cells were washed 3 times with PBS (pH 7.4), screpate and pelleted. The cell pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris (pH 7.4), 0.5 mM phenyl-methylsulfonilfluoride (PMSF) and homogenized prior to analysis by Western blot.

The cytosolic protein fraction (50 g) samples of the homogenates, obtained after centrifugation at 10,000 rpm for 5 min. at 4 ° C was used to determine the levels of expression of heat shock proteins by Western blot.

For the assay of cell viability of the cells were plated in 96-well multiwell and exposed to treatment as in Table 1 and 2.

TREATMENTS	TIME
CTRL	6h e 24h
HBB-2 (2µM)	6h e 24h
HBB-2 (5µM)	6h e 24h
HBB-2 (10µM)	6h e 24h
HBB-2 (20µM)	6h e 24h
SIN-1 (1 mM)	7h
HBB-2 (2µM)+SIN-1 (1 mM)	6h(+7) e24h (+7)
HBB-2 (5µM)+SIN-1 (1 mM)	6h(+7h) e24h (+7h)
HBB-2 (10µM)+SIN-1 (1 mM)	6h (+7h) e 24h(+7h)
HBB-2 (10µM)+SIN-1 (1 mM)	6h(+7h) e24h (+7h)

TREATMENTS	TIME
CTRL	6h e 12h
LAC (20µM)	6h e 12h
LAC (50µM)	6h e 12h
LAC (100µM)	6h e 12h
H_2O_2 (0,2mM)	-1h
LAC (20µM)+H_2O_2 (0,2mM)	6h (-1h) e 12h(-1h)
LAC (50µM)+H_2O_2 (0,2mM)	6h (-1h) e 12h(-1h)
LAC (100µM)+H_2O_2 (0,2mM)	6h (-1h) e 12h(-1h)

Vitality assay

Cell viability was assessed by MTT: 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (Cod. M2128 Sigma). The test MTT cytotoxicity assay is a colorimetric test that allows the proliferation and cell viability and in particular the

efficiency of mitochondrial. The MTT is a tetrazolium salt that in the case of viable cells is reduced by highly reducing environment of proliferating cells by the action of mitochondrial dehydrogenases. The reduction results in the formation of MTT formazan crystals which give a characteristic purple color to the mitochondria of viable cells by contrast, in non-proliferating cells, or death, then having no active mitochondria, MTT will not be reduced resulting in a less intense purplish color (188). The testing procedure requires that cell viability in each well are deposited 10µl of a solution of MTT 0.5 mg / ml and the whole is incubated for 3 hours (the incubation time should not exceed 4 hours since the MTT is cytotoxic) at 37 °C with an atmosphere of 5% CO_2 and saturated humidity. Expired the time of incubation, MTT solution is removed and addition 120 ml of acid isopropanol to dissolve the formazan crystals formed. The staining intensity of the resulting solution is directly proportional to the concentration of formazan and is therefore an expression of cell viability. The optical density (OD) is measured spectrophotometrically at a wavelength of 570 nm.
The degree of cell viability and hence the degree of toxicity of the test compound can be expressed by the following formula. % Cell viability = [OD (570 nm) test compound / OD (570 nm) negative control] x 100.
Cell viability is calculated by measuring the difference in optical density between the treated samples compared to controls.

2. Oxidative stress and type II Diabetes

Patients

All the procedures on human subjects have been conducted in accordance with the guidelines in the Declaration of Helsinki and the experimental protocol has been formally approved by the Ethical Committee of the University of Catania.
All the patients gave also their informed consent prior to undergo any procedure.
Fifteen adults with type 2 diabetes which were followed on a regular basis in the outpatient clinic of the Department of Internal Medicine of the Policlinico di Catania joined this study.
Clinical data of the participating subjects are summarized in Table 1.
Exclusion criteria were the following: renal insufficiency secondary to diabetes or to any other cause, ischemic heart disease or heart failure (stage 2 or above according to

NYHA classification), hepatic failure, endocrine disease other than diabetes mellitus, any other clinical or laboratory evidence of major organ disease. All patients underwent to a run in period of 4 to 8 weeks designed to achieve a stabilization of blood pressure levels, blood glucose control. All the subjects were not taking antioxidants, no limitation was given to the type of pharmacological therapy employed to control diabetes or hypertension, medications were maintained constant throughout the entire duration of the study.

Table 1
Clinical parameters of diabetic patients with normal renal function.

Clinical parameters (unit)	Mean ± SEM (range)
Age (years)	61 ± 4 (35–71)
Male/female	8/7
Body weight (Kg)	82 ± 17 (65–94)
Height (cm)	165 ± 10 (152–175)
Body mass index	28 ± 7 (24–34)
Plasma creatinine (mg/dl)	1,04 ± 0.52 (0.84–1.37)
Creatinine clearance (ml/min)	76 ± 10 (66–96)
MDRD-estimated GFR (ml/min)	74 ± 6 (59–89)
24 h proteinuria (mg)	77 ± 16 (44–249)
Fasting plasma glucose (mg/dl)	133 ± 12 (120–243)
Glycated hemoglobin (%)	6.4 ± 2.2 (4.6–9.7)
Sistolic blood pressure (mm Hg)	155 ± 10 (140–165)
Diastolic blood pressure (mm Hg)	85 ± 6 (70–95)

Samples

Blood was collected from controls and patients by venipuncture from an antecubital vein into tubes containing 500 mM EDTA as an anticoagulant. Immediately after sampling, 1 ml the blood was centrifuged at 10,000 g for 10 min at 4 °C to separate serum from red blood cells and 4 mL were utilized for lymphocytes purification. Lymphocytes from peripheral blood were purified using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA) (189).

Reduced and oxidized glutathione assay

Reduced glutathione (GSH) and glutathione disulfide (GSSG) were measured with the NADPH-dependent GSSG reductase method, as described in Adams et al (190).

Plasma GSH and GSSG were measured as described, except that DTNB or NEM solutions were directly added to equal volumes of whole blood and, after tilting, centrifuged at 2000 g for 6 min at 48C. GSH and GSSG standards in the ranges between 0 and 10 nM and 0.010 and 10 nM, respectively, added to control samples, were used to obtain the relative standard curves, and the results were expressed in nanomoles of GSH or GSSG, respectively, per milligrams protein.

Protein assay

The protein concentration was assayed using the bicinchoninic acid method (BCA) (Cod 23227 Pierce Protein Research Products, Rockford, IL 61101 USA) according to the method of Smith et al. (191) and using bovine serum albumin as standard.
This assay allows to determine the concentration of an unknown sample of protein (mg / ml), or can be used to determine the total amount of protein in a protein sample incognito (mg). The BCA assay has a linear range of concentration between 200-1000 μ g of protein per milliliter.
The method is based on the formation of a complex protein-Cu2 + in alkaline conditions, followed by the reduction of Cu2 + to Cu +. Cysteine, cystine, tryptophan, tyrosine and the peptide bond are able to reduce the cupric ion to cuprous. The bicinchoninic acid form a purple-blue complex with Cu + in alkaline environment, providing a basis for monitoring the reduction of Cu2 + by proteins. The assay is normally carried out at 37 ° C, as the increase of temperature favors the development of color. Is measured the absorbance at 562 nm, and the concentration of the protein is determined by comparison with a standard curve.

Western blot analysis

The expression levels of stress proteins were assessed by Western blot analysis.
Equal concentrations of protein extracted for each sample (50 g) were separated on a polyacrylamide mini gels precasting 4-20% (cod NB10420 NuSept Ltd Australia). Before being loaded on the gel, samples were boiled for 3 minutes in sample buffer(containing 40 mM Tris-HCl pH 7.4, 2.5% SDS, 5% 2-mercaptoethanol, 5% glycerol, 0.025 mg / ml of bromine phenol blue).The proteins were transferred onto

nitrocellulose membrane (0.45mM) (BIO-RAD Hercules, CA, USA) in transfer buffer containing (0.05% di SDS, 25mM di Tris, 192mM glycine and 20% v/v methanol). The transfer of the proteins on the nitrocellulose membrane was confirmed by staining with Ponceau Red which was then removed by 3 washes in PBS (phosphate buffered saline) for 5 min. each The membranes were then incubated for 1 hour at room temperature in 20 mM Tris pH 7.4, 150 mM NaCl and Tween 20 (TBS-T) containing 3% milk powder and incubated overnight with primary antibody at 4 ° C in TBS-T containing 1% milk powder.The membranes were incubated with appropriate primary antibodies overnight at 4 °C in TBS-T. Excess unbound antibodies were removed by 3 washes are with TBS-T for 5 minutes. After incubation with primary antibody, the membranes were washed 3 times for 5 min. in TBS-T and then incubated for 1 h at room temperature with the secondary polyclonal antibody conjugated with horseradish peroxidase (dilution 1:2000). The membranes were then washed 3 times with TBS-T for 5 minutes.The same membrane was incubated with a polyclonal antibody anti-beta-actin (SC 1615 Santa Cruz Biotech. Inc., CA, USA, dilution 1:1000) to verify that the concentration of protein loaded in the gel was the same in each sample. Finally, the membranes were incubated for 3 minutes with SuperSignal chemiluminiscence detection system kit (Cod 34080 Pierce Chemical Co, Rockford, USA) to display the specific protein bands for each antibody. The immunoreactive bands were quantified by capturing the luminescence signal emitted from the membranes with the Gel Logic 2200 PRO(Bioscience) and analyzed with Molecular Imaging software for the complete analysis of regions of interest for measuring expression ratios. The molecular weight of proteins analyzed was determined using a standard curve prepared with protein molecular weight.

Free and protein-bound sulfhydryl groups assay

Protein and nonprotein sulfhydryl (SH) compounds in different brain regions and in liver were estimated by the DTNB-based method of Sedlak and Lindsay (191). The content of SH groups was expressed in nanomoles per milligram of protein.

Measurement of F2-isoprostanes and pentosidine

The HPLC analysis of F2-isoprostanes was performed as described by Ritov et al. (193). Values are expressed as nM for plasma F2- isoprostanes. Plasma pentosidine was measured according to Miyata et al. (194). Values are expressed as pmol/mg protein.

Statistical Analysis

The statistical analysis was performed with the test two-way ANOVA test followed by a multiple of the control of the differences of Dunchan. The results are expressed as mean ± S.E.M. (average standard error) of 5 different experiments (n = 15). Each assay was performed in triplicate. The differences were considered significant for $P < 0.05$.

3. Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Samples

Primary Culture of Podocytes

A nephrectomy specimen was obtained from a 3-yr-old child with a minimally functioning (10% differential function) kidney, which had been detected antenatally as hydronephrosis. Cultures of primary human podocytes were infected with retrovirus containing supernatants from the packaging cell line (PA317). Cells in log-phase growth were exposed to freshly thawed filtered (0.45 μm) supernatant mixed 1:1 with growth medium plus 8 μg/ml polybrene. After 24 h, cultures were refed with fresh growth medium and grown for an additional 7 d to confluence. The culture medium was then supplemented with 0.5 mg/ml G418 (Life Technologies BRL, Life Technologies, Paisley, UK) until selection was complete (7 to 10 d). Infection, selection, and continuous culture were carried out at 33°C.The transfected cells were subcloned twice as follows. Confluent cells were trypsinized, spun down, and counted on a hemocytometer. The cells were then seeded at densities of 100, 200, 300, and 400 cells per 25-cm2 flask and grown at 33°C in growth medium as described. Irradiated

NIH 3T3 mouse fibroblast cells were added to each flask at a density of 0.5 × 106 cells/flask as not dividing feeder cells. Single cell clones were picked when visible to the naked eye (21 to 28 d) by using plastic cloning rings and transferred to individual wells of a 24 well plate. When grown to confluence, these were transferred to larger flasks, and a single clone was used for all the experiments described. Cells were used for experiments between passages 10 to 20. Cells were grown to confluence at 33°C, at which point they were trypsinized and reseeded in fresh flasks at a dilution of between 1:3 and 1:5. Before thermoswitching to 37°C, cells were grown to 70 to 80% confluence. At both temperatures, cells were fed with fresh medium 3 times per week. Cells were subsequently grown on type I collagen–coated flasks layered with glass cover slips for purposes of immunostaining. Cells were then plated onto the flasks and grown either at the permissive temperature of 33°C (in 5% CO2) to promote cell propagation as a cobblestone phenotype (undifferentiated) or at the non permissive temperature of 37°C (in 5% CO2) to inactivate the SV40 T antigen and allow the cells to differentiate (195).

4. Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes

Patients

The study was conducted according to guidelines of local Ethics Committee, and informed consent was obtained from all patients. Thirty patients (13 men and 17 women), with an age range of 69-81 years were enrolled in the study. All patients had progressive cognitive and memory impairment for at least 12 months and were diagnosed as suffering of probable AD, according to the criteria of the National Institute of Neurological and communicative Disorder and Stroke Alzheimer Disease and Related Disorder Association (NINCDSADRADA) (162). The evaluation of the stage of dementia was assessed by the Mini Mental State Examination (MMSE) (196). Status of basic and instrumental activities of daily living (Activity of Daily Living, ADL, Instrumental Activity of Daily Living, IADL) was also assessed. None of our patients had a history of major psychiatric illness or other neurological disorders (i.e. Parkinson's disease, stroke, dementia, multiple sclerosis, etc.), history of head trauma or epilepsy, acute or chronic medical illness, endocrinopathies or vitamin B deficiency

affecting cognitive functions, alcohol or drug abuse, and conditions precluding MRI or CT execution. Three patients were classified as mild and 7 as moderate. All patients were under acetylcholinesterase inhibitor (AchE-I) medication. Computed tomography (CT) or magnetic resonance imaging (MRI) scan showed widespread cortical atrophy in most patients.

In addition ten subjects (5 men and 5 women) with an age range of 60-79 years were studied as a control group. Controls showed no impairment in neuropsychological evaluation. Laboratory and neuroimaging tests were normal.

The exclusion criteria of the control subjects were in line with those of patients. Clinicodemographic characteristics and neuropsychological test scores of patients and control subjects are shown in Table 1.

Table 1 Clinical and demographic data of AD patients and control subjects

	Number of subjects	Age (mean ± SD)	Gender (F/M)	Disease duration (mean ± SD)	MMSE (mean ± SD)	ADL (mean ± SD)	IADL (mean ± SD)
Patients	30	74.6 ± 4.28	17/13	2.7 ± 1.7	17.5 ± 3.8	4.9 ± 1.2	3.7 ± 2.9
Controls	10	69.3 ± 5.77	5/5		27.9 ± 2	5.6 ± 0.5	7.9 ± 0.3

MMSE: Mini Mental State Examination (normal values: >24/30).
ADL: Activity Daily Living (normal values: 6/6).
IADL: Instrumental Activity of Daily Living (normal values: 8/8).

Sampling

Blood (5ml) collected from controls and patients into tubes containing EDTA, was divided into two aliquots,1 and 4ml respectively. One aliquots (1ml) was centrifuged at 3000 × g for 10 min at 4◦C to separate serum from red blood cells, while 4ml aliquots, were utilized for lymphocytes purification, which was accomplished by using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ,USA).

Lymphocyte purification

Lymphocytes from peripheral blood were purified by using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA).

Western Blot Analysis

Trx, Sirt-1, Sirt-2 and UCP1 were evaluated by Western blot analyses, which was accomplished as previously described in Calabrese et al. (2012) (197).

Plasma samples were processed as such, while the isolated lymphocyte pellet was homogenized (0,1 M NaCl, 0,01 M Tris Cl pH 7,6, 0,001 M EDTA pH 8, 100 μg/ml PMSF) and centrifuged at 10,000 × *g* for 10 min and the supernatant was then used for analysis after determination of protein content.

Equal concentrations of proteins extracted for each sample (40 μg) were separated on polyacrylamide mini gels precasting 4-20% (cod NB10420 NuSept Ltd Australia) using a miniprotean apparatus (BIO-RAD). Before being loaded on the gel , the samples were boiled for 3 minutes in sample buffer (containing 40 mM Tris -HCl, pH 7.4, 2.5 % SDS, 5 % 2- mercaptoethanol, 5 % glycerol, 0.025 mg / ml bromophenol blue) . The proteins were transferred onto nitrocellulose membrane (0. 45 μM) (BIO - RAD, CA , USA) in a transfer buffer containing (0.05 % SDS, 25mM Tris, 192mM of glycine and 20 % (v / v) of methanol). The transfer of the proteins on the nitrocellulose membrane was confirmed by staining with Ponceau Red, which was then removed with 3 washes in PBS (phosphate buffer saline) for 5 min. Membranes were then incubated for 1 hour at room temperature in 20 mM Tris, pH 7.4, 150 mM NaCl and Tween 20 (TBS -T) containing 2% milk powder and incubated overnight with the primary antibody at 4 ° C in TBS -T. The same membrane was incubated with a polyclonal antibody specific for β-actin (Santa Cruz Biotech Inc., CA, USA, dilution 1:1000) to verify that the concentration of protein loaded on the gel was the same in each sample. The excess unbound antibodies are removed by 3 washes you with TBS - T for 5 minutes. After incubation with the primary antibody, the membranes were washed 3 times for 5 min. in TBS -T and then incubated for 1 h at room temperature with the secondary with the secondary polyclonal antibody conjugated with horseradish peroxidase (dilution 1:500). The membranes were then washed 3 times with TBS -T for 5 minutes. Finally, the membranes were incubated for 3 minutes with SuperSignal chemiluminiscence detection kit system (Cod 34080 Pierce Chemical Co., Rockford, USA) to display the specific bands of proteins for each antibody.

Immunoreactive bands were quantified by capturing the luminescence signal emitted from the membranes with the Gel Logic 2200 PRO (Bioscience) and analyzed with

Molecular Imaging software for the complete analysis of regions of interest for measuring expression ratios.
The molecular weight of the proteins analyzed was determined using a standard curve prepared with proteins of known molecular weight .

Protein assay

The protein concentration was assayed using the bicinchoninic acid method (BCA) (Cod 23227 Pierce Protein Research Products, Rockford, IL 61101 USA) according to the method of Smith et al. (191) and using bovine serum albumin as standard.
This assay allows to determine the concentration of an unknown sample of protein (mg / ml), or can be used to determine the total amount of protein in a protein sample incognito (mg). The BCA assay has a linear range of concentration between 200-1000 μ g of protein per milliliter.
The method is based on the formation of a complex protein-Cu2 + in alkaline conditions, followed by the reduction of Cu2 + to Cu +. Cysteine, cystine, tryptophan, tyrosine and the peptide bond are able to reduce the cupric ion to cuprous. The bicinchoninic acid form a purple-blue complex with Cu + in alkaline environment, providing a basis for monitoring the reduction of Cu2 + by proteins. The assay is normally carried out at 37 ° C, as the increase of temperature favors the development of color. Is measured the absorbance at 562 nm, and the concentration of the protein is determined by comparison with a standard curve.

Statistical Analysis

The statistical analysis was performed with the test two-way ANOVA test followed by a multiple of the control of the differences of Dunchan. The results are expressed as mean ± S.E.M. (average standard error) of 5 different experiments (n = 15). Each assay was performed in triplicate. The differences were considered significant for $P < 0.05$.

5. Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

Patients

Eighteen patients (12 males and six females, mean age 60 ± 15 years) with diagnosis of hypertensive primary open-angle glaucoma (POAG), with typical optic nerve head and visual field damage, were included in the study. Twenty age-matched healthy volunteers were recruited as controls. Patients and control subjects underwent IOP measurement by Goldmann applanation tonometer, optic nerve head examination by 78 D lens at the slit lamp, and visual field test 24-2 SITA standard, by a750 Humprey perimeter (HFA II). Clinical characteristics of patients and control subjects are shown in Table1. Patients with normal tension glaucoma, previous uveitis, diabetes, arterial hypertension were excluded.

The study was conducted according to guide lines of local Ethics Committee, and informed consent was obtained from all patients.

Western Blot Analysis

HSP-70, HO-1, Trx, and Sirt-1 protein levels were estimated by Western blot analysis which was accomplished as previously described in Calabrese et al. (2012) (197). Plasma samples were processed as such, while the isolated lymphocyte pellet was homogenized and centrifuged at 10,000 × *g* for 10 min. The supernatant was then used for analysis after determination of protein content.Equal concentrations of proteins extracted for each sample (40 μg) were separated on a 12.5 % polyacrylamide gel. Before being loaded on the gel , the samples were boiled for 3 minutes in sample buffer (containing 40 mM Tris -HCl, pH 7.4, 2.5 % SDS, 5 % 2- mercaptoethanol, 5 % glycerol, 0.025 mg / ml bromophenol blue) . The proteins were transferred onto nitrocellulose membrane (0. 45 μM) (BIO -RAD, CA , USA) in a transfer buffer containing (0.05 % SDS, 25mM Tris, 192mM of glycine and 20 % (v / v) of methanol). The transfer of the proteins on the nitrocellulose membrane was confirmed by staining with Ponceau Red, which was then removed with 3 washes in PBS (phosphate buffer saline) for 5 min. Membranes were then incubated for 1 hour at room temperature in 20 mM Tris, pH 7.4, 150 mM NaCl and Tween 20 (TBS -T) containing 2% milk powder and incubated overnight with the primary antibody at 4 °

C in TBS -T . The membranes were incubated with appropriate primary antibodies, in particular with the monoclonal mouse anti- Hsp70 (SPA- 810, Stressgen, Ann Arbor, MI, USA) , which recognizes only the inducible form , while immunodetection HO -1, Trx and SIRT -1 and was performed, respectively, using rabbit polyclonal antibodies : SPA -895 (Stressgen), 07-613 (Upstate Biotechonology , Charlottesville, VA), sc-15404 and sc- 20966 (Santa Cruz Biotech . Inc., Santa Crus, CA , USA) incubated overnight at 4 ° C in TBS -T . The same membrane was incubated with a polyclonal antibody specific for β-actin (Santa Cruz Biotech Inc., CA, USA, dilution 1:1000) to verify that the concentration of protein loaded on the gel was the same in each sample. The excess unbound antibodies are removed by 3 washes you with TBS -T for 5 minutes. After incubation with the primary antibody, the membranes were washed 3 times for 5 min. in TBS -T and then incubated for 1 h at room temperature with the secondary anti -rabbit polyclonal antibody conjugated with horseradish peroxidase (1:2000 dilution) specific for HO-1, Trx, SIRT -1 , while in the case of Hsp70 has been used an anti- mouse antibody conjugated with horseradish peroxidase (1:2000 dilution) and for the beta -actin anti-goat antibody conjugated with horseradish peroxidase horseradish (1:2000 dilution). The membranes were then washed 3 times with TBS -T for 5 minutes. Finally, the membranes were incubated for 3 minutes with SuperSignal chemiluminiscence detection kit system (Cod 34080 Pierce Chemical Co., Rockford, USA) to display the specific bands of proteins for each antibody. Immunoreactive bands were quantified by scanning the plates Western blot films imaged with a laser densitometer (LKB - Ultrascan , XL model, Pharmacia, American Instruments, Haverhill, MA, USA). The molecular weight of the proteins analyzed was determined using a standard curve prepared with proteins of known molecular weight .

Measurement of F2-isoprostanes

F2-isoprostanes were determined by HPLC according to the procedure of Ritovet al. (2002). F2-isoprostane content in plasma was expressed in nM.

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RESULTS

1. Antioxidant and Neuroblastoma cells

The cytoprotective effects of antioxidant HBB2 were assessed by cell viability studies in vitro models of human neuroblastoma cells in type SH-SY5Y subjected to treatment with the oxidizing agent peroxynitrite donor SIN-1.

Figure 1 shows cell viability after treatment with a dose-response was evaluated HBB2 (2μM, 5μM, 10μM, 20 μM) for 6h and 24h.The results obtained show a significant increase in cell viability compared to controls, at a concentration of 2 μM of HBB2 and instead of a cytotoxic effect at higher concentrations (10 μM, 20 μM).

Figure 2 shows the cell viability after treatment with SIN-1 (1 mM) (7h) in the presence and absence of pretreatment with HBB2 (-24h), at various concentrations (2μM, 10μM), and HBB2 (2μM, 10μM) alone for 24 hours.

According to previous studies (198) treatment with SIN-1 (1 mM) significantly reduced cell viability by exerting a cytotoxic effect compared to untreated cells or subjected to treatment with 2μM HBB2, which reveal, however, an increase of vitality because it increases the cellular resistance to cytotoxic damage induced by SIN-1(1mM). It has detected a significant decrease in cell viability after treatment with HBB2 (10 μM), demonstrating a cytotoxic effect at that concentration.

Experimental studies suggest the key role played by Hsps in the neuroprotective effects from excitotoxic damage (199). The Figure 3 shows the Western blot analysis of protein expression levels of Hsp72 in SH-SY5Y cells treated for 7h SIN-1(1mM) in the presence and absence of pretreatment (-24h) with HBB2 (2μM).

These results demonstrate a significant increase compared with controls, protein expression of Hsp72 after treatment with SIN-1.

The cytoprotective effects of L-acetyl-canitina (LAC) were assessed by cell viability studies type in human neuroblastoma cells SH-SY5Y subjected to treatment with H2O2 oxidant. To determine the concentration required to make experiments on the cytoprotection of neuroblastoma SH-SY5Y cells with the antioxidant (LAC), was assessed cell viability after treatment with H_2O_2 dose-response (0,02mM; 0,05mM; 0,1mM, 0,2mM; 0,4mM, 0,8mM, 1mM) for 12 h. In Figure 4 shows a graph that shows the concentration of H2O2 (Equal to 0.2 mM), which has a 50% cell viability to be used for the experimental plan. Figures 40-41 shows the graph of cell viability after treatment with H_2O_2 (0,2mM) (-1h) in the presence and absence of pretreatment with LAC (6h e12h), at various concentrations (20μM, 50μM, 100μM), and with LAC (20μM, 50μM, 100μM) alone for 6h. Treatment with H_2O_2 (0.2 mM) significantly reduced cell viability by exerting a cytotoxic effect compared to untreated cells or subjected to treatment with LAC, which reveal, however, an increase of vitality.

The pretreatment with LAC-6h (20μM, 50μM, 100μM) is not sufficient to activate the cytoprotective function of the test compound (Fig. 5), while the 12h pretreatment with LAC (20μM, 50μM, 100μM) determines a marked cytoprotective effect increasing cellular resistance to cytotoxic damage induced by H_2O_2 (0,2mM) (-1h) (Fig. 6). Numerous experimental studies indicate the key role played by Hsps in neuroprotective phenomena from excitotoxic damage (200).

In agreement with the cell viability data Fig 7-8 show no activation of the HO-1 and Hsp70 in cells pretreated with LAC to 6h(20μM, 50μM, 100μM) + H_2O_2 (0,2mM)(-1h). The graphs in Fig.9 -10 show levels of protein expression of Hsp-70 and HO-1in SH-SY5Y cells treated with H_2O_2 (0.2 mM) (-1h), in presence and absence of pretreatment (12h) with LAC (20μM, 50μM, 100μM).

The results on neuroblastoma cells show significant changes in protein expression of Hsp70 compared with controls and cells treated with H_2O_2 (0,2mM) (-1h) only in cells pretreated with LAC at a concentration of 50μM (12h) and H-1 at a concentration of 20μM and 50μM of LAC.

These results support the hypothesis that L-acetylcarnitine upregulating the system of vitagenes may limit the consequences associated with nitrosative and oxidative stress to aging and age-related diseases.

1. Oxidative stress and type II Diabetes

Diabetes is a chronic inflammatory disease and this study was designed to evaluate systemic oxidative stress and cellular stress response in patients suffering from type 2 diabetes and in agematched healthy subjects. Clinical data of patients and control subjects are summarized in Table 1.

The content of reduced and oxidized GSH in plasma of type 2 diabetic patients was determined as a measure of the antioxidant status and compared with the levels in the control group (Fig. 11). We found a significant decrease (to 63% of control levels) in the content of reduced GSH and a significant increase (by 46%) in the content of oxidized GSH (GSSG) in the diabetic samples compared with the control samples (Fig. 12). These changes in absolute GSH and GSSG concentrations resulted in a 68% decrease in the GSH/GSSG ratio in type 2 diabetes compared with control patients (data not shown). Changes in the redox glutathione status were consistent with oxidative modifications of redox status in plasma proteins between the experimental and control groups investigated. Protein oxidation was evaluated by measuring the amount of protein carbonyls (DNPH).

Protein carbonyls most often are detected by two methods, i.e., derivatization with 2,4-dinitrophenylhydrazine (DNPH), followed by immunochemical detection with an antibody against the resulting protein hydrazone, or formation of the Schiff base by biotin hydrazide followed by detection of the protein-bound imine with enzyme- or fluorophore-linked avidin or streptavidin. Here we describe the DNPH-based detection method that is routinely used for detection of carbonylated proteins. In the DNPH method, samples are derivatized with DNPH. DNPH reacts with carbonyl groups to form protein-resident 2,4-dinitrophenylhydrazone (DNP), which is then detected using commercially available anti-DNP antibodies.

Fig. 13, show protein carbonyls were found at a significantly ($p<0.05$) higher level in plasma of type 2 diabetic patients than in control subjects. Furthermore, one measure of oxidative marker is lipid oxidation, indexed by HNE, which can also occur in brain and peripheral tissues under oxidative stress (201,202). HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack, was bound by Michael in addition to proteins, particularly at cysteine, histidine, or lysine residues (203). Examination of plasma content of HNE in diabetic patients showed a significant ($p<0.05$) elevation of protein-bound HNE as compared to control group (Fig. 14).

As previously reported, AGEs, such as pentosidine, are reliable markers of oxidative stress in diabetes and diabetic nephropathy, but they can trigger per se further inflammation thus creating a selfsustained vicious circle responsible for tissue damage (202,204). In accordance with this theory, we demonstrated a significant increase ($P<0.01$) in plasma levels (Fig. 15) of pentosidine in patients suffering from type 2 diabetes with respect to control subjects.

The next step was to evaluate the effects of observed systemic pro-oxidant conditions on lipids-derived circulating F2 isoprostanes. As shown in Fig. 16, a significant

elevation ($P<0.05$) of in plasma levels of total F2-isoprostanes has been found in type 2 diabetic patients ($P<0.01$) with respect to controls.

The *heat shock response* contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders.

The vitagene family is composed of the heat shock proteins HO-1 (also called Hsp32), Hsp70, by the thioredoxin system and by sirtuin proteins (179,180).We therefore evaluated the expression levels of cellular stress response proteins in plasma and lymphocytes in control and type 2 diabetes patients.

Western blot analysis of plasma probed for thioredoxin (Trx) antioxidant protein expression is reported in Fig. 17. Trx expression was significantly ($p<0.05$) decreased in the plasma of diabetic patients, compared to controls.

Heme oxygenase-1 (HO-1), also referred to as Hsp32, belongs to the Hsp family and protects brain cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin (164,205,206). This latter is then reduced by biliverdin reductase (BVR) into bilirubin (BR), a linear tetrapyrrole with antioxidant properties; very recently, BR has been shown to effectively counteract also nitrosative stress, due to its ability to bind and inactivate NO and RNS (205,207,208). We evaluated the expression of inducible HO-1 isoform in lymphocytes of type 2 diabetes patients and in controls. As shown in Fig. 5, HO-1 protein expression was higher in samples from diabetes patients compared to controls.

The 70 kDa family of stress proteins is one of the most extensively studied cytoprotective system which includes the constitutive heat shock cognate isoform (Hsc70), and the inducible isoform, Hsp70 (also referred to as Hsp72). Here we evaluated the inducible isoform and its expression is reported in Fig. 18, which shows

a significant ($p<0.05$) increase in type 2 diabetes lymphocytes with respect to healthy control subjects. A representative immunoblot is also shown (Fig. 19).

Analysis of lymphocytes in type 2 diabetes patients, compared to control group, revealed also a significant ($p<0.05$) reduction of Trx expression (Fig. 20) which was associated with increased levels of the enzyme Thioredoxin reductase-1 (Fig. 21). Interestingly, we found a significant ($p<0.05$) decrease in the levels of sirtuin-1 as well as sirtuin-2 proteins in lymphocytes of diabetic patients than in control group (Fig. 22 and Fig. 23). As far as we are concerned this is the first evidence of changes in sirtuin-1 and sirtuin-2 expression in type 2 diabetes, although at the moment we can not exclude that this might not be a specific alteration of this progressive chronic inflammatory systemic disease.

2. Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Carnosine has been demonstrated to be an effective antiglycating agent, at least in model systems and cultured cells (139,142).

The structure of carnosine closely resembles that of preferred glycation sites in proteins, that is, a target amino group with proximal imidazole and carboxyl groups. Carnosine itself could, sacrificially, react with many potential glycating agents thereby protecting other potential targets against glycation. The carnosine ability to react with toxic aldehydes (e.g. acetaldehyde, formaldehyde, malondialdehyde and methylglyoxal), and deoxyribose has been shown to protect cultured human fibroblasts and lymphocytes, rat brain endothelial, by inhibiting the protein.

Interestingly, the dipeptide has been shown to inhibit formation of protein carbonyls generated by the actions of oxygen free radicals and related species as well as aldehydes or ketones on aminoacid side chains. Moreover, it resulted also able to react with (i.e. carnosinylate) protein carbonyls to form protein carbosyl carnosine adducts, inhibiting further cross-linking to normal proteins.

Our study evaluated the expression levels of cellular stress response proteins in murine podocyte cells.

Production of heat shock proteins, including protein chaperones, is essential for the folding and repair of damaged proteins. During stressful conditions, such as elevated temperature, they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins.

The 70 kDa family of stress proteins is one of the most extensively studied cytoprotective system which includes the constitutive heat shock cognate isoform (Hsc70), and the inducible isoform, Hsp70. Here we evaluated the inducible isoform and its expression is reported in Figure 24. In Western blot analysis of podocytes probed for Hsp70, after Glucose 25 mM, Glucose 25 mM and CRSN 1 mM and CRSN 1 mM treatment, expression levels were significantly increased compared to control ($P < 0.05$).

HO-1 belongs to the Hsp family and protects cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin. This latter is then reduced by biliverdin reductase into bilirubin, a linear tetrapyrrole with antioxidant properties; very recently, bilirubin has been shown to effectively counteract also nitrosative stress, due to its ability to bind and inactivate NO and RNS. As shown in Figure 25, analysis of podocytes compared to control revealed an increase in HO-1 protein expression. We have seen after Glucose 25 mM treatment, expression levels were significantly elevated compared to control ($P < 0.05$).

In Figure 26, we were assayed for thioredoxin expression by Western blot. Trx is one of the members of a family of proteins redox-active evolutionarily conserved feature of a catalytic center sulfhydryl and thiol within the sequence of the active site, which is subjected to reversible reactions of oxidation at the level of cysteine residues following the reduction of sulfhydryl of a wide variety of target proteins oxidized. Trx has evolved similar to a protein chaperone by ensuring the maintenance of the structure sulfhydryl and thiol protein biological function. Indeed, several studies show that the scientific Trx binds to specific proteins, modulating its structural conformation. The expression levels of Trx were significantly ($P < 0.05$) increased after treatment with Glucose 25 mM compared to control, while the increase was significantly lower after Glucose 25 mM and CRSN 1 mM treatment ($P < 0.05$) compared to Glucose 25 mM treatment and only slightly increased by CRSN 1 mM treatment respect to Glucose 25 mM treatment.

Figure 27 and 28, show Sirt-1 expression by Western blot. Several studies have determined a role for the human Sirt-1 protein in cell survival. Sirt-1 specifically associates with the p53 tumor suppressor protein and deacetylates it, resulting in negative regulation of p53-mediated transcriptional activation. Importantly, p53 deacetylation by Sirt-1 also prevents cellular senescence and apoptosis induced by DNA damage and stress. The expression of Sirt-1 was significantly ($P < 0.05$) increased after CRSN 1 mM, Glucose 25 mM, ANSN 1 mM, Glucose 25 mM and ANSN 1 mM treatment, compared to control. We can see that expression levels of Sirt-1 after CRSN 1 mM treatment showed a low increase respect to ANSN 1 mM treatment and Glucose 25 mM and ANSN 1 mM treatment; This is the first evidence demonstrating CRSN 1 mM was tenfold more protective respect to ANSN 1 mM.

Protein oxidation was evaluated by measuring the amount of protein carbonyls (DNPH). In the DNPH method, samples are derivatized with DNPH. DNPH reacts

with carbonyl groups to form protein-resident 2,4-dinitrophenylhydrazone (DNP), which is then detected using commercially available anti-DNP antibodies. As shown in Figure 29, protein carbonyls were found at a significantly ($p<0.05$) higher level podocytes after Glucose 25 mM, Glucose 25 mM and ANSN 1 mM and ANSN 100 mM treatment compared to control. Carbonyls expression was higher after Glucose 25 mM treatment compared to Glucose 25 mM and ANSN 1 mM and ANSN 100 mM treatment. Whereas podocytes treated with Glucose 25 mM and ANSN 1 mM and ANSN 100 mM showed similar expression levels.

Furthermore, one measure of oxidative marker is lipid oxidation, indexed by HNE. HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack, binds by Michael addition to proteins, particularly at cysteine, histidine, or lysine residues. Examination of podocytes content of HNE showed a significant ($p<0.05$) elevation of protein-bound HNE as compared to control (Figure 30). The samples show an increase after treatment with Glucose 25 mM, Glucose 25 mM and ANSN 1 mM, ANSN 1 mM, Glucose 25 mM and ANSN 100 compared to control. However, 4-HNE expression was higher when the podocytes were treated by Glucose 25 mM, Glucose 25 mM and ANSN 1 mM, ANSN 1 mM; while increase was lower after Glucose 25 mM and ANSN 100 mM treatment .

3. Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes

Alzheimer's disease (AD) is the most common form of dementia and is characterized pathologically by senile plaques, neurofibrillary tangles and cerebral amyloid

angiopathy (209,210). Figure 31 reports brain MRI axial T2 image showing cerebral atrophy in patient with Alzheimer's disease in comparison to a normal brain.

Our laboratory previously demonstrated in the brain as well as in peripheral blood that oxidative and nitrosative stress occur in AD patients, compared to normal subjects (11) and that this can serve as a trigger for induction of the heat shock response (211,212,213).

Therefore, we evaluated the expression levels of Trx and Sirtuin in plasma and lymphocytes of AD patients and control subjects. Western blot analysis of lymphocytes probed for Sirt-1 is reported in Figure 32. Sirt-1 expression is significantly increased in AD patients, compared to controls. In contrast to Sirt-1, expression levels of Sirt-2 measured in lymphocytes did not show a significant increase in AD patients compared to controls (Figure 33).

As shown in Figure 34, analysis of lymphocytes in AD patients, compared to control group, revealed also an increase in thioredoxin protein expression.

Consistently to the observed changes in AD lymphocytes, analysis of plasma in AD patients showed higher expression levels of Sirt-1 (Figure 35).

Expression levels of Sirt-2 were also measured and results, reported in Figure 36, show an increase in AD patients, which however was not statistically significant, as compared to control group. As far as we are concerned, this is the first evidence demonstrating changes in SIRT-1 expression in AD, although at the moment we cannot exclude that this might not be a specific alteration of this progressive inflammatory neurodegenerative disease associated with oxidative stress which has emerged as a critical factor in AD.

Interestingly, we investigated the expression of Trx and we found, in the plasma, higher levels of Trx protein in AD patients compared with the control group (Figure 37). Figure 38 shows a decreased expression of UCP1 protein in plasma of AD

patients compared to controls. Analysis of lymphocytes in AD patients, compared to control group, did not allow to detect measurable levels of this protein (data not shown).

4. Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

Glaucoma is a progressive optic neuropathy characterized by degeneration of neuronal tissue due to loss of retinal ganglion cells (RGCs), with accompanying compromission of visual field over time (171).

In our study we evaluated the expression of stress proteins in plasma and lymphocytes of glaucomatous patients compared to controls.

The 70 kDa family of stress proteins is one of the most extensively studied cytoprotective system which includes the constitutive heat shock cognate isoform (Hsc70), and the inducible isoform, Hsp70. As shown in Figure 39, a significant ($P < 0.05$) increase in HSP-70 was found in lymphocytes of patients with glaucoma with respect to healthy control subjects. A representative immunoblot is reported in Figure 1B. Western blot analysis of plasma probed for HSP-70 are reported in Figure 40, which shows that HSP-70 expression increased significantly ($P < 0.05$) in patients with glaucoma, compared to controls. A representative immunoblot is shown in Figure 2B.

HO-1 or HSP-32, belongs to the Hsp family and protects cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin. This latter is then reduced by biliverdin reductase into bilirubin, a linear tetrapyrrole with antioxidant properties; was found expressed at significantly higher levels in lymphocytes of patients with glaucoma than in controls (Figure 41). A representative immunoblot is

illustrated in Figure3B. Similarly to lymphocyte finding, patients with glaucoma exhibited higher level plasma HO-1 protein then healthy controls (Figures 42).

In Figure 43, we were assayed for thioredoxin expression by Western blot. Trx is one of the members of a family of proteins redox-active evolutionarily conserved feature of a catalytic center sulfhydryl and thiol within the sequence of the active site, which is subjected to reversible reactions of oxidation at the level of cysteine residues following the reduction of sulfhydryl of a wide variety of target proteins oxidized. Trx has evolved similar to a protein chaperone by ensuring the maintenance of the structure sulfhydryl and thiol protein biological function. Indeed, several studies show that the scientific Trx binds to specific proteins, modulating its structural conformation.

Analysis of lymphocytes in patients with glaucoma, compared to control group, revealed a significant ($P < 0.05$) increase of Trx expression (Figure 43), while in the plasma there was no statistically significant difference between the two experimental groups (Figure 44). Representative immunoblots are reported in Figures 5B and 6B, respectively.

Interestingly, we found significantly ($P < 0.05$) higher levels of sirtuin-1 in lymphocytes of patients with glaucoma than in the control group (Figure 45). Consistent with the changes found in lymphocytes, analysis of plasma confirmed increased protein levels of sirtuin-1, higher in patients with glaucoma as to compare with the healthy control group (Figure 46). Representative immunoblots are shown in Figures7B and 8B, respectively. As to our knowledge this is the first evidence of changes in sirtuin-1expression in glucomatous pathology, although this finding may not be a marker specific for this progressive chronic inflammatory systemic disease.

The F2-isoprostanes are a product of lipid peroxidation from arachidonic acid catalyzed by free radicals, they are formed in situ by phospholipids and released into the circulation by the phospholipase and subsequently excreted in the urine (214,215).

The F2-isoprostanes in contrast to lipid hydroperoxides, are the end products of lipid peroxidation chemically stable, the analysis of their levels in plasma or urine may provide a sensitive and specific method for detection in *vivo* of lipid oxidative damage (216).

In figure 47, we evaluated systemic pro-oxidant conditions, by measuring lipid-derived circulating F2-isoprostanes.We found a significant increase ($P < 0.05$) of total F2-isoprostanes in the plasma of patients with glaucoma with respect to controls.

-5-

DISCUSSION

1. Antioxidants and Neuroblastoma cells

An efficient functioning of maintenance and repair processes seems to be crucial for the survival and physical quality of life. This is maintained by a complex network of so-called longevity assurance processes, made from vitagenes (2,212). Among them, The chaperonin proteins are highly conserved and responsible for the preservation and repair of the correct conformation of cellular macromolecules such as proteins, RNAs and DNA (217). The chaperonins along with heat shock proteins whose function is to protect cells from a variety of toxic conditions such as extreme temperatures, oxidative stress, viral infections, and exposure to heavy metals or cytotoxic (218,219).

Chaperone-buffered silent mutations can be activated during the aging process Phenotypic characteristics of lead exposure is not yet manifested themselves, and contribute onset of polygenic diseases such as age-related disorders, atherosclerosis and cancer (220).

In addition, Emerging experimental evidence emphasize the crucial role of heme oxygenase-1 (HO-1) as an integral system, inducible and redoxsensitive, responsible for cellular resistance to stress HO-1 induction, that occurs with the induction of other Hsps during various pathophysiological conditions, by generating the vasoactive molecule carbon monoxide (CO) and the potent antioxidant bilirubin, could represent a protective system potentially active against brain oxidative injury (221, 222).

Thus, the induction of Hsps is used by the cell in repair processes following damage to different to prevent the damage caused by the accumulation of proteins, which have lost their function. In addition, this system has recently been considered as biological

markers for the detection of physiological and stress pathophysiological conditions (223). In recent years, there has been a growing interest in identifying new pharmacological strategies to enhance the defense mechanisms through activation of multiple genes of antioxidant defense. Numerous epidemiological studies have shown a strong relationship between food intake, incidence and severity of chronic diseases, leading to the conclusion that chemicals derived from the diet (through modulation of the expression of target genes) may affect the financial healthillness playing a key role in the onset, progression, and severity of chronic (224).

Acetyl-L-carnitine has been proposed as a therapeutic agent in several neurodegenerative disorders (8, 225). It was reported that this compound, in nonhuman primates, prevents the neurological damage caused by methyl-4- phenyl-1 ,2,3,6-tetraidropiridina (MPTP) in the substantia nigra, the increase in cellular respiration, mitochondrial membrane potential and the levels of cardiolipin in hepatocytes of rats of 24 months of age (8). These data suggest that the system of carnitine is involved in repair processes and stabilization of membranes and metabolism of disintegration dell'acil-CoA as the acylcarnitine (212).

Acetyl-L-carnitine, as well as carnitine attenuates neuronal damage produced by the acid 3-nitropropionico from rotenone and MPTP (8).

In addition, the LAC induces a more rapid recovery of the levels of ATP, PCr and lactate in rats after ischemia. The results of this research have shown that in human neuroblastoma cells (SH-SY5Y) subjected to oxidative stress treatment with the LAC not only increases the percentage of cell viability, but also induces the activation of heat shock proteins. The Hsp-70 is encoded in the nucleus, but reside in both the cytosol and in mitochondria (8).

The Hsp-70 in normal form of the chaperonin complex which is involved in protein folding and nell'asseblaggio within the cytosol, but also in cell organelles as the

endoplasmic reticulum and mitochondria (226). Most mitochondrial proteins are synthesized in the cytosol, but can be transferred to the organelles in the folded state (226). During the translocation of proteins interact with Hsp-70. The ATP-dependent binding and release of Hsp-70 provides greater strength for the complete transport of polypeptides into the mitochondrial matrix . Most of the polypeptides are released from Hsp-70- soluble However, a subset of polipentidi aggregates can be transferred from the Hsp-70 Hsp-60 during the folding (227).

Having to close the functional interactions between these two systems of chaperonins, it is likely that the upregulation of Hsp-70 is a key target site for the action of LAC consequently that restores the functions of the mitochondrial complex.

These experimental results show that the LAC plays a crucial role in the regulation of vitagenes. Together, these results confirm that the cellular response to stress can be considered as targets for new therapeutic interventions and can be focused on the antioxidant capacity of some nutritional components such as polyphenols. L-carnosine and its related peptides and L-acetilcarnosina can adjust the system vitagenes to limit the deleterious consequences associated with nitrosative and oxidative stress to aging or other neurodegenerative disorders (228).

2. Oxidative stress and type II Diabetes

Prevalence of diabetes is constantly increasing in the United States, in Europe as well as in developing countries and the global burden associated with the disease is becoming one of the most relevant world health problem (229). The long term exposure to chronic hyperglycaemia along with the increase in oxidative stress that characterizes the diabetic patients results in the formation and accumulation of AGEs (118,230,231). AGEs have a wide range of chemical, cellular, and tissue effects that

contribute to the development of chronic complication of diabetes (204,231). The importance of AGEs as downstream mediators of tissue injury in diabetes has been demonstrated by animal studies in which the inhibition of the advanced glycation reaction delayed the development of microvascular complications without any direct effect on the glycemic control (204). Furthermore, AGEs-modified proteins undergo physico-chemical changes which alter charge, solubility and conformation resulting in an altered protein (204). Finally, the effects of AGEs, together with hyperglycaemia and ROS induce growth factors and cytokines causing organ hypertrophy, as the enlarged kidneys in diabetic nephropathy and accumulation of extracellular matrix components (231). Our results showing an elevation in plasma AGEs in patients with diabetes compared with healthy subjects are in accordance with current literature (232,233). Reactive carbonyl compounds, which are known precursors of carbonyl stress, can be generated during the AGEs-mediated free radical formation (234,235). Our evidence of an increased levels of carbonyls both in plasma and lymphocytes from type 2 diabetic patients are in line with clinical and experimental data showing an increased generation of ROS in diabetes (236,237).

4-hydroxy-2-nonenal (HNE) are formed from arachidonic acid or other unsaturated fatty acids following free radical attack and bind, by Michael addition, to proteins particularly at cysteine, hystidine, or lysine residues (124,201). Thus, protein-bound HNE is a reliable index of lipid oxidative stress which can also occur in different organs under oxidative stress conditions (123,125,235,238).

Isoprostanes are derived by the free radical-catalyzed peroxidation of arachidonic acid, they are formed in situ from phospholipids and subsequently released in the circulation by a phospholipase and eventually excreted in urine (126,239).

Isoprostanes, in contrast to lipid hydroperoxides, are chemically stable end products of lipid peroxidation, and the measurement of their levels in plasma or urine may permit a

sensitive and specific method for detection of lipid oxidative damage in vivo (216). Their concentration in the urine is one of the most readily available index of lipid oxidation and, more in general, of the oxidative stress status of a patient population.

The F2-isoprostane levels in type 2 diabetic patients are consitently increased (128,240,241). In a previous study we demonstrated an increased level of F2 isoprostanes in type 2 diabetic patients with incipient diabetic nephropathy (202). Our results show a significant increase in both plasma and urinary levels of F2-isoprostanes and provide further evidence about a condition of systemic rather than local pro-oxidant status.

In recent years, it was also shown that isoprostane directly affects platelet aggregation and may therefore be implicated in macrovascular complication of diabetes (131).

Eucariotic cells have developed various pathways to counteract oxidative stress-related damage. Among these stress, induced proteins, chaperones are essential to help the correct folding and maintenance of the proper conformation of other proteins and to promote cell survival after a large variety of environmental stresses. Therefore, normal chaperone function plays a pivotal role in the endogenous response of several tissues to an increased cellular stress, whereas altered chaperone function has been associated with the development of several diseases (11,213). To this regard, the expression of HO-1, Hsp70, as well as of TrxR-1 in peripheral lymphocytes of patients

with type 2 diabetes is significantly increased. Consistent to this, we show that type 2 diabetes is associated with significant perturbation of systemic redox state, as revealed by a significant decrease in both reduced glutathione and thioredoxin protein. Hsp70 induction has been proved to be an efficient system helping the recovery from a large number of diseases, such as atherosclerotic and inflammatory disease, diabetes and neurodegenerative damage-associated pathologies (11,213,242). The physiological response of HO-1 protein in human lymphocytes is also intriguing. Under oxidative

stress conditions, HO-1 is one of the early genes to be induced and it exerts cytoprotective functions by inducing the metabolic pathway of prooxidant heme degradation and the production of both the vasoactive molecule carbon monoxide and biliverdin, the latter being the precursor of the powerful antioxidant and antinitrosative molecule bilirubin (11,213,243). Thus, HO-1 increase in the lymphocytes of patients with type 2 diabetes suggests that, in response to an oxidant insult, induction of an early gene is a significant part of the antioxidant response and this is much more interesting considering the long term course of a disease such as diabetes.

The thioredoxin system (thioredoxin, thioredoxin reductase, and NADPH) regulates cellular redox balance through the reversible oxidization of its redox-active cysteine residues (−Cys-Gly-Pro-Cys-) to form a disulfide bond that in turn is reduced by thioredoxin reductase and NADPH (244). Thioredoxin plays an essential role in cell function by limiting oxidative stress directly via antioxidant effects and indirectly by protein–protein interactions with key signaling molecules, such as the thioredoxin-interacting protein (245).We have shown an increased TrxR protein expression in lymphocytes of diabetic patients which is in agreement with the well known effects induced by oxidative stress (246). Our study provides evidence also that sirtuin signal is downregulated in type 2 diabetes respect to healthy control subjects. This is consistent with the recent notion that sirtuins reduce reactive oxygen species formation by modulating the acetylation of the respiratory chain and by stimulating mithocondria superoxide dismutase (SOD-2) and isocitrate dehydrogenase which generates NADPH for the glutathione pathway. Thus, changes in sirtuin expression, as observed in our study, may contribute to explain the failure in diabetic defense mechanisms against sustained oxidative stress (132). Furthermore, given the interplay between reactive oxygen species and insulin signal transduction, it is of interest that while small physiologic amount of H2O2 mimic the action of insulin and stimulate glucose uptake,

in contrast, a large body of clinical evidence shows that sustained oxidative stress correlates directly with both mean blood glucose levels, as well as glucose variability, in response to anti-diabetic therapy (247).

Consistently, it is known that glucose and lipid levels can exert a direct toxic effect on beta cell function and insulin secretion. A direct role of oxidative stress and AGE on insulin secretion has been demonstrated in *vivo*, however, few data are available in *vivo* on a potentially advantageous antioxidative effects of therapies on beta cell function in diabetics. Data on an early positive antioxidant effect administration on insulin secretion of patients with a family history of diabetes would suggest the need of an early intervention prior that irreversible loss of beta cell mass and function have occurred (247).

The above discussed data demonstrate the role of oxidative and glycoxidative pathways in diabetes and unravel the importance of systemic cellular stress response mechanisms, in particular vitagenes which, if stimulated, may counteract this pro-oxidant status.

It is plausible to hypothesize that novel therapeutic approaches, based on combined nutritional and pharmacologic interventions, can be designed to burst cellular stress response as a mean to control and reduce oxidative stress-mediated formation of AGEs as well as to remove AGE-induced modifications, thus constituting an important component of future prophylaxis and therapy in patients with diabetes.

3. Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Carnosine has been demonstrated to be an effective antiglycating agent, at least in model systems and cultured cells. The structure of carnosine closely resembles that of

preferred glycation sites in proteins, that is, a target amino group with proximal imidazole and carboxyl groups. Carnosine itself could, sacrificially, react with many potential glycating agents thereby protecting other potential targets against glycation. The carnosine ability to react with toxic aldehydes (e.g. acetaldehyde, formaldehyde, malondialdehyde and methylglyoxal), and deoxyribose has been shown to protect cultured human fibroblasts and lymphocytes, rat brain endothelial, by inhibiting the protein. Protein and DNA protein cross linking induced by those substances. Interestingly, the dipeptide has been shown to inhibit formation of protein carbonyls generated by the actions of oxygen free radicals and related species as well as aldehydes or ketones on aminoacid side chains. Moreover, it resulted also able to react with (i.e. carnosinylate) protein carbonyls to form protein carbosyl carnosine adducts, inhibiting further cross-linking to normal proteins.

Peters et al., 2010, have therefore assessed the relevance of N-glycosylation for CN-1 protein secretion and enzyme activity, and addressed whether hyperglycemia can influence serum CN-1 secretion and activity.

N-glycosylation of CN-1 was either inhibited by tunicamycin in pCSII-CN-1–transfected Cos-7 cells or by stepwise deletion of its three putative *N*-glycosylation sites. CN-1 protein expression, *N*-glycosylation, and enzyme activity were assessed in cell extracts and supernatants. The influence of hyperglycemia on CN-1 enzyme activity in human serum was tested in homozygous (CTG)5 diabetic patients and healthy control subjects.

This study for the first time demonstrates marked alterations of renal carnosine metabolism in diabetic mice. Renal CN1 activity is increased whereas tissue anserine concentrations are tenfold reduced. Treatment with carnosine normalizes renal CN1 activity and renal anserine concentrations.

Moreover, exogenous carnosine lowered blood glucose levels, proteinuria and renal vascular permeability.

They recently showed that N-glycosylation is essential for appropriate secretion and CN1 activity and that hyperglycemia enhances CN1 secretion and enzyme activity (85). In accordance with these findings, increased CN1 activity in the diabetic mice might be the consequence of hyperglycemia due to the poor glucose control.

In accordance with these findings, increased CN1 activity in the diabetic mice might be the consequence of hyperglycemia due to the poor glucose control. Carnosine treatment of db/db mice did not affect renal tissue carnosine concentrations, but normalized anserine levels.

Anserine can be formed by methylation of carnosine via carnosine-N-methyl transferase, which explains the increase of renal anserine in carnosine treated mice. Compared to anserine, carnosine is the far better substrate for CN1 (75) and exogenous carnosine is metabolized to anserine or degraded to b-alanine and histidine, while anserine accumulates.

In carnosine-treated diabetic mice, two mechanisms could decrease CN1 activity despite increased substrate concentration. First, lowered blood glucose levels might lower CN1 activity by reduced N-glycosylation (137) second increased anserine levels are known to effectively lower CN1 activity and thus carnosine degradation (149). Inhibition of carnosine degradation by CN1 activity by homocarnosine (142) can be disregarded in kidney tissue since renal homocarnosine levels are very low. Whereas carnosine levels remain stable and do not differ between diabetic and control mice, anserine levels are clearly lower in diabetic mice. Although the function of carnosine is better described, previous studies showed that also anserine seems to have several protective functions. Similar to carnosine, anserine was described to affect renal sympathetic nerve activity (248), reduce blood glucose (249), increase the contribution

of the non-bicarbonate buffering action and decrease the bicarbonate buffering action in blood (250), act as effective transglycating agents in decomposition of aldose-derived Schiff bases (251), protect neuronal cells against reactive oxygen species, show dose-dependent angiotensin converting enzyme inhibitory activity, act as peroxyl radical scavenger to protect the protein modification and react as quencher of cytotoxic carbonyls (252). Beside these similarities, there is some evidence that both dipeptides have also different functions. Whereas carnosine facilitates NO production in endothelial cells, anserine failed to increase NO production (248).

Treatment with carnosine, but not anserine, was able to significantly reduce infarct volume and improve neurological function (253).

It is hypothesized that poor blood glucose control in diabetic patients might result in an increased CN-1 secretion even in the presence of the (CTG)5 allele.

Carnosine treatment largely prevents alterations of renal carnosine metabolism in diabetic mice.

In the present study, we evaluated the expression levels of cellular stress response proteins in podocytes.

Production of heat shock proteins, including protein chaperones, is essential for the folding and repair of damaged proteins. During stressful conditions, such as elevated temperature, they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins.

The heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders**.** Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response.

The vitagenes may therefore represent important targets for new therapeutic strategies. Modulation of cellular stress pathways and the search for neuroprotective strategies, using pharmacological interventions and food could play a key role in the treatment of diseases in general. Carnosine is an endogenously synthesized dipeptide, it acts as a free radical scavenger and possesses antioxidant properties.

In renal tissue of diabetic mice, carnosinase activity is significantly increased and anserine concentrations markedly reduced compared to controls. Carnosine treatment reverses these diabetes-associated alterations of histidine dipeptide metabolism. The concomitant reduction in proteinuria and renal vascular permeability may not only be exerted via the beneficial effects of carnosine on glucose metabolism but also via recovery of renal anserine homeostasis. The specific local mode of action of the different dipeptides and potential of pharmacological interventions deserve further analyses.

4. Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes

Alzheimer's disease (AD) is a progressive disorder characterized usually by early memory loss, however affecting all intellectual functions in the late stage and leadind to complete dependence for basic functions of life. The pathological features of AD are a variable degree of cortical atrophy, in the frontal, parietal, and temporal lobes (Figure 31).

The pathological lesions in AD include neurofibrillary tangles, neurite, plaques, the central core of which is amyloid-β peptide, derived from the transmembrane amyloid precursor protein (APP), amyloid angiopathy (156,162). AD brain has been reported to be under oxidative stress that may play an important role in the pathogenesis and

progression of AD (163,254,255). Several lines of evidence support a fundamental role for free radical mediated event in the pathogenesis of the disease. Amyloid-β peptide has been shown to induce protein oxidation in both in vitro and in vivo studies (256,257,258). As a result, amyloid-β peptide has been proposed to play a central role in the pathogenesis of AD (259). We have previously shown that increased protein oxidation and lipid peroxidation are present in the brain from patients with mild cognitive impairment (MCI), as compared to aged-matched control brain (260,261). Because many researchers consider MCI to be the transition zone between normal cognition and the dementia of early AD (262,263). Cells have evolved different adaptive responses to manage oxidative stress which includes the recognition of cellular redox potential, reactive oxygen species and protein oxidation and responding with changes in gene expression (264,265). Sirt-1 and Sirt-2 are stress induced proteins that have been implicated in defense mechanisms against agents that may induce oxidative injury, and its induction represents a common feature in a number of neurodegenerative diseases (266). In addition, another protein, thioredoxin reductase (TrxR), is emerging as critical vitagene involved in brain stress tolerance. As such, it has been demonstrated that Trx plays an important role in protecting against oxidative stress and in regulating cell growth and cell death (267,268). In the present study, the role of the vitagenes Sirt-1, Sirt-2 and Trx, was investigated in the peripheral blood of AD patients to gain further insight into the role of oxidant/ antioxidant balance as critical factors operating in the pathogenesis of AD. We found that the levels of Sirt-1 and Sirt-2 in AD lymphocytes were significantly higher than in control patients a finding associated with increased expression of Trx, and a reduced expression of UCP1, as compared to control group. The increased expression of these proteins, however, appear to be consequence of a strong oxidant environment, which can be relevant to the pathogenesis of AD. Sirt-1, has received considerable attention, as it

has been recently demonstrated that Sirt-1 induction could represent a protective system potentially active against brain oxidative injury (74,269,270). Several studies suggest that the Sirt-1 gene is redox-regulated and its expression appears closely related to conditions of oxidative stress (271,272). Another protein, in addition, thioredoxin reductase (Trx), is emerging as critical vitagene involved in brain stress tolerance. As such, it has been demonstrated that Trx plays an important role in protecting against oxidative stress and in regulating cell growth and cell death (268,273). Furthermore, we found decreased levels of UCP expression in AD patients. Uncoupling proteins (UCPs) are members of the family of mitochondrial anion carrier proteins. The UCP1 is an integral membrane protein unique to brown adipose tissue mitochondria. UCP1 separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat. UCP1 facilitates the transfer of anions from the inner to the outer mitochondrial membrane andthe return transfer of protons from the outer to the inner mitochondrial membrane. UCP1 is activated in the brown fat cell by fatty acids and inhibited by nucleotides (176). Mitochondrial uncoupling mediated by uncoupling protein 1 (UCP1) is classically associated with non-shivering thermogenesis by brown fat. UCP family proteins are also present in selected neurons. They can be activated by free radicals and free fatty acids, and their activity has a profound influence on neuronal function. By regulating mitochondrial biogenesis, calcium flux and local temperature, neuronal UCPs can directly influence neurotransmission, synaptic plasticity and neurodegenerative processes. In addition, by reducing free radical generation, UCP protein may serve a cytoprotective system. Our results demonstrate that AD is associated with increased oxidative stress, which could have an impact on mitochondrial bioenergetics affecting the function of neuronal mitochondrial complex IV and complex V (176). In this context, simultaneousreductions in cytoprotective mechanisms, such as theUCP system, could allow oxidative injury to go unchecked or

increase over time, thus representing an important factorsustaining the oxidative stress hypothesis of AD pathogenesis.Consistently, modulation of endogenous cellular defense mechanisms such as the vitagene network, including sirtuin, thioredoxin and UCP proteins may have the potential to broaden up new approaches to therapeutic interventions in diseases associated with tissue damage and cell death, such as in neurodegeneration. Our data, supporting a role for oxidative stress in the pathogenesis of AD, indicate that the stress responsive genes may represent an important target for novel cytoprotective strategies, as molecules inducing this defense mechanism, via nutritional and/or pharmacological approaches, can exploit the potential for antidegenerative therapeutic effects.

5. Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

Glaucoma is one of the leading causes of vision loss worldwide. Open-angle glaucoma, the most common form of glaucoma, is characterized by a progressive loss of RGCs and atrophy of the optic nerve, resulting in loss of visual field (177,178).

Oxidative stress is considered an important risk factor for the development of primary angle-closure glaucoma and increased levels of oxidative stress products have been documented in primary angle-closure glaucoma (180,181,182).

We have recently demonstrated that increased oxidative stress and cellular stress response are a systemic presentation of the oxidative burden occurring in AD patients, rising the conceivable possibility that Alzheimer's disease might not be exclusively a primary neurological pathology rather being a systemic oxidant disorder (274,275).

In this study we hypothesize that there may be a causal relation-ship between AD and glaucoma that may be explained by systemic oxidative stress and dysregulation of cellular stress response.

We have found in patients with glaucoma a systemic condition of oxidative stress as revealed by upregulation of lipid-derived F2 isoprostanes. This marker of oxidative stress was found in the blood of patients with glaucoma at significantly higher levels than in controls.

Similarly to other oxidant disorders, such as AD or multiple sclerosis a direct relationship, although not necessarily causal, may exist between organ specific pathology and systemic alterations underlying or reflecting the local oxidative status (197,276).

Reactive oxygen species (ROS) are an essential component of intracellular signaling network, regulated through the intrinsic antioxidant capacity of a cell, but when ROS formation exceedingly increases damage to DNA, proteins, and lipids macromolecules ensues.

Oxidative damage is one of the most important causes of brain protein damage and dysfunction in several age-related neurodegenerative disorders including Alzheimer's disease (277). RGCs and the optic nerve have demonstrated similar mechanisms of cell death in glaucoma to those of Alzheimer's disease, marking glaucoma as a neurodegenerative disease (278).

AD is a progressive neurodegenerative disorder characterized by cognitive and memory deterioration as well as changes in personality, behavioral disturbances and an impaired ability to perform activities of daily living (274). AD is known to be the most common form of dementia and is a major public health problem throughout the world (197).

In addition to synaptic degradation and extensive neuronal cell loss, neuropathological characteristics of AD include extracellular senile plaques containing β-amyloid (Aβ) derived from β-amyloid precursor protein (APP) after sequential cleavage β–secretase and c-secretase, and intracellular neurofibrillary tangles caused by abnormally phosphorylated tau protein (176).

It is intriguing to note that AD and glaucoma have many common features. Both are slow and chronic neurodegenerative disorders with a strong age-related incidence. Studies consistently report decreased levels of β-amyloid and increased levels of tau in cerebrospinal fluid from AD patients in comparison with healthy subjects. Similarly, decreased levels of β-amyloid and significantly increased levels of tau have been detected in the vitreous fluid from patients with glaucoma or diabetic retinopathy in comparison with the levels in a control group (274,275,278).

This finding corroborates a role for β-amyloid and tau in the pathogenesis of glaucoma, suggesting that the neurodegenerative process in these ocular diseases might share, at least in part, a common mechanism with AD.

Production of heat shock proteins, including protein chaperones, is essential for the folding and repair of damaged proteins. During stressful conditions, such as elevated temperature, they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins.

-6-

CONCLUSION

Cellular stress response requires the activation of pro-survival pathways which, under control of protective genes called vitagenes produce molecules (heat shock proteins, glutathione, bilirubin) endowed with anti-oxidant and anti-apoptotic activities. Vitagenes encode for heat shock proteins (Hsp) Hsp32, Hsp70, the thioredoxin and the sirtuin protein systems.

The vitagenes may therefore represent important targets for new therapeutic strategies. Having to close the functional interactions between these two systems of chaperonins, it is likely that the upregulation of Hsp-70 is a key target site for the action of LAC consequently that restores the functions of the mitochondrial complex.

These experimental results show that the LAC plays a crucial role in the regulation of vitagenes. Together, these results confirm that the cellular response to stress can be considered as targets for new therapeutic interventions and can be focused on the antioxidant capacity of some nutritional components such as polyphenols. L-carnosine and its related peptides and L-acetilcarnosina can adjust the system vitagenes to limit the deleterious consequences associated with nitrosative and oxidative stress to aging or other neurodegenerative disorders

Modulation of cellular stress pathways and the search for neuroprotective strategies, using pharmacological interventions and food could play a key role in the treatment of diseases in general. Carnosine is an endogenously synthesized dipeptide, it acts as a free radical scavenger and possesses antioxidant properties.

In renal tissue of diabetic mice, carnosinase activity is significantly increased and anserine concentrations markedly reduced compared to controls. Carnosine treatment reverses these diabetes-associated alterations of histidine dipeptide metabolism. The

concomitant reduction in proteinuria and renal vascular permeability may not only be exerted via the beneficial effects of carnosine on glucose metabolism but also via recovery of renal anserine homeostasis. The specific local mode of action of the different dipeptides and potential of pharmacological interventions deserve further analyses.

Our results, supporting a role for oxidative stress in the pathogenesis of AD, indicate that the stress responsive genes may represent an important target for novel cytoprotective strategies, as molecules inducing this defense mechanism, via nutritional and/or pharmacological approaches, can exploit the potential for antidegenerative therapeutic effects.

Consistently, modulation of endogenous cellular defense mechanisms such as the vitagene network and UCP proteins, may open a new approaches to therapeutic interventions in diseases associated with tissue damage and cell death, such as neurodegeneration.

Our data are in favor of the hypothesis linking oxidative stress to the pathogenesis of glaucoma, and indicate that stress responsive genes may represent an important target for novel cytoprotective strategies, as molecules inducing this defense mechanism, via nutritional and/or pharmacological approaches, can exploit the potential for antidegenerative therapeutic interventions.

-7-

FIGURES

1. Antioxidants and Neuroblastoma cells

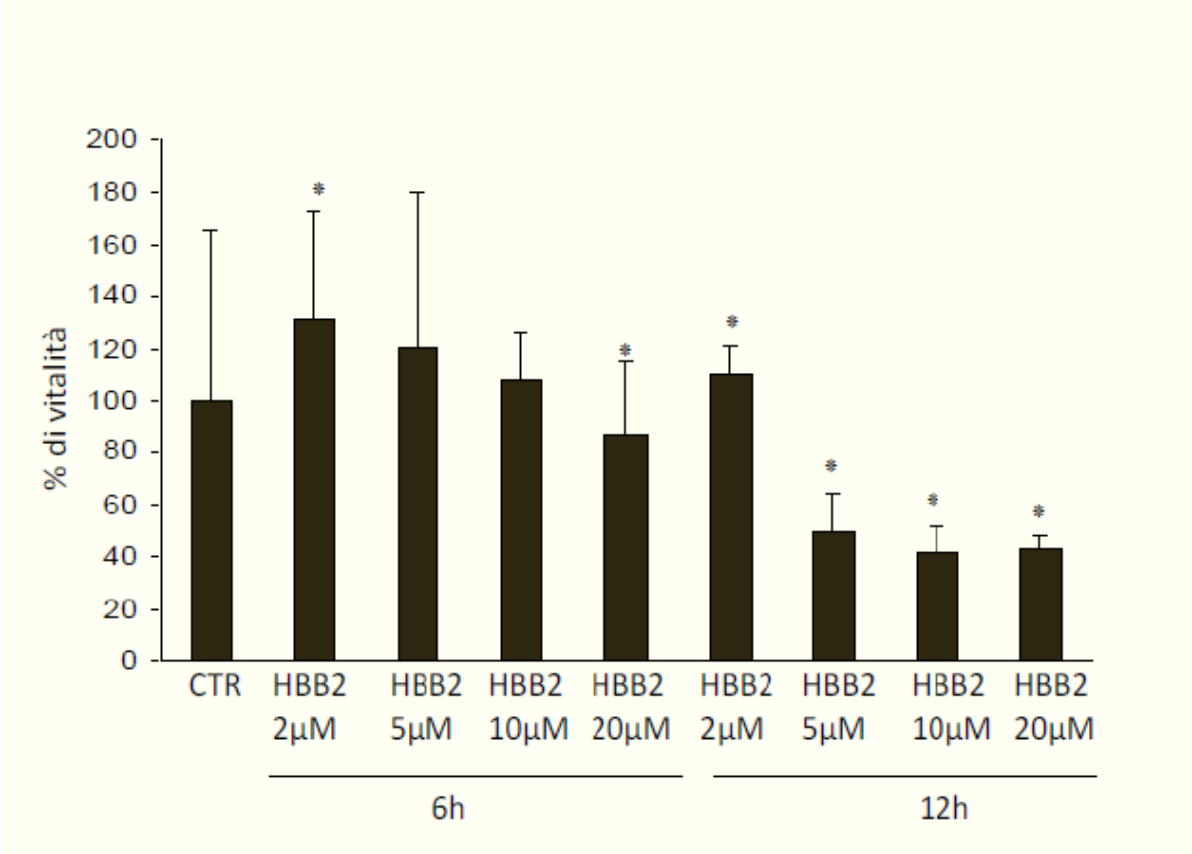

Fig. 1. Vitality cellular assay (MTT). Effect dose-response of HBB2 on cell viability. SH-SY5Y were incubated with absent or present HBB2 (2µM, 5 µM;10µM; 20 µM) for 6 and 12 h. *P < 0.05 vs. control.

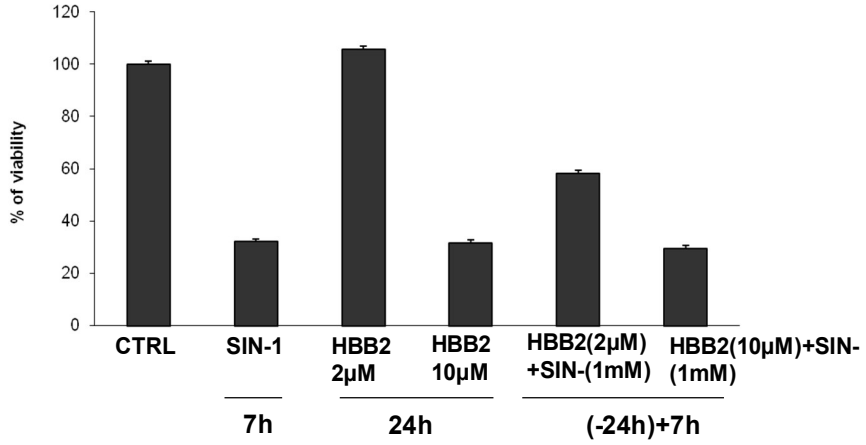

Fig. 2 Vitality cellular assay (MTT). Effect of HBB2 on cell viability after oxidative damage induced by SIN-1 treatment. SH-SY5Ys were incubated with absent or present HBB2 (2μM, 10μM) for 24 h. and only HBB2 (2μM, 10μM) (24h). SH-SY5Y *P < 0.05 vs. control;

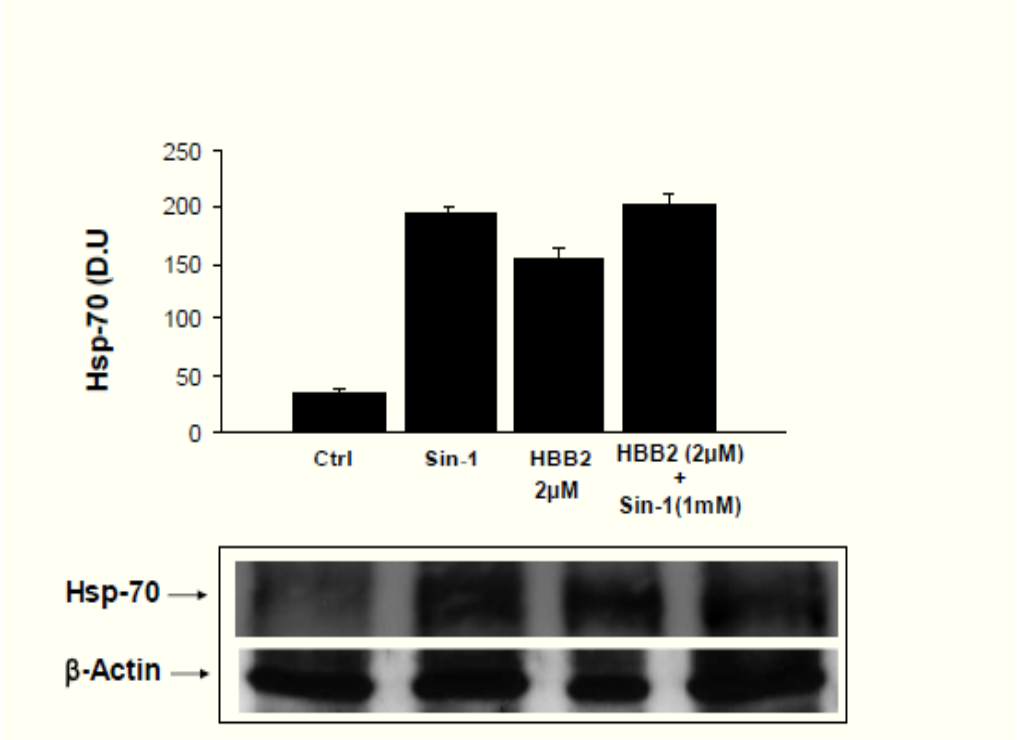

Fig. 3. Western Blot assay. Hsp-70 levels in SH-SY5Y cells after oxidative damage induced by SIN-1 (1mM) (7h) treatment. SH-SY5Y were incubated with absent or present HBB2(2μM) for 24 h. and only HBB2 (2μM) (24h)A representative immunoblot is shown in b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group. P ≤0.05 vs control. D.U., densitometric units; CTRL, control.

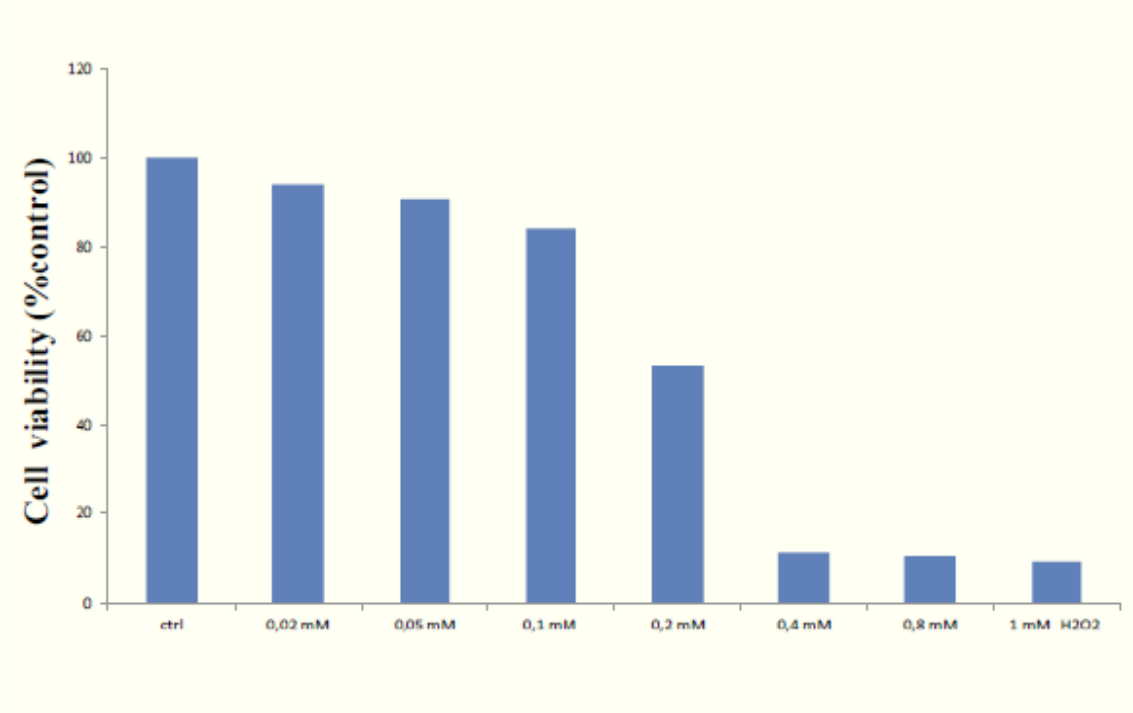

Fig. 4. Vitality cellular assay (MTT). Effect dose-response of H2O2 on cell viability. SH-SY5Ys were incubated different concentration H2O2 (0,02µM, 0,05 µM; 0,1 µM; 0,2 µM; 0,4 µM;0,8 µM; 1mM) for 24 h. *P <0.05vs Control.

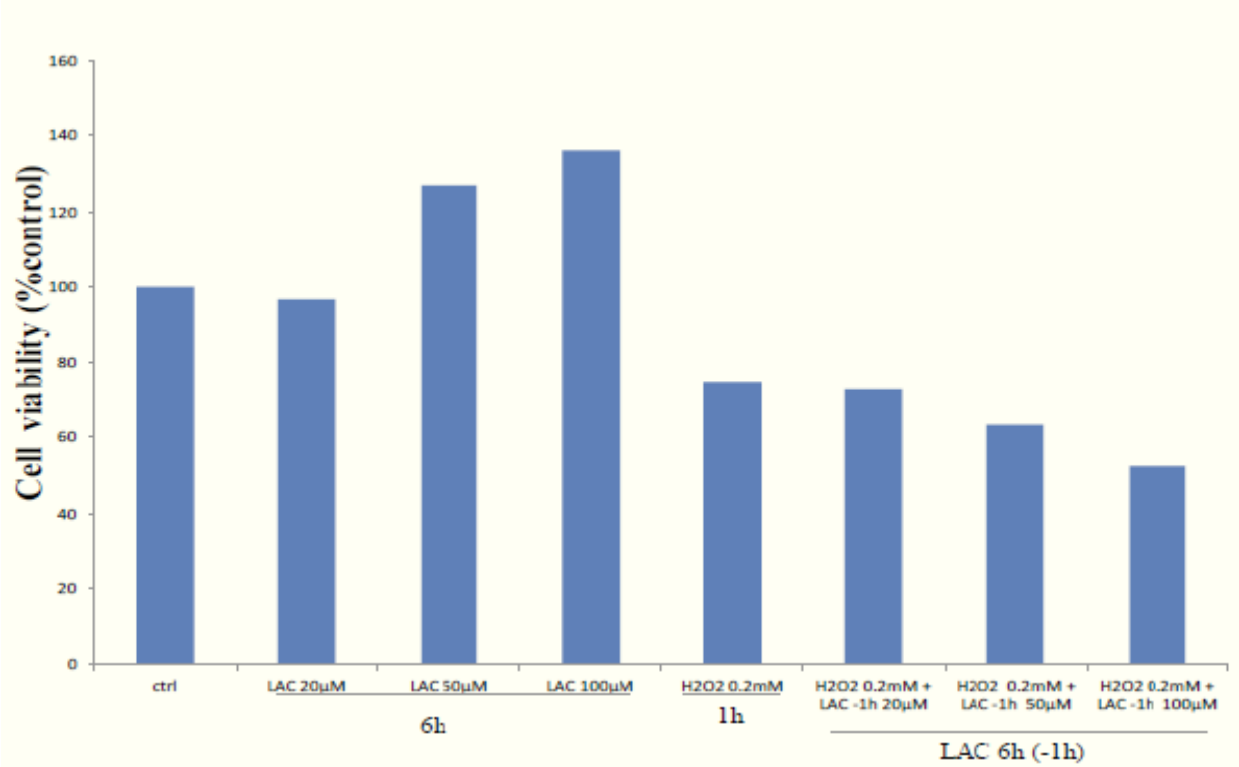

Fig. 5. Vitality cellular assay (MTT). Effect of HBB2 on cell viability after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 6 h. and only LAC (20µM, 50µM, 100µM) (6h).*P < 0.05 vs. control.

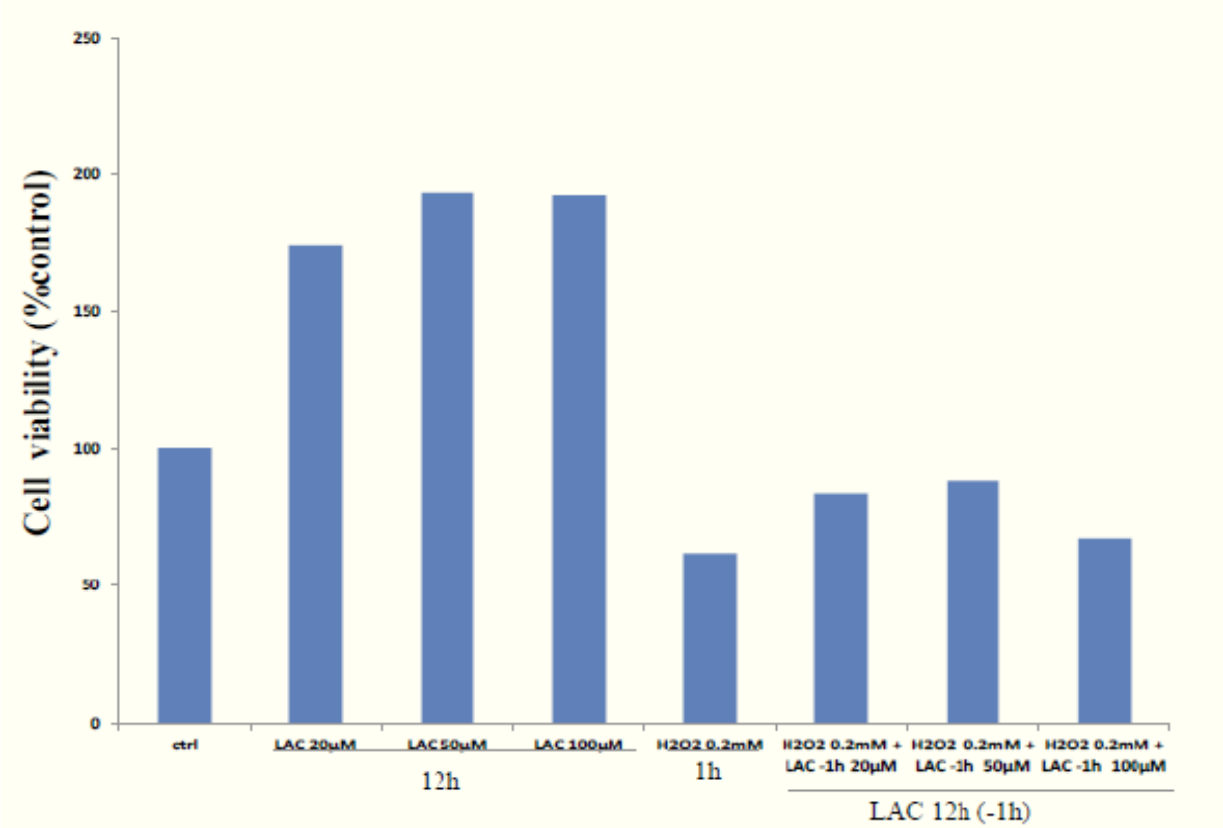

Fig. 6. Vitality cellular assay (MTT). Effect of HBB2 on cell viability after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 12 h. and only LAC (20µM, 50µM, 100µM) (12h).*P < 0.05 vs. control.

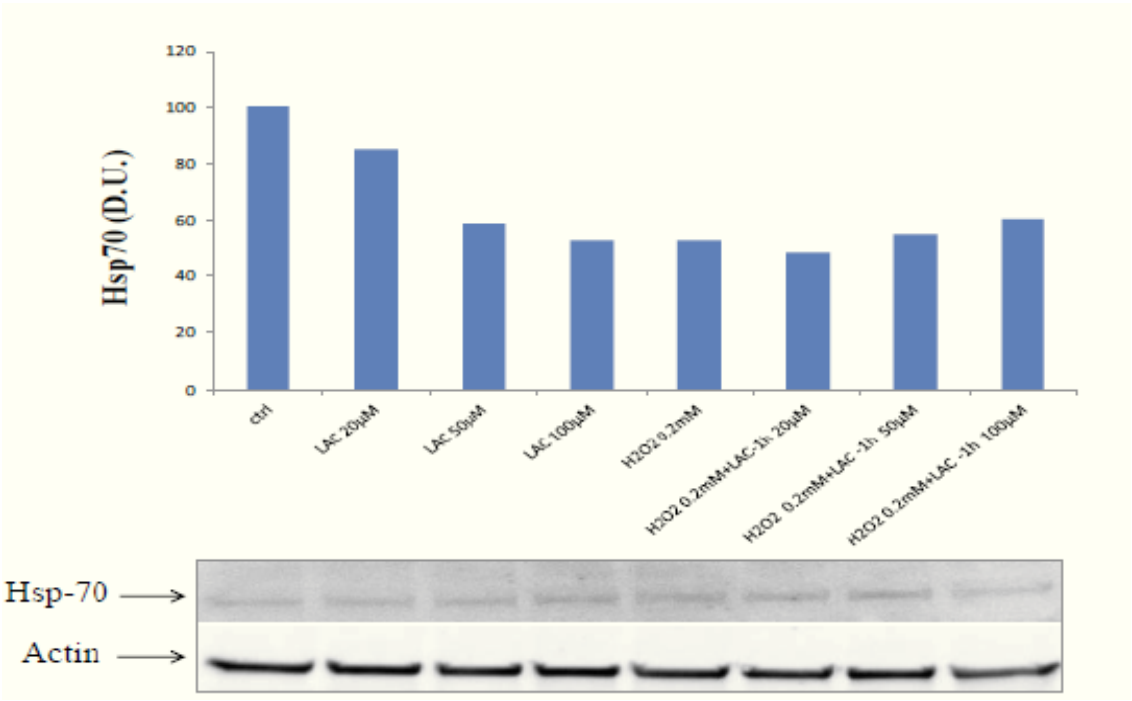

Fig. 7. Western blot assay Hsp-70 levels in SH-SY5Y cells after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 6 h. and only LAC (20µM, 50µM, 100µM) (6h)A representative immunoblot is shown in b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses. P ≤0.05 vs control. D.U., densitometric units; CTRL, control.

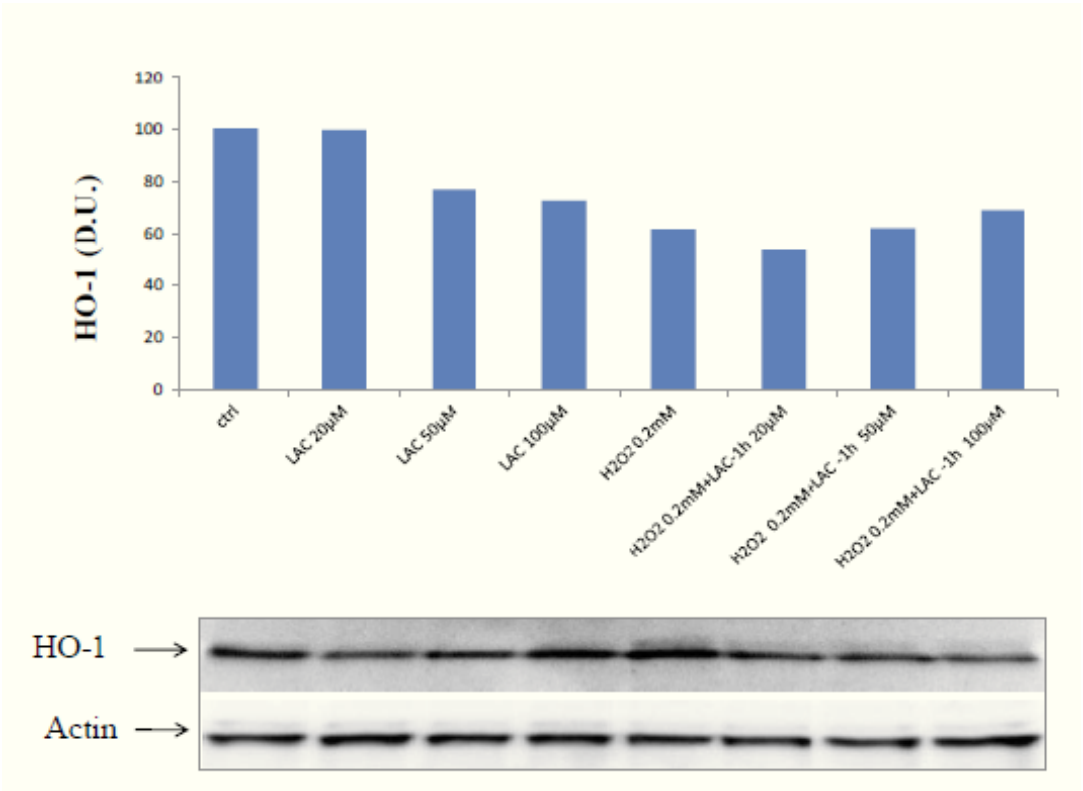

Fig. 8 Western blot assay HO-1 levels in SH-SY5Y cells after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 12 h. and only LAC (20µM, 50µM, 100µM) (12h). A representative immunoblot is shown in b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses group. P ≤0.05 vs control. D.U., densitometric units; CTRL, control.

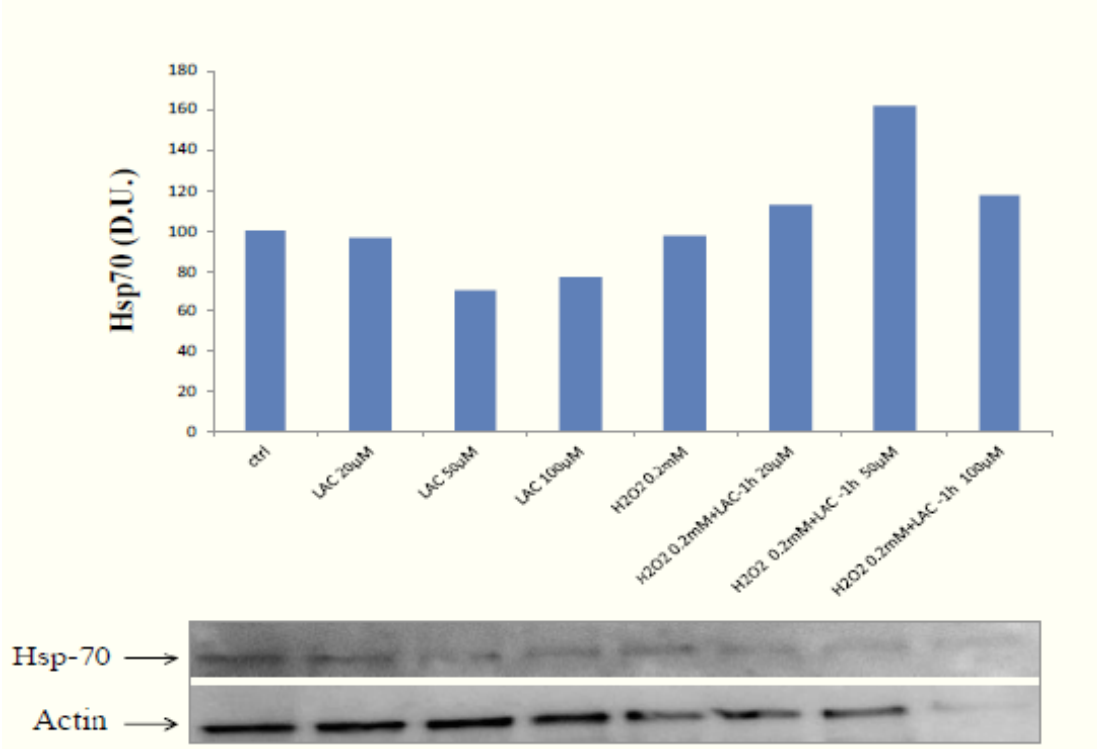

Fig. 9. Western blot assay Hsp-70 levels in SH-SY5Y cells after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 12 h. and only LAC (20µM, 50µM, 100µM) (12h)A representative immunoblot is shown in b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses . P ≤0.05 vs control. D.U., densitometric units; CTRL, control.

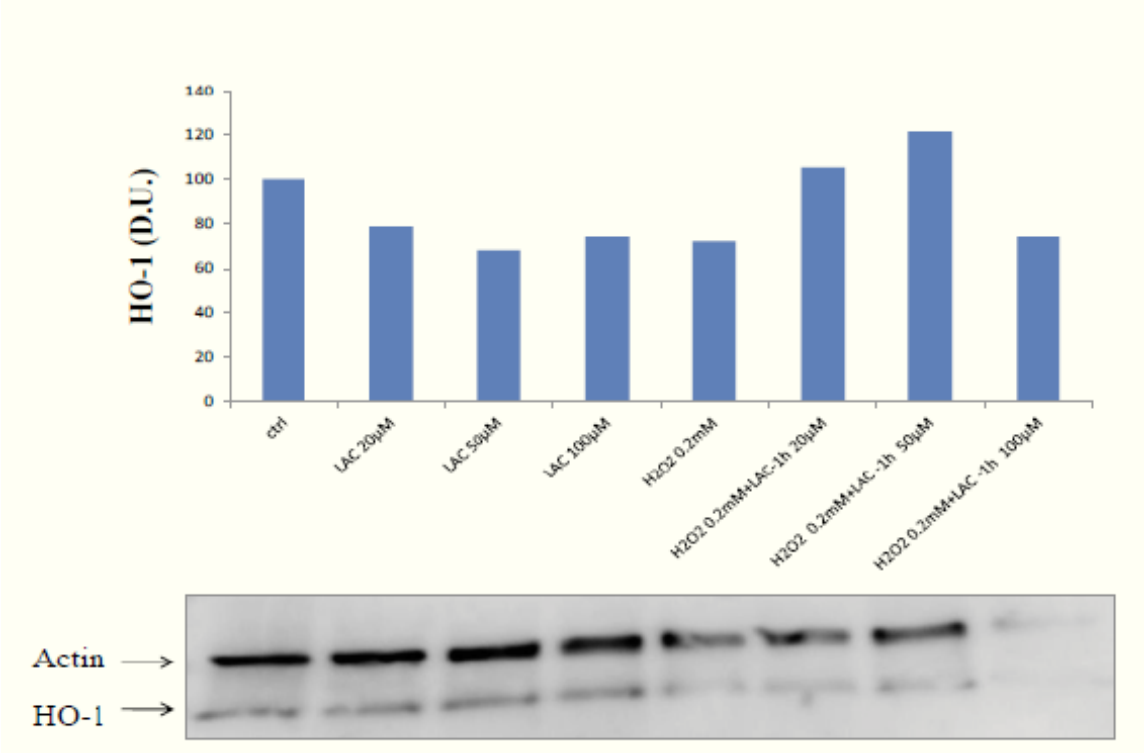

Fig. 10. Western blot assay. HO-1 levels in SH-SY5Y cells after oxidative damage induced by H2O2 (0,2mM) (-1h) treatment. SH-SY5Y were incubated with absent or present LAC (20µM, 50µM, 100µM) for 12 h. and only LAC (20µM, 50µM, 100µM) (12h)A representative immunoblot is shown in b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses. P ≤0.05 vs control. D.U., densitometric units; CTRL, control.

2. Oxidative stress and type II Diabetes

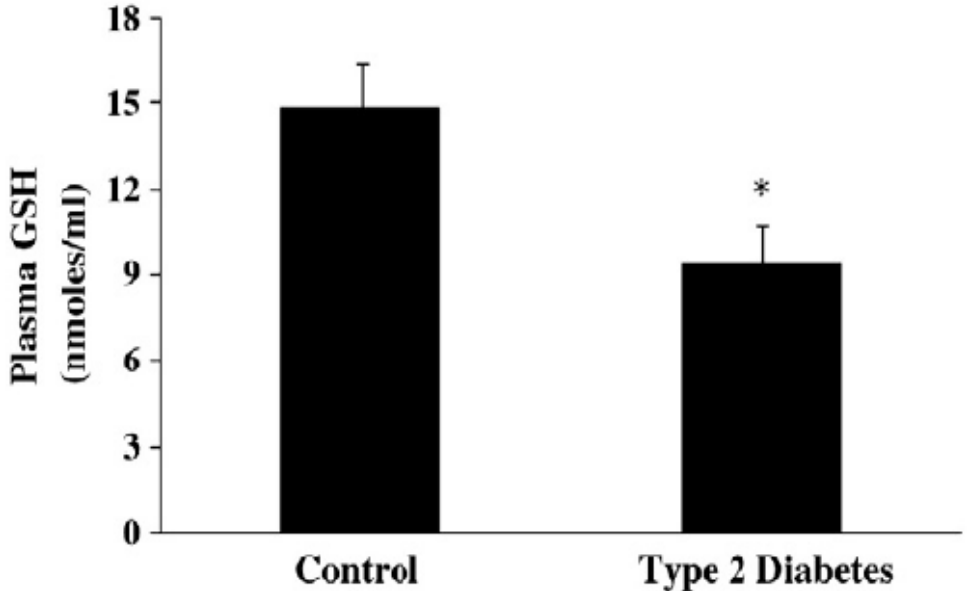

Fig. 11 Plasma levels of reduced (GSH) glutathione in type 2 Diabetic Patients and Controls. Plasma GSH and GSSG contents were assayed as described in Methods. Data are expressed asmean±SEMof 15 patients per group. *P<0.05 vs controls.

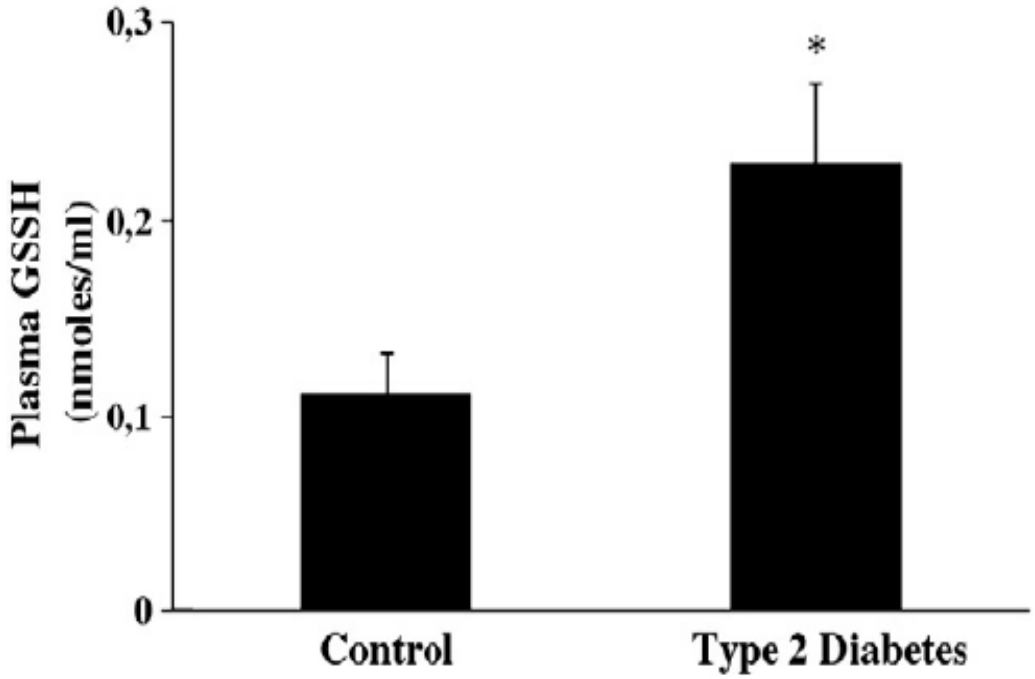

Fig. 12 Plasma levels of oxidized (GSSG) glutathione in type 2 Diabetic Patients and Controls. Plasma GSH and GSSG contents were assayed as described in Methods. Data are expressed asmean±SEMof 15 patients per group. *P<0.05 vs controls.

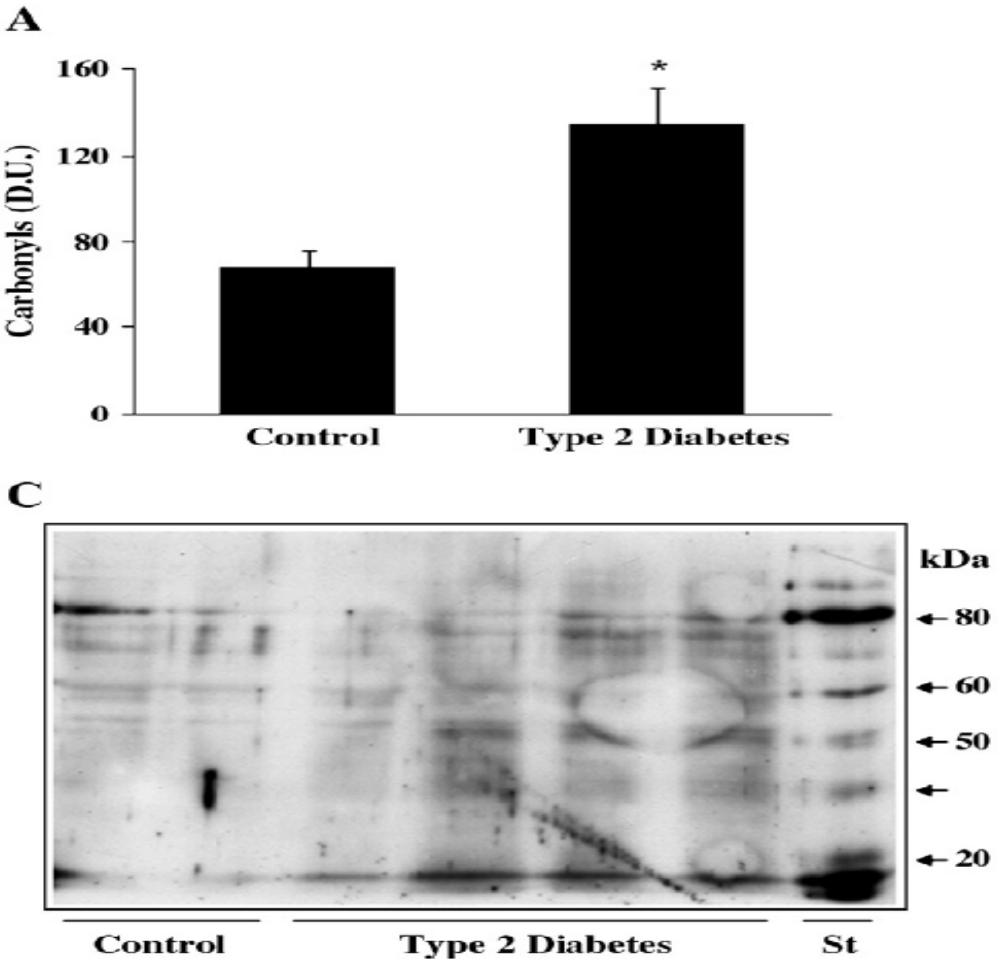

Fig.13 The western blot analysis shows a significant increase ($p < 0.05$) of the expression of proteins carbonyls (DNPH) in the plasma of patients with type 2 diabetes respect to control subjects. The (Fig. 2C) shows the densitometric values of the bands expressed in a group of 15 patients.

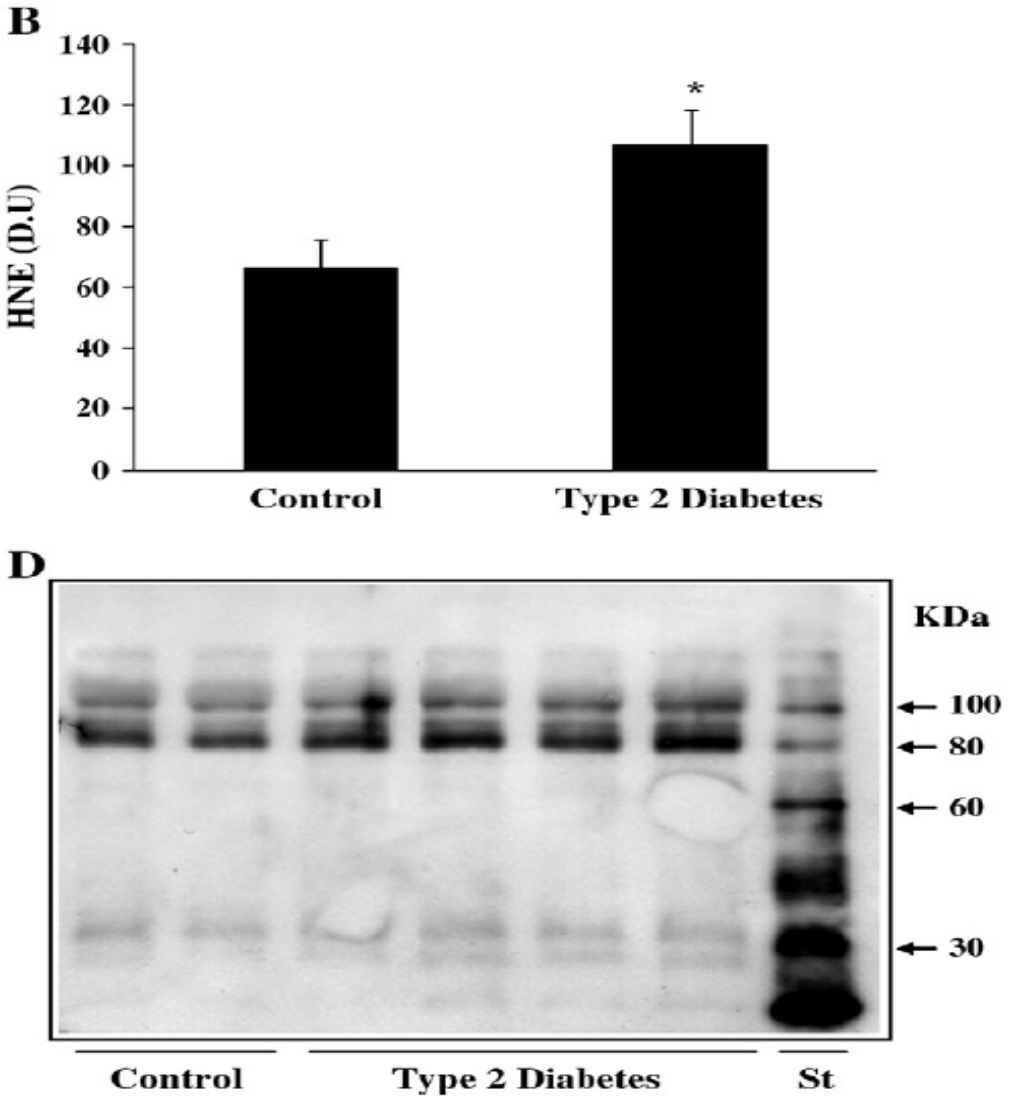

Fig. 14 Expression levels of 4-hydroxynonenal (HNE), evaluated by western blot analysis are significantly increased ($p < 0.05$) in the plasma of patients with type 2 diabetes compared with controls. The Fig. 2D shows the densitometric values of the bands expressed in a group of 15 patients**.**

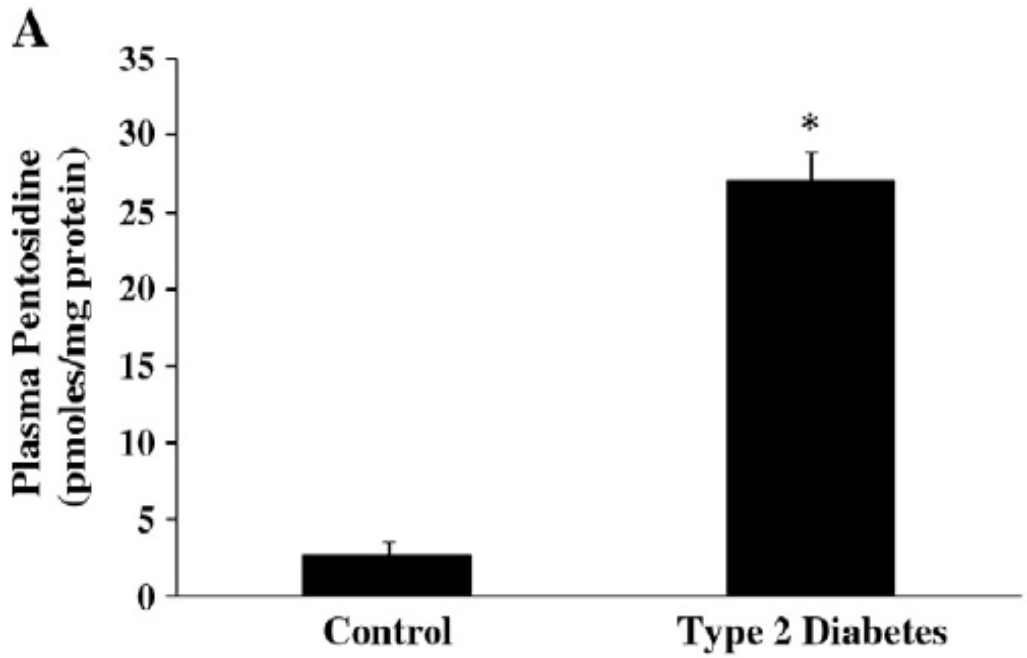

Fig. 15 Pentosidine levels in plasma from type 2 diabetic patients.
Plasma samples frompatientswith type 2 diabetes and age-matched controls were assayed for pentosidine (Fig. 3A) as indicated in Methods. Data are expressed as mean±SEM of 15 patients per group. *P<0.01 vs controls

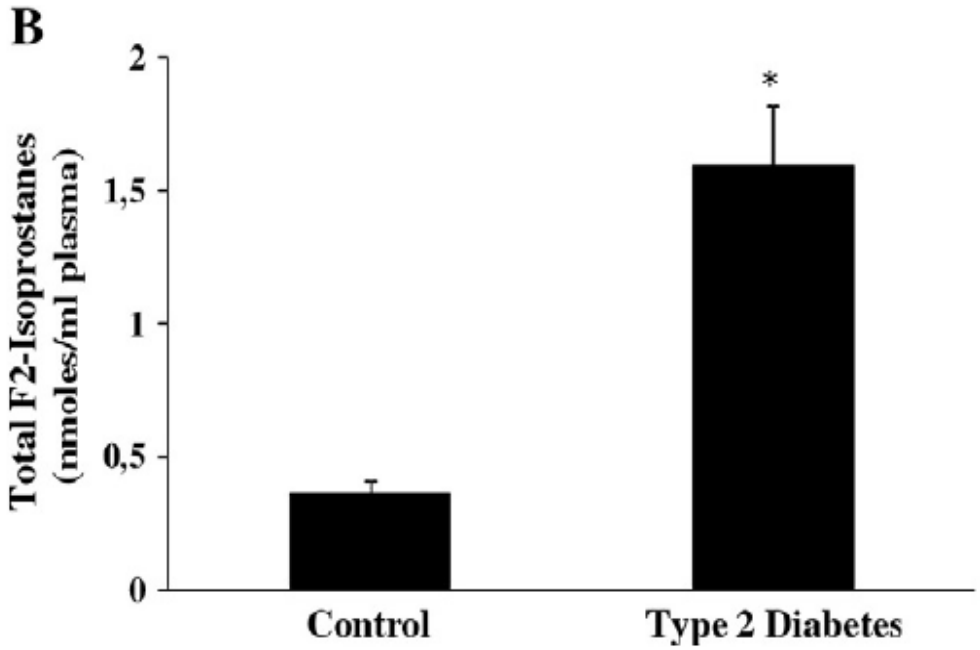

Fig. 16 Total F2-isoprostanes levels in plasma from type 2 diabetic patients. Plasma samples frompatientswith type 2 diabetes and age-matched controls were assayed for pentosidine (Fig. 3B) as indicated in Methods. Data are expressed as mean±SEM of 15 patients per group. *P<0.01 vs controls

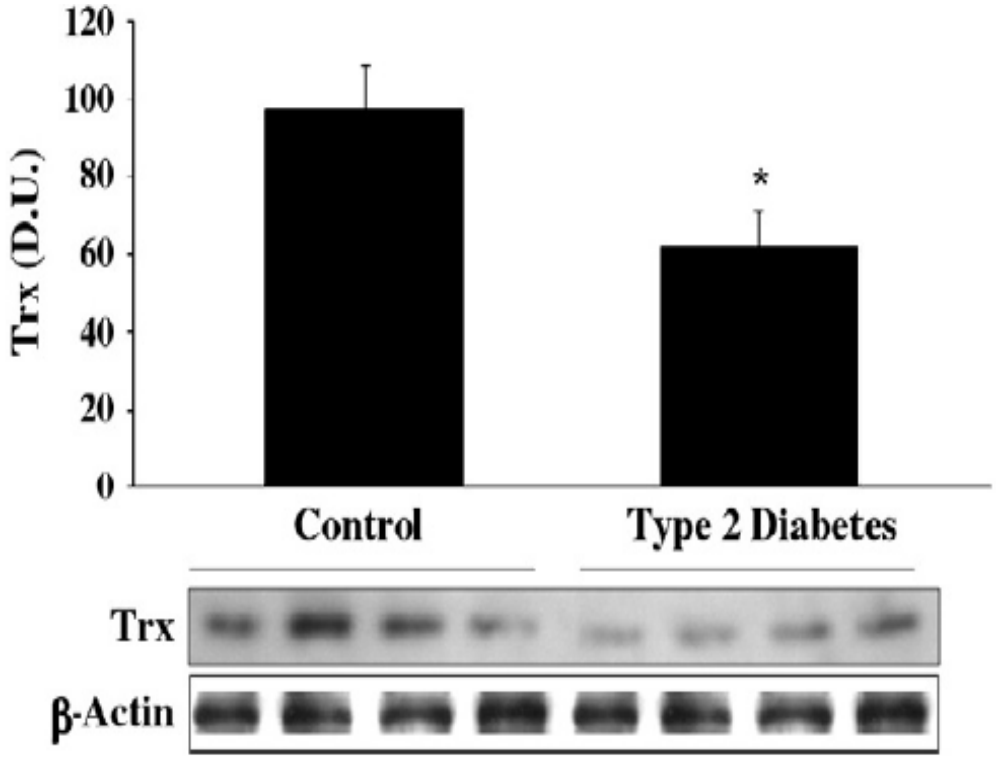

Fig. 17 Western blot analysis shows a significant decrease ($p < 0.05$), of thioredoxin protein (Trx) expression in plasma of patients with nephropathy secondary to type 2 diabetes compared with controls. The immunoblot also shows the β-actin used as a loading control.

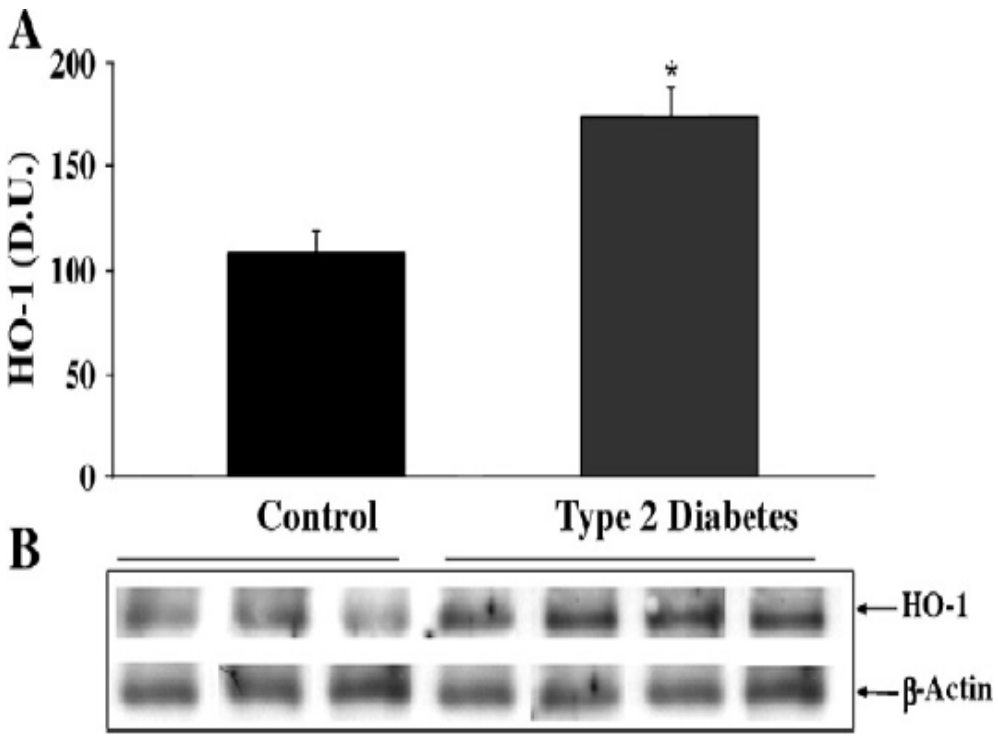

Fig. 18 Heme oxygenase-1 levels in lymphocytes from type 2 diabetic patients. Lymphocyte samples frompatientswith nephropathy secondary to type 2 diabetes and age-matched controlswere assayed for heme oxygenase-1 (HO-1) by western blot as described in Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed asmean±SEM of independent analyses on 15 patients per group. *P<0.05 vs control. D.U., densitometric units.

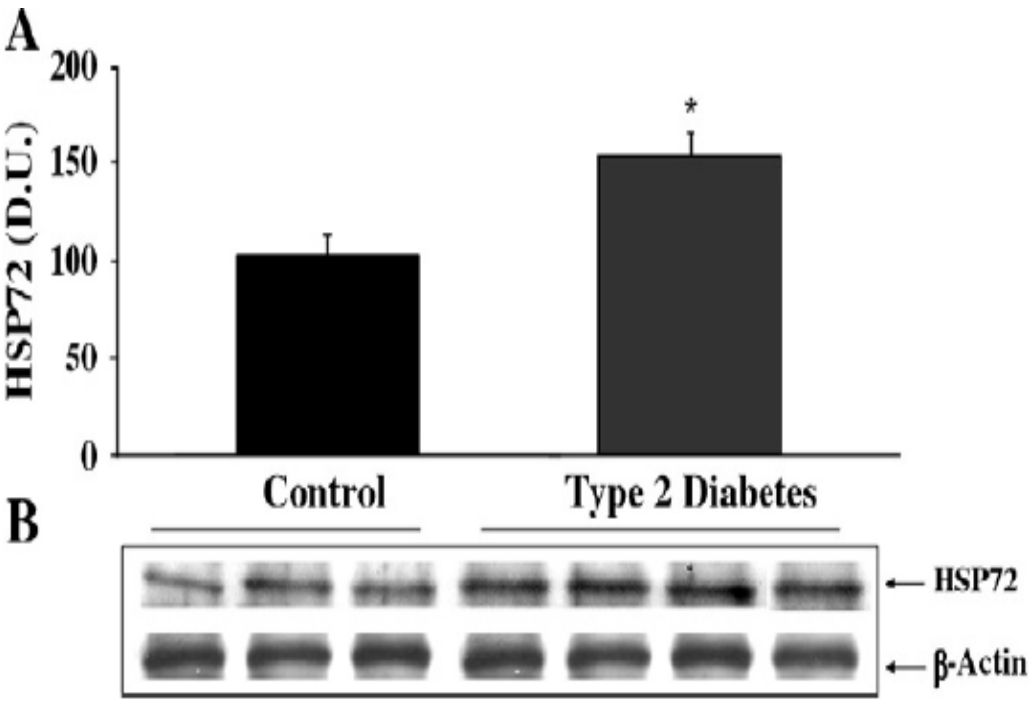

Fig. 19 Heat shock protein 70 levels in lymphocytes from type 2 diabetic patients. Lymphocyte samples from patients with nephropathy secondary to type 2 diabetes and age-matched controls were assayed for heat shock protein 70 (Hsp70) by western blot as described in Materials and Methods. A representative immunoblot is shown. β-actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean±SEM of independent analyses on 15 patients per group. *$P<0.05$ vs control. D.U., densitometric units.

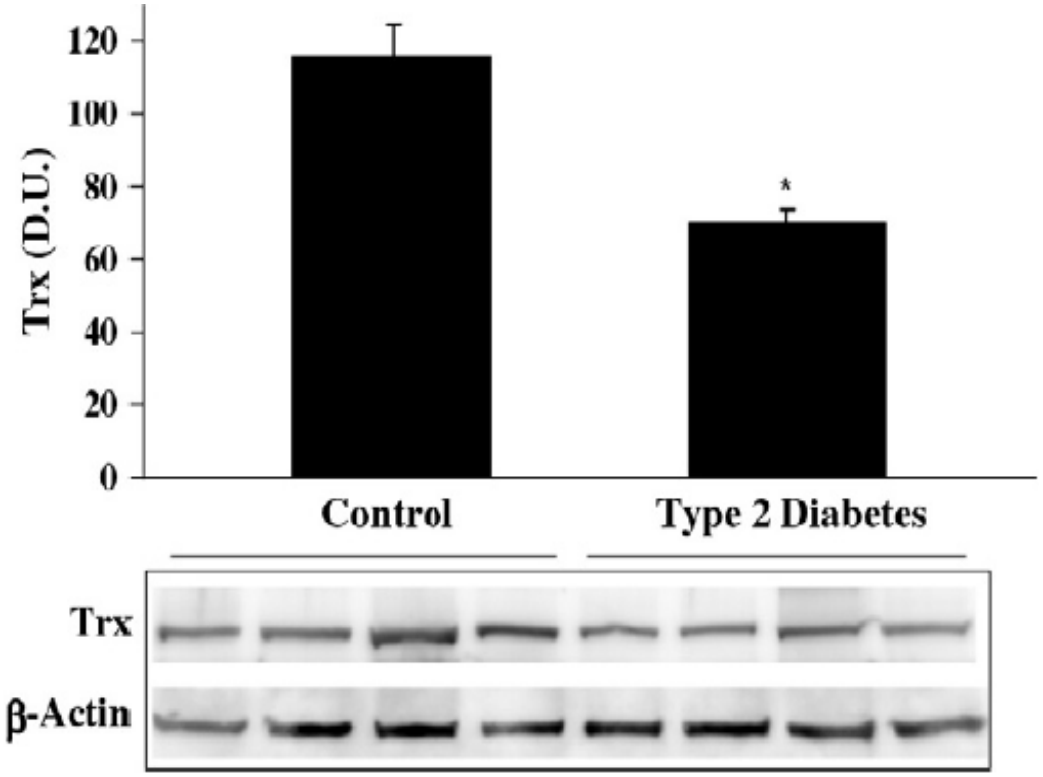

Fig. 20 The Western blot analysis shows a significant decrease ($p < 0.05$) of expression levels of thioredoxin protein (Trx) in the lymphocytes of patients with nephropathy secondary to type 2 diabetes compared to controls. The immunoblot also shows the β-actin used as a loading control.

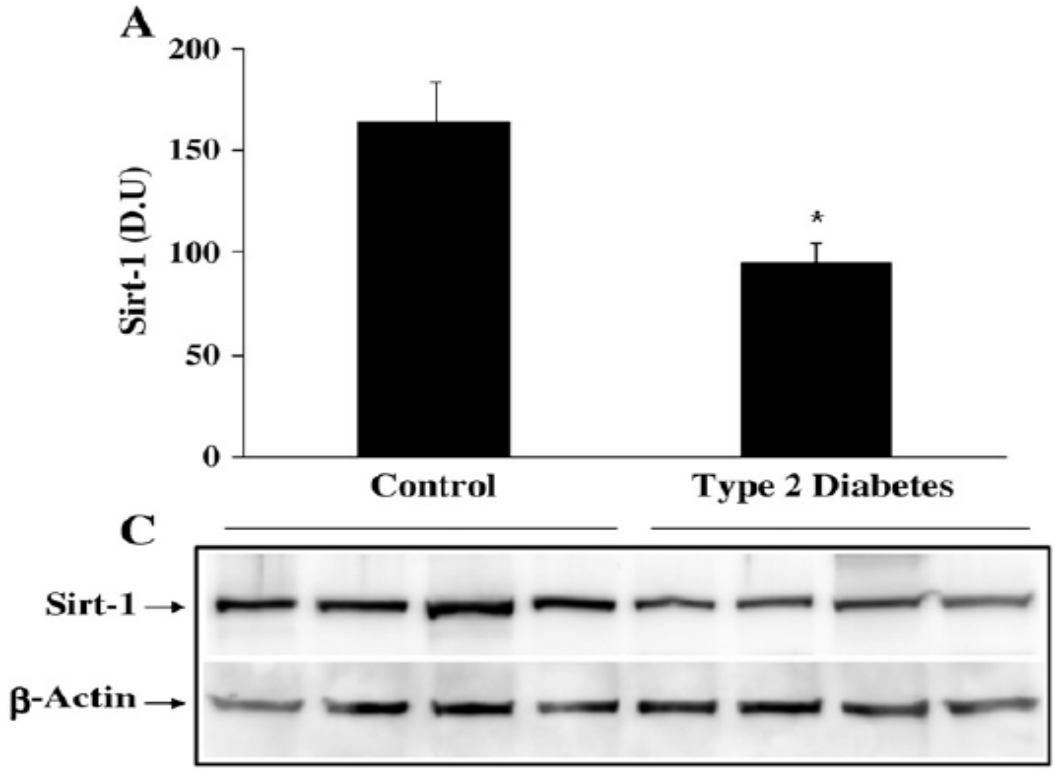

Fig. 21. The Western blot analysis shows a significant decrease ($p < 0.05$) in the expression levels of Sirtuin-1 protein (SIRT-1) in the lymphocytes of patients with type 2 diabetes compared to controls. The immunoblot shows respectively (Fig. 8C) the β-actin used as a loading control.

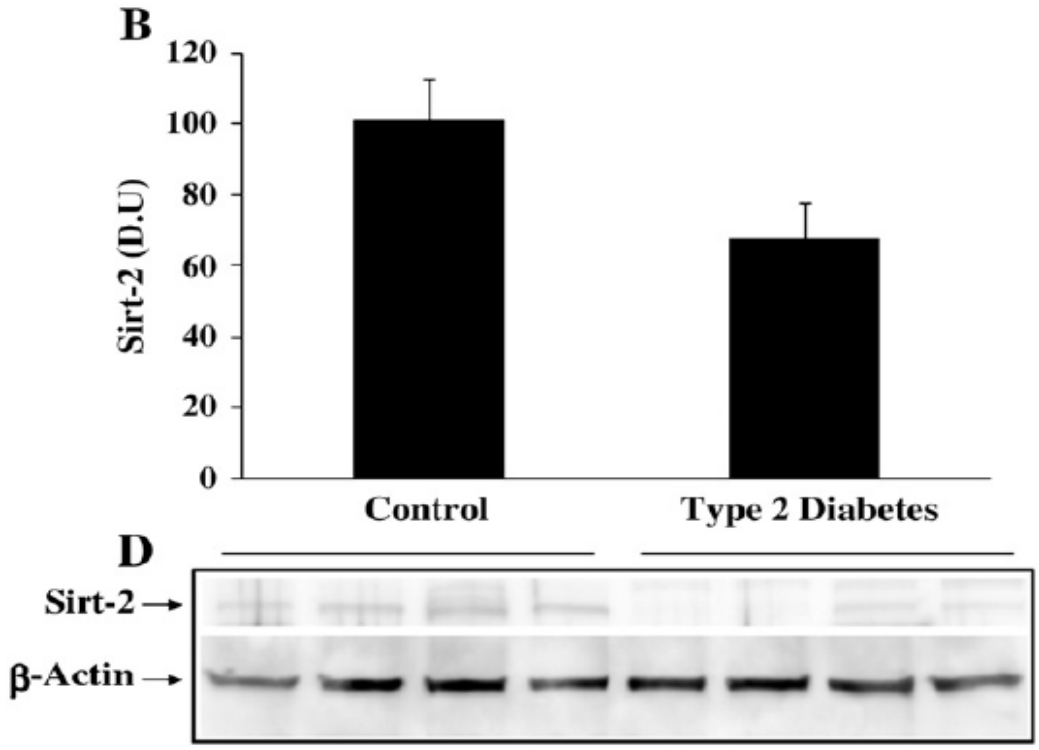

Fig. 22. The Western blot analysis shows a significant decrease ($p < 0.05$) levels of expression of Sirtuin-2 protein (SIRT-2) in the lymphocytes of patients with type 2 diabetes compared to controls. The immunoblot shows respectively (Fig. 8D) the β-actin used as a loading control.

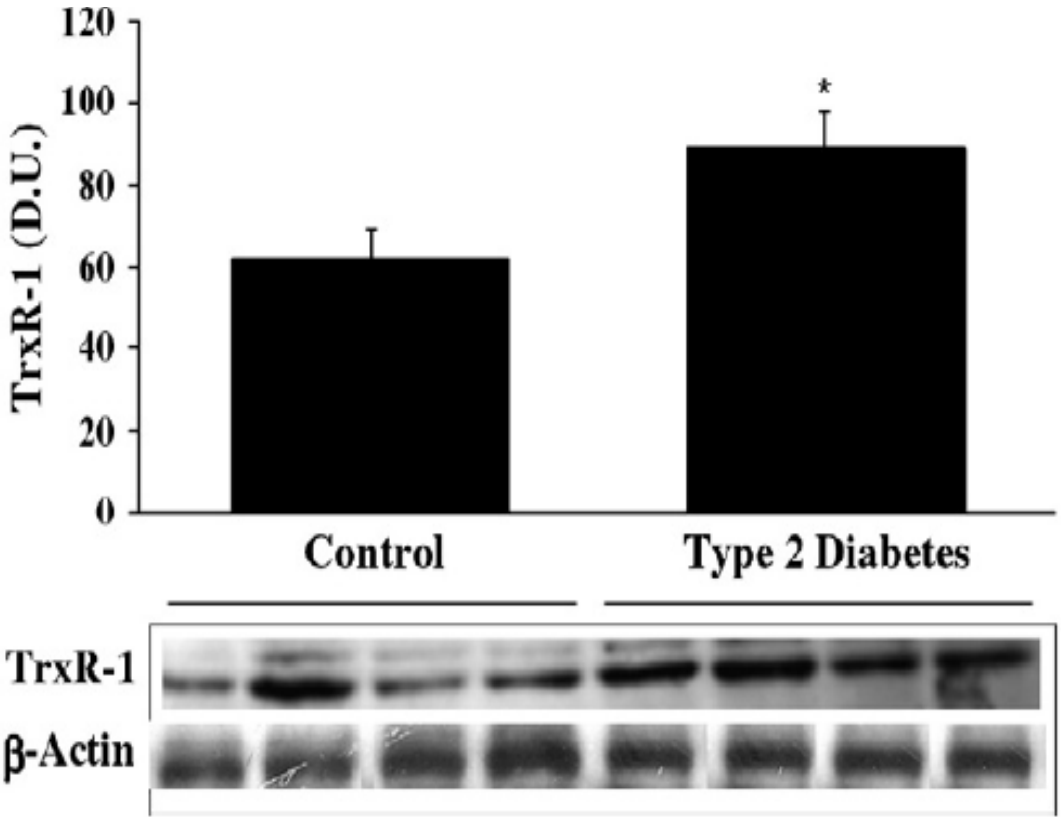

Fig. 23. The Western blot analysis shows a significant increase ($p < 0.05$) levels of expression of thioredoxin reductase-1 (TrxR-1) in the lymphocytes of patients with nephropathy secondary to type 2 diabetes compared to controls. The immunoblot also shows the β-actin used as a loading control.

3. Diabetic nephropathy: effects of carnosine and cellular stress response in podocyte cells

Fig. 1

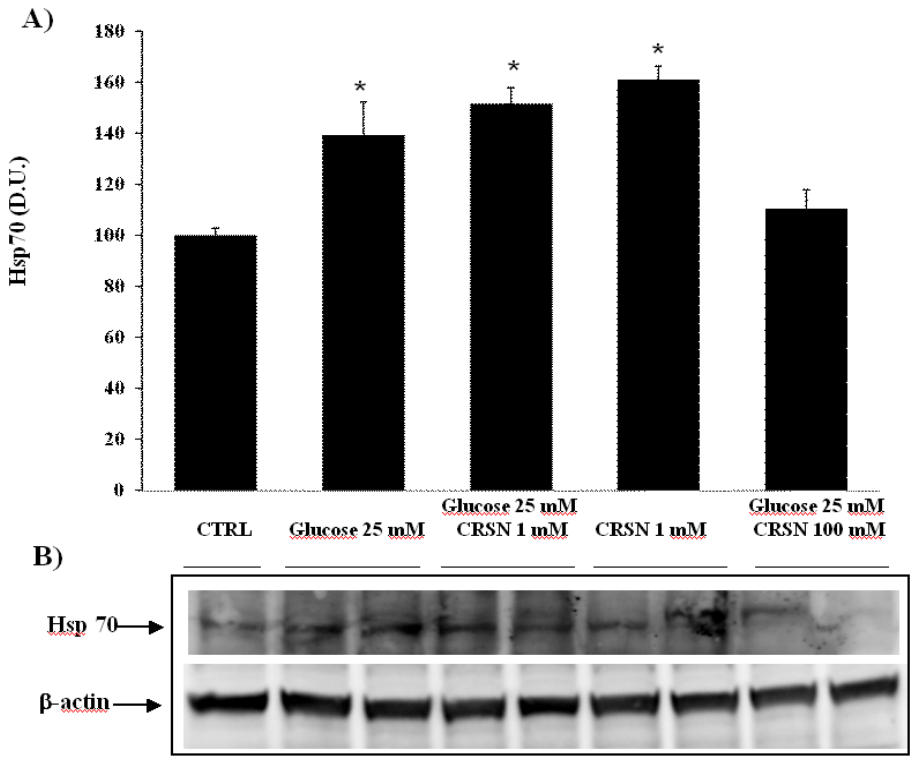

Fig 24. Samples from control and Podocytes treated with Glucose 25 mM, Glucose 25 mM and CRSN 1 mM, CRSN 1 mM and Glucose 25 mM and CRSN 100 mM were assayed for Hsp 70 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; CTRL, control. CRSN, carnosine.

Fig. 2

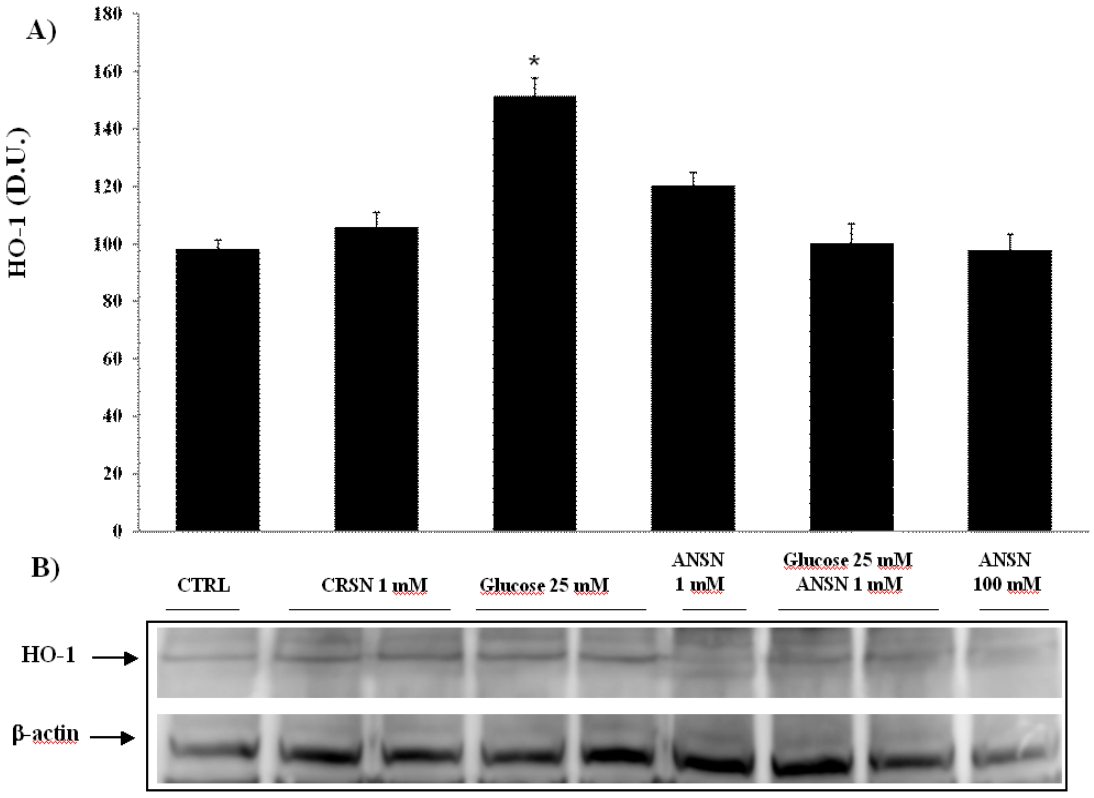

Fig. 25 Samples from control and Podocytes treated with CRSN 1 mM, Glucose 25 mM, ANSN 1 mM, Glucose 25 mM and ANSN 1 mM, ANSN 100 mM were assayed for HO-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; CTRL, control. CRSN, carnosine, ANSN, anserine.

Fig. 3

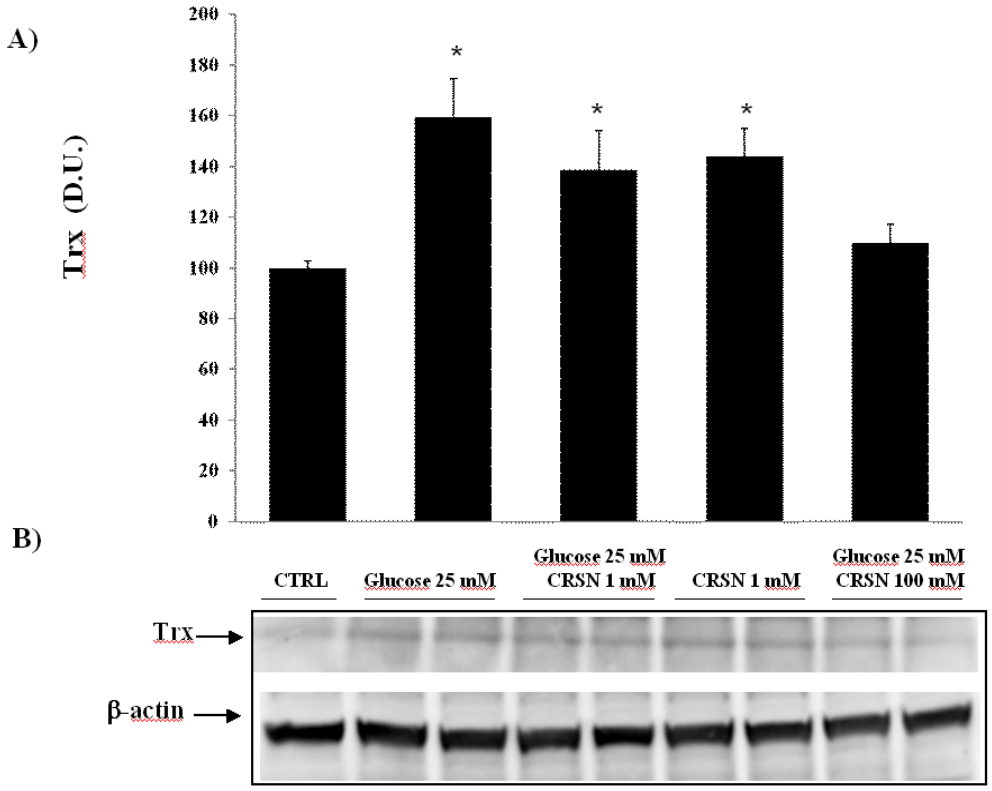

Fig. 26 Samples from control and Podocytes treated with Glucose 25 mM, Glucose 25 mM and CRSN 1 mM, CRSN 1 mM and Glucose 25 mM and CRSN 100 mM were assayed for TRX expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; CTRL, control. CRSN, carnosine.

Fig. 4

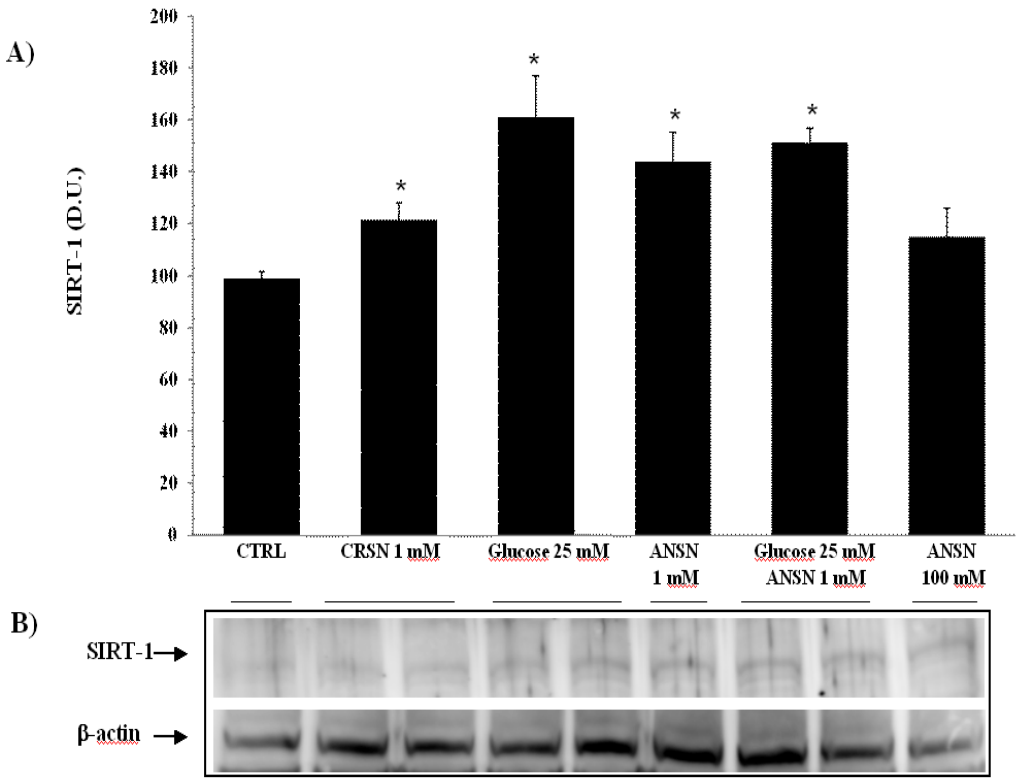

Fig. 27 Samples from control and Podocytes treated with CRSN 1 mM, Glucose 25 mM, ANSN 1 mM, Glucose 25 mM and ANSN 1 mM, ANSN 100 mM were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; CTRL, control. CRSN, carnosine, ANSN, anserine.

Fig. 5

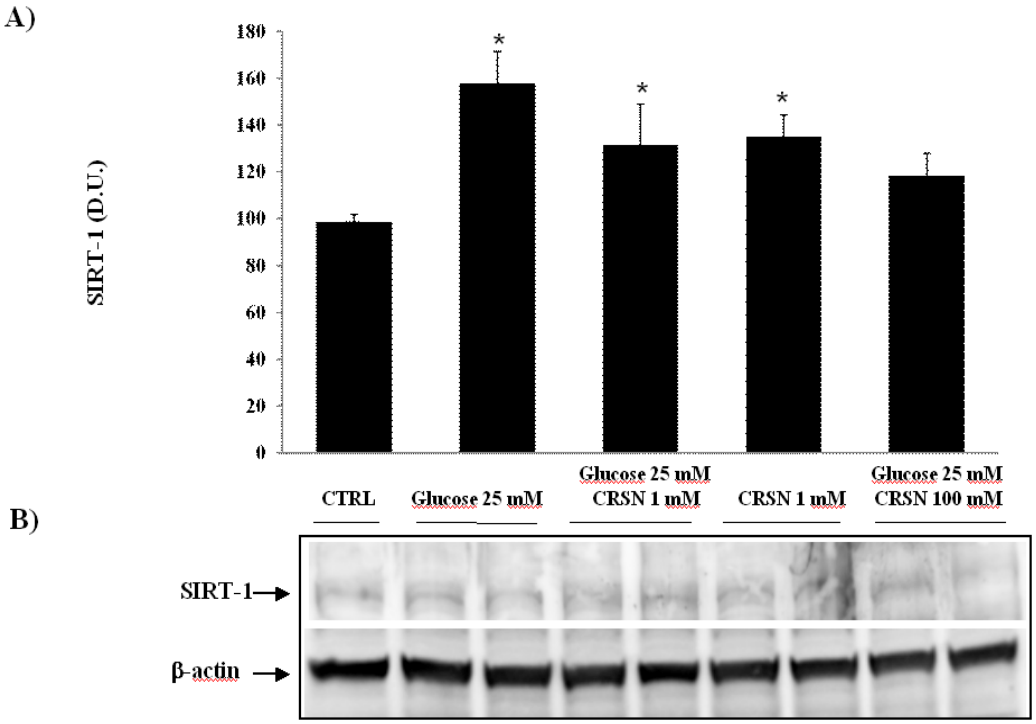

Fig. 28 Samples from control and Podocytes treated with Glucose 25 mM, Glucose 25 mM and CRSN 1 mM, CRSN 1 mM and Glucose 25 mM and CRSN 100 mM were assayed for Sirtuin 1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; CTRL, control. CRSN, carnosine.

Fig. 6

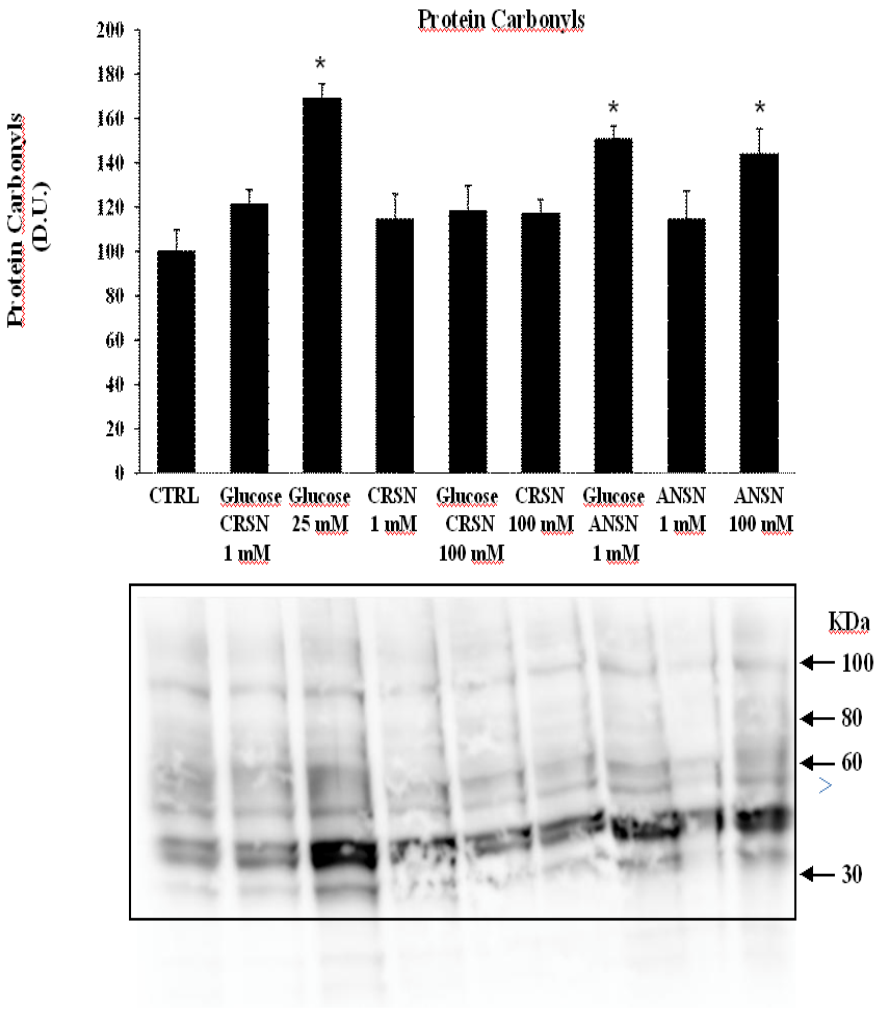

Fig. 29 Protein carbonyls levels in control and Podocytes. The samples were assayed for carbonyls by Western blot, as described in Materials and Methods. A representative immunoblot is shown. A representative immunoblot is shown. Samples from control and Podocytes treated with Glucose 25 mM and CRSN 1 mM, Glucose 25 mM, CRSN 1 mM, Glucose 25 mM and CRSN 100 mM, CRSN 100 mM, Glucose 25 mM and ANSN 1 mM, ANSN 1 mM, ANSN 100 mM were assayed for protein carbonyls expression by Western blot. Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. D.U., densitometric units; CTRL, control. CRSN, carnosine, ANSN, anserine.

Fig. 7

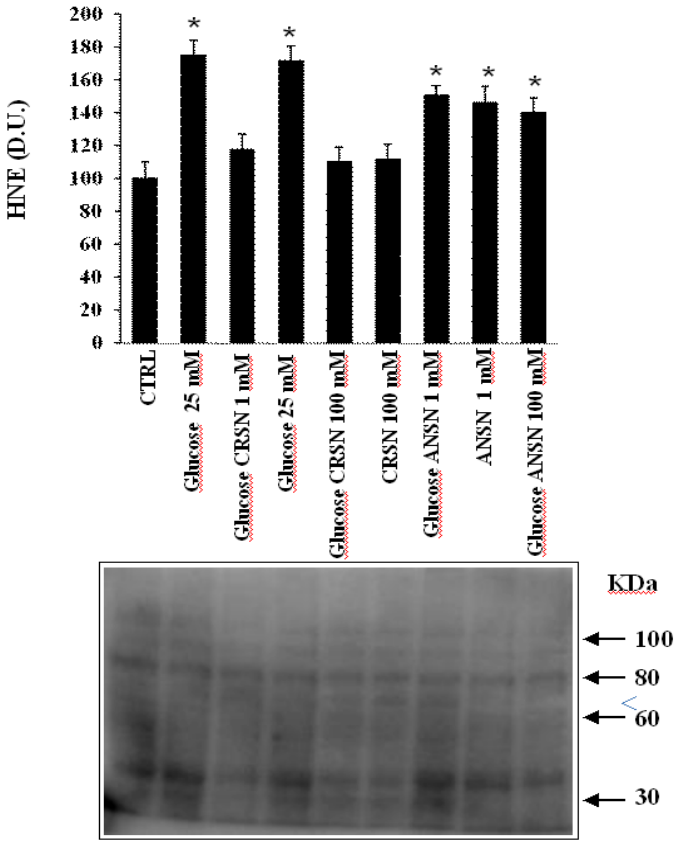

Fig. 30 4-hydroxy-2-nonenals levels in control and Podocytes. The samples were assayed for 4-hydroxy-2-nonenals by Western blot, as described in Materials and Methods. A representative immunoblot is shown. Samples from control and Podocytes treated with Glucose 25 mM, Glucose 25 mM and CRSN 1 mM, Glucose 25 mM and CRSN 100 mM, CRSN 100 mM, Glucose 25 mM and ANSN 1 mM, ANSN 1 mM, Glucose 25 mM and ANSN 100 mM were assayed for HNE expression by Western blot. Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. D.U., densitometric units; CTRL, control. CRSN, carnosine, ANSN, anserine

4. Cellular stress response, sirtuins and UCP proteins in Alzheimer disease: role of vitagenes

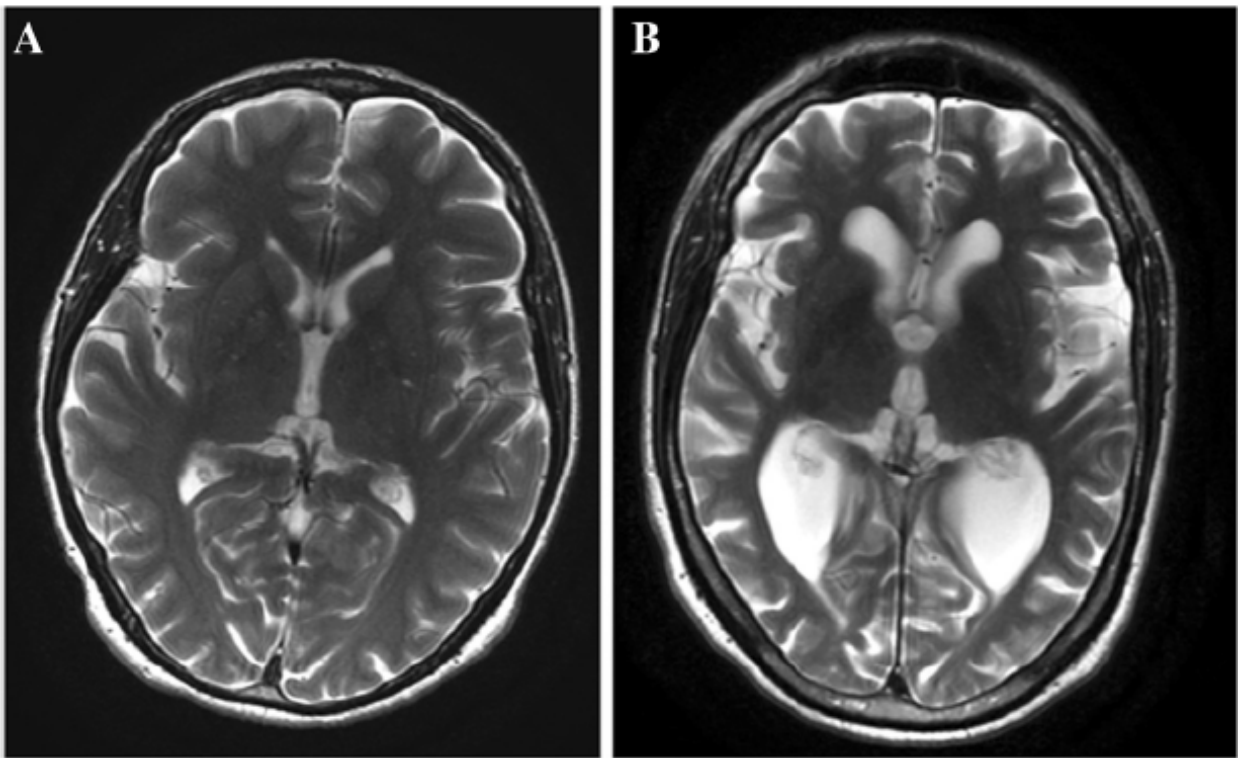

Fig. 31 brain MRI axial T2 image shows cerebral atrophy in patient with Alzheimer's disease **(A)** and normal brain in control patients of same age **(B)**.

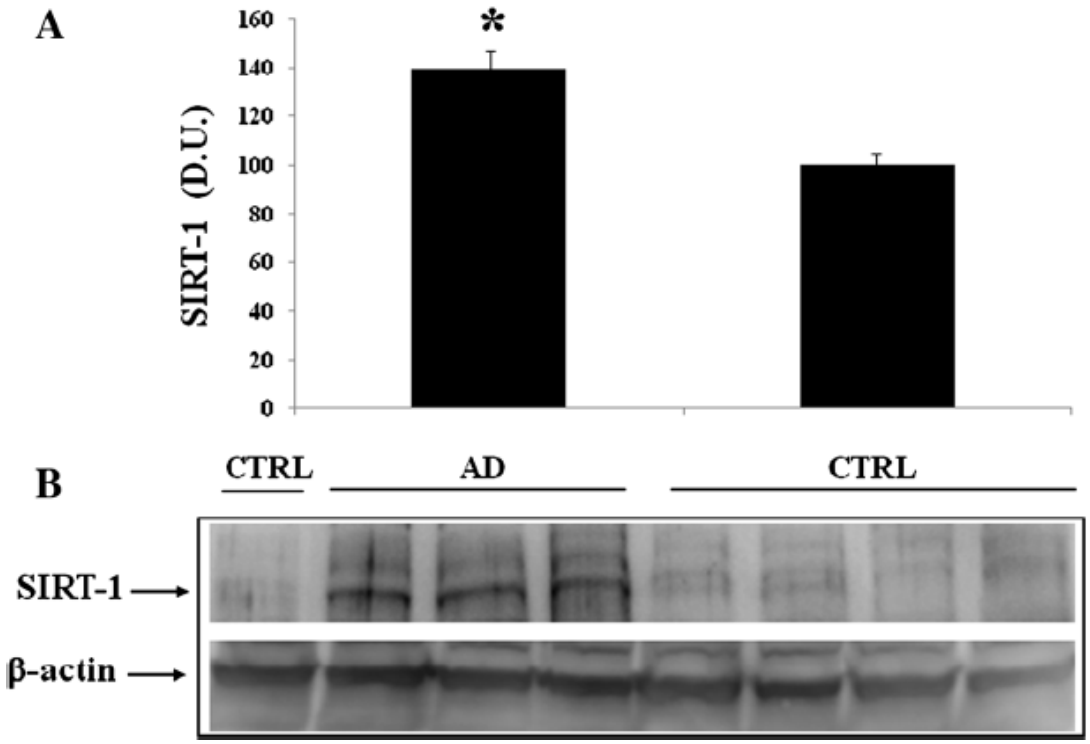

Fig. 32 Sirtuin-1 (Sirt-1) protein expression levels in lymphocytes of AD and control subjects were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

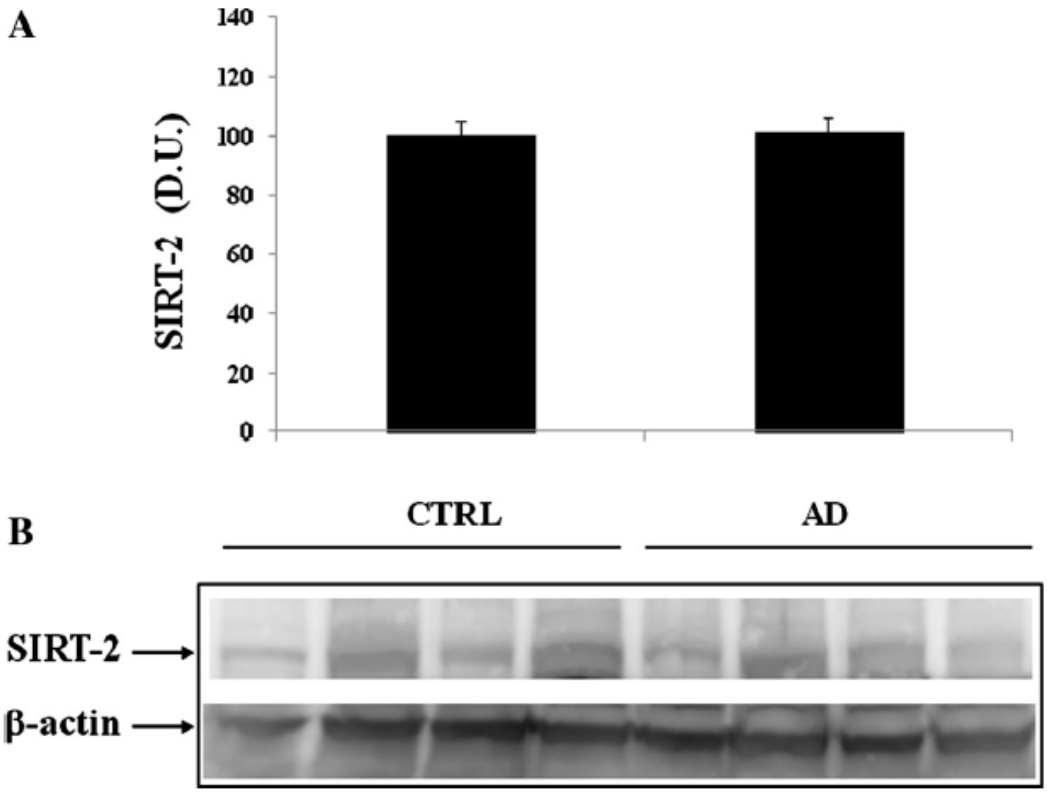

Fig. 33 Sirtuin-2 (Sirt-2) protein expression levels in lymphocytes of AD and control subjects were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

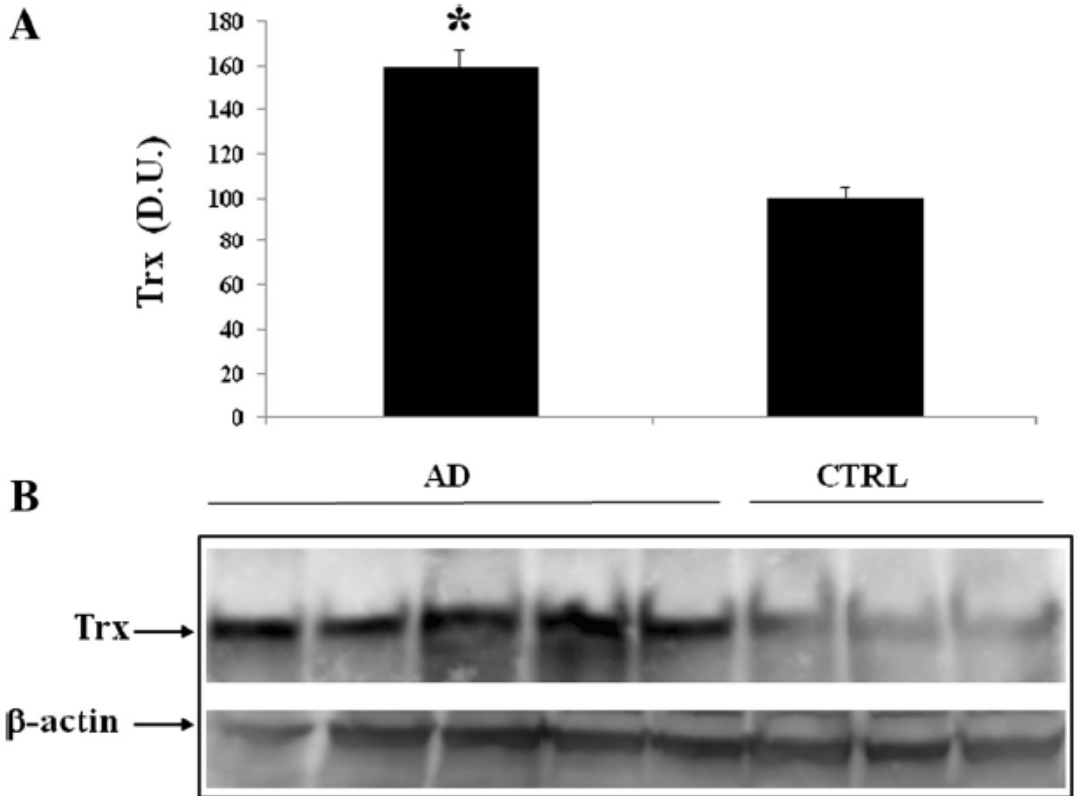

Fig. 34 Thioredoxin (Trx) protein levels in lymphocytes of AD and control subjects were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

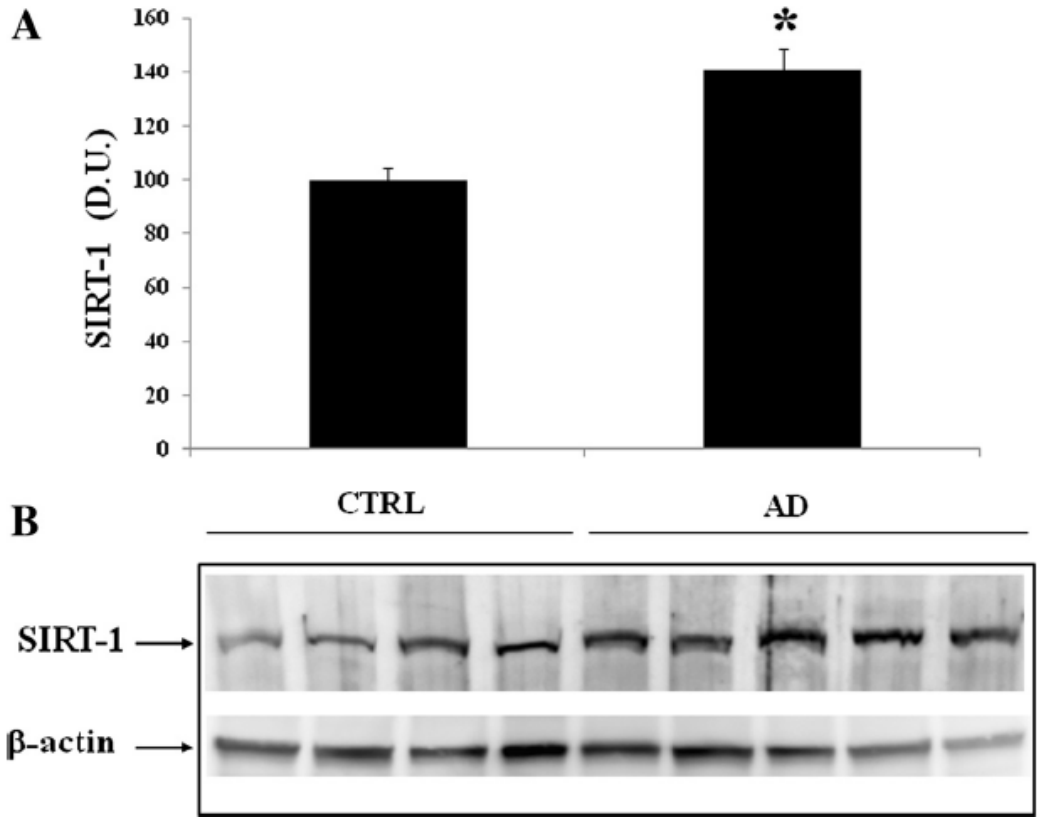

Fig. 35 Plasma levels of Sirtuin-1 (Sirt-1) in AD and control individuals. Samples from control and AD subjects were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

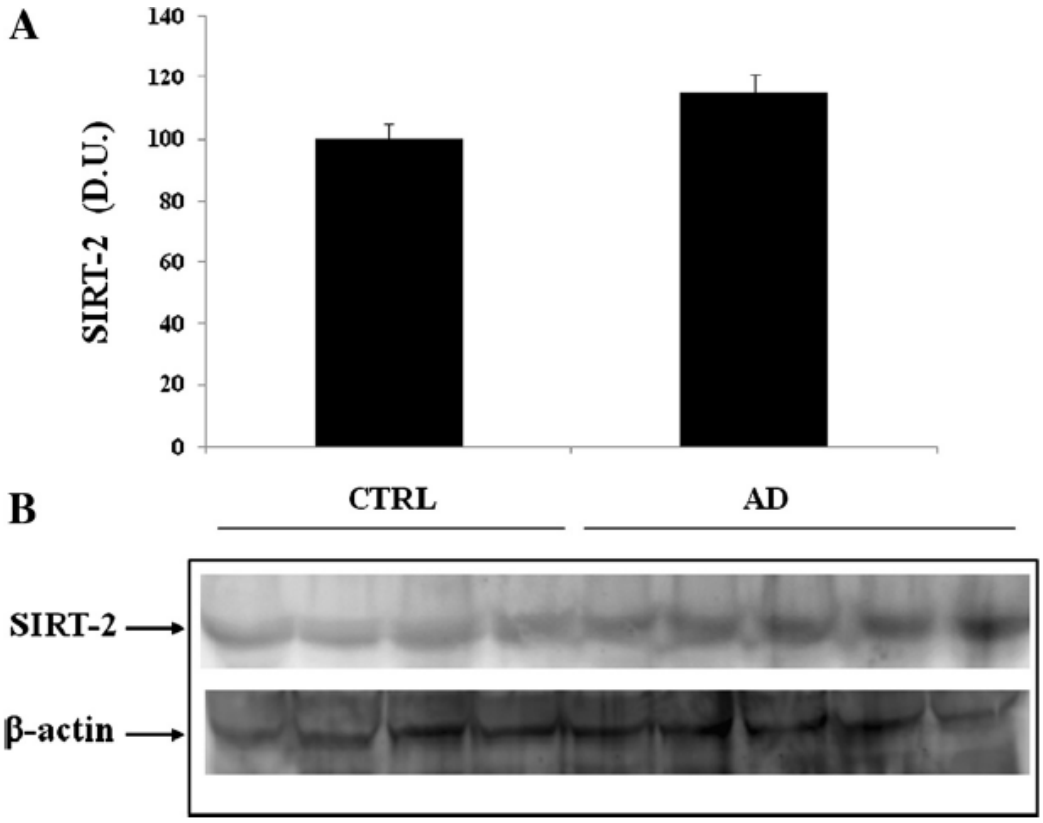

Fig. 36 Plasma expression levels of Sirtuin-2 (Sirt-2) in AD and control individuals were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

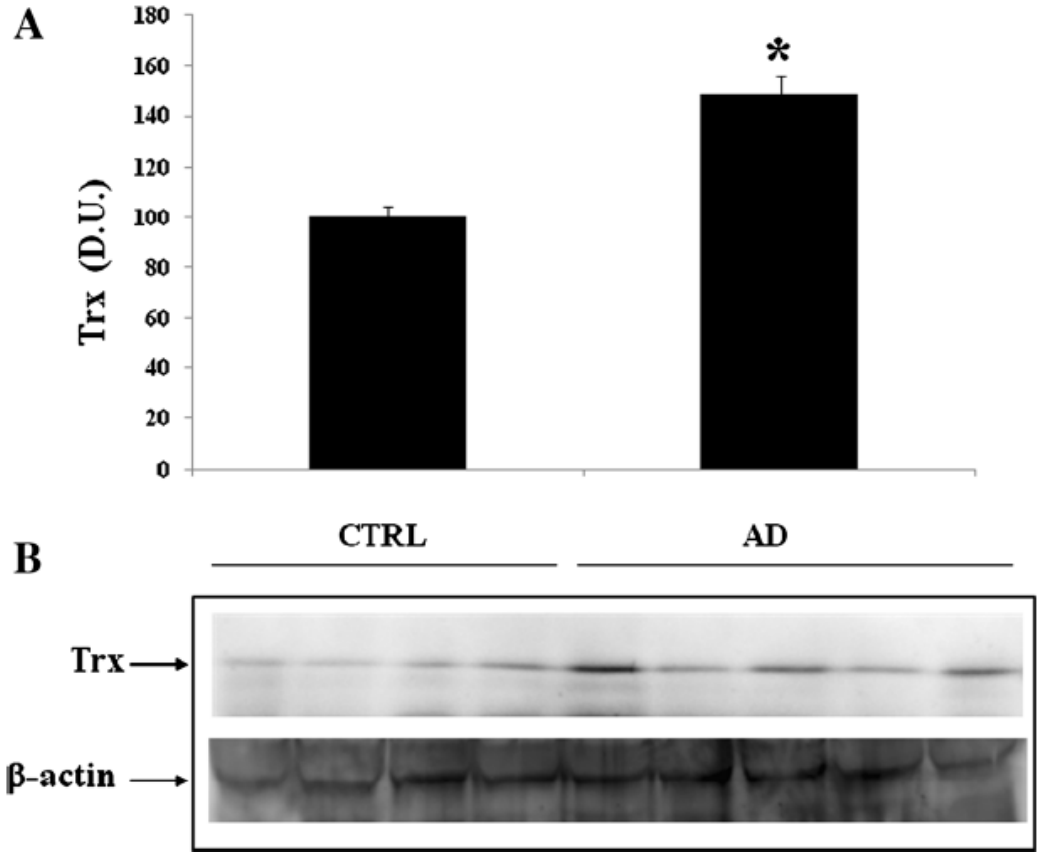

Fig. 37 Plasma expression levels of Thioredoxin (Trx) in AD and control subjects were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

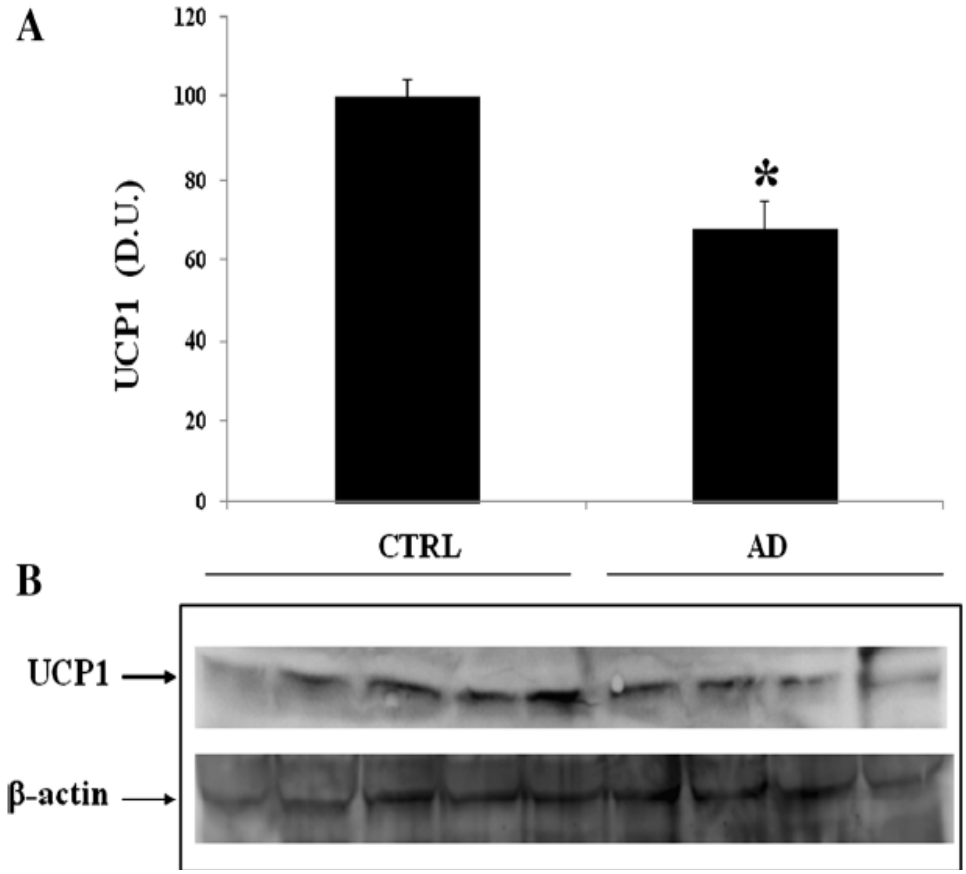

Fig. 38 Uncoupling proteins 1 (UCP1) expression levels in the plasma of AD and control individuals were assayed by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses. $P \leq 0.05$ vs control. B) A representative immunoblot is shown. β-actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

5. Oxidative stress and cellular stress response in glaucoma: implications with Alzheimer's disease

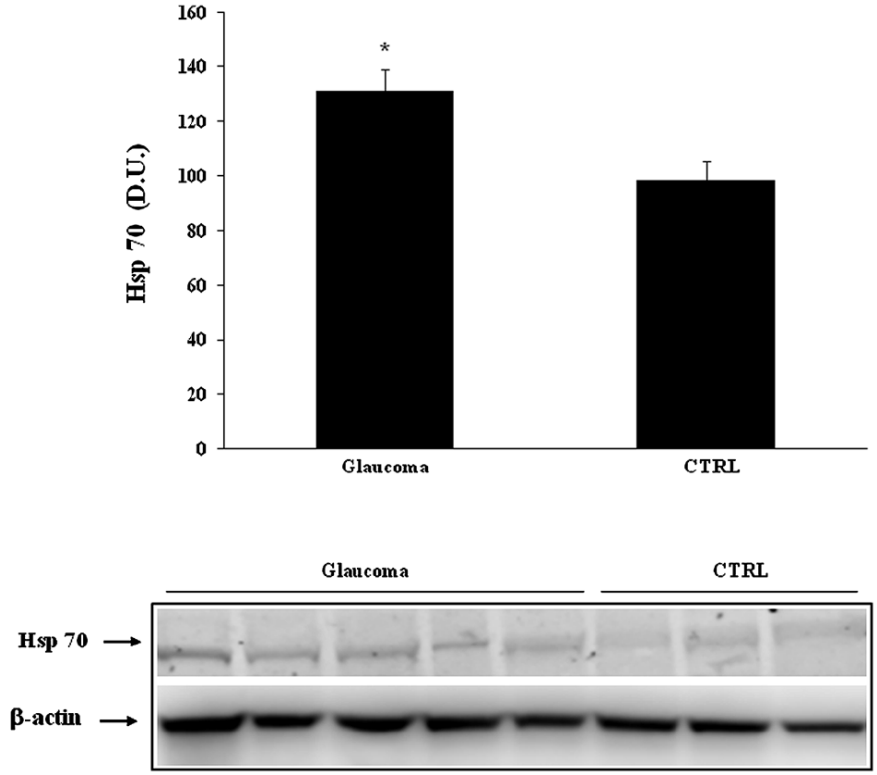

Fig. 39 HSP-70 protein levels in lymphocytes of glaucoma and control subjects. Samples from control and glaucoma patients were assayed for HSP-70 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U.,densitometric units; CTRL, control.

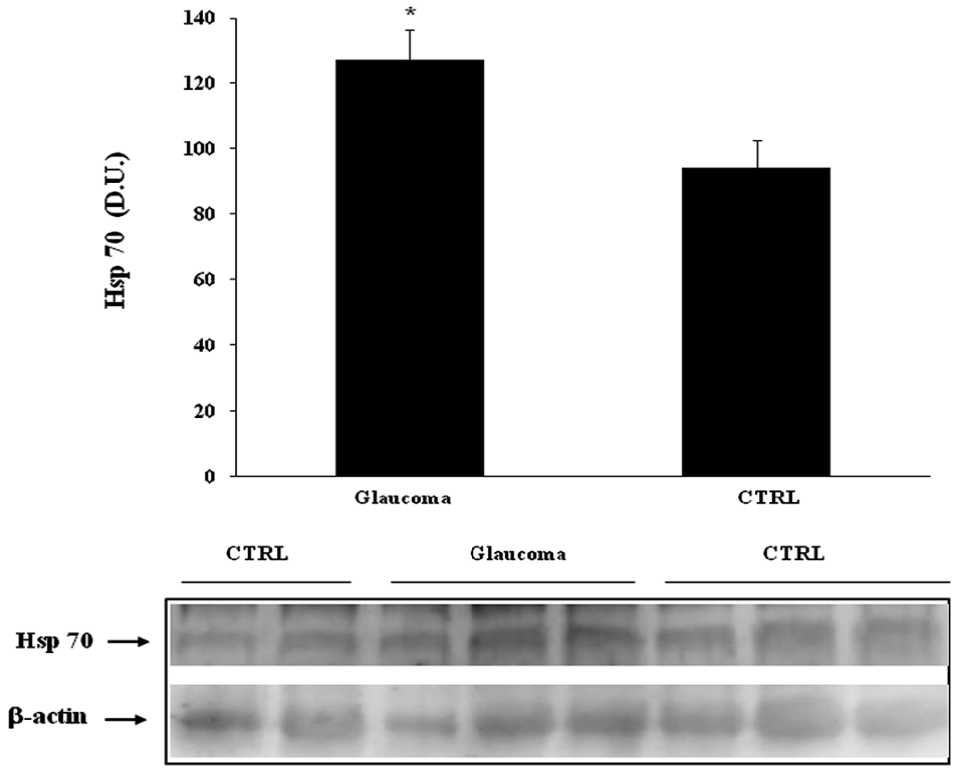

Fig. 40 HSP-70 protein levels in plasma of glaucoma and control subjects. Samples from controls and glaucoma patients were assayed for HSP-70 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U.,densitometric units; CTRL, control.

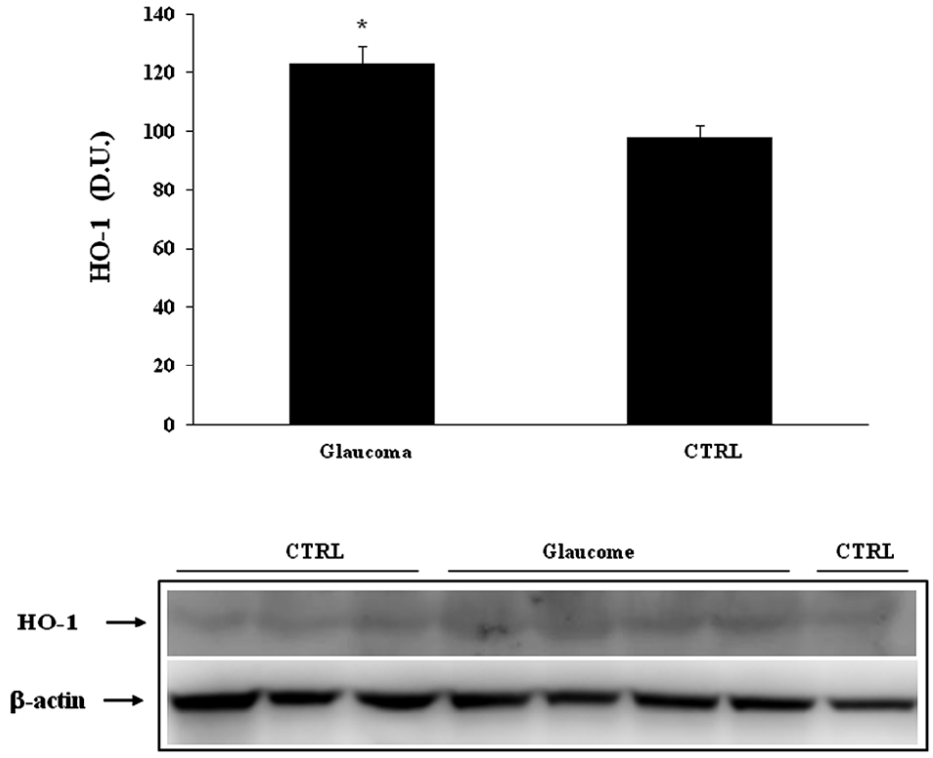

Fig. 41 HO-1 protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for HO-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U.,densitometric units; CTRL, control.

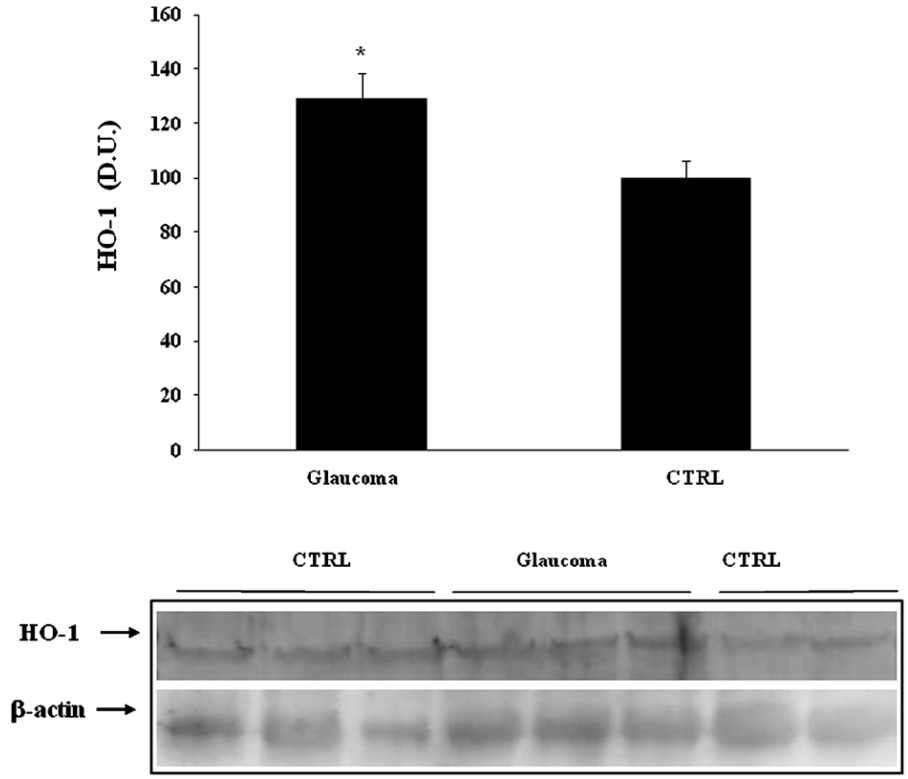

Fig. 42 HO-1 protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for HO-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U.,densitometric units; CTRL, control.

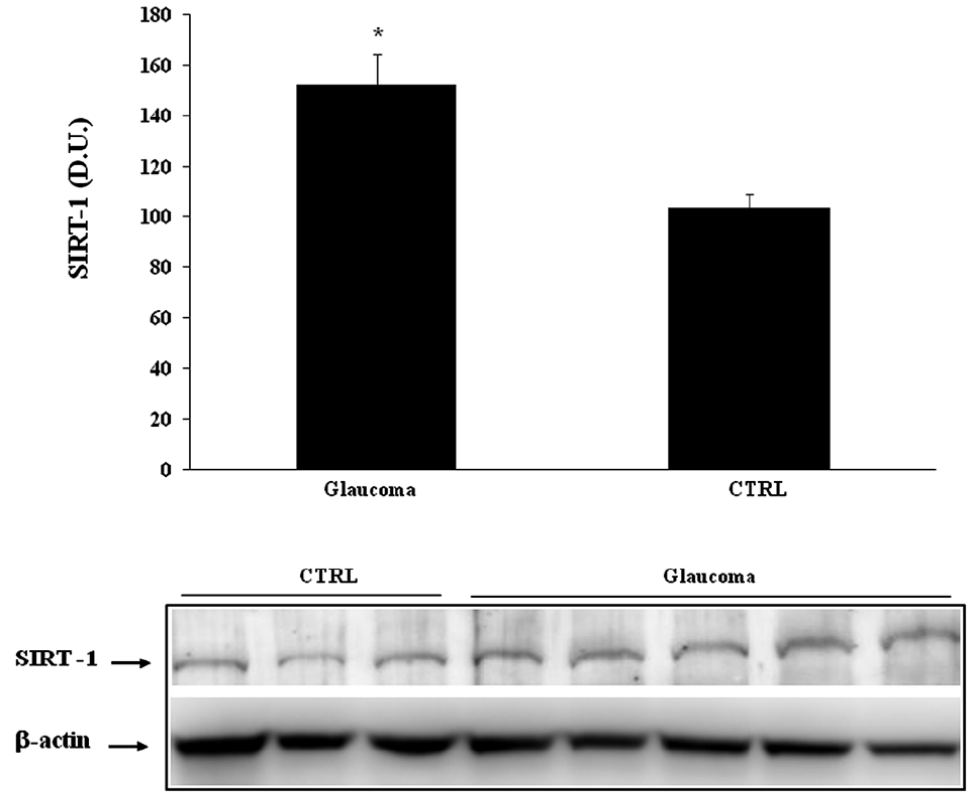

Fig. 43 Sirtuin-1 protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Sirt-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U., densitometric units; CTRL, control.

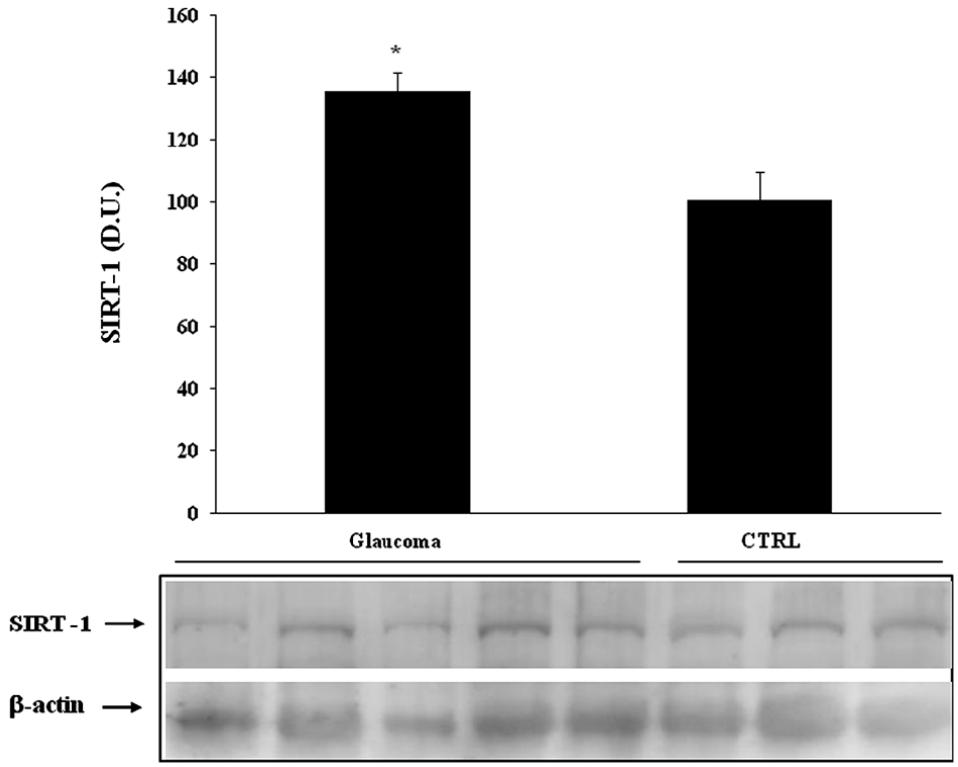

Fig. 44 Sirtuin-1 protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Sirt-1 expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*$P < 0.05$ vs. control. D.U., densitometric units; CTRL, control.

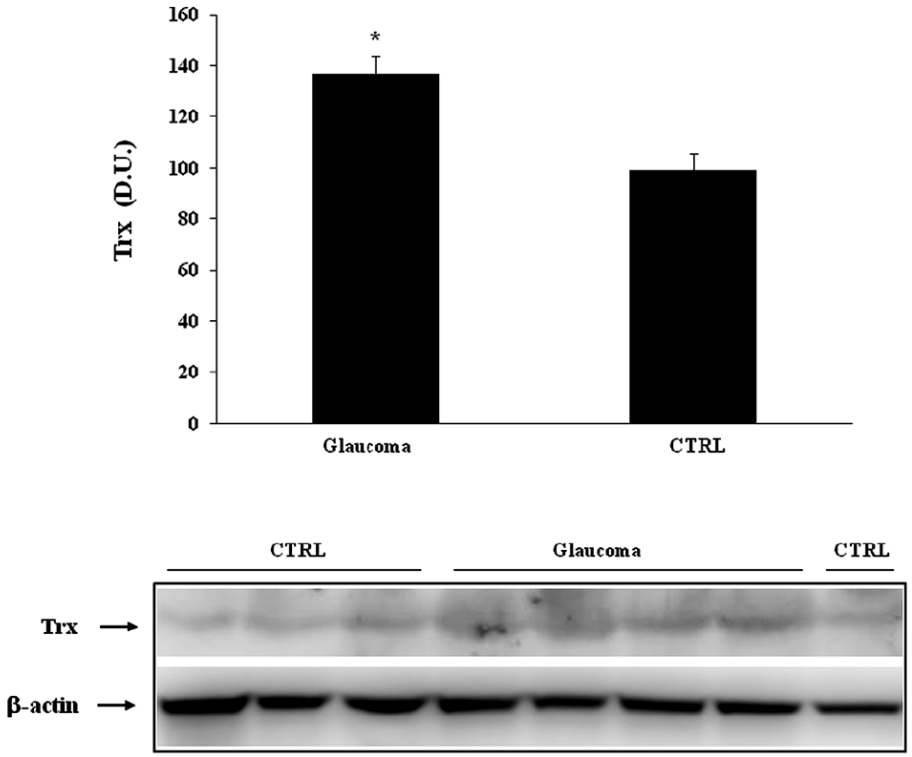

Fig. 45 Thioredoxin protein levels in lymphocytes of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Trx expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U., densitometric units; CTRL, control.

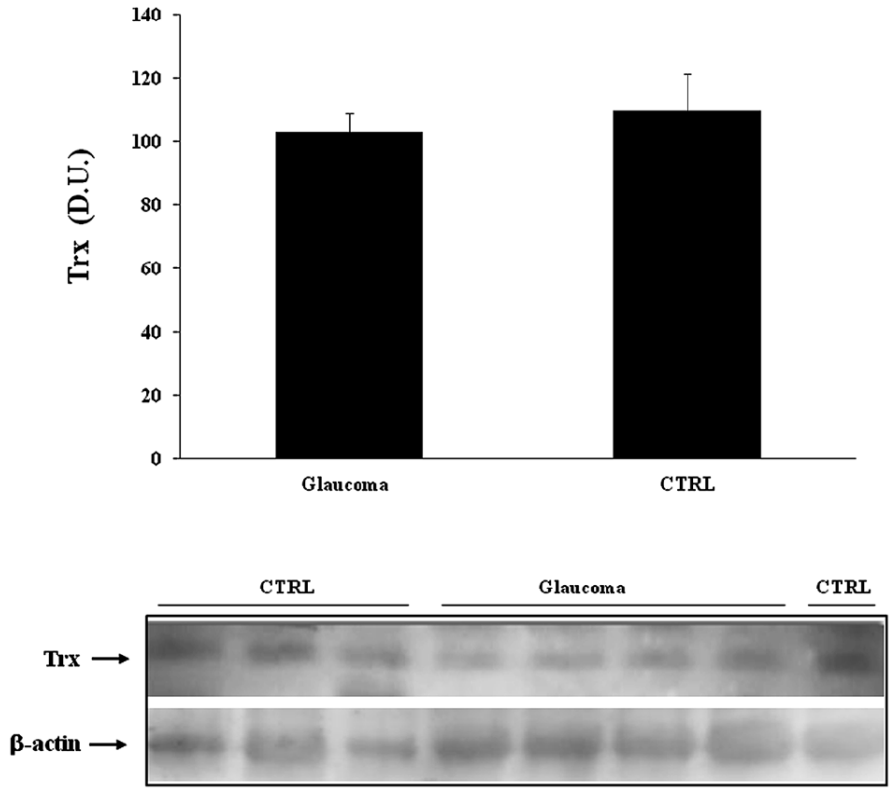

Fig. 46 Thioredoxin protein levels in plasma of glaucoma and control subjects. Samples from control and patients with glaucoma were assayed for Trx expression by Western blot. A representative immunoblot is shown. β-actin has been used as loading control. (B) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.*P < 0.05 vs. control. D.U., densitometric units; CTRL, control.

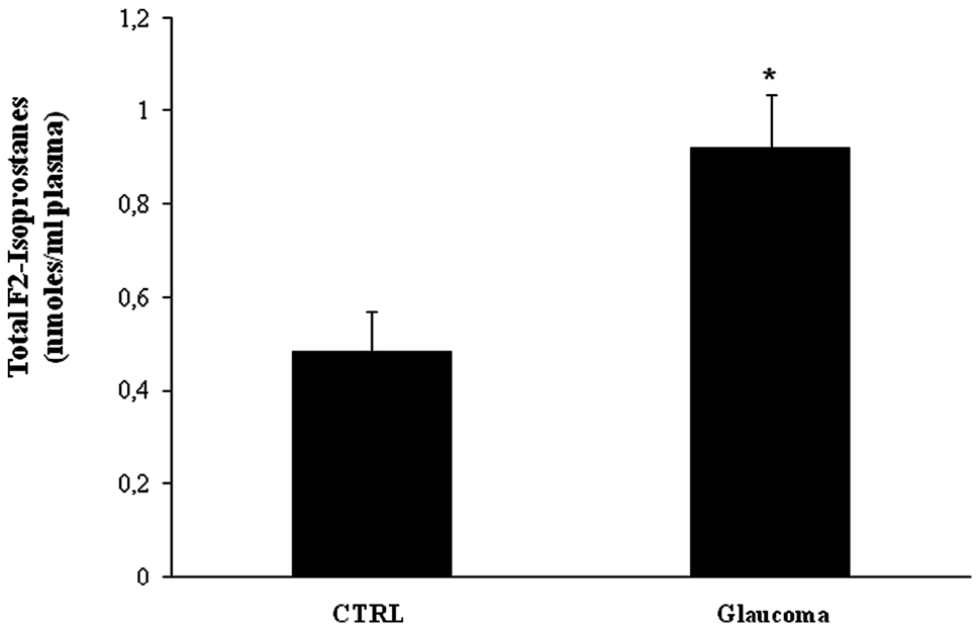

Fig. 47 Total F2-isoprostanes levels in plasma glaucoma patients. Plasma samples from patients with glaucoma and age-matched controls were assayed for total F2-isoprostanes. Data are expressed as mean ± SEM of 18 to 20 patients per group.*P < 0.05 vs. controls.

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