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Neuroinflammation and Oxidative Stress in the Traumatic Brain Injury and in the Pathogenesis of Neurodegenerative Diseases: Modulation by Nutritional Supplementation

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Chapter 1: NEUROINFLAMMATION

Neuroinflammation is a "cytokine-mediated" inflammatory process that can be caused by systemic tissue damage or, more often, associated with direct damage to the central nervous system (CNS), both of traumatic and/or neurodegenerative origin [1-3]. Neuroinflammation differs from inflammation by the low presence of lymphatic vessels inside the cerebral parenchyma, by the absence of endogenous cells capable of presenting the antigen and by the presence of the blood-brain barrier (BBB), which reduces the exchange of immune cells and mediators of inflammation with the bloodstream. The persistence in the CNS of inflammatory processes can cause serious damage to the neuronal complex up to compromising its functional integrity.

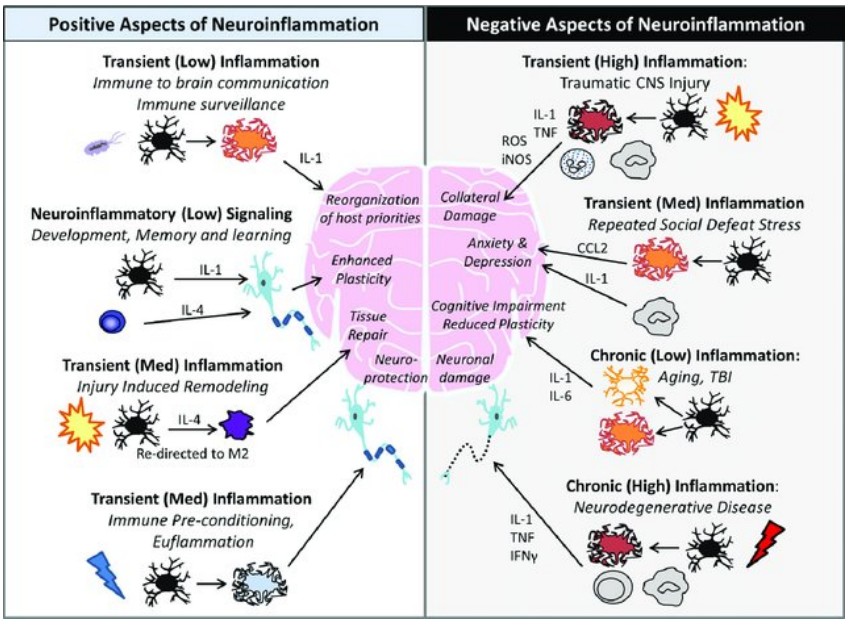

Figure 1. Positive and negative aspects of neuroinflammation

1.1 Neuroinflammation cells

1.1.1 Microglia

Microglial cells represent 5-10% of the total brain cell population. It is a population of hematopoietic derivation: during embryogenesis, in fact, a subpopulation of monocytes migrates into the nervous system and differs into resident macrophages. Microglia cells have a small cell body and long tapered processes equipped with lamellipods that give it a branched morphology. They are spread homogeneously in the cerebral parenchyma and can be found attached to neurons, but also in correspondence with blood vessels and free in gray matter. These cells are internal to the BBB and are therefore ready to receive and respond to any damage to the barrier itself. Microglia are normally inactive in the CNS, the cellular soma remains immobile while the ramifications move continuously to monitor the surrounding environment. The occurrence of changes in homeostasis in the environment, such as increase in serum proteins, toxicity from glutamate, increase in purines (ATP, ADP) or the presence of lipopolysaccharide (molecule present on the membrane of GRAM-negative bacteria) are all stimuli capable of stimulate the microglial, through different receptors and signal pathways. The microglial cells present in the perivascular areas also exercise the function of antigen-presenting cells (APC) on myelin-specific T cells, which have infiltrated the CNS and can thus initiate inflammatory processes [4]. When the microglial is activated, it passes from a branched morphology to an amoeboid, the lamellipods retract and the cell assumes phagocytic capacity, aimed at eliminating any residual dead cells or engulfing bacteria and viruses. Activated microglia has the main role of promoting and supporting the inflammatory state through the production of cytokines, reactive oxygen intermediates, proteinases, complement factors and chemokines. These inflammatory mediators promote the infiltration of immune system cells from the bloodstream, the recall of other microglial cells from the surrounding areas and the activation of astrocytes. When the inflammatory stimulus that caused the activation is lacking,

the microglia participates in the processes of suppression of the inflammatory state with the production of immunomodulatory cytokines, such as Interleukin-15 (IL-15), and anti-inflammatory cytokines, such as Interleukin-10 (IL-10); it then returns to an inactivated state, or undergoes apoptosis [5]. Microglia also have a trophic property, useful for protecting neuronal cells. This action is mediated by the production of growth factors such as glial cell-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Microglial activation and the neuroinflammatory events that follow are aimed at neuroprotection and elimination of the cause of the lack of homeostasis. In reality, both in chronic neurodegenerative diseases and in traumatic events, an uncontrolled and persistent microglial activation can have neurotoxic effects and contribute to exacerbate neuronal damage. The balance between neuroprotective and neurotoxic action of microglia is determined by many factors, including the nature of the activating stimulus and the interactions that are established between microglia, the other cells of the immune system and the neuronal network, so it is too simplistic to classify the role of microglia in an absolute way and additional studies are absolutely needed to shed light on the mechanisms that regulate this dual role [6]. Numerous evidences show that modulation of microglial activation, and in general of the inflammatory state in the brain, are able to improve the symptomatology of many pathological conditions and to decrease the phenomenon of neurodegeneration [7]. Based on these observations, microglial activation represents a potential pharmacological target for the treatment of traumatic brain injury and neurodegenerative diseases.

1.1.2 Astrocytes

Astrocytes are cells characterized by elaborate radial symmetry ramifications that give them a starry form. They can be of three types:

1. fibrous, mostly located in the white matter and characterized by long and thin processes;

2. protoplasmic, mainly located in the gray matter and with short and branched processes;
3. radial, arranged perpendicular to the axis of the ventricles.

One of the main functions of astrocytes consists in the creation of the BBB by wrapping the cerebral capillaries with their processes. They contribute to the structural integrity of the barrier and participate in exchanges between the bloodstream and the cerebral parenchyma. Astrocytes are essential for interactions with adjacent neurons, in fact, they cover the synaptic terminations, safeguarding normal neuronal excitability thanks to the maintenance of extracellular ionic homeostasis [8]. Even astrocytes have the ability to respond to pathological conditions; in these conditions, in fact, they implement a series of functional and structural changes which are called astrogliosis. The astrocytes are activated by the cytokines produced by the microglia and are in turn capable of producing pro-inflammatory molecules.

1.2 Molecules of Neuroinflammation

1.2.1 Cytokines

Cytokines are a class of very small molecules (8-80 kDa) that act in an integrated way in cell communication. In addition to the main role of stimulating the inflammatory phenomenon, they play a role in promoting cell growth, survival and differentiation. Thanks to their biological activities it is therefore possible to group cytokines into different classes: there are 18 cytokines called interleukins (IL), some of these promote inflammation and are called pro-inflammatory cytokines such as IL1β and IL1α, IL6, IL8 and TNFα; while other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines such as IL-4, IL-10, TGFβ. Cytokines are secreted by a variety of immune cells such as T lymphocytes and macrophages, as well as by non-immune cells such as fibroblasts. They are synthesized from many cell types including monocytes, neutrophils, hepatocytes and tissue macrophages, and microglia. The main function of cytokines is to regulate the differentiation of T cells from undifferentiated T-helper 1 and 2 cells, regulatory T cells and T-helper 17 cells [9]. Many of

these cytokines have already been shown to be produced by neurons or glia in central nervous system disorders where they have increased significantly. In fact, it has been shown that after the ischemic insult, and the inflammatory state that follows, there is a significant increase in the concentration of pro and anti-inflammatory cytokines aimed at protecting neuronal networks from cell damage. However, their action is not always positive, and their role in this particular type of insult is still being studied [10]. Cytokines are among the main effector molecules of inflammation, in fact, they respond to various inflammatory stimuli and are also present in auto-inflammatory diseases. The Interleukin 1 family has 11 ligands active on different specific receptors. Among the different IL-1 there are some, such as IL-1beta, which act on trans membrane receptors and others, such as IL-33, which mediate nuclear responses by acting directly from transcription factors. The parent interleukins of this subfamily are IL1-α and IL1-β.

• **IL1-α** is synthesized in the cytoplasm starting from an active precursor, and once mature it remains mostly bound to the plasma membrane. A fraction is present at the nuclear level where it acts in an autocrine way.

• **IL1-β**, the most studied of this family, differs from the α type because it is synthesized as an inactive pro-peptide and stored in vesicles. Inflammatory stimuli, such as lipopolysaccharide (LPS) and ATP, determine the maturation of the pro-peptide by the enzyme ICE (interleukin converting enzyme, also called Caspase-1) and the secretion of IL-1β therefore plays a crucial role in the response rapid. IL-1β also undergoes significant induction of gene expression. IL-1β in the brain is involved in the induction mechanism of fever, together with IL-6, and in the activation of T cells, macrophages and astrocytes. In the CNS, IL-1β can mediate the neurotoxic effects due to the induction of i-NOS and therefore of NO and radical oxygen species.

• **IL-1Ra**, it binds the receptors for IL-1β (IL-1R type I and II), but is unable to activate them, therefore it acts as a specific inhibitor. The overexpression of IL-1Ra induced in animal models

showed a reduction in the infarcted area in addition, mice with low production of this cytokine show a dramatic increase in ischemic damage [11, 12]. According to these data, the treatment with IL1-β induced a worsening of the damage. These observations clearly indicate that IL1-β has an important role in determining the severity of ischemic damage. These positive results allowed the start of clinical trials on the treatment with IL-1Ra of patients with cerebral ischemia [13].

• **Tumor necrosis factor-α (TNF-α)** is considered a fundamental cytokine in the inflammatory process; it is produced by monocytes-macrophages, dendritic cells and lymphocytes; in the brain it is produced by microglia and astrocytes and plays a fundamental role in directing the immune response. It exists both in transmembrane and soluble form, after cutting by the TACE enzyme (TNF-α converting enzyme). The balance between membrane and soluble forms depends on the activation state of the cell and is crucial for its activitỳ. Soluble TNF-α acts on transnmembrane receptors (TNFR1 and TNFR2) that activate different signal pathways, among which the main effector is the one that involves the transcription factor NF-kB, which positively regulates the transcription of numerous pro-inflammatory genes. In the TNF-α nervous system it mediates important functions including the activation of microglia and astrocytes, the regulation of the permeabilitỳ of the blood brain barrier, the induction of feverish state, glutamatergic transmission and synaptic plasticity [14]. TNF-α overexpression has neurotoxic effects: high levels of TNF-α have been measured in the serum of patients suffering from Alzheimer's Disease, Parkinson's disease and multiple sclerosis [15]. In cerebral ischemia TNF-α undergoes an increase in expression, but its role in this insult is still a matter of debate: evidence obtained in animal models shows that treatment with TNFR1 receptor inhibitor drugs determines a reduction in ischemic damage, while other studies have shown that treatment with anti-TNF-α antibodies, which do not discriminate activity on different receptors, leads to

reduced hippocampal neurogenesis. These observations suggest a different role of TNF-α receptors [14].

• **IL-6** is also a pro-inflammatory cytokine produced by macrophages, microglia, astrocytes, T lymphocytes, fibroblasts, endothelial cells and keratinocytes. Among its main functions are the induction of feverish state, the generation and coordination of the immune response. In addition, IL-6 is able to activate B lymphocytes and induce them to synthesize antibodies. Unlike IL-1, IL-6 also has anti-inflammatory functions, in particular it inhibits the synthesis of TNF-α and induces the synthesis of soluble receptors for IL-1 and TNF-α, which decrease the share of cytokines available. High serum IL-6 levels have been measured in patients with acute ischemia, and in animal models, IL-6 is induced following ischemic insult in the CNS, particularly in the peripheral region of the ischemic zone. It has a dual role: in fact, it contributes both to brain damage and to repair mechanisms that are carried out through the binding of IL-6 with the gp130 receptor, common to other neurotrophic cytokines (LIF). Although a significant improvement in ischemic damage has not been observed in the animal KO for IL-6, other studies report that direct injection of IL-6 after induction of ischemia determines a reduction in damage.

• **IL-10** is a powerful anti-inflammatory cytokine, mainly produced by monocytes-macrophages, microglia and, even if in smaller quantities, by lymphocytes. It is able to inhibit the expression of pro-inflammatory cytokines, such as TNF-α, INF-γ, IL-2 and IL-3. In the brain it plays an important role in controlling the neuroinflammatory state. It is up-regulated after ischemia, produced by the glia, and exerts a neuroprotective action. KO animals for IL-10 subjected to focal ischemia show a larger infarcted area, and other studies report that the administration of IL-10 or its over expression causes a reduction in the volume of the heart attack and inflammation [16].

Another important class of mediators of the inflammatory response is prostanoids (prostaglandins, prostacycline and thromboassane), molecules that derive from arachidonic acid. Their synthesis takes place by cyclooxygenase (COX) or PGH-synthase. There are two isoforms of this enzyme: COX-1 and COX-2; they mediate the same catalytic function, but have different physiological roles. In fact, the COX-1 enzyme is constitutively expressed in many cells of the body, including neurons, microglia and lymphocytes and performs functions of maintaining homeostasis. COX-2 is instead inducibly expressed following a pro-inflammatory stimulus. These enzymes are the main target of nonsteroidal anti-inflammatory drugs (NSAIDs). COX-2 expression is induced in many neuroinflammatory diseases. Also following the ischemic event, an increase in COX-2 expression is observed both in the area affected by heart attack and in distal regions. The role of these enzymes is mainly protective, but in the long term, the excessive production of prostaglandins has deleterious effects on the central nervous system. Recent studies suggest that the prostaglandin EP1 receptor has a role in ischemia-induced neurotoxicity. Furthermore, numerous evidences have shown that the treatment with COX-2 inhibitors improves the symptomatology after ischemic insult. COX activity leads to the production of reactive oxygen species, also accused of worsening the damage, although recent evidence has shown that the negative role of COX is mainly attributable to prostaglandins [10].

1.2.2 Chemokines

Chemokines (chemotactic cytokines) are cytokines with a predominantly chemotactic function; they are part of a superfamily of low MW proteins (6-14 kDa), active in the recall of various cell populations that participate in the immune response, such as neutrophil and eosinophil granulocytes, monocytes and lymphocytes, as well as in the cell migration processes that take place during embryogenesis. The chemokine family can be divided into three subfamilies characterized by 2-4 highly conserved cysteine residues in the sequence of the molecule. The

main families of chemokines are represented by: ***α-chemokines*** (or CXC not conserved (X); ***β-chemokines*** (or CC chemokines), which have two juxtaposed cysteine residues; and ***δ-chemokines*** (CX3C chemokines), which show the two cysteine residues separated by three amino acid residues. Chemokines are produced by a large variety of cell types, generally involved in phlogistic responses. They act on multiple cell types, carrying out numerous actions described in vitro such as chemotaxis, the release of enzymes on cell deposits, the formation of oxygen radicals, the formation of lipid mediators and the induction of adhesion to the endothelium or to the extracellular matrix [17]. In ischemic tissues, different signal pathways (such as that of reactive oxygen species, that of cytokines, the complement cascade and the NF-kB system) can regulate the synthesis of chemokines, resulting in a rapid increase in their concentration, following white blood cell infiltration and immediate inflammatory response [18]. One of the most studied chemokines belongs to the family of CC chemokines and is the Monocyte Chemoattractant Protein-1 MCP-1 / CCL2. CCL2 has a fundamental role in the attraction of monocytes, T cells and NK cells; it is also implicated in diseases characterized by monocytic infiltration.

MCP-1 also has important effects in the heart attack, on the activation and recruitment of macrophages, on the synthesis of cytokines and on the accumulation of myofibroblasts. This chemokine can therefore exert its effects in the first hours after the infarction through distinct mechanisms, such as the recruitment of monocytes in the ischemic myocardium and the modulation of macrophage differentiation, activation of phagocytes and expression of cytokines.

The up-regulation of MCP-1 / CCL2 has been observed in association with neuroinflammatory responses in animal models of cerebral ischemia and in myocardial tissues [19]. MCP-1 is responsible for the migration of leukocytes into the CNS under various pathological conditions. The synthesis of MCP-1 by astrocytes and microglia in the CNS regulates the increase in the

influence of leukocytes, which occurs following axonal damage or in association with neuroinflammatory diseases, such as autoimmune encephalopathy, a model of sclerosis multiple induced in the rat [20].

The CXC chemokines family plays a fundamental role in the regulation of chemotaxis and in the activation of neutrophils in ischemic tissues; but they are still important in inflammation induced by Th1 cell infiltration. In addition, CXCs present angiostatic and inhibitory effects on fibroblast migration.

RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted or CCL-5) is part of the CC-chemokines family, it is produced by circulating CD8+ T lymphocytes, endothelial cells, fibroblasts, platelets and in the brain by microglia and astrocytes. Its main function is the recruitment of leukocytes to inflammatory sites; it also activates the release by eosinophils of eosinophilic cationic proteins. RANTES also increases the adherence of monocytes to endothelial cells and selectively supports the migration of monocytes and T lymphocytes that express CD4 markers on the surface. Finally activates the basophils and causes the release of histamine.

Platelet Derived Growth Factor (PDGF) is a chemokine isolated from platelets and synthesized by megakaryocytes as a growth factor for connective tissue and glial cells; the biologically active protein is a dimer composed of two connected polypeptides called A and B. PDGF heterodimers are expressed by a variety of cell types, such as macrophages, endothelial cells, fibroblasts and smooth muscle cells. PDGF binds to plasma proteins and is involved in wound repair processes and angiogenesis. PDGF also stimulates the proliferation of astrocytes and inhibits the premature differentiation of progenitor cells; it is also involved in the development of the central nervous system as the PDGF-b isoform receptors are expressed in many areas of the brain. PDGF has a role in the activation of mesenchymal stem cells. PDGF-B and its PDGFR-β receptor are also expressed at the neuronal level, and an increase in

expression of these proteins has been detected in post mortem brains of patients with ischemia. PDGF has a role in addressing and regulating the differentiation of different stem cells, including neuronal stem cells present in the subventricular and sub-granular area [21].

Interferon Inducible Protein (IP-10 or CXCL10) is a member of the CXC family of chemokines. IFN-γ induces the expression of IP-10 in different cell types, such as monocytes, endothelial cells, keratinocytes, fibroblasts and microglia. IP-10 has chemoattractant activity for monocytes and T cells in humans and promotes the adhesion of T cells to endothelial cells; also in vivo it is able to inhibit angiogenesis and demonstrates anti-tumor activity. In the brain it is mainly produced by microglia and contributes to exacerbate the inflammatory state and its neutralization or depletion turns out to be neuroprotective.

Neural Regeneration Protein (NRP) is a chemokine expressed in stem cells and glial cells. It can induce migration and cell proliferation, promote neuronal survival and increase neurite development; it also induces the differentiation of neuronal stem cells into neurons. NRP exerts its effects on neuronal survival through the phosphorylation of ERK 1 and ERK 2, two cytosolic kinases [22].

Chapter 2: OXIDATIVE STRESS

The term oxidative stress represents the condition that is established when at the cellular level an imbalance is established between the production processes of reactive oxygen species (ROS) and those responsible for their removal [23]. The ROS production and removal processes are strictly regulated, so as to ensure that a reducing environment is preserved inside the cells. ROS represent a group of molecules characterized by a high tendency to react with substrates of various nature by oxidizing them. In physiological conditions, ROS are normally produced inside the cell. ROS have been shown to mediate numerous signaling pathways that take part in cell cycle control. Low or moderate intracellular levels of ROS are associated with the activation of signals involved in the processes of survival, growth, proliferation and cell migration. High quantities of ROS, on the other hand, can damage cellular structures and, consequently, activate cell death signaling pathways [24].

Proteins, nucleic acids and lipids represent the main targets of the oxidizing action of ROS. Proteins can undergo oxidation reactions especially at the level of the side chains of lysine, arginine, proline and threonine. Carbonylation is the consequence of protein oxidation and generally results in the loss of the functions of the protein itself. While moderately carbonylated proteins are directed to the proteasome and degraded, highly carbonylated proteins tend to form high molecular weight aggregates that accumulate inside the cell [25]. DNA is particularly vulnerable to oxidative damage which determines the modification of the bases, the oxidation of deoxyribose, the breakage of the filament, the formation of protein cross-links. The oxidation of the nitrogen bases can induce errors in the duplication process, which, if not corrected by the repair systems, can turn into a stable mutation [26, 27]. Lipids are much more reactive towards ROS than nucleic acids and proteins, because they have unsaturation sites in carbon-carbon double bonds. The hydroxyl radical ($OH^{\bullet}$) can react with the carbonaceous chain of the phospholipid by extracting a hydrogen atom and forming a carbon-centered radical in a reaction known as lipid peroxidation. Once irreversibly damaged, the lipid is enzymatically degraded

and resynthesized to repair the membrane. The greater the extent of peroxidative damage, the less the cell's ability to promptly repair the membrane.

The main source of intracellular ROS is the respiratory chain. It has been estimated that during breathing 1-2% of the oxygen is not completely converted to water, but only partially reduced producing the superoxide anion ($O2^{\bullet -}$), which can be converted into hydrogen peroxide (H_2O_2) and in hydroxyl radical ($OH^{\bullet}$). The main O_2 production sites • - are located at the level of complexes I and III of the electron transport chain (ETC), from which electrons escape by directly reducing molecular oxygen. The ROS generated at the ETC level do not exert their toxic action only in the mitochondria, but also in the cytosolic compartment. In fact, hydrogen peroxide is a highly diffusible species and quickly leaves the matrix and also pours into the cytosol [27].

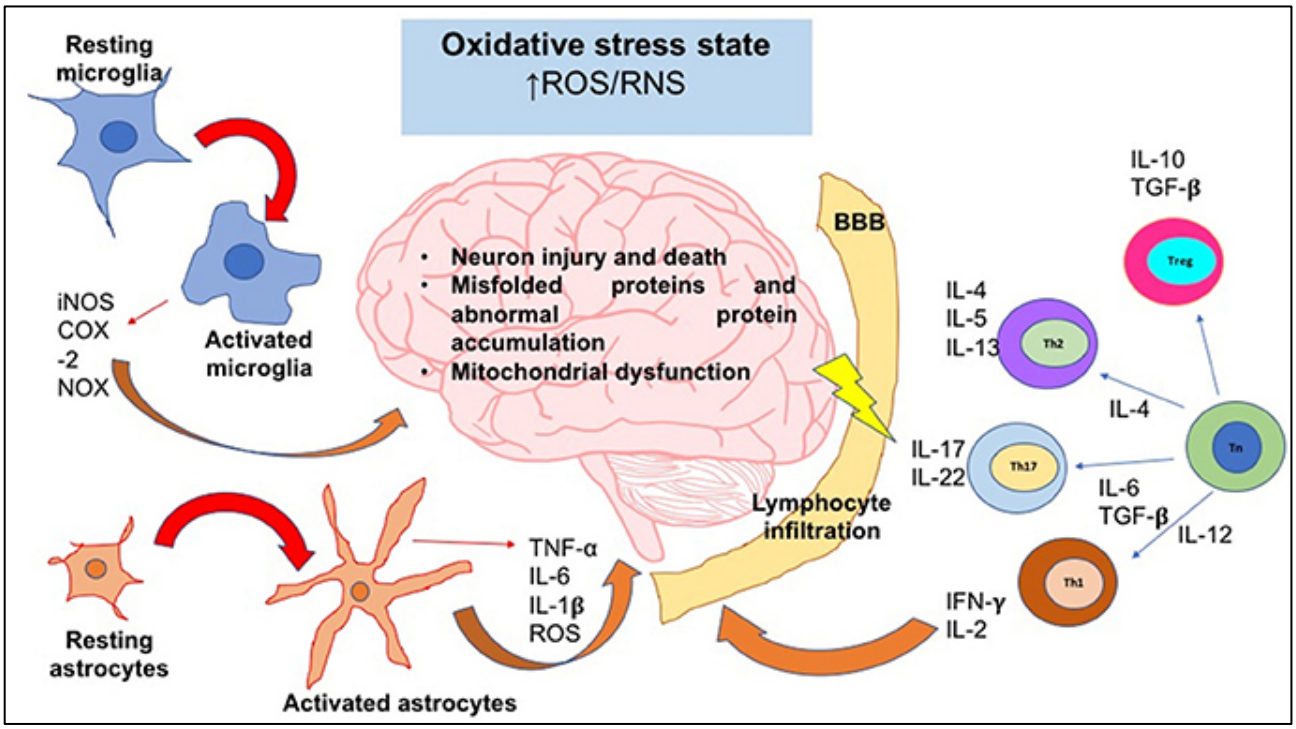

Figure 2. Oxidative stress in the brain

2.1 Endogenous Antioxidant Systems

Superoxide dismutase (SOD) (EC 1.15.1.1) is one of the most effective enzyme antioxidant systems; it catalyzes the conversion of the superoxide anion into molecular oxygen and hydrogen peroxide in the following dismutation reaction:

$$O2^{\bullet -} + O2^{\bullet -} + 2H + \rightarrow O_2 + H_2O_2$$

SOD is present in different isoforms, identifiable on the basis of the ions present in the active site, the amino acid composition and the distribution in the cellular compartments. Three isoforms have been identified in human cells: SOD1 (CuZn-SOD) represents the cytosolic isoform, SOD2 (Mn-SOD) is localized at the mitochondrial level, SOD3 (CuZn-SOD) is instead the extracellular isoform [28]. Hydrogen peroxide, deriving from the dismutation reaction, still represents a reactive species. The enzymes mainly responsible for its removal are catalase and glutathione peroxidase.

Catalase (Cat) (EC 1.11.1.6) is an enzyme present in peroxisomes and catalyzes the conversion of hydrogen peroxide to water and molecular oxygen in the following reaction:

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

Cat is one of the enzymes with the highest turnover numbers: a catalase molecule can convert about six million hydrogen peroxide molecules in a minute [29].

Glutathione peroxidase (GPx) (EC 1.11.1.9) competes with catalase for the removal of hydrogen peroxide and represents the main source of protection against low levels of oxidative stress [30]. GPx catalyzes the conversion of hydrogen peroxide or an organic peroxide (ROOH) to water or alcohol (R-OH) by using glutathione as a reducing agent.

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

$$2GSH + R\text{-}OOH \rightarrow GSSG + R\text{-}OH + H_2O$$

Glutathione (GSH) is the most potent non-enzymatic endogenous antioxidant. It is a tripeptide (cysteine-glycine-glutamate) present in large quantities in the cytosol, in the nucleus and in the mitochondria. When glutathione is used during peroxide detoxification reactions, two

molecules of reduced GSH are oxidized to form a disulfide bridge (GS-SG) [31]. Under physiological conditions, oxidized glutathione makes up about 1% of total glutathione; variations in the GSH/GSSG ratio represent an index of the state of oxidative stress [32]. A recurrent theme is reactive redox signaling based on cysteine thiol. Under physiological conditions, reactive cysteine thiols exist as thiolate anions (S−) and are more reactive towards ROS than sulfhydryl groups (-SH). Reactive thiols interact with ROS to generate a range of cysteine oxidation products, including sulfuric, sulfinic and sulfonic acids; S-nitrosothiol and thionitrates; S-glutathiolation products; and inter and intraprotein disulfides [33]. The modification of the critical cysteine thiols of Keap1 and Nrf2 by oxidants and electrophiles is a main mechanism by which ARE inductors activate Nrf2 [34, 35].

Nrf2 directly influences ROS homeostasis by regulating antioxidant defense systems through different mechanisms. Among these we find:

1. induction of superoxide and peroxide catabolism through SOD, Prx and GPx;
2. regeneration of cofactors and oxidized proteins;
3. synthesis of reducing factors, such as GSH from GCLC and GCLM, and NADPH from G6PDH and 6PGD;
4. expression of the antioxidant protein Trx and inhibition of the expression of the Trx TXNIP inhibitor;
5. increase in oxide-reduction transport;
6. metal-chelation by MT1, MT2 and ferritin;
7. induction of stress response proteins, such as HO-1.

Most of the antioxidant enzymes/proteins regulated by Nrf2 are located in specific compartments within the cell where they have the function of regulating redox signaling. Nrf2 also regulates the expression of different oxidant signaling proteins to affect a range of programmed cellular functions. Some regulators, such as p62 and DJ-1, activate Nrf2 and are

induced by oxidants through Nrf2, creating a positive feedback circuit with Nrf2. Among the proteins dependent on Nrf2 we have heme oxygenase 1 (HO-1), which in addition to removing excess toxic heme, produces iron, biliverdin and carbon monoxide ions. This protein, together with its products, have beneficial effects by protecting against oxidative lesions, regulating apoptosis, modulating inflammation and contributing to angiogenesis. Therefore, Nrf2 and HO-1 represent factors of protection from stress and anti-aging. Furthermore, recent discoveries have revealed that Nrf2 plays a role in regulating various processes such as autophagy; reporting to inflammasome; endoplasmic reticulum stress and unexplained protein response; mitochondrial biogenesis; stem cell function [36, 37].

Chapter 3: NEUROHORMESIS

The term hormesis refers to a process that places itself at the center of the responses of cells and organisms to environmental factors and biological organization [38]. This process is nothing more than the expression of integrative adaptive responses characterized by biphasic dose responses with specific quantitative response characteristics (maximum amplitude and width of the adaptive response) and induced by a direct stimulatory response or the result of compensatory biological processes following of an interruption of homeostasis [39, 40]. More simply, the term hormesis refers to those drugs, toxins or natural substances which, if administered in low doses, can give a positive response on the cell or organism, while at higher concentrations they are harmful and toxic [38].

At the cellular and molecular level, hormesis involves the activation of adaptive stress response pathways that involve, for example, a greater expression of heat shock proteins, antioxidant enzymes and anti-apoptotic proteins. All these phenomena act in most cases through hormonal mechanisms [41]. These hormone dose responses provide quantitative information on the limits of biological plasticity [42] and allow us to measure how much adaptive processes can be upregulated, which is particularly relevant for understanding the protective effects induced by plant and fungal species.

The hormonal concept is extremely important as it provides reliable estimates of the upper limit for the induction of potential therapeutic responses and should play a key role in carrying out experimental and clinical studies.

The hormesis has aroused particular interest in the toxicological community for the dose-response model, especially in vulnerable biological systems, such as the brain. We therefore define neurohormesis as the adaptive process by which nervous systems and organisms respond to a moderate level of stress by improving their ability to resist more severe stress that could be lethal or cause dysfunction or disease.

Neurohormesis affects the memory, learning, performance and neurodegenerative responses mediated by oxidative stress in cellular models for various diseases such as neurodegenerative ones [38, 43].

Common examples of neurohormesis are ischemic preconditioning [44] and adaptive responses of neurons to moderate intensity excitatory neurotransmission [45], exercise [46] and dietary restrictions [47]. Neurohormesis can also be induced by endogenous neurotoxic molecules such as nitric oxide [48], carbon monoxide [49], glutamate [50] and Ca^{2+} [51].

Both *in vitro* and *in vivo* studies have shown that the responses of neurohormesis to stressors can be mediated by several factors [52-55]:

- NF-kB;
- cAMP-response-element binding protein (CREB);
- factor related to nuclear factor E2 (Nrf2);
- factor 1 inducible by hypoxia (HIF1).

Other mechanisms could include modulating the expression of proteins involved in the regulation of oxidative stress and Ca^{2+} cellular homeostasis [56].

Chapter 4: NUTRITIONAL SUPPLEMENTATION

Epidemiological studies and experiments on animal models have revealed that the phytochemicals present in fruit and vegetables can protect the nervous system from disease [57, 58]. In particular, it has been shown that the beneficial effects of phytochemicals on health are due to the antioxidant properties of many chemical products contained in them. For example, numerous studies have reported the neuroprotective effects of compounds found in natural products, including □-tocopherol, lycopene, resveratrol, ginkgo biloba and ginsenosides [59]. Markers of oxidative stress have also been identified in the brains of patients with neurodegenerative diseases that support intervention with neuroprotective nutritional substances based on the antioxidant action and anti-inflammatory agents, such as polyphenols or fungi [60]. In fact, it is known that polyphenols and fungi activate the pathway of heat shock proteins (Hsp), which plays a crucial role in the response to cellular stress.

Therefore, the neuroprotective effects of these products are associated with their ability to reduce oxidative stress but, recently, other biological activities of phytochemicals are emerging that could be beneficial to human health [61].

4.1 Hericium erinaceus

Medicinal mushrooms have become a focus for researchers because the bioactive compounds they contain have many therapeutic properties. Hericium erinaceus has been commonly prescribed in traditional Chinese medicine (TCM), because its fruiting body and mycelia contain a high amount of structurally different bioactive and potentially bioactive components whose consumption has proven to be beneficial for human health. This mushroom species is found throughout the northern hemisphere in Europe, Asia and North America.

Figure 3. Hericium erinaceus

Fungus and fermented mycelia have been shown to produce numerous classes of bioactive molecules, including polysaccharides, proteins, lectins, phenols and terpenoids. The numerous bioactive compounds of Hericium erinaceus have been developed in food supplements and alternative medicines [62]. These compounds have been shown to have different properties such as antibiotic, antidiabetic, anti-hypertensive, cardioprotective, anticarcinogenic, anti-hyperlipidemic, anti-aging, nefroprotective, hepatoprotective, neuroprotective, anti-depression and also improves anxiety and function cognitive [63]. The antioxidant property of Hericium erinaceus was shown in a diabetic rat model in which the intraperitoneal (ip) administration of an aqueous extract of Hericium erinaceus (100 and 200 mg/ kg) led to a significant reduction in serum glucose levels, increase in insulin level and attenuated serum lipid profiles compared to the control group. These results are associated with an increase in the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), an increase in glutathione levels (GSH) and a reduction in malondialdehyde (MDA) in the liver. These data suggest that the health promoting mechanism appears to be the result of ROS inhibition [64]. Also interesting is the beneficial effect observed following the use of two

classes of terpenoid compounds, ericenones and erinacins, respectively isolated from fruiting bodies and mycelia. These compounds have been able to stimulate the synthesis of nerve growth factor (NGF) in cultured astrocytes [65]. The cholineacetyltransferase and acetyl cholinesterase enzymes are modulated by NGF which is therefore capable of influencing the cholinergic neurons of the basal midbrain. It is known that the first pathological events of Alzheimer's disease are precisely the loss and dysfunction of cholinergic neurons. Therefore, the administration of Hericium erinaceus improves cognitive dysfunction. Recent, both basic and clinical studies have shown that Alzheimer's disease is closely associated with beta amyloid-induced neuroinflammation (Aβ). This protein is responsible for activating the astrocytes and microglia that promote neuronal damage through the release of proinflammatory and cytotoxic factors, aggravating the course of the disease [66]. A new neuroprotective strategy is represented by the oral administration of a Hericium biomass preparation given for 3 months [67]. This type of intervention can represent a therapeutic goal to minimize the harmful effects related to the oxidative load, such as that which occurs in the brain during aging or neurodegenerative disorders. Hericium erinaceus treatment resulted in a significant increase in LXA4 production in most brain regions such as cortex, hippocampus, substantia Nigra, striatum and cerebellum and a modulated expression of cytoprotective proteins, such as HO-1, Hsp70 and Trx. These results are consistent with recent evidence from mice studies showing Hericium erinaceus neuroprotection on Aβ 25–35 peptide-induced cognitive dysfunction [68, 69].

4.2 Moringa oleifera

Moringa oleifera is native to India and grows in the tropical and subtropical regions of the world. It is commonly known as a "natural gift" or "miracle tree" or "mother's best friend". Moringa is able to withstand extreme weather conditions, which is why it is grown all over the world.

Figure 4. Moringa oleifera

Thanks to its high nutritional values, each part of the tree can be used for nutritional or commercial purposes. For example, the leaves are rich in minerals, vitamins and other essential compounds and for this reason their extracts are used for the treatment of malnutrition and to increase breast milk in breastfeeding mothers. As is known, most vegetables lose nutrients during cooking. However, it has been observed that Moringa leaves, fresh, cooked or stored as dried powder for months without refrigeration, have not lost their nutritional value [70]. Boiled leaves produced three times more bioavailable iron than raw leaves. These results were also observed in powdered Moringa leaves. In addition, Moringa was found to have a group of unique compounds containing sugar and rhamnose, which are unusual glucosinolates modified by sugar [71]. These compounds have been reported to exhibit some chemo preventive activity, inducing apoptosis [72].

Moringa oleifera has been used as a treatment in numerous diseases, such as obesity, diabetes, scurvy, hysteria and tumors [73, 74]. Moringa oleifera has anti-cancer [75], anti-inflammatory [76], anti-oxidant [77], hepatoprotective [78], anti-bacterial [79], cardioprotective [80], hypolipidemic [81], hypoglycemic [82] and antihypertensive [83] activity.

In a rat model of liver fibrosis, Moringa oleifera has been shown to reduce not only histopathological damage but also to modulate various biochemical markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are indicators of liver health conditions [84]. In another study, the protective ulcer property of 50% ethanolic leaf extract of Moringa oleifera was demonstrated in different induction models of gastric ulcers. The anti-inflammatory and anti-oxidant role of Moringa oleifera was initially observed in a model of diabetes induced by obesity. In this study, the ability of this natural compound to modulate cytokine levels such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), but also the inducible nitric oxide synthase (iNOS) and the nitric oxide (NO) [85-87]. Polyphenols represent the main natural compounds capable of reducing oxidative damage in tissues. Moringa oleifera leaves have a high number of polyphenols and therefore have an important antioxidant power [88]. Both mature and tender leaves of this plant have been shown to exhibit antioxidant activity against free radicals, preventing oxidative damage to major biomolecules and tissues [89]. In addition, the 50% ethanolic leaf extract has been tested to study the activities of LPO, CAT and SOD. The antioxidant properties of Moringa extract have been found to modulate the levels of SOD, CAT and LPO in the gastric mucosa of the rat [70]. In a recent study, based on the role of oxidative stress in stroke pathophysiology, it was determined whether Moringa oleifera protected against brain damage and oxidative stress in the animal model of focal stroke. The results obtained from this study showed that all the used doses of extract reduced the volume of the heart attack both in the cortex and in the sub-cortex. The protective effect of medium and low doses of extract in all areas occurred mainly through the reduction of oxidative stress [90]. Oxidative stress plays a crucial role in age-related dementia. Having Moringa oleifera antioxidant and nootropic activity, the enhancement of spatial memory and neuroprotection of Moringa oleifera leaf extract in the age-related animal model of dementia was determined. The results obtained from this study showed that the extract

is capable of improving spatial memory and neurodegeneration in the areas CA1, CA2, CA3 and in the dentate gyrus of the hippocampus. It also decreases MDA levels and AChE activity but increases that of SOD and CAT. Therefore, the extract of Moringa oleifera leaves represents a therapeutic potential against cognitive disorders. The possible mechanism is probably related to the ability to reduce oxidative stress and increase cholinergic function [91].

4.3 Hydroxytyrosol

In recent years, numerous studies support the beneficial effects of the Mediterranean diet (MD) in preventing neurodegeneration. MD predicts a high intake of cereals, vegetables, fruit, olive oil, legumes, low meat consumption, and a moderate amount of fish and seafood and alcohol. This translates into a reduction in the consumption of saturated fat and an increase in the intake of foods containing high concentrations of bioactive compounds such as polyphenols and flavonoids [92, 93]. In this regard, one of the main phytochemicals present in oil and table olives is Hydroxytyrosol (HT).

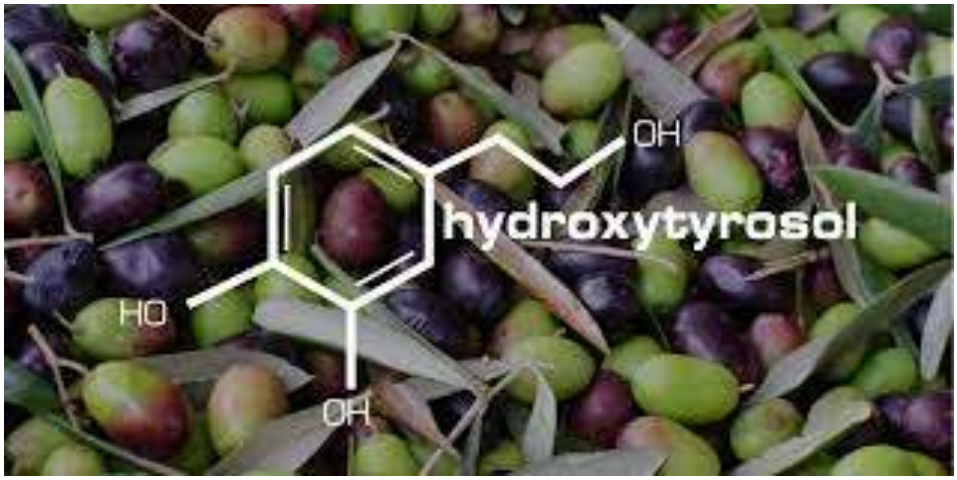

Figure 5. Hydroxytyrosol

Its importance is supported by numerous studies that highlight the high antioxidant power of HT and its ability to eliminate free radicals [92]. Therefore, interest in HT has increased in

recent years and this is also due to the possession of other biological activities including anti-inflammatory, antimicrobial, anticarcinogenic and neuroprotective effects [92, 94-97]. All of these activities could give HT a central role in the prevention of neurodegenerative diseases. Although there are still few studies on the neuroprotective effects of HT in animal models, they provide important data that encourage the evaluation of this compound in the treatment of neurodegenerative disorders. In an animal model similar to Huntington's disease, the antioxidant effect of oral administration of extra virgin olive oil (EVOO) and HT has been shown in the brain. The results show a decrease in lipid peroxidation and an increase in cellular glutathione (GSH) levels. This displays that EVOO and HT act as brain antioxidants through a natural mechanism useful for protection from oxidative damage [98]. In another study, C57BL/6 mice were treated with oligomeric acid $A\beta_{1-42}$ + ibotenic acid to induce neural behavioral dysfunction. After induction, a severe deficiency in the visuo-spatial and working memories took place. However, HT treatment appreciably ameliorated spatio-cognitive performances. Further research has shown that HT administration has been able to counteract dysregulation of signaling mechanisms in hippocampal neurons [99-101]. Recently, increasing evidence reports that HT and its derivates activate phase 2 response leading to the expression of nuclear factor erythroid 2-related factor (Nrf2) antioxidant pathway [102, 103]. Interestingly, Nrf2 represents a crucial mechanism of resistance to oxidative stress and inflammation *in vitro* and *in vivo* [104, 105]. In line with these observations, Nrf2 codifies the antioxidant pathway of vitagene that exists to counteract various forms of stress (eg oxidative, environmental and proteotoxic stress). The latter involves redox sensitive genes, such as γ-glutamyl cysteine synthetase (γ-GCS), HO-1, heat shock protein 70 (Hsp70), thioredoxin and sirtuin-1 (Sirt1), called vitagenes that help preserve the protein homeostasis and cellular redox balance in various pathological states [106, 107].

Chapter 5: TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a damage that does not occur due to a hereditary, congenital factor but can result from a wide variety of insults, such as external mechanical force. This can cause temporary or permanent impairment of both cognitive and physical functions [108]. Additionally, the TBI can lead to coma and even death. Cerebral lesions are divided into 2 subcategories: *primary lesions*, which occur at the time of the trauma, and *secondary lesions*, which occur immediately after the trauma and whose effects can be prolonged over time. What happens in the brain following TBI are a series of cellular and molecular interactions, which cause excitotoxic effects related to oxidative stress, inflammation, ionic and metabolic imbalance. The combination of these pathways induces progressive neuronal loss through necrosis and apoptosis [109, 110]. Also, very important are the changes that occur within the cells that are determined by the increased flow of calcium, which affects mitochondrial integrity. Lactate accumulation is also cytotoxic for cells as, together with the increase in vascular permeability, cerebral edema occurs, increase in intracranial pressure and decrease in brain perfusion [109-111]. People who have undergone a TBI also experience a number of stereotyped symptoms such as confusion, dizziness and sometimes loss of consciousness. In addition, about 70-80% of people with TBI, even after the resolution of the initial lesion, develop a series of long-lasting symptoms such as anxiety and depression that affect the patient's personality [112-115]. In addition, TBI may increase the risk of developing neurodegenerative diseases. An example is the repeated concussive TBI that appears to appear to lead to the development of chronic traumatic encephalopathy (CTE) in athletes. Furthermore, repeated or single TBI appears to be strongly associated with the risk of developing Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [116].

Considering the high number of cases of TBI and its association with severe neurological problems and risk of neurodegenerative diseases, there is a strong interest in developing new

therapies that not only promote cell survival immediately after injury, but also address the development of secondary pathology [117-119].

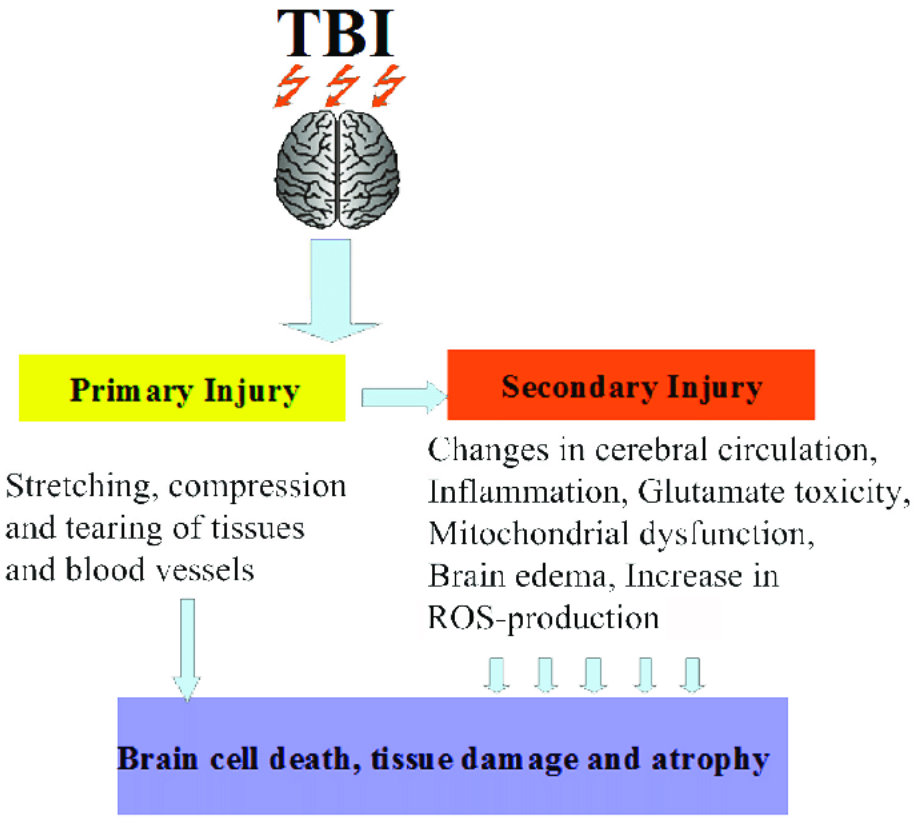

Figure 6. Schematic representation of TBI

5.1 Classification of TBI

In the United States and many other countries, a score scale known as the Glasgow Coma Scale (GCS) is used to evaluate a patient's mental and neurological status following a brain injury [120]. The score is based on the sum of three components: eye opening response, verbal response and motor response (see the following table).

Category		Best Response
Eye opening		
Spontaneous		4
To speech		3
To pain		2
None		1
Verbal	(Modified for Infants)	
Oriented	Babbles	5
Confused	Irritable	4
Inappropriate words	Cries to pain	3
Moans	Moans	2
None	None	1
Motor		
Follows commands		6
Localizes to pain		5
Withdraws to pain		4
Abnormal flexion		3
Abnormal extension		2
None		1
Glascow Coma Score		
Best possible score		15
Worst possible score		3
If tracheally intubated then verbal designated with "T"		
Best possible score while intubated		10T
Worst possible score while intubated		2T

Table 1 – Glasgow Coma Scale.

GCS can be further divided into minor injuries (13 to 15), moderate injuries (9 to 12) and serious injuries (3 to 8). The clinical features that allow us to define a mild injury are loss of consciousness for 20 minutes, absence of focal neurological changes, no intracranial injury and no intracranial surgery. The patient, on the other hand, falls into the moderate category when, regardless of mental state, he has a focal lesion of computed tomography (CT). Finally, the patient is defined as serious when, always regardless of mental state, he goes into a coma for at least 6 hours. The Glasgow scale is not the only one, however, the only scale used in terms of outcome. Another scale commonly used, especially by rehabilitation facilities, is the scale of cognitive functioning level of Rancho Los Amigos (see the following table) [120].

Levels	Clinical Signs
I. No response	Unresponsive to any stimulus
II. Generalized response	Nonpurposeful responses, usually to pain only
III. Localized responses	Purposeful; may follow simple commands
IV. Confused, agitated	Confused, disoriented, aggressive; unable to perform self-care
V. Confused, inappropriate	Nonagitated; appears alert; responds to commands; verbally inappropriate; does not learn
VI. Confused, appropriate	Can relearn old skills; serious memory defects; some awareness of self and others
VII. Automatic, appropriate	Oriented; robot-like in daily activities; minimal confusion; lacks insight or planning ability
VIII. Purposeful, appropriate	Alert and oriented; independent in living skills; capable of driving; defects may remain in judgment, stress tolerance and abstract reasoning may not be at preinjury cognitive ability

Table 2 - Rancho Los Amigos Level of Cognitive Functioning Scale.

5.2 Epidemiology, incidence and causes

Each year, approximately 30 million visits, hospitalizations and injury deaths occur in the United States alone. Among hospitalizations, approximately 16% include TBI as a primary or secondary diagnosis. About a third of deaths include the TBI as a cause of direct or underlying death. To make an estimate, of all the people who are hospitalized for accidents, about 87% are treated and subsequently released, 11% are hospitalized and discharged, and about 2% die. These data, however, are not real as they do not take into account those people who have not needed medical treatment, who have made outpatient visits, or who have received treatment at a federal facility [121].

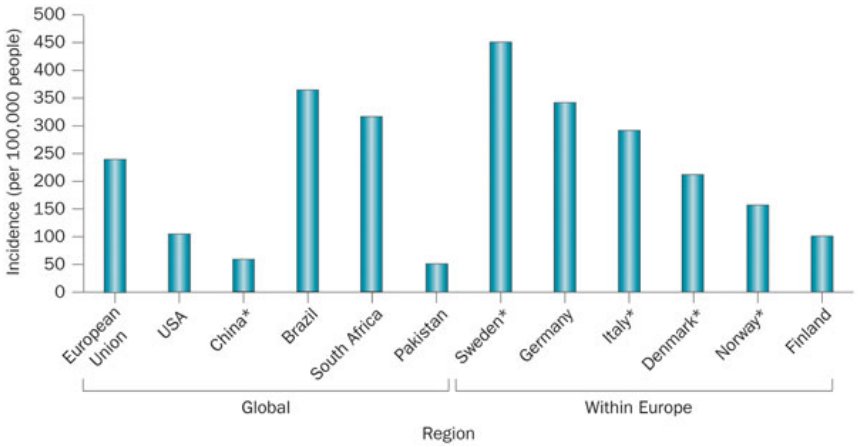

Figure 1. Estimates of the global incidence of TBI

In the United States, children (0-4 years), teenagers (15-19 years) and adults (> 75 years) are the groups most likely to undergo a visit or be hospitalized for TBI [121]. In particular, the rates of hospitalizations and deaths following TBI are very high among adults aged ≥75 years. Overall, males account for approximately 59% of all TBI-related medical visits to the United States [121]. As can be seen from Table 4, in the period 2002-2010, the main causes of TBI-related visits were falls, road accidents and hits from or against an object.

Instead, the main causes of TBI-related deaths are traffic accidents, accidental falls and suicides [122]. Other data that are not estimated are the percentages of TBI that occur during sports and recreational activities. However, according to the National Electronic Injury Surveillance System - All Injury Program, in the period 2001-2009 (CDC, 2011) the activities associated with the highest estimated number of TBI-related hospital visits were cycling, soccer, game and basketball activities among people under the age of 19.

5.3 Therapeutic Strategies of TBI

Neuroprotection, neurovascular regeneration, and neuro-restoration have been proposed to be therapeutic strategies for TBI. As we have seen before, the TBI leads to a cascade of events and a loss of neurons, which clinically manifests itself with different degrees of neurological deficit depending on the site and the severity of the loss of neurons. Numerous studies have been conducted to reduce or prevent TBI-induced neuronal loss. Generally in in vivo studies a treatment to have neuroprotective effects on TBI must be carried out within a few hours from the first impact [123-125]. However, many clinical trials using neuroprotective treatment have not shown promising results. Therefore, even today, the TBI remains an unmet medical need and an important source of disability and mortality in developed and developing societies. Below we describe the current therapeutic strategies in use.

Acetylcholinesterase inhibitors: Central acetylcholinesterase (AChEI) inhibitors have the role of increasing synaptic acetylcholine and inhibiting its breakdown in the synaptic cleft. AChEIs

are normally used for the treatment of mild to moderate Alzheimer's disease but have also been used for other cognitive disorders, including TBI. The AChEIs most used in recent years in clinical trials for chronic TBI are donepezil [126-132] rivastigmine [131, 133-137] and galantamine. These studies have suggested that these compounds may have beneficial effects, especially in patients with moderate and severe chronic TBI who have persistent cognitive deficits, by increasing synaptic ACh levels. The benefits of these compounds have also been reported in preclinical studies of TBI. In particular, it was observed that AChEI had positive effects on acute injury processes with consequent reduction of TBI-induced neuronal death, on the conservation of neurons in the hippocampus region CA1, on the reduction of the interruption of the blood-brain barrier (BBB) , on the reduction of cerebral edema and on the reduction of neurological and motor deficits [126, 138].

Amantadine: Amantadine (1-adamantamine hydrochloride) is a tricyclic amine normally used for the treatment of influenza A, and in recent years it has shown modest efficacy also for the treatment of Parkinson's disease. Studies have shown that amantadine increases extracellular dopamine (DA) concentrations by blocking the recovery of DA or facilitating the synthesis of DA [139]. It can also have post-synaptic effects, acting on the circuits of the DA and increasing the DA density of the receptor [140]. Amantadine has been studied little in the preclinical models of experimental TBI. One study showed that amantadine treatment, starting 1 day after a closed controlled cortical impact model of TBI in rats and continuing for 18 days after injury, resulted in modest improvement in Morris water maze (MWM) latencies [141]. However further studies are needed. Clinically, amantadine has been shown to be effective during the post-acute period in the patient with TBI. In another study, amantadine was shown to result in a faster improvement in the disability rating scale (DRS) than placebo, although the rate of change in DRS flattened after 4 weeks of treatment [142].

Cyclosporine A/FK 506: Cyclosporin A (CsA) is capable of maintaining the potential of the mitochondrial membrane by inhibiting the opening of the transition pore of the mitochondrial permeability after TBI. This action has been confirmed by in vivo studies that have shown that CsA is able to reduce reactive oxygen species thus preserving mitochondrial function [143, 144]. In addition, these compounds inhibit the calcineurin phosphatase protein, also having positive effects on axonal lesions and on learning and memory [145-149]. Another characteristic of CsA is their immunosuppressive power [150]. Not all studies have produced positive results. Both preclinical and clinical studies suggest that the acute treatment of patients with severe TBI is the most suitable route for the clinical development of CsA or FK 506.

Simvastatin/other statins: Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMGA) reductase inhibitors, reduce serum cholesterol but also have potent effects in the brain relevant to mechanisms of TBI injury and recovery. Such effects target mechanisms that influence both the acute and chronic phases of TBI [151, 152]. There is pre-clinical evidence of beneficial effects including those on acute injury processes such as brain edema, BBB integrity, cerebral blood flow, neuroinflammation, axonal injury, and cell death, in addition to effects on key facets of regeneration such as trophic factor production. A variety of molecular outcomes are influenced including TUNEL staining, CREB, Akt, eNOS, FOXO1, NF-κB, GSK3, cytokines, BrdU labeling, blood vessel formation, and vascular endothelial growth factor [153].

***N-acetylcysteine (NAC)*:** NAC is FDA-approved as an antidote for acetaminophen overdose and as a mucolytic for cystic fibrosis and other bronchopulmonary diseases. In animal models of TBI, NAC has shown strong antioxidant activity by increasing glutathione levels and decreasing markers of oxidative damage [154]. NAC also showed anti-inflammatory activity by decreasing the activation of NF-κB, while lowering interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and intercellular adhesion molecule (ICAM)-1 levels [155, 156]. It is unclear how the anti-inflammatory action of NAC is related to its antioxidant activity. NAC has been

shown to reduce lesion volume while simultaneously reducing levels of the putative neuroprotective enzyme heme oxidase [155, 156].

***Growth hormone (GH)*:** GH is a polypeptide that is synthesized, stored, and secreted by somatotrophic cells within the lateral wings of the anterior pituitary gland. GH deficiency/insufficiency (GHD/GHI) is the most common anterior pituitary abnormality after TBI. Manipulating the GH axis has been shown to improve motor function, enhance learning and memory retention after TBI in rats, and to improve spatial learning and memory in a mouse model of AD [157, 158].

***Erythropoietin (EPO)*:** EPO is a cytokine implicated in erythropoiesis and has a number of beneficial effects that may be important in the treatment of TBI such as attenuation of glutamate and nitric oxide toxicity, anti-apoptotic, anti-oxidant and anti-inflammatory effects , stimulation of neurogenesis and angiogenesis, and protection of mitochondria [159-161]. Numerous studies have demonstrated the efficacy of EPO in rodent models of TBI [151, 162-167]. The route of administration, dosage and therapeutic window appear to be favorable. Research indicates that any parenteral route of administration appears to be effective for the treatment of TBI. In addition, the therapeutic window can be prolonged, with some studies suggesting a benefit with a first dose for up to 24 hours after injury [159]. Similarly, the administration of EPO in humans has been shown to be effective in chronic conditions [168]. EPO has been beneficial and has reduced gray matter loss in chronic schizophrenia [169].

Chapter 6: NEURODEGENERATIVE DISEASES

Neurodegenerative diseases represent a heterogeneous set of distinct nosographic entities, united by some pathogenic and clinical characteristics. From the point of view of pathogenesis, they are characterized by a chronic and selective process of cell death affecting neurons. The exact etiology behind this pathogenetic process is not yet defined. Although in some sporadic cases some genetic mutations responsible for the development of disease have been identified in families affected by some degenerative pathologies, in the etiopathogenesis of most of them numerous risk factors, of both genetic and environmental origin, seem to play a fundamental role.

The progressive degeneration, which precedes the appearance of symptoms for a few years, concerns, at least in the initial phase, a certain population of neurons. Subsequently, during the course of the disease, other neuronal systems may be damaged. From a clinical point of view, therefore, neurodegenerative diseases begin insidiously, generally in adulthood, and have a progressive and inexorable course that culminates in a serious disability, which often follows the patient's death [170].

Although in the early stages they can take on a focal character, these pathologies generally affect a specific neuronal system bilaterally, giving rise to an extremely varied clinical symptomatology. In fact, neuronal deterioration is the cause of irreversible and inevitable damage to brain functions that occurs, depending on the type of disease, with cognitive deficits, dementia, motor alterations and behavioral and psychological disorders, more or less serious.

The definition and classification of neurodegenerative diseases, due to the overlapping of the symptoms and sometimes also the sharing of some phases of the pathogenetic process, continue to be the subject of a heated medical-scientific debate. However, it can now be said that several well-defined clinical entities are grouped under this name, of which the best known are Alzheimer's disease and Parkinson's disease. The other main neurodegenerative diseases are

Huntington's disease, amyotrophic lateral sclerosis, progressive supranuclear palsy, frontotemporal dementia and Lewy body dementia.

From a therapeutic point of view, although for some of these pathologies instruments are available which are able to delay or control, more or less effectively, the clinical symptoms, they still remain incurable diseases. Despite the significant progress made in recent years by biomedical research, in fact, there is still no therapeutic intervention that has proven capable of reversing or stopping the pathological process underlying these disorders. This situation largely depends on the fact that the cellular and molecular mechanisms underlying the neuronal damage observed in the various neurodegenerative diseases are still poorly understood [170].

6.1 Parkinson's disease

It is a chronic progressive disease, defined clinically by the association of tremor, rigidity, brady-akinesia and postural instability, and neuropathologically characterized by severe degenerative changes in the substantia nigra (SN) and in the pigmented nuclei of the brain stem, with the presence of specific cell inclusions (Lewy bodies) in residual neurons. Described initially as "agitating paralysis" by James Parkinson ("Essay on the Shaking Palsy", 1817) and later by Charcot (1872-1873) who specified its clinical characteristics, the disease found its first neuropathological identity with the studies by Tretiakoff (1919) who underlined the constancy of the alterations of the SN. Biochemical studies have made it possible to demonstrate the existence of a severe dopamine depletion of the striatum, as a consequence of the neuronal depletion of the SN, thus providing a pathophysiological interpretation of the disease [171].

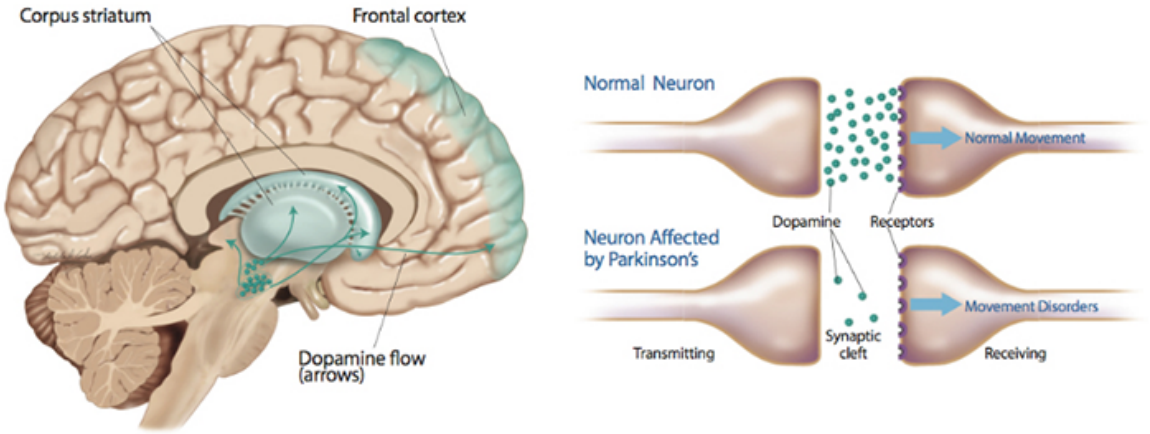

Figure 7. Main feature of Parkinson's disease.

6.1.1 Epidemiology and Etiology

The disease begins insidiously in the second half of life (usually between 50-60 years); cases that begin before the age of 40 are relatively rare. The frequency is high: it is estimated that every year a new case appears for every 4000 inhabitants and, if referred to subjects over 50 years of age, a new case for every 1000. In Italy the prevalence has been indicated between 95 and 199 /100,000 in different epidemiological studies [172], with progressive growth over 50 years. It can be assumed that there are currently around 100,000 Parkinsonians in Italy. The disease affects males and females (with a slight preponderance for the male sex) and is ubiquitously widespread, albeit less frequently in China and Africa than in western nations.

The cause of the disease is unknown, although studies on its etiology have intensified especially after the discovery of a toxic substance (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or MPTP; rotenone) capable of reproducing, in humans and in some animal species, a clinical and neuropathological picture similar to that of Parkinson's [173]. The toxicity of MPTP develops as a result of its metabolization by glial monoamine oxidase B (MAO B) with the formation of the MPP + compound which is transported within dopaminergic neurons, where it accumulates in the mitochondria by inhibiting complex I of the respiratory chain and causing ATP depletion and therefore the onset of cell death.

The discovery of parkinsonism from MPTP or rotenone has supported the hypothesis that Parkinson's idiopathic disease may depend on exposure to toxic substances of environmental origin with variable distribution (the risk of disease would be higher in a rural environment, in relation to the use of pesticides and herbicides). However, research in this direction has not been conclusive and the hypothesis has been put forward that the responsible toxic substances are more widely distributed, but that only some individuals are vulnerable to potential toxic damage in relation to a genetic predisposition. Alternatively, it has been suggested that the toxic action on the nigral cells may be of endogenous origin: in particular, some products of the normal dopamine catabolism (similarly to any environmental toxins) would be responsible for the formation of free radicals and would induce oxidative stress damage. Free radical theory has been the subject of heated controversy [174, 175], but there is no doubt that a specific deficiency of mitochondrial complex I and an increase in iron levels have been documented in the SN of parkinsonian subjects, not compensated by increases in ferritin [176]. In addition, it has recently been shown that the toxic mechanisms mentioned above are capable of inducing the phenomenon of apoptosis which could represent the main factor responsible for neuronal death. The possible role of heredity in Parkinson's disease has also been the subject of conflicting opinions: the finding of a positive family history only in 10-15% of cases has suggested the existence of an autosomal dominant predisposition with reduced penetrance , which is difficult to highlight due to the marked variability of the preclinical phase of the disease [177]. The recent study by Tanner et al. (1999) on the degree of concordance between monozygotic and dizygotic twins suggests that early-onset parkinsonisms would be characterized by a strong genetic component, while in parkinsonisms with onset over 50 years the latter would be considered irrelevant.

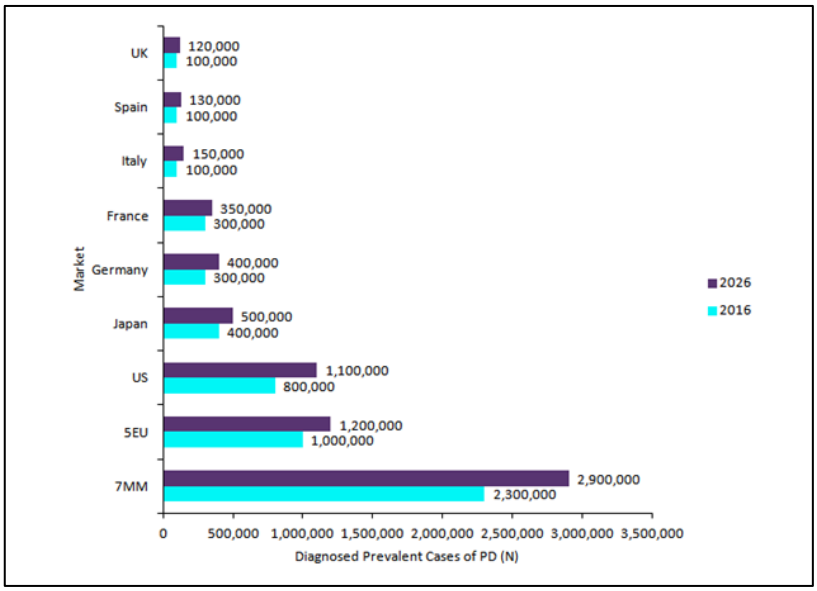

Figure 8. Diagnosed prevalent cases of PD

6.1.2 Pathogenesis

The consequence of the loss of dopaminergic neurons of the SN is the reduction of dopamine at the striatal level, the main biochemical alteration of Parkinson's disease [171]. There is a close parallel between the extent of neuronal depletion, the degree of depletion of striatal dopamine and the severity of clinical symptoms: the preclinical phase of disease has a variable duration, probably less than 7 years according to recent PET data [178], during which the loss of dopaminergic neurons and the reduction of the striatal dopamine content are accompanied by a minor reduction of the homovanillic acid (and of the enzymes tyrosine hydroxylase and dopa-decarboxylase) in relation to an increased dopaminergic turnover and to the compensatory development of receptor hypersensitivity from denervation (i.e. an increase in the number of dopaminergic receptors). Parkinsonian symptomatology occurs when the number of dopamine neurons and the content of striatal dopamine have fallen below a critical level (70-80%) [179]. As the disease progresses, the reduction of dopaminergic neurons is associated with a progressive reduction in the number of postsynaptic receptors. However, the biochemical alterations that characterize Parkinson's disease are much more complex and widespread,

indicating a prevalent, but not exclusive, compromise of dopaminergic systems. Starting from the observation that the striatal neurons also receive a cholinergic innervation in addition to the dopaminergic innervation, the existence of an alteration of the normal neurotransmitter balance between dopamine and acetylcholine has been hypothesized, therefore a relative hyperfunction would be associated with the reduced activity of the dopaminergic neurons of cholinergic neurons. Although not without important practical findings, the theory of the altered "dopamine-acetylcholine balance", based on outdated anatomical data, is no longer considered valid. Other neurotransmitters (norepinephrine, serotonin) are reduced, albeit to a lesser degree. The concentration of the glutamic acid decarboxylase enzyme (GAD), responsible for the synthesis of GABA, is reduced in the black substance and in the cortex of parkinsonian subjects: this lays down for a functional impairment of the strio-nigric GABAergic projections involved in the inhibitory regulation of neurons dopaminergic. At present, therefore, Parkinson's disease cannot be related exclusively to a dopamine deficiency secondary to the degeneration of pigmented neurons of the SN and of the brain stem, but must rather be considered as a complex disease, characterized biochemically by an imbalance in the relationships between the various neurotransmitters.

6.1.3 Neuropathology

The main alterations concern the midbrain region. The most characteristic and constant histopathological changes are localized in the pars compacta of the black substance (ventro-lateral region), where there is a serious neuronal rarefaction with almost complete disappearance of the pigmented neurons, followed by a mild or moderate glial reaction [180]. The melanin pigment is found free in the nervous parenchyma or engulfed by macrophages. In surviving neurons, hyaline cytoplasmic inclusions (rounded in shape with a clear peripheral halo and a strongly acidophilic central part) called "Lewy bodies" are observed in variable numbers, in the composition of which the presence of α-synuclein polymers stands out [181].

Similar alterations are also observed in the pigmented formations of the brainstem, in particular in the locus coeruleus and in the dorsal motor nucleus of the vagus, as well as in the unnamed substance, in the basal nucleus of Meynert and in the cells of the intermediolateral column of the spinal cord. "Lewy bodies" do not constitute a pathognomonic finding of Parkinson's disease, being also present in other degenerative diseases and in about 5% of the brains of normal people over the age of 65, but in fact they represent such a characteristic neuropathological aspect (they are present in about 76% of the brains of Parkinsonian patients) to be considered indispensable for confirming the diagnosis of disease [182]. The "Lewy bodies" also characterize a rare morbid form, called "Lewy body disease", in which they are located in large numbers in the cerebral cortex and in the gray subcortical nuclei, giving this condition a nosographic identity on a neuropathological basis. More discrete alterations of uncertain significance (some of which may represent senile type changes) are found in the striatum and in the pale and may reflect a trans-synaptic degeneration linked to the serious and persistent compromise of the nigro-striated dopaminergic pathway. Neurons in neurofibrillary degeneration and senile plaques have been described in the frontal cortex and hippocampus of Parkinsonian patients to a greater extent than controls of the same age: it is not yet certain, however, whether these alterations represent the pathological substrate of mental deterioration which, after years of illness, can be found in a variable percentage of cases [180].

6.1.4 Symptomatology

The disease occurs insidiously and in about 60% of cases the onset symptom is represented by tremor; other initial manifestations can be constituted by a motor hindrance or a feeling of stiffening of a limb, but also nonspecific disturbances (pains, fatigue, depression of mood, gait disturbances, changes in vocal timbre or writing) may be present for months before the disease fully develops. Onset symptoms tend to be one-sided (or asymmetric) and can remain so for many years (very rarely definitively), even if minimal contralateral objective signs can be

highlighted by neurological examination. The cardinal signs of the disease are represented by the triad: tremor, rigidity, akinesia, and later by alterations in posture and balance, variously combined in intensity and order of appearance.

- ***Tremor*** - Parkinsonian tremor occurs at rest and is caused by the rhythmic contraction of an alternating type of antagonist muscles with a frequency of 4-6 shocks per second (Hz.). It disappears during the execution of voluntary movements, even if in the most serious and advanced forms, the voluntary movement attenuates, but may not completely suppress the tremor. External circumstances are able to influence and modify it: fatigue, emotions, mental calculation and all the situations in which the patient believes to be observed, determine an increase in the intensity of the tremor, which is instead attenuated in conditions of tranquility, to completely disappear during sleep. It is preferentially located, especially in the initial stages of the disease, in the limbs and in particular in the distal segments of the upper limb: the thumb has abduction-adduction movements and the other fingers of flexion-extension, creating a movement described as "counting coins" or "pack pills". The hand can also be the seat of flexion-extension and prono-supination movements; the lower limb is affected less frequently, rarely in the initial phase, with distal movements of flexion-extension of the foot. The head is rarely involved, although the tremor can spread to the jaw and lips. The pathophysiological mechanisms responsible for resting tremor are still controversial. It is hypothesized that segmental mechanisms (oscillation phenomena transmitted by hyperfunctioning long-loop reflex circuits) and supraspinal mechanisms (rhythmic activity of a central generator, identified in the nucleus ventralis intermedius of the thalamus) influenced by peripheral afferents contribute to the genesis of resting tremor. It should be remembered that postural tremors are commonly observed in parkinsonians (with various frequencies: 6-7 and 9.5-11 Hz.) During the maintenance

of specific attitudes of the upper limbs, often in the initial stages of disease and even in the absence of the typical tremor at rest, which can pose differential diagnostic problems with respect to the various forms of "essential tremor" -

- ***Rigidity*** - Characteristic and constant symptom, rigidity can be the only clinical sign of the disease for a long period of time. It consists of an increase in muscle tone appreciated by the patient as muscle "stiffening" and by the examiner as a continuous resistance to passive movement, of similar intensity in antagonistic muscle groups, constant for the whole area of manipulation, defined as "plastic" (reminiscent of the flexibility of wax or a lead pipe). These semeiological characteristics contrast with the selective distribution and dependence on the stretching speed of "spasticity". The pathophysiological mechanisms responsible for rigidity are currently not fully known. The prevailing hypothesis is that rigidity derives from an altered cortical control of the spinal segmental circuits (where the interneurons Ib would be mainly involved), responsible for modifying the threshold, gain and duration of the EMG activity related to muscle stretching. The changes in the pale ouput directed to the pedicle-pontine nucleus would play an important role. Stiffness affects all muscle groups: initially localized to the axial, cervical and proximal limb muscles, it also tends to affect the distal extremities and to prevail in the flexor and adductor muscle groups, determining the particular postural attitude of the Parkinsonian, called "camptocormia" : the head and trunk in slight flexion, the shoulders forward, the arms adhering to the chest, the semi-flexed and rounded forearms, the thighs adduced and in modest flexion with respect to the trunk, the legs slightly flexed and the feet in the initial position varus. Superimposed on rigidity, during passive mobilization (especially on the wrist and elbow), small, regular and rhythmic sagging of muscle hypertonia described by Negro as a phenomenon of the "toothed wheel" or "trochlea", attributed to a reaction of

elongation, are appreciated shortening, although it has recently been suggested that this phenomenon may express the superimposition of activity discharges of a subclinical action tremor. Parkinsonian stiffness, such as tremor, can also be influenced by numerous factors: it is increased by emotions, cold, fatigue and exertion, while it subsides during sleep.

- ***Akinesia*** - This term is referred to a global reduction of motility (voluntary, automatic, associated and reflex), while the term bradykinesia refers to the slowness and fatigue with which voluntary movements are performed. The associated and automatic movements, which are normally performed in a regular and harmonious way without the intervention of the will, are compromised and require attention and concentration. For example, the movements spontaneously performed during a conversation such as the use of the hands, cross the legs, change the position on the chair, are reduced or absent. The voluntary movement is quantitatively reduced and altered in all the phases of its realization: repetitive movements, of rapid alternating type, such as rhythmically beating the fingers of the hand or foot, opening and closing the fist, pronating and supine the forearm is performed slowly with a gradual reduction in breadth and completeness. The pathophysiological bases of bradykinesia have been identified in the thalamo-cortical facilitation deficit of the supplementary motor area and in the inappropriate activation of the primary motor areas which would follow the hyperactivity of the "indirect route" from putamen to pale (external segment) and subthalamic nucleus. Walking occurs slowly and in small steps, the feet are crawled on the ground mainly with the toe, the pendulum movements of the upper limbs are significantly reduced or abolished. Starting to walk is particularly difficult: the patient feels his feet as glued to the floor and only after repeated attempts to start does he start walking. Similarly, in attempts to change direction (behind-front), when encountering an obstacle, when

passing through narrow spaces (for example, a doorway) the patient hesitates and freezes (paradoxical or "freezing" akinesia phenomenon) . In the more advanced stages of the disease, the spontaneous attitude in flexion of the trunk favors a constant tendency to anteropulsion, which can be evidenced with a progressive acceleration of the pace, "as if chasing its own center of gravity" (phenomenon of "festination"). The loss of normal facial expression (hypo-amimia) is often an early sign: the physiognomic features are fixed (having lost the expressive capacity of emotions and feelings) giving the subject an impassive and expressionless aspect, the eyelid rhyme is slightly wider than the Normally, blinking is rare. The language is monotonous, slow, without inflection; the speech loses its normal prosody, the articulation is irregular and intertwined, the voice is hypophonic. In some cases, after the difficult and slow start, the discussion shows a trend towards progressive acceleration, a phenomenon called "festination of language". More rarely, in advanced forms, palilalia appears, that is, iterative repetition of a word several times, or more often of a syllable or a syllabic fragment. As the disease progresses, dysarthria can make the word unintelligible. Even the alterations of the writing often represent one of the initial clinical signs: the graphic characters are irregular, uneven, disturbed by the tremor and they are getting smaller and smaller, creating the typical Parkinsonian micrograph. In the advanced stages, writing can become totally illegible.

- ***Postural alterations*** - The prevalence of hypertonia in the flexor and adductor muscle groups involves the development of a specific postural attitude ("camptocormia") which can be associated, over time, with skeletal changes (kypho-scoliosis); sometimes the unilateral prevalence of the symptoms affects the development of postural alterations on the frontal plane (lateral inclination of the column). Postural deformations also occur in the hands (flexion of the metacarpophalangeal joints with hyperextension of the

interphalangeal joints) and in the feet (extension of the big toe with a "hammer" attitude of the other fingers, which constitutes the so-called "striatal foot", wrongly interpreted, sometimes, as an extensor plantar response). As the disease progresses, the deformations described above are associated with a progressive patient compromise tends to fall backwards on the chair. This postural instability (evidenced by the "thrust test") can become responsible for antero-retropulsion and falls to the ground, severely limiting autonomy.

- ***Other signs or symptoms*** - While objective sensitivities are not compromised, patients often complain of subjective sensory disturbances, such as: paraesthesia or dysesthesia of various types, muscle pain and cramps (very annoying, but often indefinite and poorly localized). The eye movements are normal, except for a deficit of convergence, a limitation of the conjugate gaze of verticality upwards and a reduced speed of the saccadic movements. Blepharospasm is possible. Sialorrhea is a frequent symptom in the advanced stages of the disease and is the expression of inadequate spontaneous swallowing. Sometimes mild dysphagia may be present. An important vegetative dysfunction is always present: increased sweating (hyperhidrosis), with sebaceous secretion of the skin (especially the face), constipation; urination disorders (pollakiuria, nocturia, incontinence) are rarer and alterations of thermoregulation are possible. Orthostatic hypotension occurs clinically in about 10% of cases with dizziness and presynopal phenomena, without assuming the severity and earliness that distinguishes Shy-Drager syndrome. Sleep disturbances can be considered a frequent occurrence (60-90% of parkinsonism cases) [183] and include, among other things, parasomnias (vivid dreams, nightmares, REM sleep behavioral disorders) and motor disorders (myoclonus , restless legs syndrome, periodic sleep movements).

- ***Cognitive alterations*** - The detection of disturbances of affect (dysphoric states, anxiety crisis, depressive states, asthenia, abulia, inertia) is a frequent and long-known observation. More complex, however, is the problem of impaired cognitive functions: the presence of cognitive deficits has been demonstrated by epidemiological and neuropsychological studies, but important controversies remain regarding the precise nature of neuropsychological alterations, their neuropathological and neurochemical substrate, the actual prevalence. Despite the large percentage differences reported in the literature, it can be assumed that about 20% of parkinsonian subjects also present a picture of dementia [184]. In view of the possible development of an impairment of cognitive functions, the use of anticholinergic drugs (especially in patients of older age) must always be considered with caution. The neuropsychological characteristics of parkinsonian intellectual deterioration have been analyzed not only with respect to normal control subjects, but above all with respect to dementia with Alzheimer's disease. Although there are some similarities, the type of neuropsychological impairment is very different in these two pathologies suggesting in Parkinson's disease a prevalent involvement of the functions related to the frontal lobes (visual-spatial impairment, impaired verbal fluency, attention deficit, reduced ability to late memory, perseveration, alterations of the sequential and temporal order) [184]. The neuropathological and neurochemical bases of intellectual deterioration are highly controversial. The hypothesis of a correlation between mental deterioration and neuropathological alterations typical of Alzheimer's disease (presence of neurons in neurofibrillary degeneration and senile plaques) is not currently receiving consensus, even if the possible concomitance between Alzheimer's disease and Parkinson's disease is accepted. Alternative hypotheses are constituted by the dopaminergic deafferentation of the frontal cortex and by the cortical deafferentation following the depletion of

cholinergic neurons in the basal nucleus of Meynert and noradrenergic in the locus coeruleus. It is possible that, more than a single form of dementia, Parkinsonians suffer from different intellectual impairments, linked to different lesion sites and specific neurotransmitter deficits.

However, it should be remembered that a correct assessment of cognitive impairment in parkinsonians is made difficult by the existence of numerous factors of variability related to motor impairment, bradyphrenia, the effects of therapy, and socio-environmental isolation.

6.1.5 Therapy

Failure to identify the cause of the disease makes causal therapy impossible in the current state of knowledge. The treatment is therefore symptomatic, that is, aimed at compensating for the dopaminergic deficit underlying the main symptoms. Alongside it, neuroprotective therapies have recently been proposed, aimed at slowing the progression of the disease, and neurosurgical approaches.

- ***Symptomatic treatment*** - Many drugs can have a positive effect on parkinsonian symptoms, among which levodopa remains the most reliable and effective drug. A heated controversy, however, arose (as a consequence of the possible development of the "prolonged treatment syndrome with levodopa") and the type of drugs and the time of use. In general, the therapeutic choices must be adapted to the individual in relation to his specific conditions and expectations. In the initial stages of the disease (when motor impairment is mild, such as not to interfere with daily activities), symptomatic treatment may be unnecessary, or drugs with potential neuroprotective (selegiline), anticholinergic and amantadine significance may be used. Anticholinergics (trihexyphenidyl, biperidene, orfenadrine, bornaprine) were the first drugs used in the treatment of the disease: blocking muscarinic receptors at the level of striatal interneurons are able to attenuate the relative cholinergic hyperfunction and induce a

clinical benefit (quantifiable, however, , to an extent not exceeding 20%), in particular, on rigidity, tremor, sialorrhea. The most frequent side effects of anticholinergic substances are xerostomia, nausea, dizzy sensations, accommodation disorders, sometimes urinary retention and confused-hallucinatory states; also hypersensitization is frequent. The use of anticholinergics, currently much less widespread, can interfere with the absorption of levodopa, is contraindicated in glaucoma and not recommended in subjects over 65-70 years of age due to the possibility of inducing memory, cognitive and psychic disorders. Amantadine has a modest anticholinergic action and also has a partial indirect dopamine agonist effect by promoting the release of dopamine from the accumulation sites. The therapeutic effect is early, affecting both akinesia and rigidity, but usually of limited size and duration. Side effects (edema, livedo reticularis) are infrequent. Its current use is linked to the demonstration of a distinct anti-dyskinetic effect. As the disease progresses, a more adequate symptomatic treatment becomes necessary, and the problem therefore arises of the choice between the use of levodopa and that of dopamine agonists (or their association). Levodopa which, as mentioned, constitutes the immediate natural precursor of dopamine in which it is converted by the aromatic decarboxylase enzyme, is currently administered in association with peripheral decarboxylase inhibitors (carbidopa or benserazide) which have allowed a significant reduction of gastro side effects enteric (nausea, vomiting) and cardiovascular (orthostatic hypotension, rhythm disturbances). Levodopa induces in the vast majority of patients a rapid and significant improvement (quantifiable in excess of 50%) of symptoms, in particular, of rigidity and akinesia, much less than tremor. The limit to the use of levodopa is constituted by the fact, already mentioned, that a large percentage of patients (with an incidence between 5-10% per year of treatment) undergo a "variable syndrome" after a variable number of years. protracted treatment with levodopa

"(inadequate and declining therapeutic response, clinical fluctuations, dyskinesias). In this regard, the introduction on the market of delayed decommissioning levodopa preparations may allow for a more physiological dopaminergic stimulation, but does not seem to be able to reduce the risk of developing clinical fluctuations. Dopaminergic agonists are able to directly stimulate postsynaptic receptors (with different specificity towards the five subtypes, D1-D5), do not induce the formation of free radicals, are characterized by a longer duration of action and some can be administered via parenterally. They are divided into ergotderivatives (bromocriptine, pergolide, lysuride, cabergoline) and non-ergolinics (apomorphine, piribedil, pramipexole, ropinirole). These drugs demonstrate a definitely lower efficacy than that of levodopa if used as monotherapy: only a minority of patients, around 25%, retains sufficient symptomatic benefit over time from their use. Direct dopaminergic agonists, however, are associated with a lower risk of clinical fluctuations or dyskinesias and indeed their early use can prevent or delay their appearance [185]. A therapeutic strategy based on the early association of levodopa and dopaminergic agonists is also possible. Among the main side effects of dopaminergic agonists, it is worth mentioning: nausea, orthostatic hypotension, mental disorders, edema of the lower limbs, erythromelalgia. The appearance of the clinical phenomena associated with the continued use of levodopa and the long duration of illness always represents a painful (and sometimes dramatic for the sick) event and a therapeutic problem that is very difficult to solve. The reduction of single doses of levodopa, increasing the number of administrations, despite the apparent initial benefit, accentuates the instability of the plasma levels of levodopa (favoring the formation of sub-therapeutic concentrations) and makes the patient more exposed to fluctuations related to peripheral pharmacokinetic factors (delayed gastric emptying, competition with food proteins). Partial results can be obtained with delayed

disposal levodopa preparations, the association with dopaminergic agonites, the infusion of lysuride and apomorphine or the association with catechol-methyl-transferase (COMT) inhibitor drugs (tolcapone, entacapone) which result in an increase in the plasma half-life of levodopa. Despite these strategies, many patients evolve towards a full-blown picture of clinical fluctuations, which cannot be adequately managed on a pharmacological level. The problem of dyskinesias can be tackled by reducing the dose of levodopa, but often at the expense of an accentuation of akinesia, only partially compensated by the increase in the dose of dopaminergic agonists. The use of amantadine [186] has recently been proposed in order to reduce dyskinesias. Sialorrhea can be alleviated by the use of anticholinergics or by the use of infiltration of the salivary glands with botulinum toxin, orthostatic hypotension may require the use of fluorohydrocortisone (not commercially available in Italy), alterations in the sphincter lines involve measures specific. Other symptoms of the more advanced stages of the disease are insensitive to drug treatment, in particular: the accentuation of the "freezing" phenomena, the increasing postural impairment (with possible falls to the ground), the alterations of the word's articulation. Some neuropsychiatric pictures, which occur frequently, can be treated effectively. The depressive symptoms (not only reactive to one's condition of disability) are positively affected by the use of tricyclic and serotonergic drugs; anxious manifestations can be attenuated by diazepam or beta blockers. Hallucinatory phenomena can be controlled with atypical neuroleptics (clozapine, olanzapine, quetiapine) which act on D4 receptors and are characterized by a lower incidence of extrapyramidal side effects. The appearance of confusional states can be tentatively addressed with the suspension of anticholinergic preparations and, possibly, with short transitory suspensions of dopamine replacement therapy. At the

moment, however, there are no therapeutic aids to deal with the progressive appearance of cognitive disorders.

- ***Neuroprotective treatment*** - Neuroprotection is a therapeutic intervention aimed at slowing down or blocking the progression of neuronal degeneration by acting on pathogenic mechanisms. Numerous drugs (antioxidants, anti-excitotoxic, anti-apoptotic, neurotrophic) have been proposed or tested. In particular, it has been suggested that the use of selegiline (Deprenyl), an irreversible inhibitor of the monoamine oxidase type B enzyme, could modify the natural evolution of the disease, postponing the use of levodopa; it is likely, however, that the observed results are largely attributable to a delayed symptomatic effect. Other substances (e.g. NMDA antagonists, dopamine agonists) are currently under study.

6.2 Alzheimer's disease

Alzheimer's disease is the most common cause of dementia in the elderly population of western countries, accounting for around 50-60% of cases of mental deterioration. The term Alzheimer identifies a primary degenerative brain disease, due to the progressive and irreversible death of brain cells, which leads to the loss and ability to communicate with each other. In other words, it is the progressive loss of the associative capacity of the different areas of the cerebral cortex. Therefore, there is deterioration of cognitive abilities and the appearance of behavioral and affective disorders that inexorably lead the patient to a loss of functional autonomy and the inability to maintain relationships with the surrounding environment.

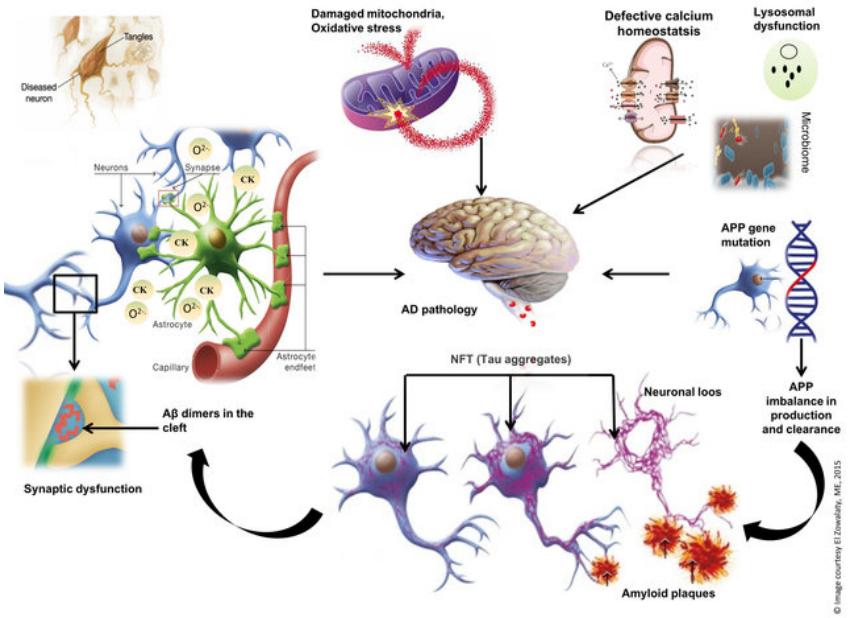

Figure 9. Schematic representation of the pathology of Alzheimer's disease

6.2.1 Epidemiology

AD is the most frequent cause of dementia over the age of 65 and is believed to represent around 60% of all cases of dementia.

The prevalence of dementia in industrialized countries is around 8% in the over-65s and rises to over 20% after the age of eighty. It is estimated that currently around 600 thousand people in Italy are affected by AD, equal to 4% of the over 65 population [187].

As a consequence of the aging of the world population, the prevalence of this disease is expected to increase by about four times in the coming decades. Recent estimates predict that more than 74 million individuals will suffer from this type of dementia by 2030 and 131 million by 2050 [187]. Therefore, the CEO is destined to become one of the most important social and health emergencies in the world, with a dramatic increase in the costs of dementia worldwide.

Women have an almost double risk of developing AD compared to men and the prevalence of the disease is much higher, especially in the older age groups. Although higher male mortality may partially explain these differences, it is clear that other factors come into play [188]. In fact, females live significantly longer than males [189]. In women, however, the survival

advantage is associated with a worse quality of life in old age, characterized by an increase in disability and in the prevalence of degenerative diseases [190]. Therefore men and women undergo a qualitatively different aging process [191].

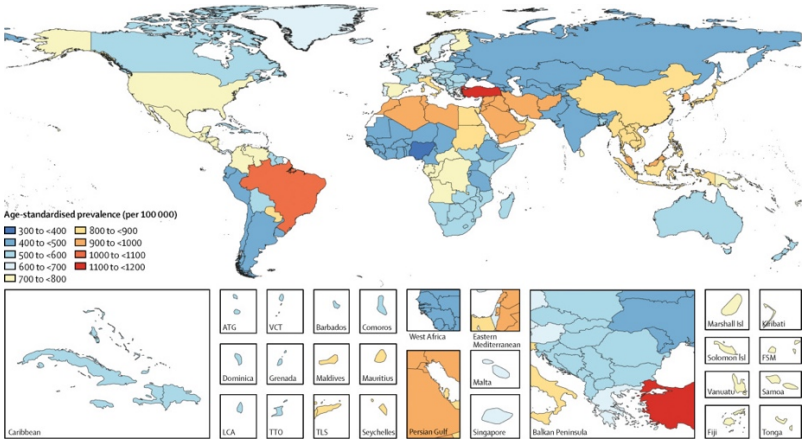

Figure 10. Global, regional, and national burden of Alzheimer's disease and other dementias

6.2.2 Clinical features and pathological changes

AD is a primitive, irreversible and progressive neurodegenerative disease, characterized by the loss of multiple cognitive functions of an amount that interferes with the patient's usual social activities. In addition to cognitive symptoms, alterations affecting the sphere of personality, affectivity, ideation, perception, vegetative functions and behavior are present, especially in the moderate-advanced stages of the disease.

In AD, the first changes in the cerebral cortex are found mainly in the entorhinal region, located in the anterior portions of the hippocampal gyrus of the temporal lobe [192]; these modifications subsequently affect other areas of the cerebral cortex and specific groups of subcortical nuclei. On macroscopic examination, the brain of a patient with AD shows a variable degree of cortical atrophy, which can be found in the autopsy both with the decrease in the weight and volume of the organ, and with the thinning of the neocortical layers and

convolutions, which involves regions involved in memory and learning processes, such as the temporal, parietal, frontal cortex, the hippocampus and the amygdala.

Atrophy is mainly due to neuronal degeneration; this is accompanied by a compensatory ventricular enlargement, secondary to the loss of parenchyma. In reality, all macroscopic changes found in AD patients are also found in healthy elderly subjects and are an expression of physiological brain aging. However, AD is characterized by their greater quantity and their peculiar distribution.

At the histological level, there are the accumulation of two different proteins: the β-amyloid protein (Aβ), which is deposited at the extracellular level, and the phosphorylated Tau protein (pTau) which constitutes intracellular neurofibrillary clusters. Aβ is organized in different forms, the senile plaques, the diffuse plaques and the so-called amyloid angiopathy.

Senile plaques are made up of a compact central part of insoluble fibrils of the Aβ protein, surrounded by a phlogistic agglomerate with degenerating neurites, reactive astrocytes and activated microglia, activated monocytes or macrophages derived from the reticuloendothelial system residing in the central nervous system (CNS); they are found above all at the hippocampus and limbic structures, the amygdala, the subiculum and the entorhinal cortex. The diffuse plaques are instead composed of poorly structured material and lack the neuritic component. Amyloid angiopathy is caused by the deposition of Aβ at the level of the walls of the cerebral and meningeal arteries.

The neurofibrillary tangles are made up of compact bundles of anomalous filaments made up mainly of the Tau protein; they mainly affect the soma, but can also extend to the dendrites. The tangles are found mainly at the level of the medium-sized pyramidal neurons of the entorhinal cortex, the limbic cortex, the hippocampus, the amygdala, the neocortical layers of the frontal and temporal lobes and the cholinergic systems of the nuclei of the base.

6.2.3 Pathogenesis

The etiopathogenesis of AD is still unknown, despite the efforts made by the scientific community in recent decades. The most accredited theory is the amyloid one, supported by the autoptic observation of plaques containing this molecule at the brain level of patients with AD. The cerebral senile plaques are made up of Aβ, which derives from APP by means of a proteolytic process [193]. It has functions of adhesion to the extracellular matrix, receptor and of modulation of gene expression. Furthermore, secreted APP molecules participate in the formation of synapses and probably play a role in the integrity of the mnemonic process.

The fragments of Aβ40 and Aβ42 aggregate spontaneously to form a β-toxic structure for neurons and synapses, both in its early, monomeric, dimeric or oligomeric form, and as a deposit in aggregates, the senile plaques. The toxic effects of Aβ are believed to cause dementia development [194].

In the healthy subject the two pathways of APP processing are in balance with each other, while it is believed that in the individual with Alzheimer's disease an unbalance condition is created, with the prevalence of the amyloidogenic pathway over the non-amyloidogenic pathway. This determines a greater production of Aβ40 and Aβ42 and a consequent oversaturation of the structures responsible for its disposal, which are already compromised due to aging.

Three observations would not support the amyloid hypothesis. The first is functional: Aβ is normally present in the brain and the concentration of soluble Aβ is higher in the brain of young people than in non-demented elderly people [195] and a possible role of Aβ could be to modulate neuroplasticity [196, 197]. The second observation regards the fact that 20-30% of the cognitively healthy subjects have a significant number of pre or post-mortem amyloid plaques [198, 199]. Therefore, their presence cannot alone justify the onset of AD, suggesting that the deposition of plaques is actually a paraphysiological phenomenon and that discriminating for the onset of AD may be the site where they are deposited, as well as interaction with other cofactors, equally important for the development of the disease. In

addition, it should be noted that immunotherapy can remove Aβ plaques, but does not affect the progression of dementia [200, 201]. The third observation highlights that individuals with fully penetrating mutations of both APP and γ-secretase do not develop dementia until 30-40 years of age, thus suggesting that the toxic effect is due to the accumulation and deposition of amyloid at the brain level, accompanied by other factors [202]. Therefore, in recent years more and more evidence indicates that Aβ acts as a trigger in the early disease process and appears to be necessary but not sufficient in the late AD phase [203].

The neurofibrillary tangles, containing pTau protein, together with the senile plaques, constitute a typical anatomic-pathological finding in AD patients and therefore make the involvement of this protein in the etiopathogenesis of the disease highly probable. The Tau protein is the main neuronal protein associated with microtubules, whose function is to assemble tubulin into microtubules in the cytoskeleton supporting structure. The dysfunction of the Tau protein in AD is due to its hyperphosphorylation, mediated by the activation of a series of protein kinases. This anomaly has two important repercussions: on the one hand, the pTau sequesters the normal one and the other proteins associated with the microtubules, causing disassembly of the microtubules and alterations in axonal transport and neuronal plasticity, on the other it is prone to aggregation with the formation neurofibrillary tangles, which compromise the neuron and synaptic function.

The neurofibrillary tangles are not, however, specific for AD, having been observed in many other neurodegenerative diseases, to the point that the generic term "Taupatie" has been coined. The inflammatory reaction that characterizes most neurodegenerative disorders is called "neuroinflammation" and mainly involves components of innate immunity. Activated microglia and astrocytes are the main cells that take part in this response in AD. Chronic activation of the immune response is observed in AD, which probably plays a harmful role and contributes to the progression of the disease. On the other hand, the involvement of the immune system could

play a positive role in the acute, for example through processes of phagocytosis and production of trophic and repair factors, helping to limit the progression of the disease itself.

The presence of Aβ plaques could keep microglia constantly activated, leading to a condition of chronic inflammation in the CNS; various studies on glial cell cultures and macrophages have shown that Aβ stimulates the synthesis and release of pro-inflammatory cytokines [204] and overexpression of the same cytokines has been observed in the brains of patients with AD [205]. Polymorphisms present in the genes of some cytokines (IL-1, IL-6, TNF-α) and acute phase proteins (α1-antichymotrypsin) have been shown to be associated with an increased risk of AD [206]. In addition, recent genetic and transcriptome studies have supported the hypothesis of the involvement of pathways from microglia in the pathogenesis of AD [207-209] and numerous evidences have shown that microglia play a central role from the early stages of the pathology. Microglia, activated by the trigger receptor expressed on type II myeloid cells (TREM2) and by the complement system, is responsible for the synaptic alteration found in AD [210, 211]. Furthermore, an alteration, both centrally and peripherally, of the expression of TREM2 has been demonstrated in patients with AD and in the preclinical phases of the disease, which has a peculiar role in the activation of microglia [212]. The demonstration of an activation of inflammatory processes in the early stages of the disease would support the hypothesis of inflammation as "primum movens", although inflammation could play a key role in the etiopathogenesis of the disease even if it intervened later.

Aβ plaques also induce the proliferation of astrocytes, glial cells, which would participate in the clearance and degradation of Aβ by forming a protective barrier between Aβ deposits and neurons. The neurons themselves, reacting to toxic stimuli, could say that they themselves are a source of complement factors, prostanoids and various cytokines. Many of these factors can promote neurodegenerative mechanisms, while others can counteract the spread of inflammation or have beneficial neurotropic effects.

"Neuroinflammation" could therefore be the primary cause of AD or be a consequence of it.

Oxidative stress is the result of a dysregulation between the amount of free and non-free radicals produced. This can be attributed to the loss of homeostasis caused by the mitochondrial overproduction of oxidants compared to the production of antioxidants [213]. The brain uses more oxygen than other tissues, which increases the potential for ROS exposure. ROS, as well as RNS, are produced in physiological conditions by the mitochondria, act on the second messengers and can influence the signal pathways [214]. Similarly, mitochondria are able to produce antioxidants that neutralize the harmful effects of reactive oxygen species, to maintain the balance between ROS production and detoxification.

The development of oxidative stress in AD has been linked to mitochondrial dysfunction, which leads to the overproduction of ROS and RNS and which leads to synaptic damage. Several authors have observed a decrease in mitochondrial activity in transgenic mice for AD and mitochondrial dysfunction that could induce an increase in the production of Aβ [215, 216]. The authors suggested that the progressive increase in oxidant production related to a decrease in antioxidant components could cause the loss of cerebral homeostasis observed in AD patients. Although the role of oxidative stress in neurodegenerative diseases is not fully understood, increased oxidation and nitration of proteins, glycosylation and lipid peroxidation have been observed and it has been seen that the accumulation of Aβ can induce oxidative stress [217, 218]. Furthermore, there is a modest level of oxidative damage to RNA in the neurons of patients in the early stage of cognitive decline [219] and studies have shown alterations in the oxidative markers in the hippocampus and in the lower parietal cortex, regions mainly compromised in AD [220].

Glucose hypometabolism is an early pathogenetic event observed in the prodromal phase of AD and associated with the cognitive and functional decline of patients affected by the disease [221, 222]. At the basis of AD there may be a decrease in the synaptic / metabolic brain activity.

Glucose is essential for the release and re-uptake of neurotransmitters, for post-synaptic terminal activity, for depolarization and repolarization of axons. Some data indicate that synaptic activity and brain metabolism seem to regulate the activity and expression of β-secretases and APP-derived proteins, which increase in response to a decrease in metabolic activity. Decreased metabolic brain activity has been shown to be associated with AD [223].

This interpretation would allow to combine the different risk factors for dementia in a single hypothesis and would direct research on the identification of the factors that determine a decrease in brain metabolic activity, in order to maintain it adequate and prevent the onset of the disease [194].

AD is conventionally considered a CNS disorder. However, growing experimental, epidemiological and clinical evidence has suggested that the manifestations of AD may extend beyond the brain. In particular, research in recent years reveals that the intestinal microbiota has a profound impact on the formation of the blood-brain barrier, on myelination, on neurogenesis and on the maturation of microglia [224, 225]. In particular, tests on animals exposed to pathogenic microbial infections, antibiotics, probiotics or faecal microbiota transplantation have shown that the intestinal microbiota modulates many behavioral aspects of animals, suggesting its role in AD cognition and pathogenesis [226-228] . The mechanisms underlying the influence of the intestinal microbiota in the brain could involve the immune system, the endocrine system, the vagus nerve and the metabolites derived from bacteria.

6.2.4 Therapy

Currently, there is still no pharmacological treatment capable of curing Alzheimer's disease, that is, of restoring cognitive functions and memory to the patient. The most commonly used drugs are those that increase acetylcholine levels, since Alzheimer's leads to a decrease in this. They are called "acetylcholinesterase", have the function of blocking an enzyme that demolishes acetylcholine and would seem to be able to slow down the evolution of the disease,

the loss of autonomy and improve the control of some behavioral disorders such as apathy and psychotic symptoms. Memantine is used for patients with moderate and / or severe Alzheimer's disease: memantine works by blocking the harmful effect of glutamate, while the three cholinesterase inhibitors are donepezil, galantamine, rivastigmine and are used in the mild or moderate phase of the disease. Furthermore, antidepressant drugs can also be useful to the patient as frustration with the event and the continuous and incessant loss of memory pieces often leads to depression and closure in themselves.

Both acetylcholinesterase inhibitors and memantine are distributed free of charge by the National Health Service, after an evaluation of the patient at the Alzheimer's Assessment Units. Unfortunately, not all patients respond positively to treatment, but an attempt must always be made with everyone unless there are important contraindications. Non-pharmacological therapies can also help the patient a lot, such as music therapy and / or dance therapy, where patients are made to listen to music, sometimes accompanied by the rhythmic clapping of the hands. Often with this method you get participation and attention from the participants that usually is not there. Pet therapy is also widely used, which is based on the presence of trained animals that keep the patient’s assistance, arousing interest and satisfaction. However, we must always remember that Alzheimer's dementia cannot be cured and that is why it is essential to guarantee assistance and personalization of care to all affected subjects, not only with drugs but also by learning to listen to them and offering them a safe place.

Chapter 7: EXPERIMENTAL MODEL OF TRAUMATIC BRAIN INJURY

7.1 Materials and Methods

7.1.1 Animals

Male CD1 mice (25 to 30 g, Envigo, Italy) were used for this study. Mice were accommodated in cages (five in each) and kept under a light/dark cycle at controlled humidity. Standard diet and water were provided ad libitum. The Review Board for the care of animals of the University of Messina approved the study. We respected the legislation for the protection of laboratory animals (D.Lgs 2014/26 and EU Directive 2010/63).

7.1.2 Controlled cortical impact (CCI) experimental TBI

TBI was induced in mice by a controlled cortical impact (CCI) as previously described [229]. Briefly, a craniotomy was made encompassing bregma and lambda and between in the sagittal suture and the coronal ridge the right hemisphere, using a Micro motor hand piece and drill. The ensuing bone flap was removed and on the exposed cortex a cortical contusion was produced using the controlled impactor device Impact OneTM Stereotaxic impactor for CCI (Leica, Milan, Italy). The impact tip was lowered over the exposed cortex until it touched the dura mater. The rod was then retracted and the tip was advanced to induce a brain injury of moderate severity (tip diameter: 2mm; cortical contusion depth: 1mm; impact velocity: 3.6 m/s) [230]. Subsequently, the skin incision was sutured and 2% lidocaine jelly was applied to the lesion to minimize any possible discomfort.

Experimental design

Animals were randomized in the indicated groups (10 mice for each group):

- Sham + vehicle group*:* the described surgical procedures were applied except that the impact was not applied and animals were administered o.s. with vehicle (saline);

- Sham + Hericium erinaceus the described surgical procedures were applied except that the impact was not applied and animals were administered o.s. with *Hericium erinaceus* 300 mg/kg (data not shown);
- Sham + Moringa oleifera: the described surgical procedures were applied except that the impact was not applied and animals were administered o.s. with *Moringa oleifera* 150 mg/kg (data not shown);
- Sham + Hericium erinaceus + Moringa oleifera*:* the described surgical procedures were applied except that the impact was not applied and animals were administered o.s. with *Hericium erinaceus* (300mg/kg) + *Moringa oleifera* (150mg/kg) (data not shown);
- TBI + vehicle group: the described surgical procedures were applied and animals were administrered o.s. with vehicle;
- TBI + Hericium erinaceus: the described surgical procedures were applied and animals were administrered o.s. with *Hericium erinaceus* (300 mg/kg) 1h after TBI and once a day for 10 days for the behavioral test;
- TBI + Moringa oleifera: the described surgical procedures were applied and animals were administrered o.s. with *Moringa oleifera* (150 mg/kg) 1h after TBI and once a day for 10 days for the behavioral test;
- TBI + Hericium erinaceus+Moringa oleifera: the described surgical procedures were applied and animals were administrered o.s. with *Hericium erinaceus* (300mg/kg) + *Moringa oleifera* (150mg/kg) 1h after TBI and once a day for 10 days for the behavioral test.

The tested doses were chosen based on previous experiments executed in our laboratories. Mice were sacrificed 1 day after surgical procedures brain was collected for histological and biochemical investigation. For behavioral testing mice were sacrificed 10 days after traumatic brain injury.

7.1.3 Behavioral assessment

Elevated pluz-maze Test: The Elevated pluz-maze Test (EPMT) test was performed as described previously [231]. The EPMT apparatus (Panlab, S.L.- Harvard Apparatus, Spain) was employed. The test was based on rodents' aversion of open spaces that leads to thigmotaxis. The percentage of time spent on the open arms and total numbers of arm entries and closed-arm entries was recorded.

Elevated Biased Swing Test: The Elevated Biased Swing Test (EBST) comprised of 20 trials with the number of swings contralateral and ipsilateral to the injured hemisphere recorded and expressed in percentage to evaluate the biased swing activity. This analysis was performed as previous described [232].

Open Field Test: Anxiety-like behavior and locomotor activity were evaluated for 5 min using the Open Field Test (OFT). To perform the test a white Plexiglas box 50 × 50 cm with its floor divided into 16 squares was used. The animals' behavior was videotaped. The line crossings and the time spent in the center were counted and scored [233, 234].

7.1.4 Histology

An experienced histopathology evaluated coronal sections of 5-µm thickness from the perilesional brain area of each animal. Histopathologic changes of the gray matter were evaluated on a six-point scale [235]: 0, no lesion observed; 1, gray matter contained one to five eosinophilic neurons; 2, gray matter contained five to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections of each

brain were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion [236].

7.1.5 Western Blot Analysis for IkB-α, NF-kB, Bax, Bcl-2, GFAP and Iba-1

Western Blots was made as previously described [237]. Filters were blocked with 1× PBS, 5% (w/v) non-fat dried milk (PM) for 40 min at room temperature and then probed with one of the next primary antibodies: anti-IkB-α (1:500, Santa Cruz Biotechnology), or anti-NF-kB (1:500, Santa Cruz Biotechnology), or anti-Bax (1:500, Santa Cruz Biotechnology), or anti-Bcl-2 (1:500, Santa Cruz Biotechnology), or anti-GFAP (1:500, Santa Cruz Biotechnology), or anti-Iba-1 (1:500, Santa Cruz Biotechnology), in 1× PBS, 0.1% Tween-20, 5% w/v no fat dried milk (PMT) at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Blots were also incubated with primary antibody against β-actin protein (1:10,000; Sigma-Aldrich Corp.) or laminin (1:10,000; Sigma-Aldrich Corp), used as internal standards. The relative expressions of the protein bands of IkB-α (37 kDa), NF-kB p65 (65 kDa), Bax (23 kDa), Bcl-2 (29 kDa), GFAP (50 kDa) and Iba-1 (17 kDa) were detected and quantified by densitometry as previously explained by Cordaro et al [238]. In the experiments including western blot, a representative blot is displayed and densitometric analysis is related in each figure.

7.1.6 Malondialdehyde Content, Superoxide Dismutase, and Glutathione Peroxidase Activity

Levels of malondialdehyde (MDA) content (nmoL/mg), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity (U/mg protein) were measured using a spectrophotometer (Nanjing Jiancheng Biochemistry Co, China).

7.1.7 Cytokines measurement

Tumor Necrosis Factor-(TNF-α) and interleukin 1β (IL-1β) levels were evaluated using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy).

7.1.8 Materials

All composites were gotten from Sigma-Aldrich. All other substances were of the uppermost profitable grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Milan, Italy).

7.1.9 Statistical evaluation

All values in the images and text are expressed as mean ± standard error of the mean (SEM) of N observations. For *in vivo* studies, N represents the number of animals. In experiments involving histology the illustrations represent the outcomes of at least three independent experiments. A *p*-value of less than 0.05 was considered significant. The results were analyzed by one- or two-way ANOVA, followed by a Bonferroni post-hoc test for multiple comparisons. * p>0,05 vs Sham; *** p>0,001 vs Sham; # p<0,05 vs vehicle; ## p< 0,01 vs vehicle; ### p< 0,001 vs vehicle.

7.2 Results

7.2.1 Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on behavioral alterations TBI-induced.

Using EBST test we estimate motor function deficiency in mice 24 h after TBI. Sham group did not display any issue in swing behavior (Fig. 1A) whereas Moringa oleifera and the association between Hericium erinaceus and Moringa oleifera treatment appreciably ameliorated influence on the swing bias (Fig. 1A) compared to the vehicle group. Analysis of the OFT during the days showed a significantly rise in the total distance traveled in vehicle treated animals compared to sham group (Fig. 1B). On the other hands only Moringa oleifera treatment was able to ameliorated anxiety status. Additionally, to have further confirmation of

the anxious state, a decisive component of behavioral change after brain injury, CCI injured animals were subjected to the EPMT at 1, 2, 3, 6, and 10 days; also, mice were treated with Moringa oleifera, every day, observing an improved latency compared to vehicle group. No significantly amelioration were found in animals treated with Hericium erinaceus or Hericium erinaceus plus Moringa oleifera (Fig. 2).

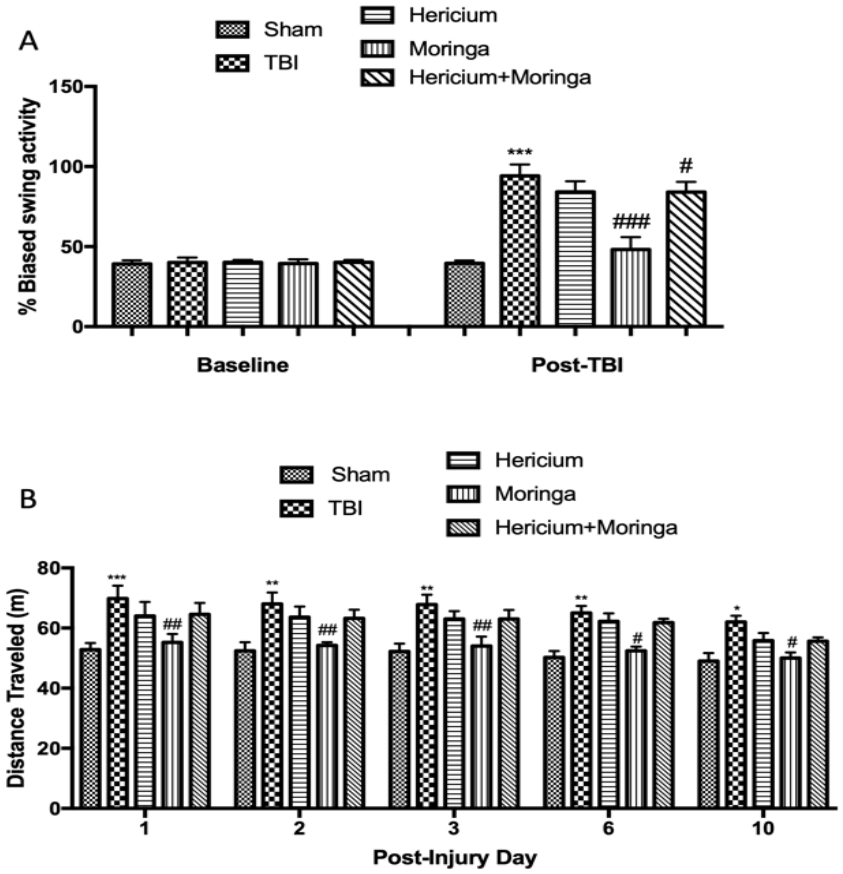

Figure 1: Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on motor functions alterations TBI-induced. Elevated Biased Swing Test (A) and Open Field Test (B).

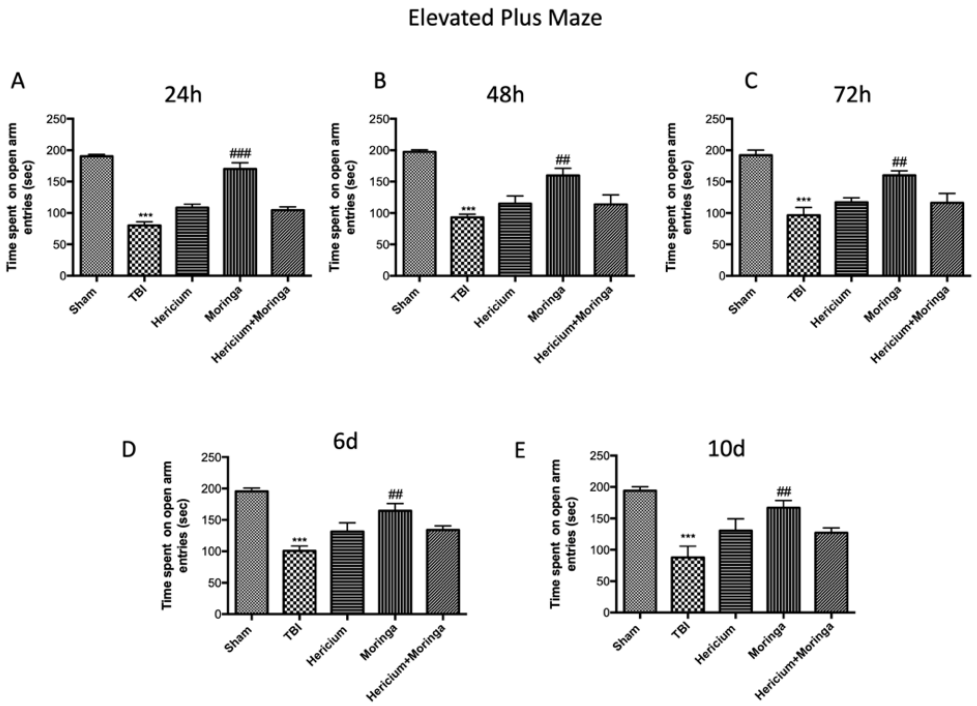

Figure 2: Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera on anxious state alterations TBI-induced. Elevated Plus Maze Test at different time point: (A) 24h, (B) 48h, (C) 72h, (D) 6 days and (E) 10 days.

7.2.2 Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on histological injury TBI-induced.

Histological examination of the brain collected by TBI animals, at 24 h post-impact, showed significant tissue disorganization and white matter alteration (Fig. 3 B, see histological score 3F) compared to sham mice (Fig. 3 A, see histological score 3F). We showed that the treatment with Moringa oleifera (150 mg/kg) or Hericium erinaceus plus Moringa oleifera (300 mg/kg+150 mg/kg) notably reduced the degree of brain injury (Fig. 3D and 3F, see histological score 3F). No significant improvements were found after Hericium erinaceus treatments (Fig. 3C see histological score 3F).

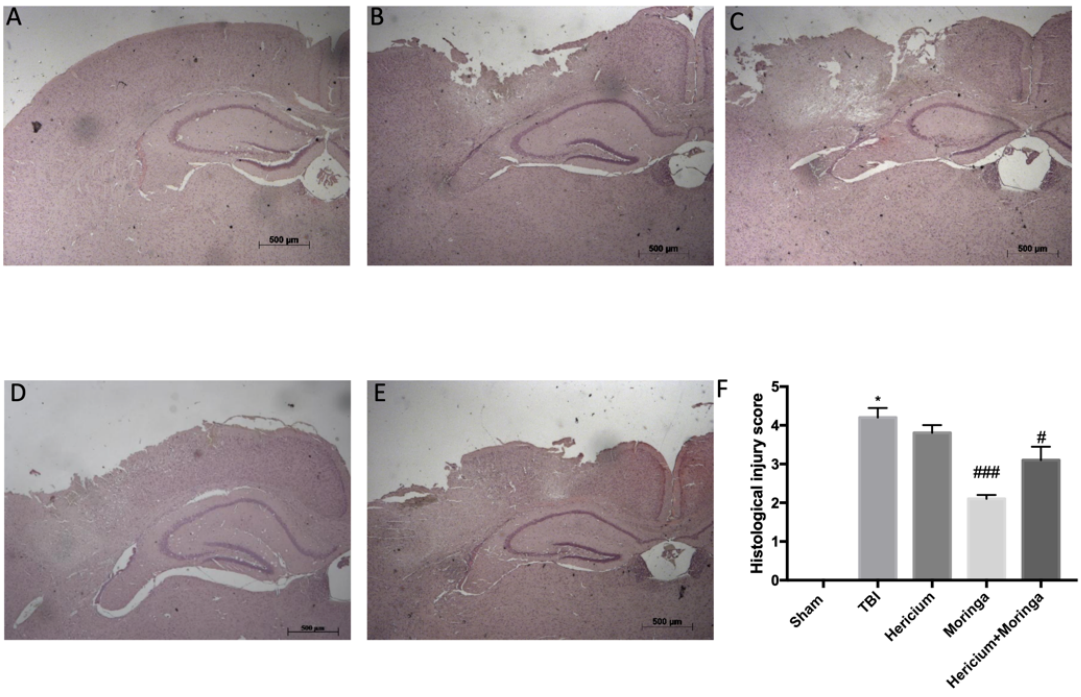

Figure 3: Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on histological injury TBI-induced. H/E of brain sections: (A) Sham; (B) TBI; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F) histological score.

7.2.3 Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, decreased TBI-induced IκB-α degradation, NF-κB p65 nuclear translocation and pro-inflammatory cytokines expression.

To enhanced our information on the molecular mechanism underlying the anti-inflammatory effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera actions IκB-α cytosolic expression and NF-κB p65 nuclear expression were evaluated by Western blot analysis. The expression of IκB-α significantly diminished in brain tissue from vehicle group, compared to sham group (Fig. 4A see densitometric analysis C); oral treatment with Moringa oleifera, or Hericium erinaceus plus Moringa oleifera significantly limited TBI-induced IκB-α expression. *Viceversa*, nuclear translocation of the NF-κB subunit p65 increased in brain tissue from vehicle administered mice, when compared to sham (Fig. 4B see densitometric analysis D), while only orally treatment with Moringa oleifera significantly decreasing p65 nuclear expression. Additionally, in this study, we evaluated whether pro-

inflammatory cytokines are associated with TBI. High levels of TNF-α (Fig. 4E) and IL-1β (Fig. 4F) were found in vehicle-group compared to sham. Moringa oleifera or Hericium erinaceus plus Moringa oleifera orally administration substantially reduce these proinflammatory cytokines levels (Fig. 4E and 4F).

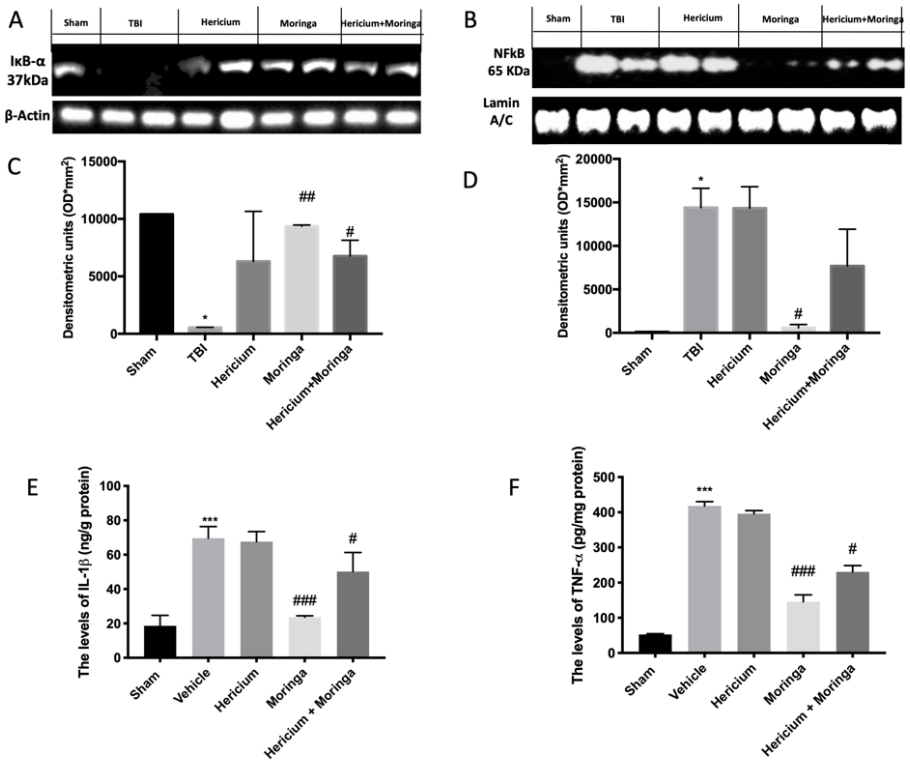

Figure 4: Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera decreased TBI-induced IκB-α degradation, NF-κB p65 nuclear translocation and pro-inflammatory cytokines expression. Representative Western blots of brain tissues showed the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on: (A) IκB-α degradation, (B) NF-κB p65 translocation after TBI, densitometric analysis (C) and (D) respectively. Additionally, IL-1β (E) and TNF-α (F) levels were detected.

7.2.4 Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on oxidative stress.

Oxidative stress was evaluated by malondialdehyde (MDA) (Fig. 5A), superoxide dismutase (SOD) (Figure 5B) and glutathione peroxidase (GPx) (Fig. 5C) levels. MDA levels were found increased in vehicle-treated group compared to the sham mice, while SOD and GPx were down-regulated. Moringa oleifera or Hericium erinaceus plus Moringa oleifera administrations

reduced MDA levels and increased SOD and GPx. In addition, we wanted to evaluate the effect of these three natural compounds on Nrf2. We observed no significantly modifications in the brain of vehicle group compared to sham group (Fig. 5D see densitometric analysis E); while a considerable activation of Nrf-2 was found after Hericium erinaceus or Hericium erinaceus plus Moringa oleifera treatment and in particular after the administration of Moringa oleifera.

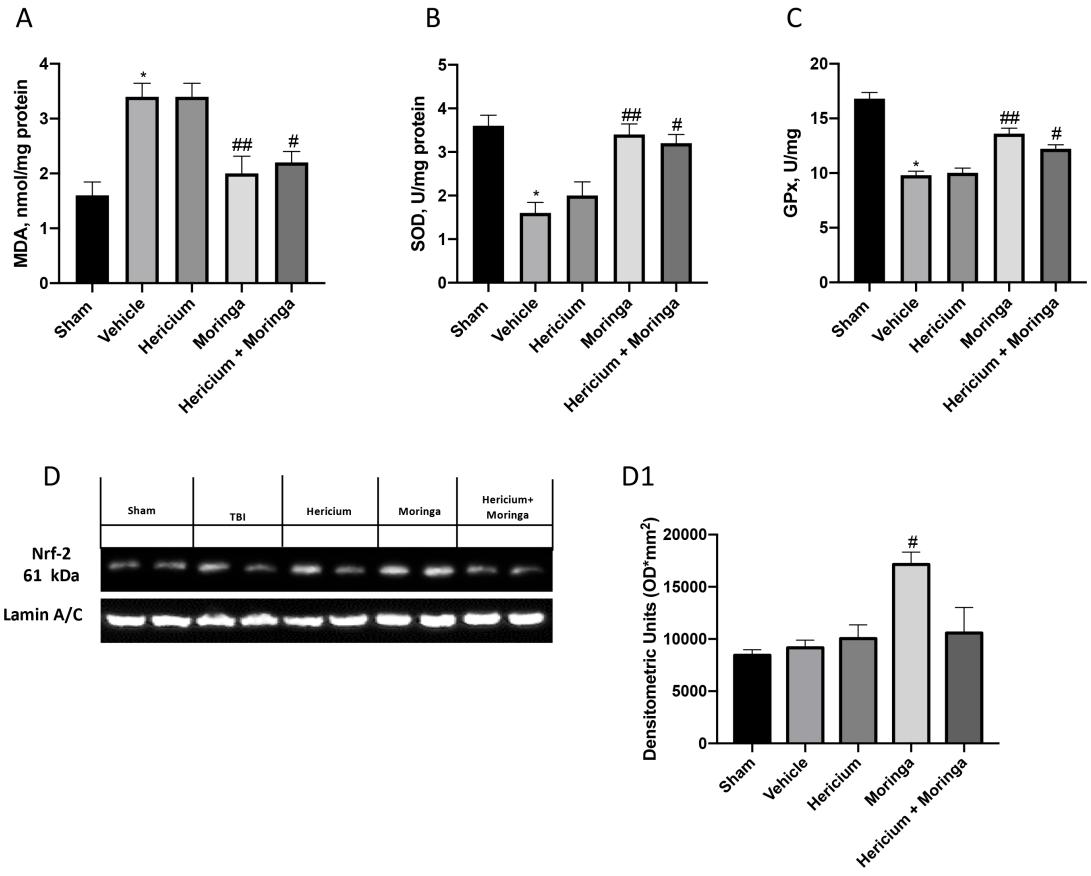

Figure 5: Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on oxidative stress. (A)MDA, malondialdehyde; (B) SOD, superoxide dismutase; (C) GPx, glutathione peroxidase; (D) Nrf-2 and densitometric analysis (D1).

7.2.5 Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on astrocytes and microglial activation.

To analyze the activation of astrocytes and microglia TBI-induced, GFAP and Iba-1 were evaluated by Western blot analysis. Brain from vehicle-treated group showed a significantly increased astrogliosis (Fig. 6A see densitometric analysis 6B) as well as microgliosis (Fig. 6C

see densitometric analysis 6D). Orally treatment with Moringa was able to significantly decrease astrogliosis as well as microgliosis (Fig. 6A,C see densitometric analysis B,D).

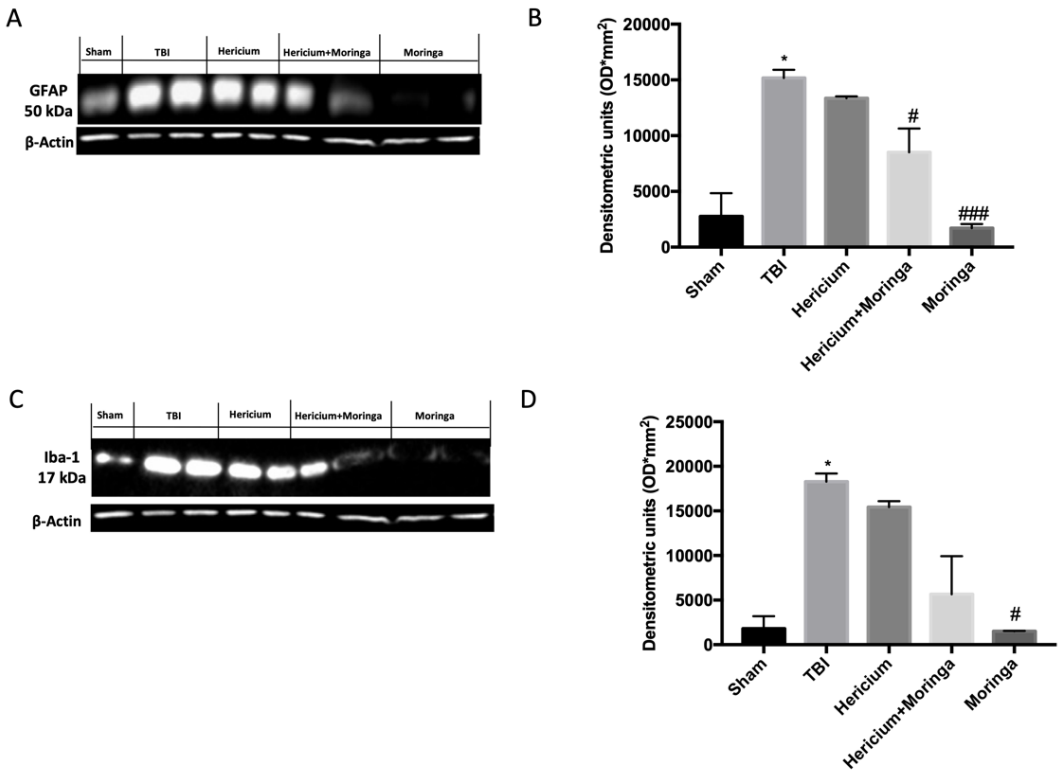

Figure 6: Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on astrocytes and microglial activation. Representative Western blots of brain tissues showed the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on: (A) GFAP and (C) Iba-1 after TBI, densitometric analysis (B) and (D) respectively.

7.2.7 Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on apoptosis pathway.

To evaluate the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera actions on apoptosis pathway, Bax and Bcl-2 were evaluated by Western blot analysis. The expression of Bax significantly increase after TBI, compared to sham group (Fig. 7A see densitometric analysis B); oral treatment with Moringa oleifera, or Hericium erinaceus plus Moringa oleifera significantly limited Bax expression TBI-induced. *Viceversa*, Bcl-2 diminished in brain tissue from vehicle treated mice, when compared to sham (Fig. 7C see

densitometric analysis D), while only orally treatment with increasing Bcl-2 expression. No significant amelioration was found in the apoptosis pathway after Hericium erinaceus treatment alone.

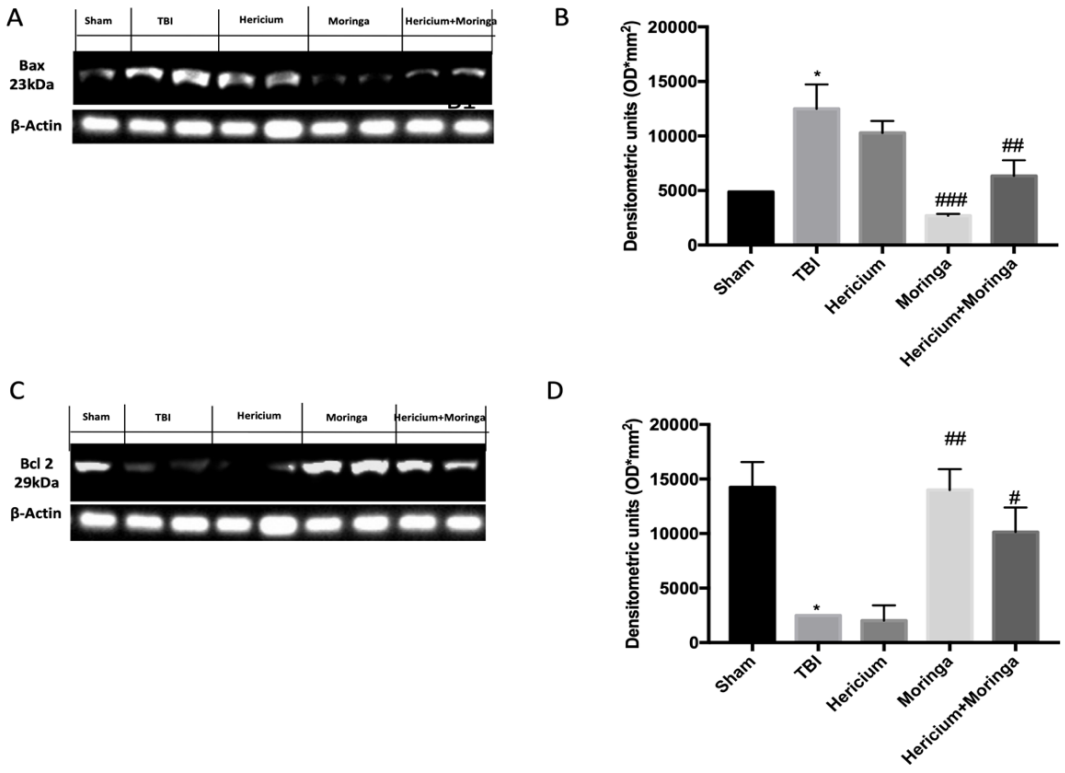

Figure 7: Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment on apoptosis pathway. Representative Western blots of brain tissues showed the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on: (A) Bax and (C) Bcl-2 after TBI, densitometric analysis (B) and (D) respectively.

Chapter 8: EXPERIMENTAL MODEL OF ALZHEIMER'S DISEASE

8.1 Materials and Methods

8.1.1 Animals

Male Wistar rats (250 - 280 g) were sited in an ordered location (room 22 ± 1 °C 12-h dark/light cycles) with standard rodent chow and water. Body weight (BW) was recorded weekly during the experimental period. The animals were adapted to these conditions for one week. Messina University Review Board for the carefulness of animals permitted the research. All animal experiments agree with the new regulations in Italy (D.Lgs 2014/26), EU regulations (EU Directive 2010/63).

Experimental design

Respectively, rats were randomly assigned in different groups of 10 animals as described below:

- Sham: rats were injected intraperitoneally with saline.
- Vehicle: rats were administered orally with $AlCl_3$ (100 mg/kg b.w.) and intra-peritoneally with D- galactose (60 mg/kg b.w.) daily for 60 days;
- Hericium erinaceus: rats received $AlCl_3$ plus D-galactose as described above and were treated orally for 60 days with Hericium erinaceus (300 mg/kg b.w.) dissolved in water.
- Moringa oleifera: rats received $AlCl_3$ plus D-galactose as described above and were treated orally for 60 days with Moringa oleifera (150 mg/kg b.w.) dissolved in water.
- Hericium erinaceus+Moringa oleifera: rats received $AlCl_3$ plus D-galactose as described above and were treated orally for 60 days with an association of Hericium erinaceus and Moringa oleifera (300 mg/kg + 150 mg/kg b.w.) dissolved in water.

8.1.2 Behavioral assessment

Morris Water Maze (MWM): MWM was selected as a method of evaluation of spatial learning and memory and was made as previously described by Mahmoodzadeh et al [239]. Briefly, a circular blank water tank (152 cm in diameter and 60 cm in height) was filled with water (23°C) to a depth of 30 cm. An escape platform (10 cm in diameter) was placed inside the tank, with the top sinking 2 cm below the water surface. The platform was in the middle of the target quadrant and its position remained fixed during the experiment. Above the tank, a white floor-to-ceiling cloth curtain was drawn around the pool and four types of black cardboard (circular, triangular, rhombus, and square) were hung equidistantly on the interior of the curtain to serve as spatial cues. Each rat had daily sessions of four trials for four consecutive days. For each trial, a rat was placed into the water facing the wall at one of the four standard start locations selected at random (N, S, W, and E) and then released. When the rat succeeded, it was allowed to stay on the platform for 20 seconds. When the rat failed to find the platform within 60 seconds, it was assisted by the experimenter and allowed to stay there for the same time.

Novel Object Recognition (NOR): NOR investigation was used to evaluate various aspects of learning and memory [240]. Briefly, animals are presented with two identical objects to explore for a period of time. After a delay following the removal of the objects, the rats are again presented with two objects, one of which is from the initial exposure and one of which is novel. Depending in part on the length of the delay between the two presentations, the rats will either explore the novel object differentially from the familiar object, a measure of short-term working memory in recognizing the initial presentation of identical objects, or for the same amount of time, which indicates no recollection of the encounter with the initial object presented and now presented again [241].

8.1.3 Histopathological examination

At the end of experiment, brains were processed and embedded in paraffin wax. Sections of 7 µm thickness were cut into longitudinal sections and stained and with hematoxylin and eosin (H&E) for routine histopathological examination, and Congo red for the demonstration of amyloid plaques. Five sections per group were examined using a Leica DM6 microscope. Representative images were shown.

8.1.4 Western Blot Analysis for IkB-α, NF-kB, Cox-2, iNOS and Nrf-2.

Western Blots was made as previously described [237]. Filters were blocked with 1× PBS, 5% (w/v) nonfat dried milk (PM) for 40 min at room temperature and then probed with one of the next primary antibodies: anti-IkB-α (1:500, Santa Cruz Biotechnology), or anti- NF-kB (1:500, Santa Cruz Biotechnology), or anti-Cox-2 (1:500, Santa Cruz Biotechnology), or anti-iNOS (1:500, Santa Cruz Biotechnology), or anti-Nrf-2 (1:500, Santa Cruz Biotechnology), in 1× PBS, 0.1% Tween-20, 5% w/v no fat dried milk (PMT) at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Blots were also incubated with primary antibody against β-actin protein (1:10,000; Sigma-Aldrich Corp.) or laminin (1:10,000; Sigma-Aldrich Corp), used as internal standards. The relative expressions of the protein bands of IkB-α (37 kDa), NF-kB p65 (65 kDa), Cox-2 (72 kDa), iNOS (130 kDa) and Nrf-2 (57 kDa) were detected and quantified by densitometry as previously explained by Cordaro et al [238]. In the experiments including western blot, a representative blot is displayed and densitometric analysis is related in each figure.

8.1.5 Immunohistochemical localization of Nitrotyrosine.

The immunohistochemical techniques used have been previously described [242]. Brain tissue slices were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:200 in PBS, v/v, Millipore). Sections were cleaned with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA, 1:2,000). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The counter-stain was developed with diaminobenzidine (DAB; brown color) and nuclear fast red (red background). To verify antibody-binding specificity, control slices were incubated with only the primary antibody or the secondary antibody, neither of which gave positive staining. Images were collected using a Leica DM6 microscope and Leica DM software.

The digital images were opened in ImageJ, followed by deconvolution using the color deconvolution plug-in. When the IHC profiler plug-in is select, it mechanically plots a histogram profile of the deconvoluted DAB image, and a corresponding scoring log is exhibited [243]. The histogram profile relates to the positive pixel intensity value gotten from a computer program [244]. All immunohistochemical analyses were carried out by two observers masked to the treatment.

8.1.6 Malondialdehyde (MDA) levels

Thiobarbituric acid-reactant substances evaluation, a suitable indicator of lipid peroxidation, was determined on brain tissues [245]. The absorbance of the supernatant was detected at 650 nm by spectrophotometry and indicated as nmol/g of tissue.

8.1.7 Cytokines measurement

Tumor Necrosis Factor-(TNF-α) and interleukin 6 (IL-6) levels were evaluated using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy).

8.1.8 Materials

All composites were gotten from Sigma-Aldrich. All other substances were of the uppermost profitable grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Milan, Italy).

8.1.9 Statistical evaluation

All values in the images and text are expressed as mean ± standard error of the mean (SEM) of N observations. For *in vivo* studies, N represents the number of animals. In experiments involving histology and immunohistochemistry, the illustrations represent the outcomes of at least three independent experiments. A *p*-value of less than 0.05 was considered significant. The results were analyzed by one- or two-way ANOVA, followed by a Bonferroni post-hoc test for multiple comparisons.

8.2 Results

8.2.1 Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on body weight loss and behavioral alterations

Daily administration of $AlCl_3$ significantly increase body weight loss compared to sham group (Fig. 1A). that was recovered after daily orally administration Hericium erinaceus or Moringa oleifera or the association of both.

Using the NOR test (Fig. 1B) we evaluated changes in cognitive function. After $AlCl_3$ administration animals showed significantly reduced preference for the novel and familiar object, indicating compromise of cognitive function; while in mice treated with Hericium erinaceus or Moringa oleifera or the association of both the function within the novel object recognition test returned to normal values.

The MWM test was performed to evaluate the effect of Hericium erinaceus or Moringa oleifera or the association of both on memory impairments. The time taken to find the platform during training was increased in $AlCl_3$ administered animals compared to the controls (Fig. 1C). The treatment Hericium erinaceus or Moringa oleifera or the association of both reduced this escape latency (Fig. 1C). In addition, time spent in target quadrant of the platform during probe trials was reduced in $AlCl_3$ administered animals (Fig. 1D). Hericium erinaceus or Moringa oleifera or the association of both treatments increased the frequency time, ameliorating the cognitive deficits (Fig. 1D).

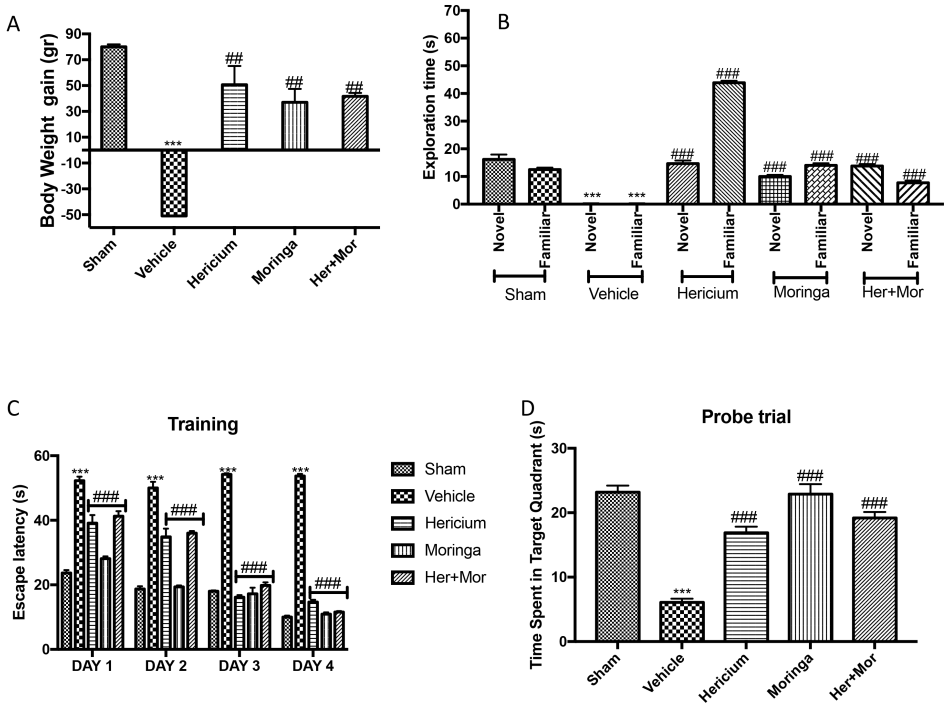

Figure 1. Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on body weight loss and behavioral alterations. (A) Body weight gain; (B) Object Recognition test; (C) Training of Morris Water Maze test; (D) Probe trial of Morris Water Maze Test; *** p>0,001 vs Sham; ### p< 0,001 vs vehicle.

8.2.2 Daily treatment with Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, reduced histopathological modifications and β-amyloid depositions $AlCl_3$-induced

Sham group showed a normal histological structure with normal CA1 hippocampal region and cerebral cortex (Figs. 2A and 3A, see respectively count 2F and 3F). However, the brain of $AlCl_3$-injured group showed severe histopathological alterations in the hippocampal CA1 as well as in the cerebral cortex, which revealed extensive neuronal degeneration wherein the degenerated neurons looked shrunken (Figs. 2B and 3B, see respectively count 2F and 3F).

Daily treatment with Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera demonstrated a significantly ability to counteract neuronal degeneration $AlCl_3$-induced (Figs. 2C,2D, 2E and 3C, 3D and 3E, see respectively count 2F and 3F).

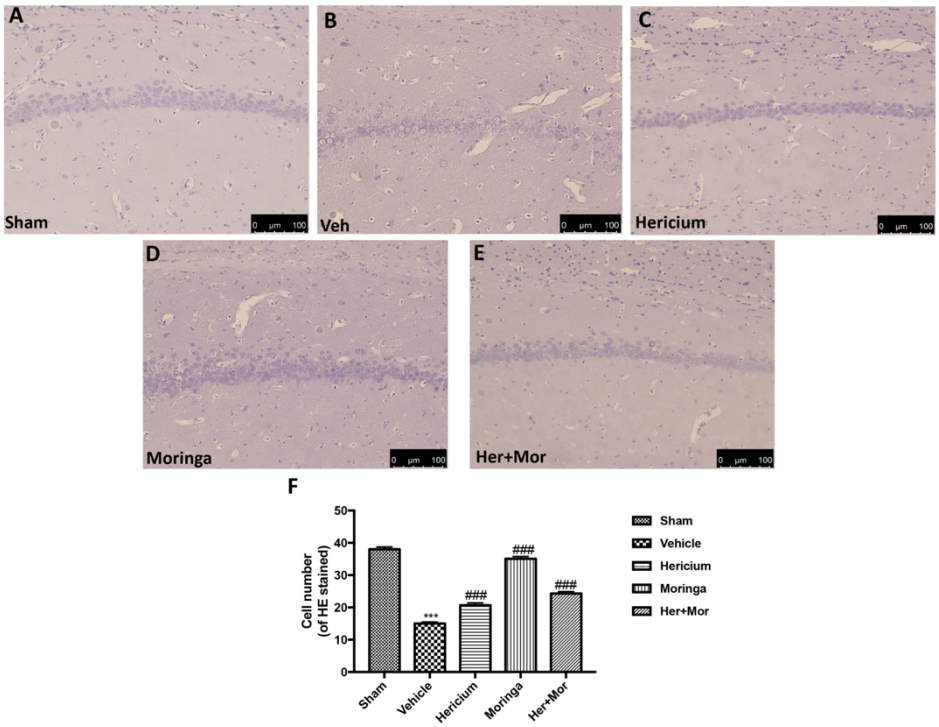

Figure 2. Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on hippocampal histological alteration. H/E of CA1 hippocampal sections: (A) Sham; (B) Vehicle; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F) cell number count of hippocampal damage; *** p>0,001 vs Sham; ### p< 0,001 vs vehicle.

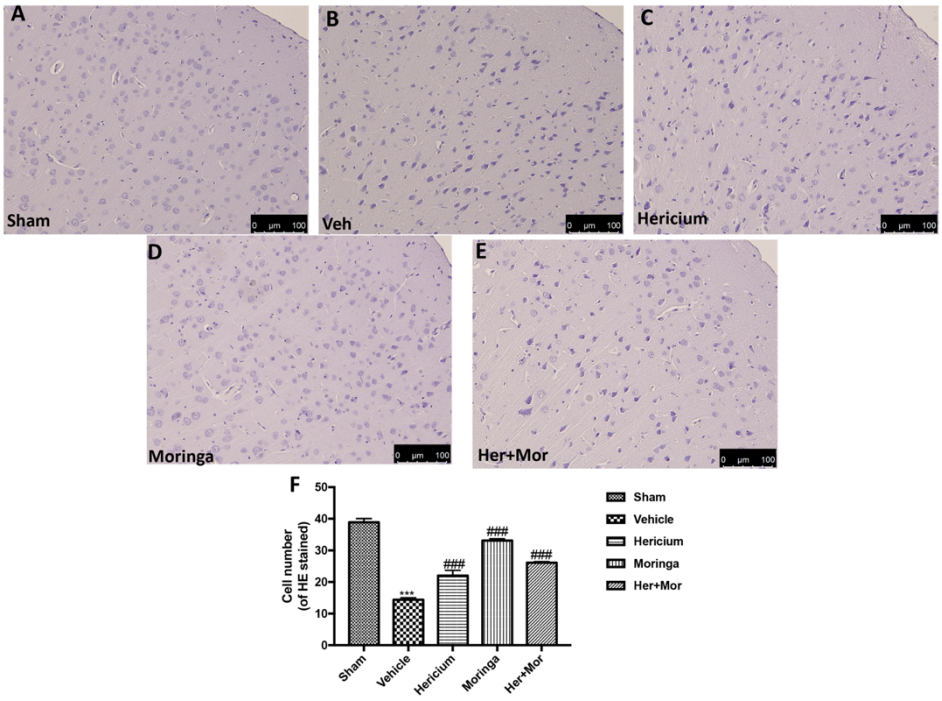

Figure 3. Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on cerebral cortex histological alteration. H/E of cerebral cortex sections: (A) Sham; (B) Vehicle; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F) cell number count of cerebral cortex injury; *** p>0,001 vs Sham; ### p< 0,001 vs vehicle.

Additionally, sham group, was negative for Congo Red staining both in the hippocampus and in the cortex (Figs. 4A and 5A, see respectively count 4F and 5F). On the other hand, significantly depositions of β-amyloid were founded in the hippocampus and in the cortex of $AlCl_3$-group (Figs. 4B and 5B, see respectively count 4F and 5F). Daily orally administration of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, showed a considerably capability to counteract β-amyloid depositions $AlCl_3$-induced (Figs. 4C,4D, 4E and 5C, 5D and 5E, see respectively count 4F and 5F). In particular we found that treatment with Moringa oleifera alone was able to counteract neuronal loss and the accumulation β-amyloid in a better way than Hericium erinaceus alone or the association Hericium erinaceus plus Moringa oleifera.

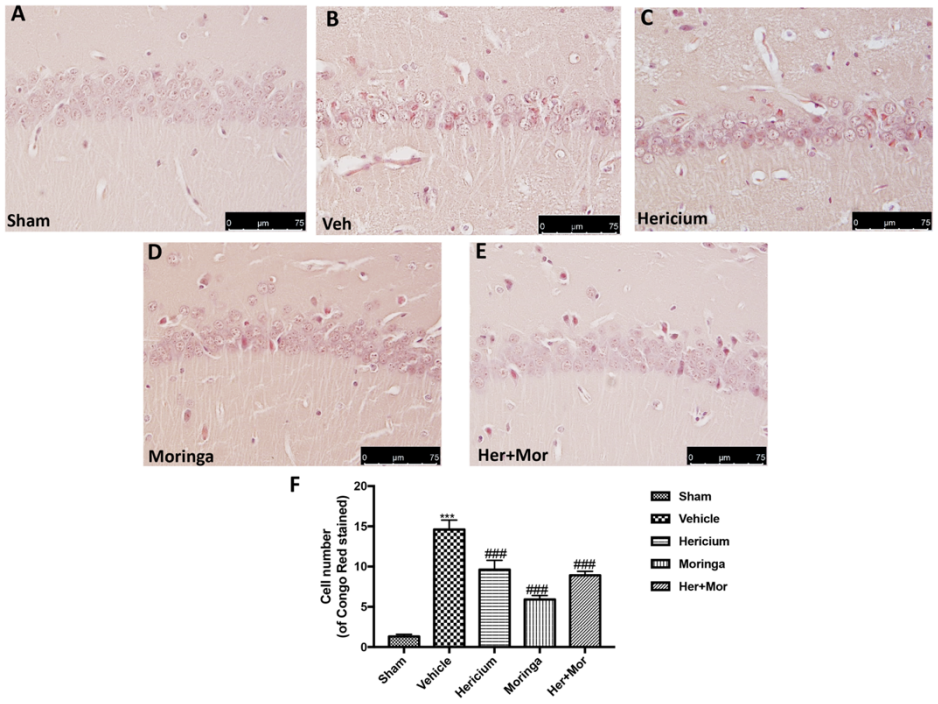

Figure 4. Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on hippocampal β-amyloid depositions. Congo Red of CA1 hippocampal sections: (A) Sham; (B) Vehicle; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F) Congo Red positive stained cell number counted; *** p>0,001 vs Sham; ### p< 0,001 vs vehicle.

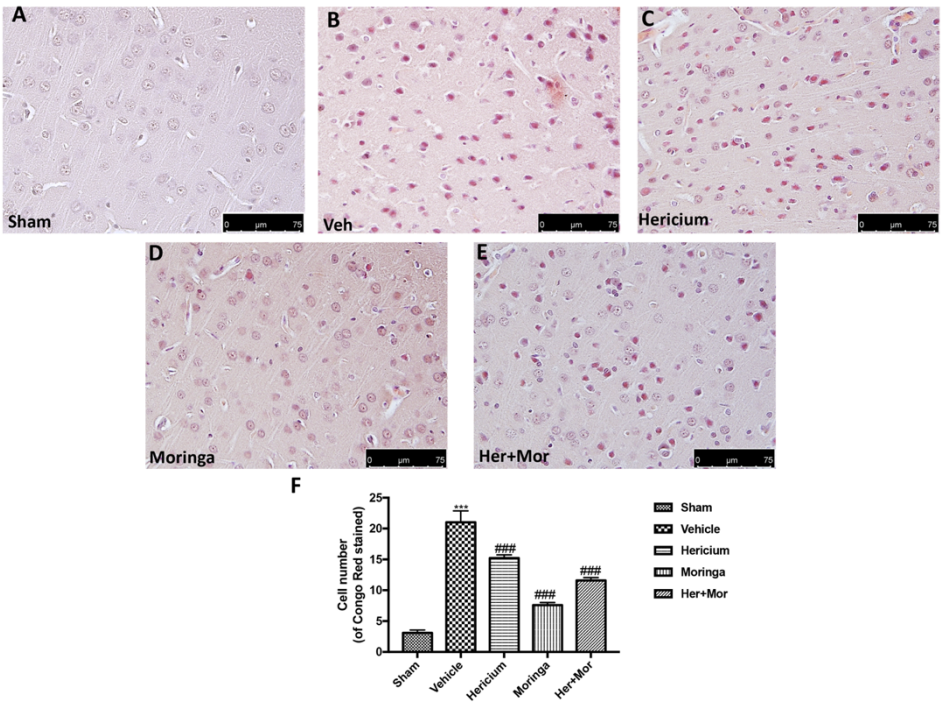

Figure 5. Effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on β-amyloid depositions in cerebral cortex. Congo Red of cerebral cortex

sections: (A) Sham; (B) Vehicle; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F). Congo Red positive stained cell number counted; *** p>0,001 vs Sham; ### p< 0,001 vs vehicle.

8.2.3 Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, decreased $AlCl_3$-induced IκB-α degradation, NF-κB p65 nuclear translocation, COX-2 and iNOS expressions in rat brain tissue

To improved our knowledge on the molecular mechanism underlying the anti-inflammatory effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera actions IκB-α degradation and NF-κB p65 nuclear translocation were evaluated by Western blot analysis. The expression of IκB-α significantly diminished in rat brain tissue from $AlCl_3$-administred rats, as compared to sham group (Fig. 6A; A1, densitometric analysis), and oral treatment with Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera significantly limited $AlCl_3$-induced IκB-α degradation. *Viceversa*, nuclear translocation of the NF-κB subunit p65 increased in rat brain tissue from $AlCl_3$-administred rats, when compared to sham rats (Fig. 6B; B1, densitometric analysis), while only daily orally treatment with Moringa oleifera, or Hericium erinaceus plus Moringa oleifera significantly decreasing p65 nuclear translocation (Fig. 6B; B1, densitometric analysis). Assumed the COX-2 role in lipid degradation and subsequent production of leukotrienes and prostaglandins, we investigated its expression by Western blot analysis. COX-2 significantly increased in rat brain tissue from $AlCl_3$-injured rats as compared to the sham group (Fig. 6C; C1 densitometric analysis), and was significantly decreased after orally treatment with Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera (Fig. 6C; C1 densitometric analysis). Furthermore, to establish the role of ·NO produced after $AlCl_3$ administration, iNOS expression was studied using Western blot analysis. A substantial intensification in iNOS expression was identified in brain from rats exposed to $AlCl_3$ administration; while a significant reduction in iNOS expression was detected after Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment (Fig. 6D, D1 densitometric analysis).

Also, in this evaluation we found that treatment with Moringa oleifera alone was more able to counteract AlCl3-induced IκB-α degradation, NF-κB p65 nuclear translocation, COX-2 and iNOS expressions in rat brain tissue in a better way than Hericium erinaceus alone or the association Hericium erinaceus plus Moringa oleifera.

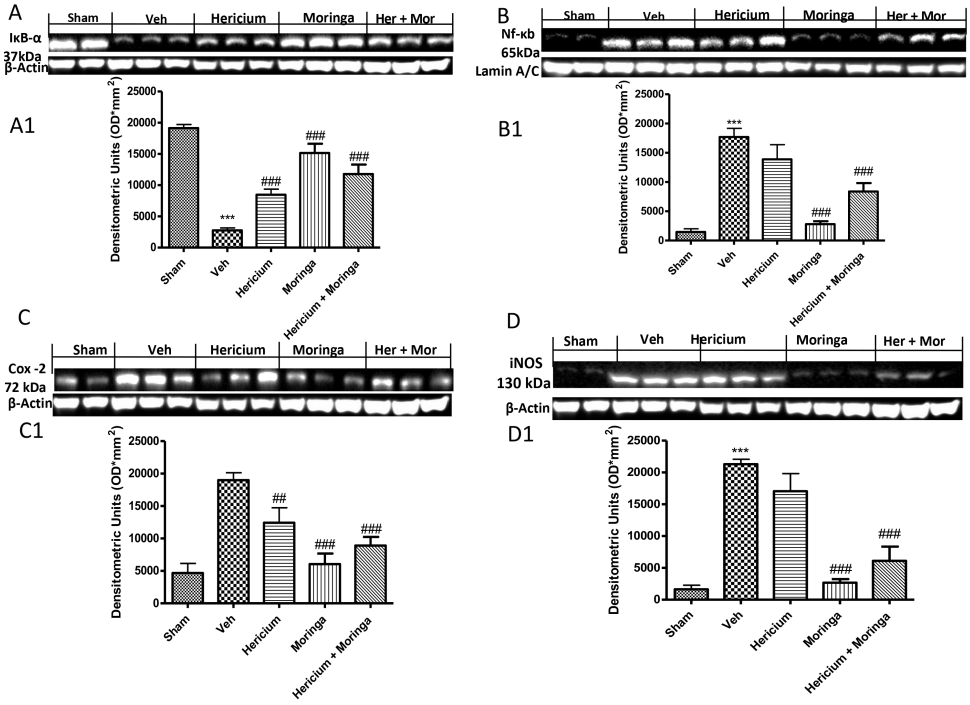

Figure 6. Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment decreases $AlCl_3$-induced IκB-α degradation, NF-κB p65 nuclear translocation, COX-2 and iNOS expressions in rat brain tissue. Representative western blots of brain tissues showed the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on: (A) IκB-α degradation, (B) NF-κB p65 translocation, (C) Cox-2 expression, (D) iNOS expression after $AlCl_3$ administration. *** p>0,001 vs Sham; ## p< 0,01 vs vehicle; ### p< 0,001 vs vehicle.

8.2.4 Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on Nrf-2 expression, MDA levels and TNF-α and IL-6 release.

Additionally, in this study, we evaluated whether pro-inflammatory cytokines are associated with $AlCl_3$ administration. Elevated levels of TNF-α (Fig. 7 A) and IL-6 (Fig. 7 B) were found in $AlCl_3$-group compared to sham. Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera daily orally administration substantially reduce these

proinflammatory cytokines levels (Fig. 7A, B). Furthermore, we also assessed MDA level as indicator of lipid peroxidation. $AlCl_3$-group exhibited amplified levels of MDA that was significantly reduced after Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera treatment (Fig. 7 C).

Moreover, to investigate if Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, possess neuroprotective effects dependent on Nrf-2 pathway we explored Nrf-2 expression by Western blot analysis. No appreciably modifications was found in the brain of vehicle group compared to sham rats (Figure 7D,D1 see densitometric analysis); *viceversa* a considerable intensification in Nrf-2 expression was found after Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment (Figure 7D,D1 see densitometric analysis).

Moringa oleifera administered alone at the doses of 150mg/kg was more competent to neutralize $AlCl_3$-induced pro-inflammatory cytokines release and MDA augmentations and was in grade to better stimulate the activation of Nrf-2 pathways in rat brain tissue in a better way than Hericium erinaceus administered alone at the dose of 300 mg/kg or the association Hericium erinaceus plus Moringa oleifera at the doses of 300 mg/kg plus 150 mg/kg.

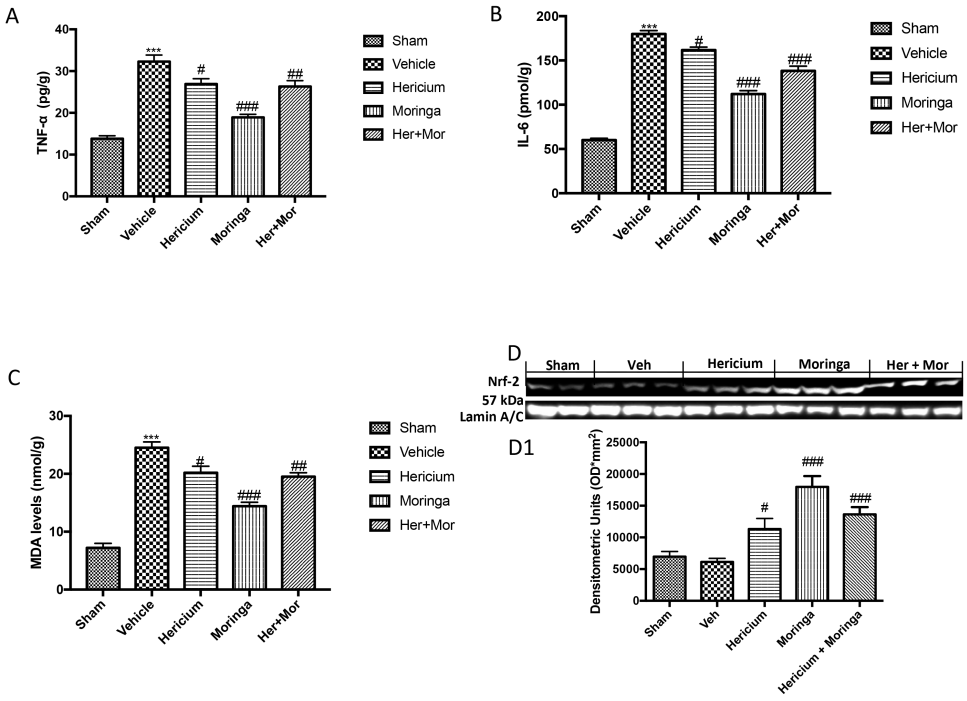

Figure 7. Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment treatment on Nrf-2 expression, MDA levels and TNF-α and IL-6 release. TNF-α (A), IL-6 (B) proinflammatory cytokines levels were detected. Brain MDA levels (C); Representative western blots of brain tissues showed the effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on Nrf-2 expression. *** p>0,001 vs Sham; # p< 0,05 vs vehicle; ## p< 0,01 vs vehicle; ### p< 0,001 vs vehicle.

8.2.5 Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment on nitrotyrosine expression

At the end of experiment, nitrotyrosine, a detailed marker of nitrosative stress, was examined by immunohistochemical staining in the brain slices. No positive staining for nitrotyrosine was identified in hippocampal CA1 region obtained from Sham rats (Figures 8A, and relative quantification 8F); however, brain sections obtained from rats, received only $AlCl_3$, displayed a significantly positive staining for nitrotyrosine (Figures 8B and relative quantification 8D). Treatment with Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, significantly reduced the positive nitrotyrosine staining in hippocampal CA1 region (Figures 8C, 8D and 8E and relative quantification 8F). Moringa oleifera treatment was in grade to better neutralize the expression of nitrotyrosine in the CA1 hippocampal region in a better

way than Hericium erinaceus administered alone or the association Hericium erinaceus plus Moringa oleifera.

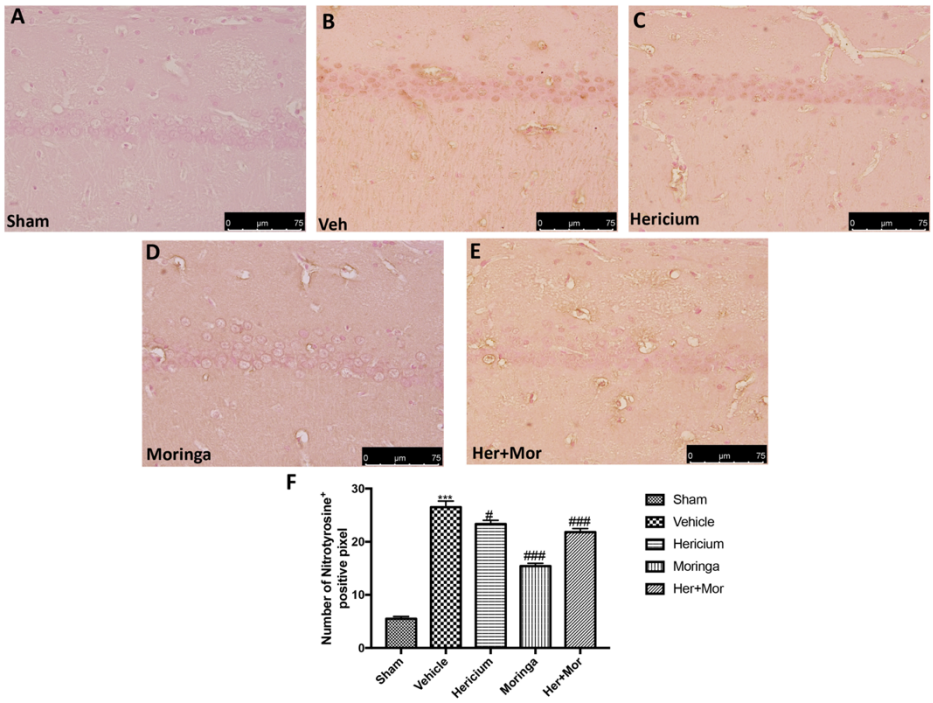

Figure 8. Effects of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera, treatment treatment on nitrotyrosine expression. Immunohistochemical localization on hippocampal nitrotyrosine expression: (A) Sham; (B) Vehicle; (C); Hericium erinaceus; (D) Moringa oleifera; (E) Hericium erinaceus plus Moringa oleifera; (F) The results are expressed as % of positive pixels; *** p>0,001 vs Sham; # p< 0,05 vs vehicle; ### p< 0,001 vs vehicle.

Chapter 9: EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

9.1 Materials and Methods

9.1.1 Animals

For the study we used CD1 male mice of about 25-30g (Harlan Nossan, Milan, Italy). They were first adapted to the habitat for a week and then were kept in a controlled environment (22 ± 1 ° C with a 12-h dark, 12-h light cycle) and had water and food for rodents available. Before the execution of the study, we received the approval of the Review Board of the University of Messina for the care of animals. In addition, the experiments on mice complied with U.S. regulations (Animal Welfare Insurance No. A5594-01, Department of Health and Human Services, United States), Europe (OJ of EC L 358/1 12/18/1986) and Italy (DM 116192).

9.1.2 Rotenone-induced PD and treatment

8 weeks old male CD1 mice were treated with rotenone or saline. For rotenone intoxication, mice received daily oral gavage (o.s.) of rotenone (5 mg/kg in 4 % carboxymethylcellulose, CMC; Sigma, St. Louis, MO) in saline. For polyphenol treatment (10 mg/kg) mice received by intraperitoneal injections (i.p.) "Hidrox®", daily till the end of the experiment. HD which was kindly provided by Oliphenol LLC., (Hayward, CA USA) is a freeze-dried powder prepared from the aqueous portion of olives extracted from the defatted olive pulps, a derivative during the processing of Olea europaea L. for olive oil extraction [246]. 12% of the HD extract is made up of polyphenols. 12% of the HD extract is made up of polyphenols. Among these, the most abundant in HD is Hydroxytyrosol with 40–50%, while with 5-10% there is oleuropein, with 0.3% tyrosol and with about 20% oleuropein aglycone and gallic acid [247] (Fig. S1).

On the 28th day the mice were anesthetized with ketamine (2.6 mg / kg) and xylazine (0.16 mg / kg) and subsequently beheaded. The brains were taken for various analyzes. The doses of rotenone (5 mg / kg) and HD (10 mg / kg) were chosen based on studies in the literature [248, 249].

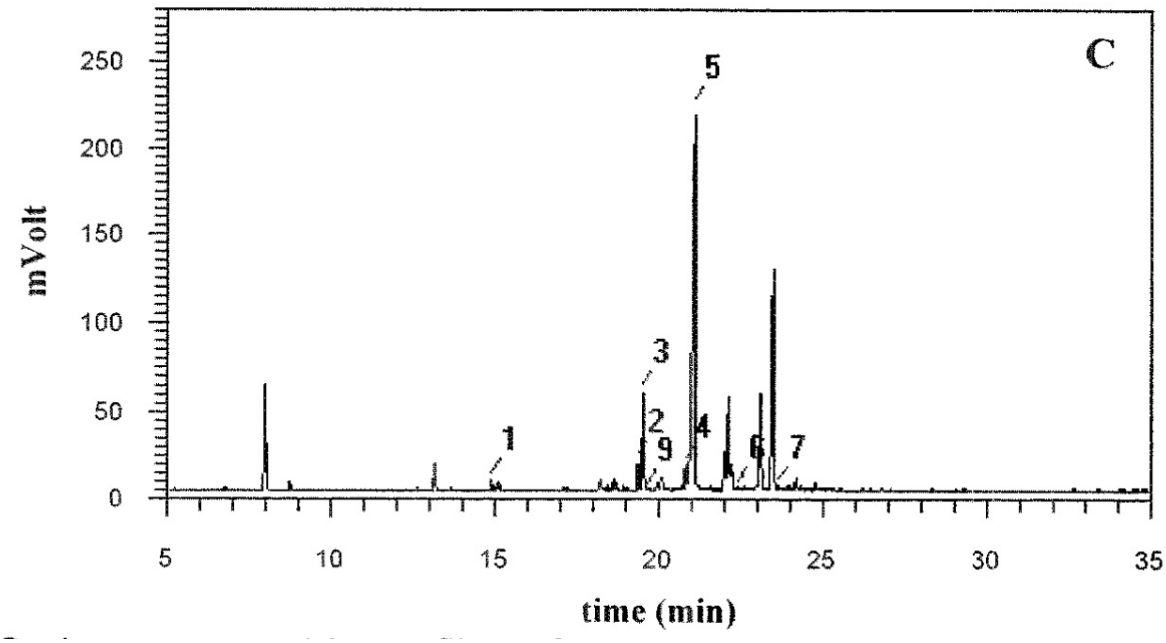

Figure S1. Gas chromatography mass spectrometry (GC-MS) analysis and phenolic compound identification, by spectrum matching and library searching in the NIST/EPS/NIH Mass Spectral Library Database, of HD. Peaks: 1, tyrosol; 2, Vanilic acid; 3, Hydroxytyrosol; 4, 3,4-dihydroxybenzoic acid; 5, citric acid (hydrolysis acid to obtain HD from OVW, olive acqueous vegetation water); 6, syringic acid; 7, gallic acid; 8, caffeic acid (trace); 9, 3-hydroxy,4-methoxyphenylacetic acid; 10, gentisic acid (trace).

Experimental group:

Animals were casually distributed into the following groups:

Group 1: Sham = vehicle solution (saline) was administrated daily, as rotenone protocol, o.s. (N=10)

Group 2: Sham + HD = HD solution was administrated by i.p. for 28 days (data not shown). (N=10)

Group 3: Rotenone + vehicle = rotenone solution was administrated daily o.s. and vehicle solution (saline) was administrated i.p. daily. (N=10)

Group 4: Rotenone + HD = rotenone solution was administrated daily o.s. and HD solution was administrated by i.p. daily for 28 days and 1 hour after rotenone administration. (N=10)

9.1.3 Behavioral testing

Behavioural evaluations on all animals (N= 10 mice for each group) were made 1 day prior and 28 days after to rotenone injection:

Pole test: PT was performed to detect motor alteration such as bradykinesia as previously described [250, 251].

Rotarod test: Motor activity was assessed with a rotary rod apparatus using a protocol previously described [252, 253].

Catalepsy test: Catalepsy, demarcated as a reduced capability to start movement and a failure to correct posture, was measured as previously described [254, 255].

9.1.4 Histology

The brains of 5 mice for each group were fixed, cut and colored with Hematoxylin and Eosin (H&E, Bio-Optica) and subsequently the sections were analyzed using an optical microscope connected to an imaging system (AxioVision, Zeiss, Milan, Italy) as previously described [30, 35]. Histopathological evaluation was performed blindly using a semi-quantitative five point rating: 0=normal, no death neuron observed; 1=insignificant pathology, SN contained one to five death neurons; 2=modest pathology, SN contained five to 10 death neurons; 3=severe pathology, SN contained more than 10 death neurons; 4=more severe pathology, SN contained only death neurons [235]. The scores from all slides were averaged to give a final score for each individual mouse. Images are representative of all animals in every group.

9.1.5 Stereological analysis

Impartial counting of TH-positive DA neurons within the SN was carried out as previously described [256]. For use, the polyclonal rabbit anti-TH antibody (1: 400, Merck-Millipore, AB152) was used which was incubated at 4 ° C O / N. Describes the sections are treated state with the ABC (Vector Labs) method. The images were taken blind and for each sample 5

representative sections of the SN were analyzed using the StereoInvestigator software (Microbrightfield, Williston, VT).

9.1.6 Immunohistochemical localization of tyrosine hydroxylase (TH), dopamine transporter (DAT) and α-synuclein (α-syn).

The immunohistochemical techniques used have been previously described [257, 258]. The antibodies that have been incubated O/N on the brain sections are: anti-TH (Millipore, 1: 500 in PBS, v/v, AB152), anti-DAT (Santa Cruz Biotechnology, 1: 300 in PBS, v/v, 65G10 sc-32258), anti-α-syn (Santa Cruz Biotechnology, 1:50 in PBS, v/v, LB509 sc-58480). To verify the specificity of the antibodies, the brain sections of 5 mice for each group were treated either with primary or only with secondary antibody. The images were taken using a Zeiss microscope and Axio Vision software. The ImageJ IHC profiler plug-in was used for densitometric analysis. When this is selected, it automatically traces a histogram profile of the deconstructed DAB image and a corresponding score log is shown[243]. The histogram profile corresponds to the positive pixel intensity value obtained from the computer program [244]. Immunohistochemical analyzes were performed by experienced people who did not know the treatment.

9.1.7 Immunofluorescence co-localization of TH/α-syn

Immunofluorescence analysis was performed with the protocol that we previously described [257, 258]. The antibodies that have been incubated in a humidified chamber at 37°C O/N on the brain sections of 5 mice for each group are: anti-TH (1:250; Merck-Millipore, AB152), anti-α-syn (1:50; Santa Cruz Biotechnology, LB509 sc-58480). The sections were analyzed using a fluorescence microscope (Leica DM2000). Each photograph taken was digitized with an 8-bit resolution in an array of 2560 × 1920 pixels. The images obtained were subsequently cut out and prepared for the assembly of the figures using Adobe Photoshop 7.0 (Adobe Systems, Palo Alto, CA).

9.1.8 Western blot analysis for IkB-α, NF-kB, Bax, Bcl-2, iNOS, NRLP3, ASC, Caspase-1, IL-18, IL-1β, Hsp70, Sirt-1 and HO1

Western blot analysis was performed on brains of 5 mice for each group with the protocol that we previously described [259, 260]. The levels of IkB-α, Bax, Bcl-2, iNOS, NRLP3, ASC, Caspase-1, IL-18 and IL-1β were quantified in cytosolic, while NF-kB p65 levels were quantified in nuclear fraction. The specific primary antibodies that have been incubated at 4° C O/N are: anti-IkB-α (1:500; Santa Cruz Biotechnology, C-21: sc-371), anti-NF-kB p65 (1:500; Santa Cruz Biotechnology, F-6: sc-8008), anti-Bax (1:500; Santa Cruz Biotechnology, P-19: sc-526), anti-Bcl-2 (1:500; Santa Cruz Biotechnology, N-19: sc-492), anti-iNOS (1:1000; Transduction Laboratories, 610432), anti-NRLP3 (1:500; Santa Cruz Biotechnology, sc-66846), anti-ASC (1:500; Santa Cruz Biotechnology, N-15: sc-22514-R) anti-Caspase-1 p20 (1:1000; Santa Cruz Biotechnology, G-19: sc-1597), anti-IL-18 (1:500; Santa Cruz Biotechnology, H-173: sc-7954), anti-IL-1β (1:500; Santa Cruz Biotechnology, H-153: sc-7884), anti-Hsp70 (1:500; Santa Cruz Biotechnology, 3A3 sc-32239), anti-Sirt1 (1:500; Santa Cruz Biotechnology, B7 sc-74465), anti-HO-1 (1:500; Santa Cruz Biotechnology, A3 sc-136960), anti-γGCs (1:500; Santa Cruz Biotechnology, H5 sc-390811). Protein lysates were also incubated with β-actin antibody or laminin antibody (1: 5000; Santa Cruz Biotechnology, C4 sc-47778, E1 sc-376248) in order to verify that all samples had been loaded in equal quantities. The signals were captured with BIORAD ChemiDocTM XRS + software thanks to the use of a reagent that emits chemiluminescence (Super Signal West Pico, Pierce chemiluminescent substrate). The relative expression of the bands was subsequently normalized to β-actin levels. Image analysis was performed using Image Quant TL software, v2003.

9.1.9 Protein Carbonyl Assay

Oxidized proteins were analyzed on tissues of 5 mice for each group by means of the OxyBlotTM Protein Oxidation (Merck Millipore, Darmstadt, Germany) conceding to the manufacturer's instructions. Briefly, 15 µg protein of each sample were denatured by adding 5 µl of 12% SDS (Sigma-Aldrich). Samples were derivatized by adding 10 µl of 1x of 2,4 dinitrophenolhydrazine (DNPH, Sigma-Aldrich) solution and incubated for 15 minutes at RT. Subsequently, samples were neutralized with 7.5 µl of neutralization solution and the proteins were separated by SDS/PAGE. The primary antibody used was against DNPH and revealed by luminescence (SuperSignal detection system kit: Pierce Chemical, Dallas, TX, USA). The bands were quantified normalizing pixels in each lane to the loading control band (Gel-Logic 2200-PRO Bioscience, London, UK) and analyzed (Molecular Imaging software).

9.1.10 Statistical Evaluation

In the figures and in the text the values have been expressed as an average ± SEM and are representative of at least 3 experiments carried out at different times. 10 mice per group were used in each experiment unless otherwise noted. Data review was performed by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. A p value of less than 0.05 was considered significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

9.2 Results

9.2.1 Effect of HD treatment on behavioral impairments and on the neuronal degeneration of dopaminergic tract induced by rotenone administration

To investigate the relationship between the degeneration of dopaminergic neurons rotenone-induced and recovery processes we analyzed the motor activity 1 day prior and 28 days after the rotenone induction. The data at time point 0 are not shown as no significant differences between different groups have been observed. The pole test was used to assess whether the rotenone-induced mouse model efficaciously induced bradykinesia [261]. "Time to turn" and

“Total time” notably increased following injection of rotenone compared with the Sham group (Fig. 1 A and A1). HD treatment significantly reduced “Total time” (45%) and “Time to turn” (61%) (Fig. 1 A and A1), suggesting a substantial reduction of bradykinesia. In addition, through the Rotarod test we evaluated the motor function. 28 days after the induction of PD, the mice showed significant motor changes evidenced by the reduction in the time spent on the Rotarod and by the greater number of falls. In contrast, HD-treated mice had a significant reduction in motor deficits (Fig. 1 B). Moreover, the rotenone produced an important cataleptic effect in mice. In fact, at 28 days after rotenone injection, mice exhibited a significant increase of cataleptic symptoms. Otherwise, the daily HD administration significantly reduced the catalepsy duration induced by rotenone (Fig. 1 C).

To evaluate the histopathological alteration induced by the administration of rotenone, H&E staining was performed on the brain sections. The mice treated with saline or only with HD for 28 days showed normal brain architecture and a normal number of neurons in the SN (Fig. 1 D and E, graph H). Instead, mice treated with rotenone showed evident alterations such as cytoplasmic vacuolization, vascular degeneration and nigrostriatal neuronal cell loss. The architecture of brain in rotenone mice was altered compared with control mice (Fig. 1 F, graph H). In contrast, mice treated with rotenone and HD showed a marked reduction in vascular degeneration and cytoplasmic vacuolization. It also reduced the cell with pyknotic nuclei in the SN (Fig. 1 G, graph H).

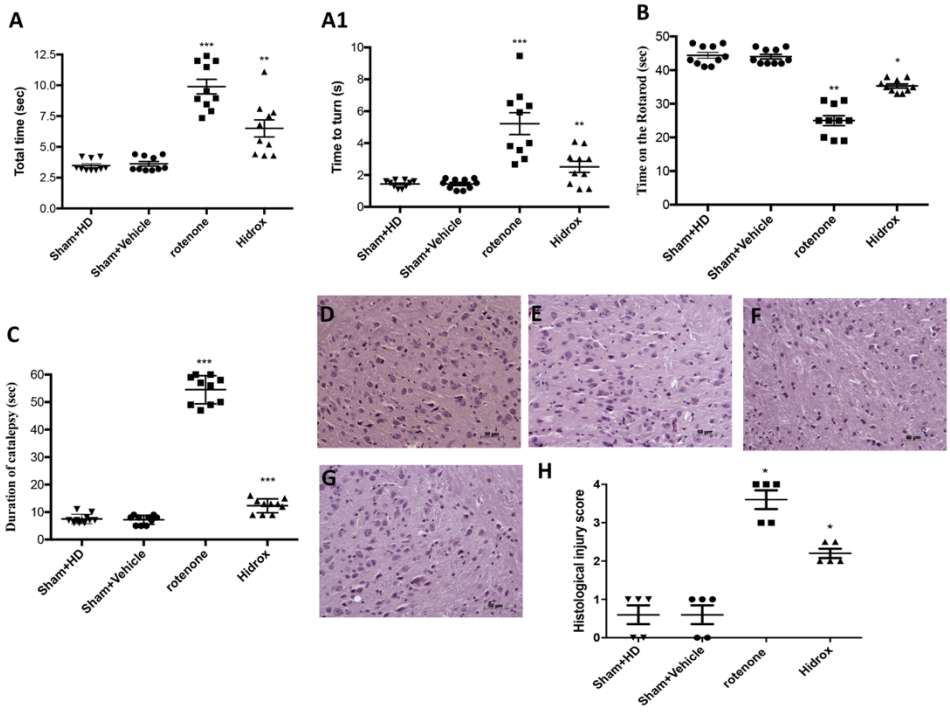

Figure 1. Effect of HD on behavioral impairments and on histological parameters induced by rotenone intoxication. A, A1) Motor function was assessed using a Pole test. At 28 days, mice exhibited a significant motor dysfunction as indicated by an increase in "Time to turn" and "Total time" spent to descend to the floor following injection of rotenone compared with the Sham group. HD administration notably reduced "Total time" and "Time to turn". A) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone; A1) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone. B) At 28 days, using a Rotarod apparatus, mice exhibited a significant motor dysfunction as indicated by a decrease in time spent on the Rotarod. HD treatment blunted the motor dysfunction in mice. B) ** $p<0.01$ *vs* Sham; # $p<0.05$ *vs* rotenone. C) Catalepsy was evaluated according to the standard bar hanging procedure; this motor test showed that HD treatment reduced behavioral impairment induced by rotenone. C) *** $p<0.001$ *vs* Sham; *** $p<0.001$ *vs* rotenone. Values are mean ± SEM of 10 mice for each group. Sham+HD and Sham+vehicle groups showed no evidence of degenerating cells in the SN (D, E), whereas degeneration of neuromelanin-pigmented cells was evident in the SN of the rotenone-treated animals (F). HD treatment restored the architecture comparable to control mice (G). The data are representative of at least three independent experiments and are expressed as mean ± SEM of 5 mice for each group. H) * $p<0.05$ *vs* Sham; * $p<0.05$ *vs* rotenone. Scale bar 50 µm.

9.2.2 HD treatment reduced loss of TH, DAT and α-synuclein expression in the SN induced by rotenone administration

The expression of TH and DAT was evaluated to prove the effect of HD treatment on the DA pathway. The group of animals treated only with rotenone showed a significant loss of TH-positive cells in the SN (Fig. 2 B and graph D) compared to the control group (Fig. 2 A and graph D). Instead, HD administration has been shown to significantly reduce the loss of TH-

positive neurons in SN (Fig. 2 C and graph D). Stereology analysis of nigral TH-positive neurons displayed important neuroprotection by HD treatment. We observed a considerably decline in the number of TH-positive neurons in mice after rotenone injection (Fig. S2 B and graph D), compared to Sham group (Fig. S2 A and graph D). This loss decreased following HD treatment (Fig. 2S C and graph D). Similarly, Nissl stained neurons were depleted significantly by rotenone lesioning but not in mice that had been treated with HD (Fig. 2S A-B and graph E).

Furthermore, we showed a critical loss of DAT in rotenone-injected mice at the level of the midbrain (Fig. 2 F and graph F) comparable to Sham group (Fig. 2 E and graph H); while HD treatment considerably restored the levels of DAT (Fig. 2 G and graph H).

α-syn is the highest constituent of the intraneuronal protein aggregates known as Lewy bodies. Since the accumulation of α-syn is a characteristic of PD, we wanted to evaluate the expression of this protein in order to determine the ability of HD to counteract the neurodegenerative process. We observed an important immuno-reactivity in rotenone-injured mice (Fig. 2 J and graph L) compared to Sham group (Fig. 2 I and graph L). Instead, the treatment with HD reduced notably α-synuclein expression in the SN after rotenone-intoxication (Fig. 2 K and graph L).

Furthermore, to prove that the accumulation of α-synuclein occurred in dopaminergic neurons, we carried out by immunofluorescence analysis a double coloring between TH (green) and α-syn (red). In the Sham group we did not find α-syn in TH-positive dopaminergic neurons (Fig. 3 G), while after rotenone intoxication there was an increase in the accumulation of α-syn in TH-positive neurons (Fig. 3 H). HD treatment prevented α-syn aggregation in dopaminergic neurons. (Fig. 3 I).

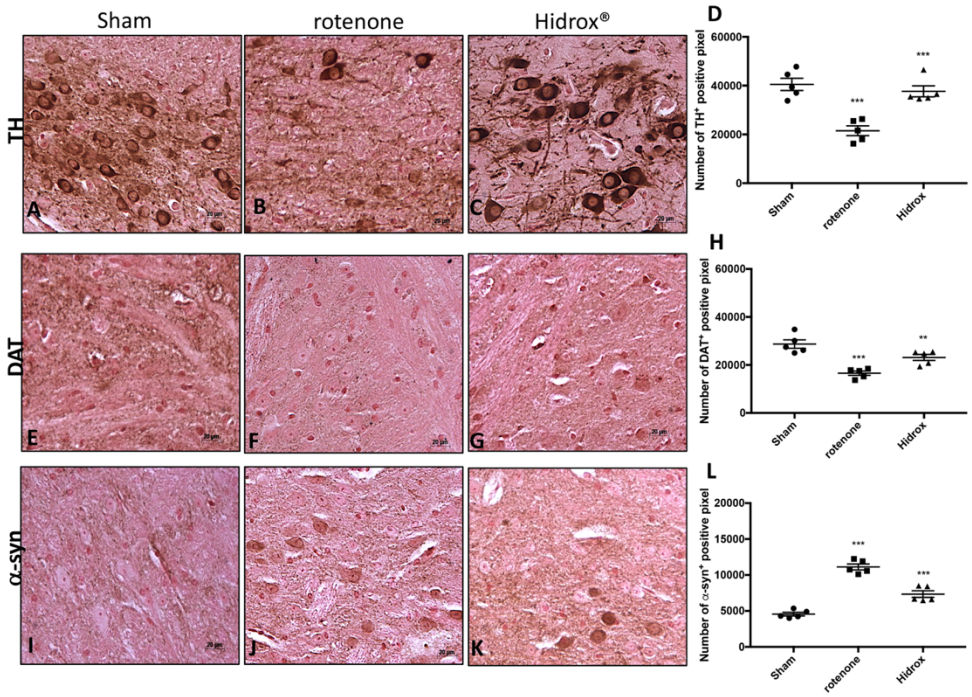

Figure 2. Effects of HD on TH, DAT and α-synuclein expression in SN of rotenone-treated mice. Immunohistochemical analysis shown, compared with Sham mice (A), a noticeable loss of TH-positive cells (B). Animals treated with HD revealed an increase of TH expression (C). *** $p < 0.001$ *vs* Sham; *** $p < 0.001$ *vs* rotenone (D). Immunohistochemical analysis revealed, compared with Sham group (E), an evident loss of DAT-positive cells (F). Animals subjected to treatment with HD revealed a positive stain for DAT (G) compared with rotenone group. *** $p < 0.001$ *vs* Sham; ** $p < 0.01$ *vs* rotenone (H). Midbrain was marked with antibodies against α–synuclein aggregation (I-K). Immunohistochemical analysis revealed, compared with Sham animals (I), a positive staining for α-synuclein (J). HD treatment appreciably reduced a positive staining for α-synuclein in the SN (K). *** $p < 0.001$ *vs* Sham; *** $p < 0.001$ *vs* rotenone (L). Data are expressed as % of TH+ positive pixel and are means ± SEM of 5 mice/group. Scale bar 20 μm.

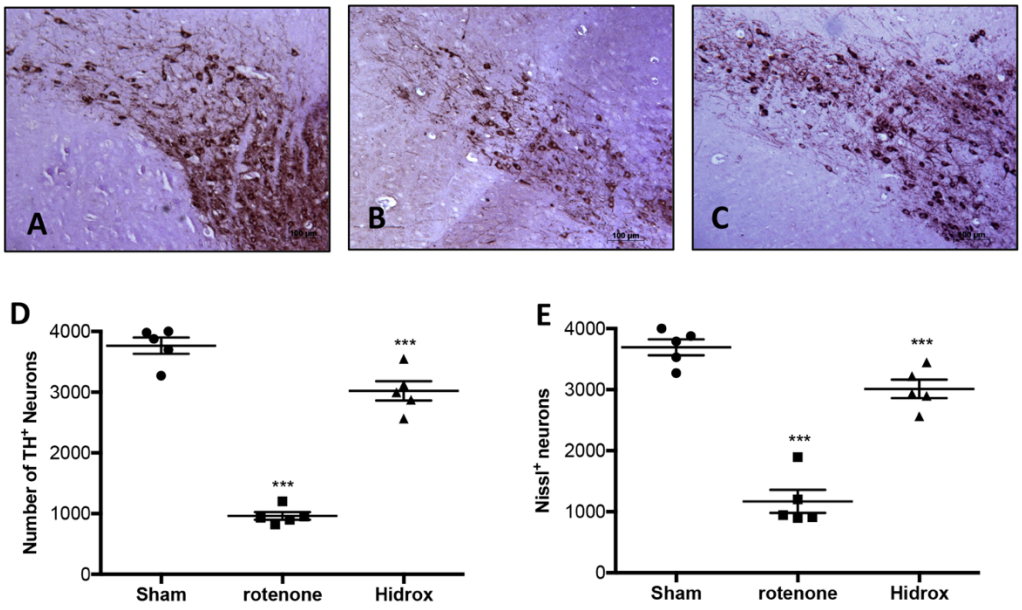

Figure S2. Stereological analysis in SN of rotenone-treated mice. Sham (A), rotenone-mice (B), and HD-treated mice (C), respectively, stained for stereological counting of TH-positive and cresyl violet positive neurons in sections of the SN from one hemisphere (D, E). Each data is expressed as a number of TH^+ and $Nissl^+$ neurons and are mean ± SEM from 5 mice/group. *** $p < 0.001$ *vs* Sham; *** $p < 0.001$ *vs* rotenone (D, E). Scale bar 100 µm.

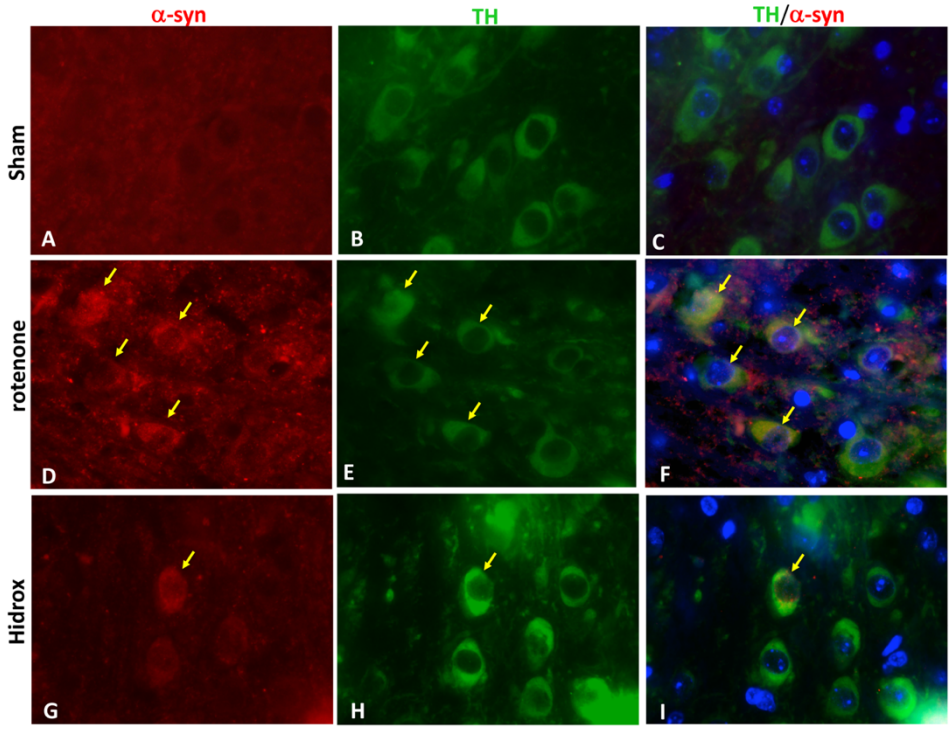

Figure 3. Effects of HD on α-synuclein expression and co-localization of TH/ α–synuclein after rotenone-intoxication. Results are shown for Sham (A-C), mice after rotenone-intoxication (D-F), mice

treated with HD (G-I). Midbrain sections were double stained with antibodies against TH (B, E, H green)/α-syn (A, D, G red). Midbrain sections revealed an important co-localization of TH with α-syn in rotenone group (F), compared with Sham group (C). TH/α-syn co-localization was increased after HD (I). The photographs are illustrative of at least three experiments performed on different days, and are representative of all animals in every group (N = 5 mice/group). Images were digitalized at a resolution of 8 bits into an array of 2048 x 2048 pixels. Scale bar 100 μm.

9.2.3 Effect of HD on cellular stress response after rotenone administration

Oxidative stress in the brain is accompanied by augmented expression of genes contributing to free radicals' detoxification, rescue of mitochondrial function and cell survival stress responsive genes called *vitagenes*. Vitagenes encode for Hsps, HO-1, and sirtuin protein systems whose activation is most likely dependent on Nrf2. In agreement with previously reported data from our laboratory demonstrating beneficial effects by HD against PD [102], we wanted to evaluate the effect of HD treatment on the activation of Nrf2 and consecutively on HO-1, Hsp-70 and Sirt-1 expression. Western blot analysis revealed a significant increase of Nrf2 in rotenone-treated mice. Treatment with HD furtherly increased the levels of this protein (Fig 4 A and graph A1). In addition, we demonstrated that HO-1, Hsp-70 and Sirt-1 expression also began to increase in mice treated with rotenone but we observed more significant levels of this protein in HD-treated mice (Fig. 4 C, D, E and graphs C1, D1 and E1)

In addition, we investigated rotenone effect in absence or in presence of HD in different areas of the brain. Levels of Hsp70, measured by Western blot analysis in the cortex (CX), substantia nigra (SN), striatum (ST) and hippocampus (HP) in rotenone treated animal in absence and presence of HD administration and compared to untreated control animals are shown in Figure 5. Treatment of rotenone injected animals with HD resulted in a significant increase in the synthesis of Hsp70 in all brain regions examined, whereas this effect was not observed in the group of mice receiving rotenone alone, as compared to controls, in all brain regions examined, except for SN, where rotenone-induced increase in Hsp70 was significant vs both control and rotenone plus HD treatment group. A representative Western blot, obtained probing the different brain regions for Hsp70 proteins, is shown in the same figure (Fig. 5 A, A1; B, B1; C,

C1 and D, D1). We also examined regional expression of γ-GGCS in the same experimental conditions. Rotenone administration, given alone, significantly increased γ-GCS expression in the brain regions of SN and striatum, but not in the cortex or hippocampus, as compared to control group of animals. In response to HD administration the animals with the damage showed increased synthesis of γ-GCS protein, as compared to controls, and this increase was higher than the levels found in the group of mice receiving only rotenone, in all brain regions examined. A representative Western blot, obtained probing brain homogenate obtained from a specific region with an antibody specific for γ-GCS protein is also shown (Fig. 6 A, A1; B, B1; C, C1 and D, D1).

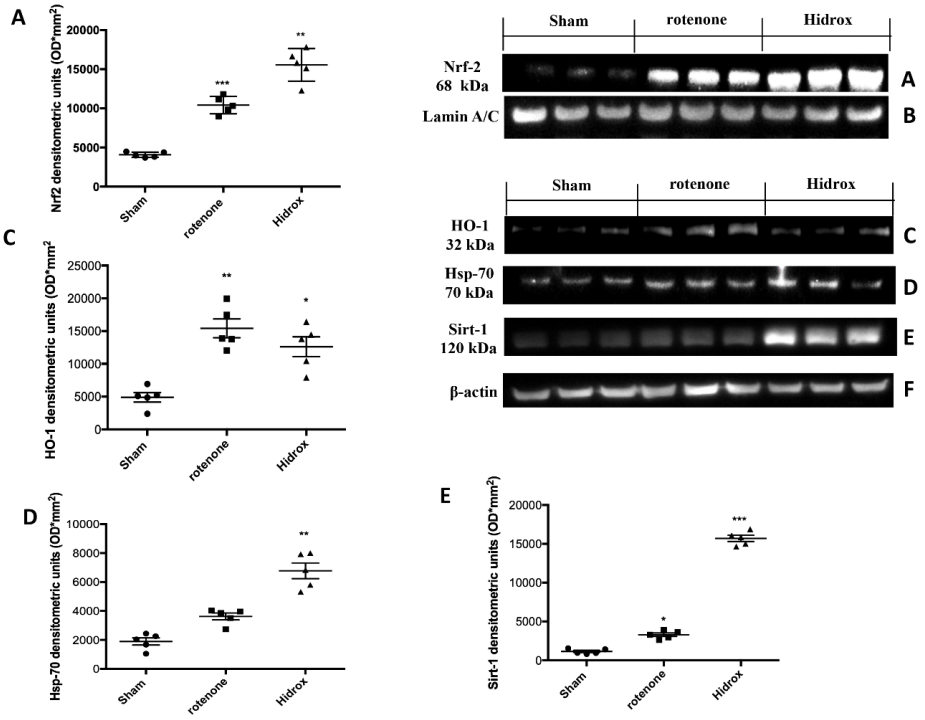

Figure 4. Effects of HD on Nrf2, HO-1, Hsp-70 and Sirt-1 proteins after rotenone intoxication. Western blot analysis demonstrated Nrf2 expression Sirt-1 expression to be significantly increased in the rotenone group, whereas treatment with HD significantly increased further Nrf2 expression (A). HO-1 expression was increased after rotenone intoxication; treatment with HD decreased levels of this protein (C). Hsp-70 expression was increased after rotenone intoxication; treatment with HD maintained high levels of this protein (D). Sirt-1 expression was increased after rotenone intoxication; treatment with HD decreased levels of this protein (E). The data are expressed as mean ± SEM from N = 5 mice/group. A) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone; C) ** $p<0.01$ *vs* Sham; * $p<0.05$ *vs* rotenone; D) ** $p<0.01$ *vs* rotenone; E) * $p<0.05$ *vs* Sham; *** $p<0.001$ *vs* rotenone.

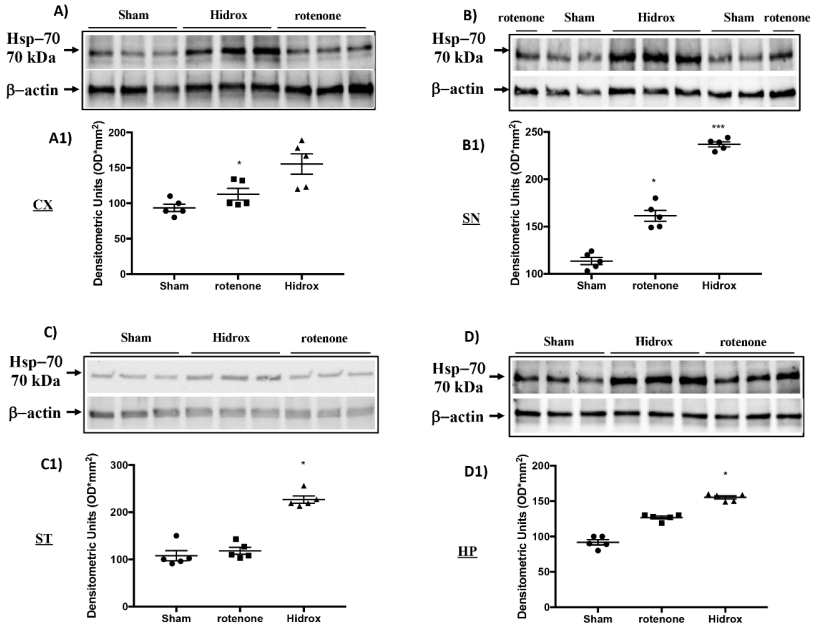

Figure 5. Effects of HD on Hsp-70 protein after rotenone intoxication. Western blot analysis showed that the expression of Hsp-70 increased insignificantly in the rotenone group in CX (A and A1), ST (C and C1), HP (D and D1) regions, while there was a significant increase in SN (B and B1) area. HD treatment further significantly increased the expression of Hsp-70 (A, A1; B, B1; C, C1; D, D1). The data are expressed as mean ± SEM from N = 5 mice/group. A1) * $p<0.05$ *vs* rotenone; B1) * $p<0.05$ *vs* rotenone; *** $p<0.001$ *vs* Sham; C1) * $p<0.05$ *vs* rotenone; D1) * $p<0.05$ *vs* rotenone.

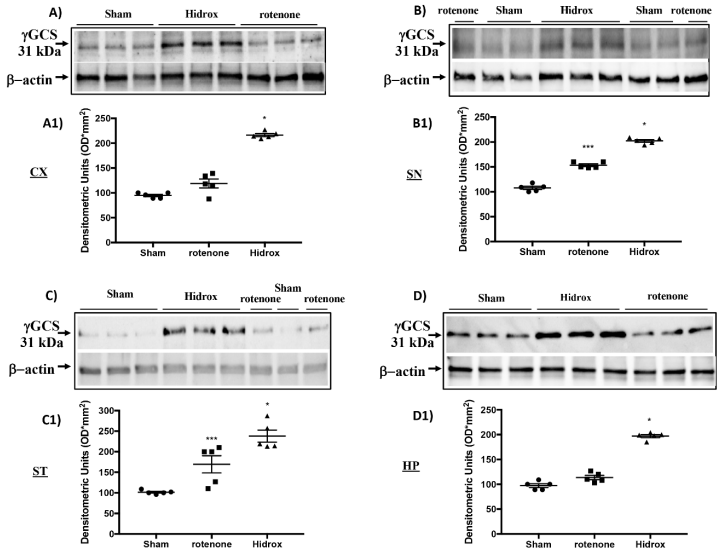

Figure 6. Effects of HD on γ-GCS protein after rotenone intoxication. Western blot analysis showed that the expression of γ-GCS increased insignificantly in the rotenone group in CX (A and A1), HP (D and D1) areas, while there was a significant increase in ST (C and C1), SN (B and B1) regions. HD treatment further significantly increased the expression of Hsp-70 (A, A1; B, B1; C, C1; D, D1). The data are expressed as mean ± SEM from N = 5 mice/group. A1) * $p<0.05$ *vs* rotenone; B1) * $p<0.05$ *vs* rotenone; *** $p<0.001$ *vs* Sham; C1) * $p<0.05$ *vs* rotenone; *** $p<0.001$ *vs* Sham; D1) * $p<0.05$ *vs* rotenone.

9.2.4 Modulation of protein carbonyls in mice brain after rotenone treatment and HD supplementation

Following oxidative stress, the accumulation of oxidation products of proteins and lipids occurs, which are measured respectively by the protein carbonyl and the 4-hydroxynonenal (HNE) [262, 263]. The formation of carbonyl groups in the residues of amino acids and HNE from arachidonic acid or other unsaturated fatty acids following the oxidation of proteins and lipids, represents a clear sign of oxidative attack of free radicals and damage to proteins and lipids [262]. Protein carbonylation and HNE exerts negative effects on cell function and viability, being generally unrepairable and leading to production of potentially dangerous protein aggregates and to cellular dysfunction [262]. Examination of brain protein carbonyls revealed in the group of rotenone-treated mice a significant elevation in all brain regions examined respect to the control group; while in rotenone-treated animals supplemented with HD we observed a significant reduction of protein carbonyl (Fig. 7 A, B and graph C).

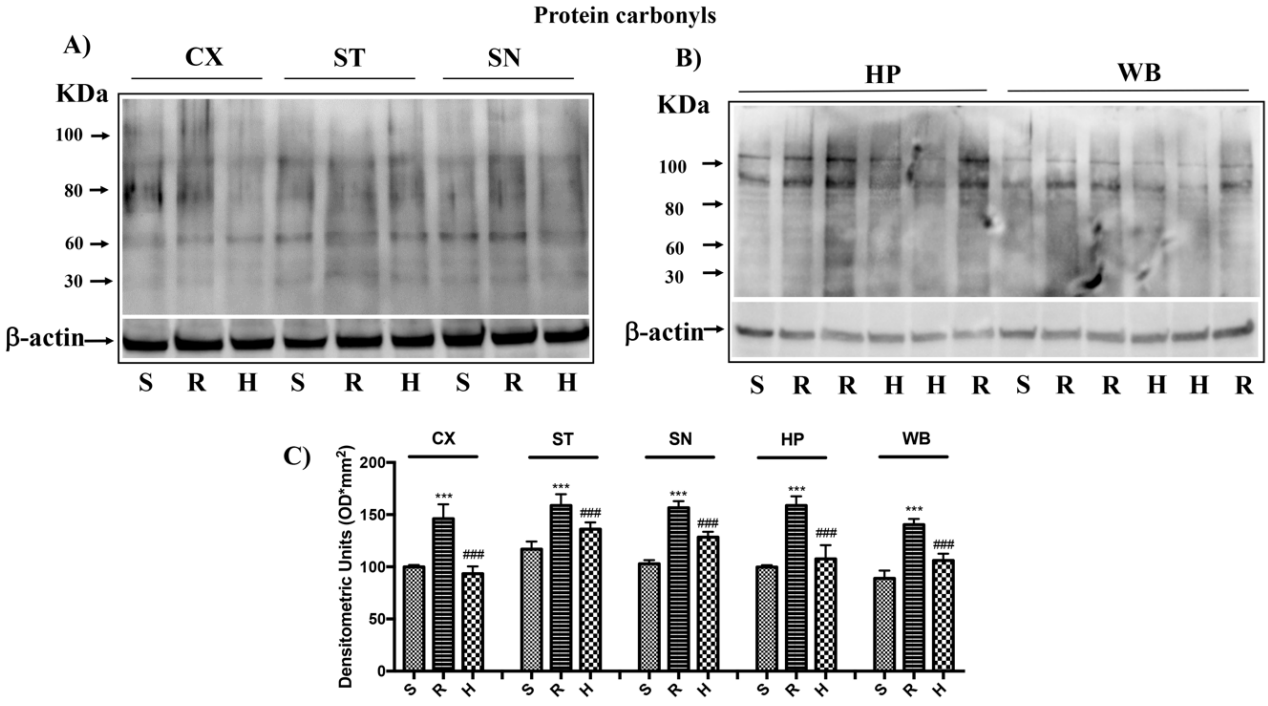

Figure 7. Brain protein carbonyls analysis after rotenone intoxication. (A, B) The examination of the carbonyls of brain proteins showed a significant increase of these in all brain regions examined after induction of rotenone (R), compared to the control (S) and animals treated with HD (H). The data are expressed as mean ± SEM from N = 5 mice/group. C) *** $p<0.001$ *vs* Sham; ### $p<0.001$ *vs* rotenone.

9.2.5 Effect of HD treatment on NF-κB, IκB-α, iNOS expression and on apoptosis induced by rotenone administration

In order to evaluate the anti-neuroinflammatory effect by which HD treatment may attenuate the development of PD, we investigated the expression of NF-κB and IκB-α by Western blot analysis in the midbrain samples. NF-κB nuclear translocation was significantly increased in rotenone group.; while HD treatment attenuated NF-κB expression (Fig. 8 A). IκB-α degradation was lowered in rotenone-injured mice vis-a-vis to the Sham mice, while HD treatment increased IκB-α cytosolic activity (Fig. 8 C). In addition, to determine the role of nitric oxide (NO), iNOS expression was assessed 28 days after rotenone administration. Analysis evidenced a significant increase in iNOS levels in the SN of the rotenone group, which was considerably reduced by HD treatment (Fig. 8 D). In order to evaluate the effect of the HD treatment on the rotenone-induced apoptosis we analyzed the expression of Bax and Bcl-2. Tissues collected from rotenone-treated animals showed an increased Bax expression, compared to the control mice. HD treatment reduced this expression (Fig. 8 E). Samples taken from Sham mice showed basal levels of Bcl-2, rotenone administration reduced this expression. HD treatment restored Bcl-2 expression to basal levels (Fig. 8 F).

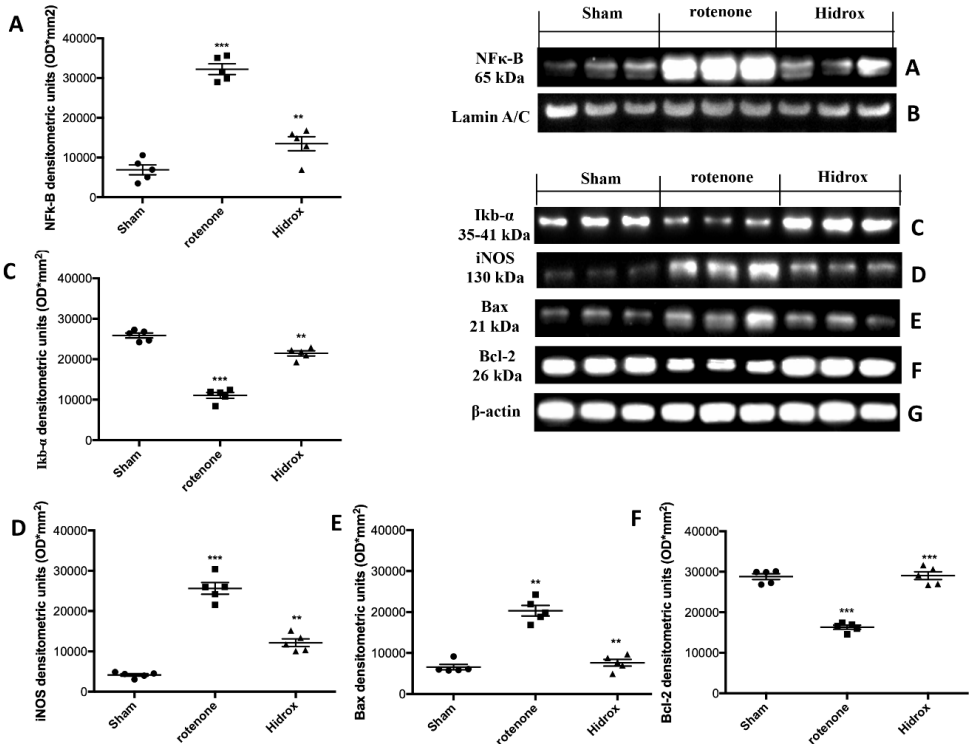

Figure 8. Effects of HD on expression of IκB-α and nuclear translocation of NF-κB p65 and on iNOS after rotenone intoxication. NF-kB levels were significantly increased in the nuclear fraction compared to the Sham animals (A). HD treatment significantly reduced NF-kB translocation (A). Basal levels of IkB-α were found tissues from Sham group, they were notably reduced in samples from rotenone-treated mice (C). HD administration significantly reduced IkB-α degradation (C). Western blot analysis of tissue lysates from rotenone-treated mice shows significant increases in iNOS expression after 28 days. HD significantly lowered the expression of iNOS in the SN after rotenone intoxication (D). Western blot analysis demonstrated Bad expression to be significantly increased in the rotenone group, whereas treatment with HD significantly limited the rise in Bad expression (E). Finally, Bcl-2 expression was reduced after rotenone intoxication; however, treatment with HD restored basal levels (F). The data are expressed as mean ± SEM from N = 5 mice/group. A) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone; C) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone; D) *** $p<0.001$ *vs* Sham; ** $p<0.01$ *vs* rotenone; E) ** $p<0.01$ *vs* Sham; ** $p<0.01$ *vs* rotenone; F) *** $p<0.001$ *vs* Sham; *** $p<0.01$ *vs* rotenone.

9.2.6 Effect of HD treatment on inflammasome pathway and Caspase-1, IL-1β, IL18 expression induced by rotenone administration

To evaluate the effect of the HD treatment on the activation of the inflammasome pathway we investigated by Western blot analysis the levels of expression of NRLP3 and ASC. We showed an increased NRLP3 expression in the rotenone-treated group, compared to the Sham mice and a down-regulation of this expression in samples collected from HD treated mice (Fig. 9 A). Western blot analysis revealed an up-regulation of the ASC levels in samples collected from

rotenone-treated mice, compared to the control group. HD-treated mice showed an inhibition of the ASC expression (Fig. 9 B). Brain tissues from rotenone-treated mice showed an increased expression of the Caspase-1, compared to the control mice. HD treatment reduced this expression (Fig. 9 C). Western Blot analysis displayed an up-regulation of IL-1β levels, compared to the Sham animals. HD administration down-regulated IL-1β expression (Fig. 9 D). HD treatment also reduced IL-18 expression induced by rotenone-administration. Samples collected from sham mice showed s basal expression of IL-18 (Fig. 9 E).

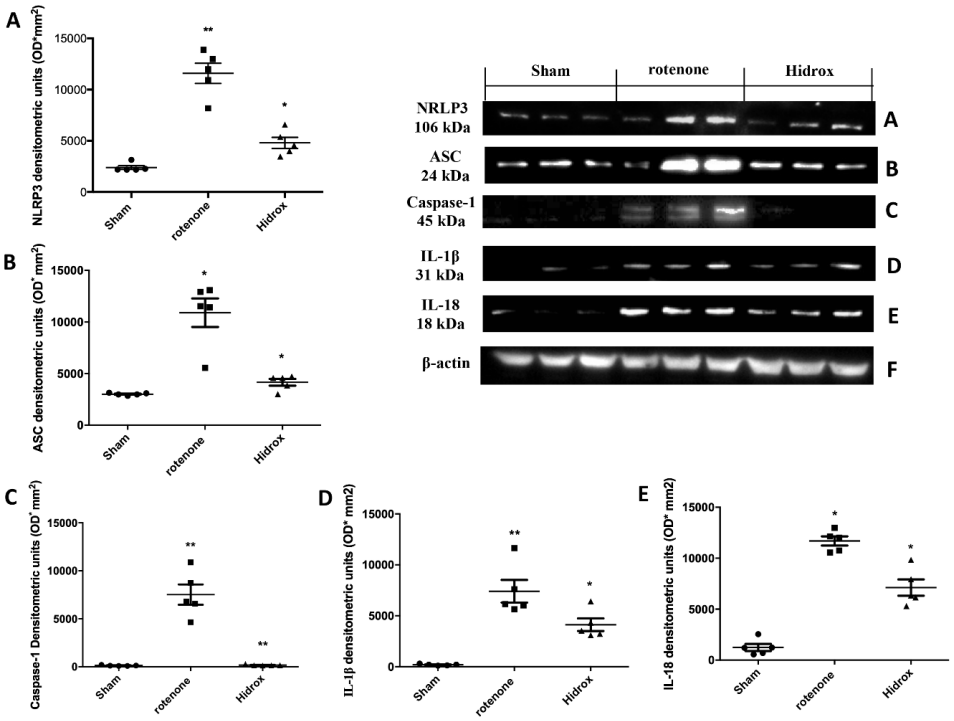

Figure 9. Effects of HD on Inflammasome pathway after rotenone administration. Brain tissues collected from rotenone treated-animals displayed increased levels of NRLP3 expression, compared to the Sham mice (A). HD treatment reduced NRLP3 expression (A). Western blot analysis also showed an increased expression of ASC levels in rotenone treated-mice, compared to the control mice (B). HD treatment decreased ASC expression (B). Western blot analysis showed significant expression levels of Caspase-1 in SN of rotenone-treated mice which were reduced significantly by HD (C). The increase of IL-1β expression observed in the rotenone group was likewise limited to a significant extent with HD (D). Furthermore, Western blot analysis displayed an up-regulation of IL-18 expression compared to the Sham mice. HD administrations notably reduced this expression (E). The data are expressed as mean ± SEM from N = 5 mice/group. A) ** $p<0.01$ *vs* Sham; * $p<0.05$ *vs* rotenone; B) * $p<0.05$ *vs* Sham; * $p<0.05$ *vs* rotenone; C) ** $p<0.01$ *vs* Sham; ** $p<0.01$ *vs* rotenone; D) ** $p<0.01$ *vs* Sham; * $p<0.05$ *vs* rotenone; E) * $p<0.05$ *vs* Sham; * $p<0.05$ *vs* rotenone.

Chapter 10: DISCUSSIONS AND CONCLUSIONS

The successes of research, technology and social development have led to a lengthening of the life duration, leading researchers to address the many problems related to the management of an ever-increasing number of people suffering from complex diseases, and in most cases, disabling. Particular attention is now paid to TBI and neurodegenerative diseases, which are disorders in strong growth in the most developed countries and of great impact for its economic and social repercussions. Neuroinflammation has a critical role in the pathophysiology of neurodegenerative diseases, pain, neuropsychiatric disorders, brain stroke and brain trauma. Tissue injury and associated inflammatory mediators release may trigger heterogeneous events, including cognitive decline and neurodegeneration [264]. Furthermore, numerous studies have shown that oxidative stress is a mediator of the damage associated with TBI and neurodegenerative process [265-268]. In recent years, many studies have focused on the analysis of natural phyto-components present in food as important bioactive molecules against chronic age-related diseases such as neurodegenerative diseases or against inflammatory disorders [269-274]. This therapeutic approach appears to be advantageous for several aspects including the reduction of side effects and greater tolerability by patients. Mushrooms are nutraceutical foods with important nutritional values and are sources of novel therapeutic compounds [275, 276]. Hericium erinaceus is a culinary-medicinal mushroom commonly found in East Asian countries [277]. It is widely used in medicine [277] for its ability to promote brain health. In particular it has been described that it contains neurotrophic substances that are able to pass the blood-brain barrier [278, 279]. Bioactive compounds obtained by its mycelium of fruiting body have antidiabetic [280], antioxidant [281], anticancer [282, 283], antimicrobial [283], anti-inflammatory [284], hypolipidemic [285] and antihyperglycemic properties [64]. Moreover, its efficacy has been described in cognitive impairments [284], presbycusis [287] and ischemic stroke [288]. Recently, Moringa oleifera has been extensively studied for its important potential as a source of healthy diet of medicinal value. Moringa oleifera contains

high nutraceutical compounds in the leaves [289]. Minerals, protein and β-carotene present in the leaves can be stored for long time as dried powder. Several international relief organizations are proposing the leaves of Moringa oleifera as nutritional supplement for Africa countries [290]. Moreover, it has been used as treatment in numerous diseases, such as obesity, diabetes, scurvy, hysteria and tumors [73, 74]. Moringa oleifera has anti-cancer [75], anti-inflammatory [76], anti-oxidant [77], hepatoprotective [78], anti-bacterial [79], cardioprotective [80], hypolipidemic [81], hypoglycemic [82] and anti-hypertensive [83] activities. Based on the results reported in the literature on the beneficial effects of Hericium erinaceus and Moringa, we wanted to evaluate these properties in two experimental models of TBI and AD. Regarding TBI model, animals receiving Hericium erinaceus administration showed no significant differences with vehicle-treated mice with respect to histological analysis and behaviors. Treatment with Moringa oleifera and Hericium erinaceus plus Moringa oleifera significantly improved not only histological alterations, but also led to improved trauma-induced motor function and behavioral changes.

In the AD model, daily treatment with these compounds and in particular with Moringa demonstrated a significant ability to counteract neuronal degeneration and $AlCl_3$-induced β–amyloid depositions. Furthermore, treatment with Hericium erinaceus or Moringa oleifera or the combination of both also improved the cognitive deficits observed after $AlCl_3$ administration.

Inflammation, as mentioned above, is implicated in both brain injury and AD [206, 291]. Consequently, the search for compounds capable of preventing or countering the inflammatory response that is activated in both pathologies is important.

The most studied inflammatory pathway is that of NFkB. It can be activated by various stimuli such as oxidative stress, hypoxia, extracellular signals, infections and inflammation. These events cause IκB phosphorylation by IκB kinase, which in turn leads to the release of the NFκB

dimer and translocation to the nucleus. In particular, the phosphorylation of the p65 subunits in Ser^{536} and its translocation into the nucleus is required for the transcriptional activity of NFκB **[292]**.

In the TBI study we found an upregulation of IκB-α expression in the cytoplasm and an increase in NFkB expression in the nucleus in vehicle treated animals and also in Hericium erinaceus treated animals. Conversely, treatment with Moringa oleifera and Hericium erinaceus plus Moringa oleifera were able to reduce the activation of the NFkB pathway. The inflammatory pathway triggered by traumatic injury also induces the release of the of proinflammatory cytokines and recruitment of peripheral immune cells **[293]**. TNF-α and IL-1β expressions in the brain were reduced in Moringa oleifera and Hericium erinaceus plus Moringa oleifera treated animals compared to the vehicle treated mice. Hericium erinaceus administered by itself did not show any significant difference compared to the animals only administered with vehicle. Molecular changes induced by TBI also includes the increase of the oxidative stress [10, 294]. These reactive oxygen species elicits the damage of DNA and protein, and rises membrane permeability. Our results indicate that Moringa oleifera and Hericium erinaceus plus Moringa oleifera administrations reduced oxidative stress activation as demonstrated via decrease of MDA, increase of SOD and glutathione peroxidase, and activation of Nrf2. The axonal injury produced by TBI induce secondary injury cascades, which in turn produce glial activation [295, 296]. Our experiments confirm the increased expression of GFAP, used as marker of reactive astrocytes [297], and Iba-1, used as marker of microglia activation [298], in vehicle treated animals and Hericium erinaceus administered mice as well. In groups treated with Moringa oleifera and Hericium erinaceus plus Moringa oleifera there was a reduced glia activation. One of the major factors of brain damage induced by TBI is the neuronal cell death. It is well described that apoptosis is a molecular characteristic of damage induced by TBI [299]. Vehicle and Hericium erinaceus treated animals showed up-regulation of Bax and reduction of Bcl-2

expression. In this study Moringa oleifera and Hericium erinaceus plus Moringa oleifera administration as well displays protective role against TBI improving neuronal survival.

Regarding the anti-inflammatory effect of Hericium erinaceus or Moringa oleifera, or Hericium erinaceus plus Moringa oleifera in the AD study, we have shown that these treatments, especially the one with Moringa, were capable of significantly reducing the degradation of IκB-α and consequently to inhibit $AlCl_3$-induced NF−κB p65 nuclear translocation. Furthermore, Hericium erinaceus, Hericium erinaceus plus Moringa oleifera and in particular Moringa have been shown to act on COX-1 and iNOS by significantly reducing their expression. To consolidate the effect of treatments on the inflammatory process induced by $AlCl_3$, we evaluated the levels of proinflammatory cytokines such as TNF-α and IL-6. Our AD study found that all three treatments were able to significantly reduce pro-inflammatory cytokine production. Furthermore, we have shown that treatment with Hericium erinaceus or Moringa oleifera or the combination of both can also reduce the levels of MDA, an important indicator of lipid peroxidation, and stimulate the activation of the Nrf2 pathway. Also, in these analyzes a greater effect of Moringa was shown compared to the other treatments.

For the study on Parkinson's disease we thought of a preventive approach linked to the potential beneficial properties of the Mediterranean diet (MD). Numerous studies support the beneficial effects of the MD in the prevention of neurodegeneration. MD expects a high consumption of grains, vegetables, fruits, olive oil, legumes, a low consumption of meat and a moderate amount of fish, seafood and alcohol. This results in a reduction in the consumption of saturated fats and in an increase in the intake of foods containing high concentrations of bioactive compounds such as polyphenols and flavonoids [92, 93]. In particular, many recent data highlight the importance of phenols contained in extra virgin olive oil (EVOO) to counteract the misfolding and toxicity of proteins, with particular attention to the mechanisms that lead to the onset and progression of PD [300, 301]. The main component of EVOO and olive is hydroxytyrosol

which is an important protective factor. HT has been shown to have the highest antioxidant activity ever measured compared to any other known natural antioxidant [302, 303]. HT in addition to reducing oxidative stress by inhibiting free radicals and eliminating reactive oxygen and nitrogen, has been shown to have anti-inflammatory and antimicrobial effects on humans and animals and to stimulate the immune system [304-306]. The properties of HD were evaluated in an in vitro study on neuroinflammation which highlighted the ability of this compound to reduce the production of pro inflammatory cytokines such as TNF-α, IL-1β and IL-6 [307]. Fascinating is also the in vivo study with olive polyphenols which showed that oleuropein and its derivatives, in particular HD, exert neuroprotective effects, a general improvement in the duration of health and longevity, as well as resistance to stress in the model of PD in C. elegans stressed by rotenone [308].

Based on this evidence, the aim of our study was to evaluate whether the aqueous extract of olive pulp containing 40-50% of HT, known as Hidrox® (HD), prevents the neurodegenerative process in an animal model of rotenone induced PD. In particular, we hypothesize that HD is able to act not only on the oxidative stress through the Nrf2 pathway but also on the neuroinflammation and other important mechanisms involved in the pathogenesis of PD.

Here, thanks to our PD model induced by rotenone on male mice, we have shown that administration of HD during the entire period of the PD induction by rotenone rises the redox potential correlated with induction of *vitagenes* and helps susceptible neurons to resist to proteotoxic insults and therefore to neurodegeneration. Restoration of normal proteostasis seems to be crucial for neuronal survival. Therefore, our study does not aim to evaluate a treatment for the cure of PD but to demonstrate that the use of natural compounds such as HD could prevent the neurodegenerative process typical of this pathology. Several statistical studies have shown that more men than women are diagnosed with PD [309]. The differences between gender of symptoms, course and cognitive aspect have not yet been extensively examined. A

source of cognitive differences could be the effect of estrogen on dopaminergic neurons and pathways in the brain. For this reason, we decided to carry out our research on male mice, but the study on females would also be interesting. Our results have shown that HD is able to act not only on oxidative stress but also on the inflammatory response, apoptosis and inflammasome, thus containing the accumulation of α-synuclein, the loss of dopaminergic neurons and the behavioral deficits.

Nrf2, one of the most important transcription factors that activates several genes with cytoprotective function, participates in antioxidant and anti-inflammatory reactions [310-315]. These cytoprotective genes encode a wide variety of phase II detoxification enzymes such as NAD (P) H: quinone oxidoreductase 1 (NQO1), HO-1, γ-GCS, glutathione S-transferase (GST) and glutamate-cysteine ligase, thioredoxin, thioredoxin reductase, thermal shock proteins and many others [106, 316-319].

Cell culture studies have shown that HT was the only olive oil phenol capable of increasing the transactivation of Nrf2, which suggests that this polyphenol could be responsible for the induction of Nrf2 dependent gene expression [320].

Therefore, our hypothesis is that HD carries out its neuroprotective action through the activation of the Nrf2 / HO-1 axis. As an effect of oxidative stress in tissues and organs, protein and lipid oxidation occur. Introduction of carbonyl groups into amino acid residues is a hallmark for oxidative injury to proteins by ROS. Protein carbonylation can have harmful effects on cell function and viability, since it is generally unrepairable by cells and can lead to protein dysfunction and to the production of potentially harmful protein aggregates [262]. Analysis of the carbonyls in the brain proteins revealed significantly higher levels in the rotenone group compared to that treated with HD. Moreover, our results showed the ability of HD to significantly increase the levels of Nrf2, HO-1, Hsp-70, Sirt-1 and γ-GCS compared to the group treated only with rotenone. As observed in several studies [321, 322] and confirmed by

our results, we can indicate a relationship between Nrf2 and NF-kB, a transcription factor responsible for the inflammatory response that activates many genes coding for pro-inflammatory cytokines and immunoregulatory mediators.

In our study we have shown that HD is capable of preventing the nuclear translocation of NF-κB and the degradation of IkB-α. In addition, iNOS levels have also been significantly reduced by HD. This suggests that regulation of redox homeostasis by Nrf2 probably leads to modulation of NF-kB activity and the inflammatory response characteristic of PD. Another complex that could be modulated by Nrf2 is the NLRP3 inflammasome whose activation is linked to various stressors. Therefore, in our study we evaluated the Caspase-1, IL-1β, IL-18, ASC and NLRP3 levels by demonstrating that HD was actually able to significantly reduce the expression of these proteins which was instead increased by rotenone. Our study also showed that HD protects neuronal cells from rotenone-induced apoptosis. The levels of the Bax pro-apoptosis marker were significantly reduced by HD, conversely the anti-apoptotic protein Bcl-2 was significantly increased. Overall, the modulation of the processes just described allowed a reduction in the death of dopaminergic neurons evidenced by the restoration of the levels of two specific markers, TH and DAT, in the brains of HD treated mice. In addition, HD prevented the α-synuclein from aggregating and forming accumulations in in dopaminergic neurons which is also reflected in a reduction in the motor deficits induced by rotenone.

In conclusion, our results demonstrated that natural compounds such as Hericium erinaceus, Moringa and Hydroxytyrosol are powerful anti-inflammatories and antioxidants. Therefore, Hericium erinaceus and Moringa represent a nutritional product that could be used as therapies against TBI and AD, while Hidrox® could be used as a preventive agent in the neurodegenerative process characteristic of PD.

References

1. Mueller, K. (2013) Inflammation. Inflammation's yin-yang. Introduction, *Science.* **339**, 155.

2. Neumann, H., Kotter, M. R. & Franklin, R. J. (2009) Debris clearance by microglia: an essential link between degeneration and regeneration, *Brain : a journal of neurology.* **132**, 288-95.

3. Di Filippo, M., Chiasserini, D., Tozzi, A., Picconi, B. & Calabresi, P. (2010) Mitochondria and the link between neuroinflammation and neurodegeneration, *J Alzheimers Dis.* **20 Suppl 2**, S369-79.

4. Ransohoff, R. M. & Perry, V. H. (2009) Microglial physiology: unique stimuli, specialized responses, *Annu Rev Immunol.* **27**, 119-45.

5. Garden, G. A. & Moller, T. (2006) Microglia biology in health and disease, *J Neuroimmune Pharmacol.* **1**, 127-37.

6. Harry, G. J. & Kraft, A. D. (2008) Neuroinflammation and microglia: considerations and approaches for neurotoxicity assessment, *Expert Opin Drug Metab Toxicol.* **4**, 1265-77.

7. Morganti-Kossmann, M. C., Rancan, M., Stahel, P. F. & Kossmann, T. (2002) Inflammatory response in acute traumatic brain injury: a double-edged sword, *Curr Opin Crit Care.* **8**, 101-5.

8. Nagele, R. G., Wegiel, J., Venkataraman, V., Imaki, H., Wang, K. C. & Wegiel, J. (2004) Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease, *Neurobiol Aging.* **25**, 663-74.

9. Steinman, L. (2007) A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage, *Nat Med.* **13**, 139-45.

10. Wang, Q., Tang, X. N. & Yenari, M. A. (2007) The inflammatory response in stroke, *Journal of neuroimmunology.* **184**, 53-68.

11. Yang, G. Y., Zhao, Y. J., Davidson, B. L. & Betz, A. L. (1997) Overexpression of interleukin-1 receptor antagonist in the mouse brain reduces ischemic brain injury, *Brain research.* **751**, 181-8.

12. Pinteaux, E., Rothwell, N. J. & Boutin, H. (2006) Neuroprotective actions of endogenous interleukin-1 receptor antagonist (IL-1ra) are mediated by glia, *Glia.* **53**, 551-6.

13. Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family, *Annu Rev Immunol.* **27**, 519-50.

14. McCoy, M. K. & Tansey, M. G. (2008) TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease, *Journal of neuroinflammation.* **5**, 45.

15. Fillit, H., Ding, W. H., Buee, L., Kalman, J., Altstiel, L., Lawlor, B. & Wolf-Klein, G. (1991) Elevated circulating tumor necrosis factor levels in Alzheimer's disease, *Neuroscience letters.* **129**, 318-20.

16. Planas, A. M., Gorina, R. & Chamorro, A. (2006) Signalling pathways mediating inflammatory responses in brain ischaemia, *Biochem Soc Trans.* **34**, 1267-70.

17. Rebenko-Moll, N. M., Liu, L., Cardona, A. & Ransohoff, R. M. (2006) Chemokines, mononuclear cells and the nervous system: heaven (or hell) is in the details, *Current opinion in immunology.* **18**, 683-9.

18. Frangogiannis, N. G. (2007) Chemokines in ischemia and reperfusion, *Thromb Haemost.* **97**, 738-47.

19. Kakio, T., Matsumori, A., Ono, K., Ito, H., Matsushima, K. & Sasayama, S. (2000) Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart, *Lab Invest.* **80**, 1127-36.

20. Huang, D., Han, Y., Rani, M. R., Glabinski, A., Trebst, C., Sorensen, T., Tani, M., Wang, J., Chien, P., O'Bryan, S., Bielecki, B., Zhou, Z. L., Majumder, S. & Ransohoff, R. M. (2000) Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation, *Immunol Rev.* **177**, 52-67.

21. Renner, O., Tsimpas, A., Kostin, S., Valable, S., Petit, E., Schaper, W. & Marti, H. H. (2003) Time- and cell type-specific induction of platelet-derived growth factor receptor-beta during cerebral ischemia, *Brain Res Mol Brain Res.* **113**, 44-51.

22. Gorba, T., Bradoo, P., Antonic, A., Marvin, K., Liu, D. X., Lobie, P. E., Reymann, K. G., Gluckman, P. D. & Sieg, F. (2006) Neural regeneration protein is a novel chemoattractive and neuronal survival-promoting factor, *Exp Cell Res.* **312**, 3060-74.

23. Harman, D. (1981) The aging process, *Proceedings of the National Academy of Sciences of the United States of America.* **78**, 7124-8.

24. Patten, D. A., Germain, M., Kelly, M. A. & Slack, R. S. (2010) Reactive oxygen species: stuck in the middle of neurodegeneration, *J Alzheimers Dis.* **20 Suppl 2**, S357-67.

25. Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R. & Milzani, A. (2006) Protein carbonylation, cellular dysfunction, and disease progression, *J Cell Mol Med.* **10**, 389-406.

26. Das, D. K., Maulik, N., Sato, M. & Ray, P. S. (1999) Reactive oxygen species function as second messenger during ischemic preconditioning of heart, *Molecular and cellular biochemistry.* **196**, 59-67.

27. Poyton, R. O., Ball, K. A. & Castello, P. R. (2009) Mitochondrial generation of free radicals and hypoxic signaling, *Trends Endocrinol Metab.* **20**, 332-40.

28. Landis, G. N. & Tower, J. (2005) Superoxide dismutase evolution and life span regulation, *Mech Ageing Dev.* **126**, 365-79.

29. Mates, J. M., Perez-Gomez, C. & Nunez de Castro, I. (1999) Antioxidant enzymes and human diseases, *Clin Biochem.* **32**, 595-603.

30. Chaudiere, J. & Ferrari-Iliou, R. (1999) Intracellular antioxidants: from chemical to biochemical mechanisms, *Food Chem Toxicol.* **37**, 949-62.

31. Masella, R., Di Benedetto, R., Vari, R., Filesi, C. & Giovannini, C. (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes, *J Nutr Biochem.* **16**, 577-86.

32. Droge, W. (2002) Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants, *Exp Gerontol.* **37**, 1333-45.

33. Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., Stamler, J. S., Rhee, S. G. & van der Vliet, A. (2008) Redox-based regulation of signal transduction: principles, pitfalls, and promises, *Free radical biology & medicine.* **45**, 1-17.

34. Kensler, T. W., Wakabayashi, N. & Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway, *Annu Rev Pharmacol Toxicol.* **47**, 89-116.

35. Ma, Q. & He, X. (2012) Molecular basis of electrophilic and oxidative defense: promises and perils of Nrf2, *Pharmacol Rev.* **64**, 1055-81.

36. Finkel, T. (2012) Signal transduction by mitochondrial oxidants, *The Journal of biological chemistry.* **287**, 4434-40.

37. Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A. & Dulak, J. (2016) Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism, *Cellular and molecular life sciences : CMLS.* **73**, 3221-47.

38. Calabrese, E. J. & Mattson, M. P. (2017) How does hormesis impact biology, toxicology, and medicine?, *NPJ Aging Mech Dis.* **3**, 13.

39. Calabrese, E. J. & Baldwin, L. A. (2001) The frequency of U-shaped dose responses in the toxicological literature, *Toxicol Sci.* **62**, 330-8.

40. Calabrese, E. J. & Baldwin, L. A. (2002) Defining hormesis, *Human & experimental toxicology.* **21**, 91-7.

41. Calabrese, E. J., Bachmann, K. A., Bailer, A. J., Bolger, P. M., Borak, J., Cai, L., Cedergreen, N., Cherian, M. G., Chiueh, C. C., Clarkson, T. W., Cook, R. R., Diamond, D. M., Doolittle, D. J., Dorato, M. A., Duke, S. O., Feinendegen, L., Gardner, D. E., Hart, R. W., Hastings, K. L., Hayes, A. W., Hoffmann, G. R., Ives, J. A., Jaworowski, Z., Johnson, T. E., Jonas, W. B., Kaminski, N. E., Keller, J. G., Klaunig, J. E., Knudsen, T. B., Kozumbo, W. J., Lettieri, T., Liu, S. Z., Maisseu, A., Maynard, K. I., Masoro, E. J., McClellan, R. O., Mehendale, H. M., Mothersill, C., Newlin, D. B., Nigg, H. N., Oehme, F. W., Phalen, R. F., Philbert, M. A., Rattan, S. I., Riviere, J. E., Rodricks, J., Sapolsky, R. M., Scott, B. R., Seymour, C., Sinclair, D. A., Smith-Sonneborn, J., Snow, E. T., Spear, L., Stevenson, D. E., Thomas, Y., Tubiana, M., Williams, G. M. & Mattson, M. P. (2007) Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework, *Toxicology and applied pharmacology.* **222**, 122-8.

42. Calabrese, E. J. & Mattson, M. P. (2011) Hormesis provides a generalized quantitative estimate of biological plasticity, *J Cell Commun Signal.* **5**, 25-38.

43. Calabrese, V., Giordano, J., Ruggieri, M., Berritta, D., Trovato, A., Ontario, M. L., Bianchini, R. & Calabrese, E. J. (2016) Hormesis, cellular stress response, and redox homeostasis in autism spectrum disorders, *Journal of neuroscience research.* **94**, 1488-1498.

44. Kirino, T. (2002) Ischemic tolerance, *J Cereb Blood Flow Metab.* **22**, 1283-96.

45. Jiang, X., Zhu, D., Okagaki, P., Lipsky, R., Wu, X., Banaudha, K., Mearow, K., Strauss, K. I. & Marini, A. M. (2003) N-methyl-D-aspartate and TrkB receptor activation in cerebellar granule cells: an in vitro model of preconditioning to stimulate intrinsic survival pathways in neurons, *Ann N Y Acad Sci.* **993**, 134-45; discussion 159-60.

46. Ding, Y., Li, J., Luan, X., Ding, Y. H., Lai, Q., Rafols, J. A., Phillis, J. W., Clark, J. C. & Diaz, F. G. (2004) Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin, *Neuroscience.* **124**, 583-91.

47. Mattson, M. P. (2005) Energy intake, meal frequency, and health: a neurobiological perspective, *Annu Rev Nutr.* **25**, 237-60.

48. Huang, P. L. (2004) Nitric oxide and cerebral ischemic preconditioning, *Cell Calcium.* **36**, 323-9.

49. Dore, S., Goto, S., Sampei, K., Blackshaw, S., Hester, L. D., Ingi, T., Sawa, A., Traystman, R. J., Koehler, R. C. & Snyder, S. H. (2000) Heme oxygenase-2 acts to prevent neuronal death in brain cultures and following transient cerebral ischemia, *Neuroscience.* **99**, 587-92.

50. Jiang, X., Tian, F., Mearow, K., Okagaki, P., Lipsky, R. H. & Marini, A. M. (2005) The excitoprotective effect of N-methyl-D-aspartate receptors is mediated by a brain-derived neurotrophic factor autocrine loop in cultured hippocampal neurons, *Journal of neurochemistry.* **94**, 713-22.

51. See, V., Boutillier, A. L., Bito, H. & Loeffler, J. P. (2001) Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* **15**, 134-144.

52. Mattson, M. P. & Meffert, M. K. (2006) Roles for NF-kappaB in nerve cell survival, plasticity, and disease, *Cell death and differentiation.* **13**, 852-60.

53. West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., Takasu, M. A., Tao, X. & Greenberg, M. E. (2001) Calcium regulation of neuronal gene expression, *Proceedings of the National Academy of Sciences of the United States of America.* **98**, 11024-31.

54. Jeong, W. S., Jun, M. & Kong, A. N. (2006) Nrf2: a potential molecular target for cancer chemoprevention by natural compounds, *Antioxid Redox Signal.* **8**, 99-106.

55. Liu, J., Narasimhan, P., Yu, F. & Chan, P. H. (2005) Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin, *Stroke.* **36**, 1264-9.

56. Mattson, M. P. (2003) Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders, *Neuromolecular Med.* **3**, 65-94.

57. Liu, R. H. (2003) Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals, *Am J Clin Nutr.* **78**, 517S-520S.

58. Joseph, J. A., Shukitt-Hale, B. & Casadesus, G. (2005) Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds, *Am J Clin Nutr.* **81**, 313S-316S.

59. Ikeda, K., Negishi, H. & Yamori, Y. (2003) Antioxidant nutrients and hypoxia/ischemia brain injury in rodents, *Toxicology.* **189**, 55-61.

60. Calabrese, V., Giordano, J., Signorile, A., Laura Ontario, M., Castorina, S., De Pasquale, C., Eckert, G. & Calabrese, E. J. (2016) Major pathogenic mechanisms in vascular dementia: Roles of cellular stress response and hormesis in neuroprotection, *Journal of neuroscience research.* **94**, 1588-1603.

61. Trovato Salinaro, A., Pennisi, M., Di Paola, R., Scuto, M., Crupi, R., Cambria, M. T., Ontario, M. L., Tomasello, M., Uva, M., Maiolino, L., Calabrese, E. J., Cuzzocrea, S. & Calabrese, V. (2018) Neuroinflammation and neurohormesis in the pathogenesis of Alzheimer's disease and Alzheimer-linked pathologies: modulation by nutritional mushrooms, *Immunity & ageing : I & A.* **15**, 8.

62. Mattson, M. P. & Cheng, A. (2006) Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses, *Trends Neurosci.* **29**, 632-9.

63. Friedman, M. (2015) Chemistry, Nutrition, and Health-Promoting Properties of Hericium erinaceus (Lion's Mane) Mushroom Fruiting Bodies and Mycelia and Their Bioactive Compounds, *J Agric Food Chem.* **63**, 7108-23.

64. Liang, B., Guo, Z., Xie, F. & Zhao, A. (2013) Antihyperglycemic and antihyperlipidemic activities of aqueous extract of Hericium erinaceus in experimental diabetic rats, *BMC Complement Altern Med.* **13**, 253.

65. Lai, C. L., Lin, R. T., Liou, L. M. & Liu, C. K. (2010) The role of event-related potentials in cognitive decline in Alzheimer's disease, *Clin Neurophysiol.* **121**, 194-9.

66. Mori, K., Inatomi, S., Ouchi, K., Azumi, Y. & Tuchida, T. (2009) Improving effects of the mushroom Yamabushitake (Hericium erinaceus) on mild cognitive impairment: a double-blind placebo-controlled clinical trial, *Phytotherapy research : PTR.* **23**, 367-72.

67. Trovato, A., Siracusa, R., Di Paola, R., Scuto, M., Ontario, M. L., Bua, O., Di Mauro, P., Toscano, M. A., Petralia, C. C. T., Maiolino, L., Serra, A., Cuzzocrea, S. & Calabrese, V. (2016) Redox modulation of cellular stress response and lipoxin A4 expression by Hericium Erinaceus in rat brain: relevance to Alzheimer's disease pathogenesis, *Immunity & ageing : I & A.* **13**, 23.

68. Wu, J., Wang, A., Min, Z., Xiong, Y., Yan, Q., Zhang, J., Xu, J. & Zhang, S. (2011) Lipoxin A4 inhibits the production of proinflammatory cytokines induced by beta-amyloid in vitro and in vivo, *Biochemical and biophysical research communications.* **408**, 382-7.

69. McGeer, P. L. & McGeer, E. G. (2002) Innate immunity, local inflammation, and degenerative disease, *Sci Aging Knowledge Environ.* **2002**, re3.

70. Abdull Razis, A. F., Ibrahim, M. D. & Kntayya, S. B. (2014) Health benefits of Moringa oleifera, *Asian Pacific journal of cancer prevention : APJCP.* **15**, 8571-6.

71. Fahey, J. W., Zalcmann, A. T. & Talalay, P. (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants, *Phytochemistry.* **56**, 5-51.

72. Brunelli, D., Tavecchio, M., Falcioni, C., Frapolli, R., Erba, E., Iori, R., Rollin, P., Barillari, J., Manzotti, C., Morazzoni, P. & D'Incalci, M. (2010) The isothiocyanate produced from glucomoringin inhibits NF-kB and reduces myeloma growth in nude mice in vivo, *Biochemical pharmacology.* **79**, 1141-8.

73. Gupta, S., Jain, R., Kachhwaha, S. & Kothari, S. (2018) Nutritional and medicinal applications of Moringa oleifera Lam.—Review of current status and future possibilities, *Journal of Herbal Medicine.* **11**, 1-11.

74. Kasolo, J. N., Bimenya, G. S., Ojok, L., Ochieng, J. & Ogwal-Okeng, J. W. (2010) Phytochemicals and uses of Moringa oleifera leaves in Ugandan rural communities, *Journal of Medicinal Plants Research.* **4**.

75. Jung, I. L. (2014) Soluble extract from Moringa oleifera leaves with a new anticancer activity, *PLoS One.* **9**, e95492.

76. Coppin, J. P., Xu, Y., Chen, H., Pan, M.-H., Ho, C.-T., Juliani, R., Simon, J. E. & Wu, Q. (2013) Determination of flavonoids by LC/MS and anti-inflammatory activity in Moringa oleifera, *Journal of Functional Foods.* **5**, 1892-1899.

77. Verma, A. R., Vijayakumar, M., Rao, C. V. & Mathela, C. S. (2010) In vitro and in vivo antioxidant properties and DNA damage protective activity of green fruit of Ficus glomerata, *Food Chem Toxicol.* **48**, 704-9.

78. Atta, A. H., Nasr, S. M., Almaweri, A. H., Sedky, D., Mohamed, A. M., Desouky, H. M. & Shalaby, M. A. (2018) Phytochemical, antioxidant and hepatoprotective effects of different fractions of Moringa oleifera leaves methanol extract against liver injury in animal model, *Asian Pacific Journal of Tropical Medicine.* **11**, 423.

79. Peixoto, J. R., Silva, G. C., Costa, R. A., de Sousa Fontenelle, J. R., Vieira, G. H., Filho, A. A. & dos Fernandes Vieira, R. H. (2011) In vitro antibacterial effect of aqueous and ethanolic Moringa leaf extracts, *Asian Pac J Trop Med.* **4**, 201-4.

80. Choudhary, M. K., Bodakhe, S. H. & Gupta, S. K. (2013) Assessment of the antiulcer potential of Moringa oleifera root-bark extract in rats, *J Acupunct Meridian Stud.* **6**, 214-20.

81. Helmy, S. A., Morsy, N. F. S., Elaby, S. M. & Ghaly, M. A. A. (2017) Hypolipidemic Effect of Moringa oleifera Lam Leaf Powder and its Extract in Diet-Induced Hypercholesterolemic Rats, *J Med Food.* **20**, 755-762.

82. Jaiswal, D., Rai, P. K., Kumar, A., Mehta, S. & Watal, G. (2009) Effect of Moringa oleifera Lam. leaves aqueous extract therapy on hyperglycemic rats, *Journal of ethnopharmacology.* **123**, 392-396.

83. Dangi, S., Jolly, C. I. & Narayanan, S. (2002) Antihypertensive activity of the total alkaloids from the leaves of Moringa oleifera, *Pharmaceutical biology.* **40**, 144-148.

84. Hamza, A. A. (2010) Ameliorative effects of Moringa oleifera Lam seed extract on liver fibrosis in rats, *Food Chem Toxicol.* **48**, 345-55.

85. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A. & Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance, *The Journal of clinical investigation.* **112**, 1821-30.

86. Ferrante, A. W., Jr. (2007) Obesity-induced inflammation: a metabolic dialogue in the language of inflammation, *J Intern Med.* **262**, 408-14.

87. Bhargava, P. & Lee, C. H. (2012) Role and function of macrophages in the metabolic syndrome, *The Biochemical journal.* **442**, 253-62.

88. Verma, A. R., Vijayakumar, M., Mathela, C. S. & Rao, C. V. (2009) In vitro and in vivo antioxidant properties of different fractions of Moringa oleifera leaves, *Food Chem Toxicol.* **47**, 2196-201.

89. Siddhuraju, P. & Becker, K. (2003) Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves, *J Agric Food Chem.* **51**, 2144-55.

90. Kirisattayakul, W., Wattanathorn, J., Tong-Un, T., Muchimapura, S., Wannanon, P. & Jittiwat, J. (2013) Cerebroprotective effect of Moringa oleifera against focal ischemic stroke induced by middle cerebral artery occlusion, *Oxid Med Cell Longev.* **2013**, 951415.

91. Sutalangka, C., Wattanathorn, J., Muchimapura, S. & Thukham-mee, W. (2013) Moringa oleifera mitigates memory impairment and neurodegeneration in animal model of age-related dementia, *Oxid Med Cell Longev.* **2013**, 695936.

92. Robles-Almazan, M., Pulido-Moran, M., Moreno-Fernandez, J., Ramirez-Tortosa, C., Rodriguez-Garcia, C., Quiles, J. L. & Ramirez-Tortosa, M. (2018) Hydroxytyrosol: Bioavailability, toxicity, and clinical applications, *Food Res Int.* **105**, 654-667.

93. Hoffman, R. & Gerber, M. (2015) Food Processing and the Mediterranean Diet, *Nutrients.* **7**, 7925-64.

94. Angeloni, C., Malaguti, M., Barbalace, M. C. & Hrelia, S. (2017) Bioactivity of Olive Oil Phenols in Neuroprotection, *Int J Mol Sci.* **18**.

95. Peyrol, J., Riva, C. & Amiot, M. J. (2017) Hydroxytyrosol in the Prevention of the Metabolic Syndrome and Related Disorders, *Nutrients.* **9**.

96. Feart, C., Samieri, C. & Barberger-Gateau, P. (2010) Mediterranean diet and cognitive function in older adults, *Curr Opin Clin Nutr Metab Care.* **13**, 14-8.

97. Scarmeas, N., Stern, Y., Mayeux, R., Manly, J. J., Schupf, N. & Luchsinger, J. A. (2009) Mediterranean diet and mild cognitive impairment, *Archives of neurology.* **66**, 216-25.

98. Tasset, I., Pontes, A. J., Hinojosa, A. J., de la Torre, R. & Tunez, I. (2011) Olive oil reduces oxidative damage in a 3-nitropropionic acid-induced Huntington's disease-like rat model, *Nutr Neurosci.* **14**, 106-11.

99. Grossi, C., Rigacci, S., Ambrosini, S., Ed Dami, T., Luccarini, I., Traini, C., Failli, P., Berti, A., Casamenti, F. & Stefani, M. (2013) The polyphenol oleuropein aglycone protects TgCRND8 mice against Ass plaque pathology, *PLoS One.* **8**, e71702.

100. Casamenti, F., Grossi, C., Rigacci, S., Pantano, D., Luccarini, I. & Stefani, M. (2015) Oleuropein Aglycone: A Possible Drug against Degenerative Conditions. In Vivo Evidence of its Effectiveness against Alzheimer's Disease, *J Alzheimers Dis.* **45**, 679-88.

101. Arunsundar, M., Shanmugarajan, T. S. & Ravichandran, V. (2015) 3,4-dihydroxyphenylethanol attenuates spatio-cognitive deficits in an Alzheimer's disease mouse model: modulation of the molecular signals in neuronal survival-apoptotic programs, *Neurotox Res.* **27**, 143-55.

102. Leri, M., Scuto, M., Ontario, M. L., Calabrese, V., Calabrese, E. J., Bucciantini, M. & Stefani, M. (2020) Healthy Effects of Plant Polyphenols: Molecular Mechanisms, *Int J Mol Sci.* **21**.

103. Montoya, T., Aparicio-Soto, M., Castejon, M. L., Rosillo, M. A., Sanchez-Hidalgo, M., Begines, P., Fernandez-Bolanos, J. G. & Alarcon-de-la-Lastra, C. (2018) Peracetylated hydroxytyrosol, a new hydroxytyrosol derivate, attenuates LPS-induced inflammatory

response in murine peritoneal macrophages via regulation of non-canonical inflammasome, Nrf2/HO1 and JAK/STAT signaling pathways, *J Nutr Biochem.* **57**, 110-120.

104. Peng, S., Zhang, B., Yao, J., Duan, D. & Fang, J. (2015) Dual protection of hydroxytyrosol, an olive oil polyphenol, against oxidative damage in PC12 cells, *Food & function.* **6**, 2091-100.

105. Kim, S., Indu Viswanath, A. N., Park, J. H., Lee, H. E., Park, A. Y., Choi, J. W., Kim, H. J., Londhe, A. M., Jang, B. K., Lee, J., Hwang, H., Lim, S. M., Pae, A. N. & Park, K. D. (2020) Nrf2 activator via interference of Nrf2-Keap1 interaction has antioxidant and anti-inflammatory properties in Parkinson's disease animal model, *Neuropharmacology.* **167**, 107989.

106. Calabrese, V., Cornelius, C., Dinkova-Kostova, A. T., Calabrese, E. J. & Mattson, M. P. (2010) Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders, *Antioxid Redox Signal.* **13**, 1763-811.

107. Calabrese, E. J., Calabrese, V., Tsatsakis, A. & Giordano, J. J. (2020) Hormesis and Ginkgo biloba (GB): Numerous biological effects of GB are mediated via hormesis, *Ageing Res Rev*, 101019.

108. Topal, N. B., Hakyemez, B., Erdogan, C., Bulut, M., Koksal, O., Akkose, S., Dogan, S., Parlak, M., Ozguc, H. & Korfali, E. (2008) MR imaging in the detection of diffuse axonal injury with mild traumatic brain injury, *Neurological research.* **30**, 974-978.

109. Kalia, L. V., Kalia, S. K. & Salter, M. W. (2008) NMDA receptors in clinical neurology: excitatory times ahead, *Lancet Neurol.* **7**, 742-755.

110. Morganti-Kossmann, M. C., Satgunaseelan, L., Bye, N. & Kossmann, T. (2007) Modulation of immune response by head injury, *Injury-International Journal of the Care of the Injured.* **38**, 1392-1400.

111. Bains, M. & Hall, E. D. (2012) Antioxidant therapies in traumatic brain and spinal cord injury, *Bba-Mol Basis Dis.* **1822**, 675-684.

112. Whitnall, L., McMillan, T. M., Murray, G. D. & Teasdale, G. M. (2006) Disability in young people and adults after head injury: 5-7 year follow up of a prospective cohort study, *J Neurol Neurosur Ps.* **77**, 640-645.

113. Kiraly, M. A. & Kiraly, S. J. (2007) Traumatic brain injury and delayed sequelae: A review - Traumatic brain injury and mild traumatic brain injury (Concussion) are precursors to later-onset brain disorders, including early-onset dementia, *TheScientificWorldJournal.* **7**, 1768-1776.

114. Draper, K. & Ponsford, J. (2008) Cognitive functioning ten years following traumatic brain injury and rehabilitation, *Neuropsychology.* **22**, 618-625.

115. Ponsford, J., Draper, K. & Schonberger, M. (2008) Functional outcome 10 years after traumatic brain injury: Its relationship with demographic, injury severity, and cognitive and emotional status, *J Int Neuropsych Soc.* **14**, 233-242.

116. Gyoneva, S. & Ransohoff, R. M. (2015) Inflammatory reaction after traumatic brain injury: therapeutic potential of targeting cell-cell communication by chemokines, *Trends in pharmacological sciences.* **36**, 471-480.

117. Das, M., Mohapatra, S. & Mohapatra, S. S. (2012) New perspectives on central and peripheral immune responses to acute traumatic brain injury, *Journal of neuroinflammation.* **9**.

118. Giunta, B., Obregon, D., Velisetty, R., Sanberg, P. R., Borlongan, C. V. & Tan, J. (2012) The immunology of traumatic brain injury: a prime target for Alzheimer's disease prevention, *Journal of neuroinflammation.* **9**.

119. Woodcock, T. & Morganti-Kossmann, M. C. (2013) The role of markers of inflammation in traumatic brain injury, *Front Neurol.* **4**.

120. Gouvier, W. D., Blanton, P. D., Laporte, K. K. & Nepomuceno, C. (1987) Reliability and Validity of the Disability Rating-Scale and the Levels of Cognitive-Functioning Scale in Monitoring Recovery from Severe Head-Injury, *Archives of physical medicine and rehabilitation.* **68**, 94-97.

121. Faul, M., Xu, L., Wald, M.M., and Coronado, V.G. (2010) Traumatic brain injury in the United States: Emergency department visits, hospitalizations, and deaths 2002–2006, *Atlanta, GA: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control*.

122. Coronado, V. G., McGuire, L. C., Sarmiento, K., Bell, J., Lionbarger, M. R., Jones, C. D., Geller, A. I., Khoury, N. & Xu, L. K. (2012) Trends in Traumatic Brain Injury in the US and the public health response: 1995-2009, *J Safety Res.* **43**, 299-307.

123. Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Zhang, Z. G., Morris, D. C. & Chopp, M. (2012) Neuroprotective and neurorestorative effects of thymosin beta4 treatment following experimental traumatic brain injury, *Ann N Y Acad Sci.* **1270**, 51-8.

124. Hagg, T. (2005) Molecular regulation of adult CNS neurogenesis: an integrated view, *Trends Neurosci.* **28**, 589-95.

125. Marklund, N. & Hillered, L. (2011) Animal modelling of traumatic brain injury in preclinical drug development: where do we go from here?, *British journal of pharmacology.* **164**, 1207-29.

126. Ballesteros, J., Guemes, I., Ibarra, N. & Quemada, J. I. (2008) The effectiveness of donepezil for cognitive rehabilitation after traumatic brain injury: a systematic review, *J Head Trauma Rehabil.* **23**, 171-80.

127. Bourgeois, J. A., Bahadur, N. & Minjares, S. (2002) Donepezil for cognitive deficits following traumatic brain injury: a case report, *J Neuropsychiatry Clin Neurosci.* **14**, 463-4.

128. Foster, M. & Spiegel, D. R. (2008) Use of donepezil in the treatment of cognitive impairments of moderate traumatic brain injury, *J Neuropsychiatry Clin Neurosci.* **20**, 106.

129. Hayashida, K., Parker, R. & Eisenach, J. C. (2007) Oral gabapentin activates spinal cholinergic circuits to reduce hypersensitivity after peripheral nerve injury and interacts synergistically with oral donepezil, *Anesthesiology.* **106**, 1213-9.

130. Kaye, N. S., Townsend, J. B., 3rd & Ivins, R. (2003) An open-label trial of donepezil (aricept) in the treatment of persons with mild traumatic brain injury, *J Neuropsychiatry Clin Neurosci.* **15**, 383-4; author reply 384-5.

131. Tenovuo, O. (2005) Central acetylcholinesterase inhibitors in the treatment of chronic traumatic brain injury-clinical experience in 111 patients, *Prog Neuropsychopharmacol Biol Psychiatry.* **29**, 61-7.

132. Wheaton, P., Mathias, J. L. & Vink, R. (2011) Impact of pharmacological treatments on cognitive and behavioral outcome in the postacute stages of adult traumatic brain injury: a meta-analysis, *J Clin Psychopharmacol.* **31**, 745-57.

133. Blount, P. J., Nguyen, C. D. & McDeavitt, J. T. (2002) Clinical use of cholinomimetic agents: a review, *J Head Trauma Rehabil.* **17**, 314-21.

134. Noble, J. M. & Hauser, W. A. (2007) Effects of rivastigmine on cognitive function in patients with traumatic brain injury, *Neurology.* **68**, 1749; author reply 1750.

135. Silver, J. M., Koumaras, B., Chen, M., Mirski, D., Potkin, S. G., Reyes, P., Warden, D., Harvey, P. D., Arciniegas, D., Katz, D. I. & Gunay, I. (2006) Effects of rivastigmine on cognitive function in patients with traumatic brain injury, *Neurology.* **67**, 748-55.

136. Silver, J. M., Koumaras, B., Meng, X., Potkin, S. G., Reyes, P. F., Harvey, P. D., Katz, D. I., Gunay, I. & Arciniegas, D. B. (2009) Long-term effects of rivastigmine capsules in patients with traumatic brain injury, *Brain Inj.* **23**, 123-32.

137. Tenovuo, O., Alin, J. & Helenius, H. (2009) A randomized controlled trial of rivastigmine for chronic sequels of traumatic brain injury-what it showed and taught?, *Brain Inj.* **23**, 548-58.

138. Chen, Y., Shohami, E., Bass, R. & Weinstock, M. (1998) Cerebro-protective effects of ENA713, a novel acetylcholinesterase inhibitor, in closed head injury in the rat, *Brain research.* **784**, 18-24.

139. Bales, J. W., Wagner, A. K., Kline, A. E. & Dixon, C. E. (2009) Persistent cognitive dysfunction after traumatic brain injury: A dopamine hypothesis, *Neurosci Biobehav Rev.* **33**, 981-1003.

140. Gianutsos, G., Chute, S. & Dunn, J. P. (1985) Pharmacological changes in dopaminergic systems induced by long-term administration of amantadine, *European journal of pharmacology.* **110**, 357-61.

141. Dixon, C. E., Kraus, M. F., Kline, A. E., Ma, X., Yan, H. Q., Griffith, R. G., Wolfson, B. M. & Marion, D. W. (1999) Amantadine improves water maze performance without affecting motor behavior following traumatic brain injury in rats, *Restorative neurology and neuroscience.* **14**, 285-294.

142. Giacino, J. T., Whyte, J., Bagiella, E., Kalmar, K., Childs, N., Khademi, A., Eifert, B., Long, D., Katz, D. I., Cho, S., Yablon, S. A., Luther, M., Hammond, F. M., Nordenbo, A., Novak, P., Mercer, W., Maurer-Karattup, P. & Sherer, M. (2012) Placebo-controlled trial of amantadine for severe traumatic brain injury, *The New England journal of medicine.* **366**, 819-26.

143. Brustovetsky, N. & Dubinsky, J. M. (2000) Limitations of cyclosporin A inhibition of the permeability transition in CNS mitochondria, *The Journal of neuroscience : the official journal of the Society for Neuroscience.* **20**, 8229-37.

144. Sharov, V. G., Todor, A., Khanal, S., Imai, M. & Sabbah, H. N. (2007) Cyclosporine A attenuates mitochondrial permeability transition and improves mitochondrial respiratory function in cardiomyocytes isolated from dogs with heart failure, *J Mol Cell Cardiol.* **42**, 150-8.

145. Alessandri, B., Rice, A. C., Levasseur, J., DeFord, M., Hamm, R. J. & Bullock, M. R. (2002) Cyclosporin A improves brain tissue oxygen consumption and learning/memory performance after lateral fluid percussion injury in rats, *Journal of neurotrauma.* **19**, 829-41.

146. Colley, B. S., Phillips, L. L. & Reeves, T. M. (2010) The effects of cyclosporin-A on axonal conduction deficits following traumatic brain injury in adult rats, *Exp Neurol.* **224**, 241-51.

147. Kilbaugh, T. J., Bhandare, S., Lorom, D. H., Saraswati, M., Robertson, C. L. & Margulies, S. S. (2011) Cyclosporin A preserves mitochondrial function after traumatic brain injury in the immature rat and piglet, *Journal of neurotrauma.* **28**, 763-74.

148. Sullivan, P. G., Sebastian, A. H. & Hall, E. D. (2011) Therapeutic window analysis of the neuroprotective effects of cyclosporine A after traumatic brain injury, *Journal of neurotrauma.* **28**, 311-8.

149. Turkoglu, O. F., Eroglu, H., Gurcan, O., Bodur, E., Sargon, M. F., Oner, L. & Beskonakli, E. (2010) Local administration of chitosan microspheres after traumatic brain injury in rats: a new challenge for cyclosporine--a delivery, *Br J Neurosurg.* **24**, 578-83.

150. Singleton, R. H., Stone, J. R., Okonkwo, D. O., Pellicane, A. J. & Povlishock, J. T. (2001) The immunophilin ligand FK506 attenuates axonal injury in an impact-acceleration model of traumatic brain injury, *Journal of neurotrauma.* **18**, 607-14.

151. Chauhan, N. B. & Gatto, R. (2010) Synergistic benefits of erythropoietin and simvastatin after traumatic brain injury, *Brain research.* **1360**, 177-92.

152. Li, B., Mahmood, A., Lu, D., Wu, H., Xiong, Y., Qu, C. & Chopp, M. (2009) Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1beta level after traumatic brain injury, *Neurosurgery.* **65**, 179-85; discussion 185-6.

153. Chen, G., Zhang, S., Shi, J., Ai, J., Qi, M. & Hang, C. (2009) Simvastatin reduces secondary brain injury caused by cortical contusion in rats: possible involvement of TLR4/NF-kappaB pathway, *Experimental neurology.* **216**, 398-406.

154. Hicdonmez, T., Kanter, M., Tiryaki, M., Parsak, T. & Cobanoglu, S. (2006) Neuroprotective effects of N-acetylcysteine on experimental closed head trauma in rats, *Neurochemical research.* **31**, 473-81.

155. Chen, G., Shi, J., Hu, Z. & Hang, C. (2008) Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine, *Mediators of inflammation.* **2008**, 716458.

156. Yi, J. H. & Hazell, A. S. (2005) N-acetylcysteine attenuates early induction of heme oxygenase-1 following traumatic brain injury, *Brain research.* **1033**, 13-9.

157. Saatman, K. E., Contreras, P. C., Smith, D. H., Raghupathi, R., McDermott, K. L., Fernandez, S. C., Sanderson, K. L., Voddi, M. & McIntosh, T. K. (1997) Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury, *Experimental neurology.* **147**, 418-27.

158. Doulah, A. H., Rohani, A. H., Khaksari Haddad, M., Motamedi, F., Farbood, Y., Badavi, M., Malek, M. & Sarkaki, A. (2009) The effect of peripheral administration of growth hormone on AD-like cognitive deficiency in NBM-lesioned rats, *Neuroscience letters.* **466**, 47-51.

159. Xiong, Y., Mahmood, A., Zhang, Y., Meng, Y., Zhang, Z. G., Qu, C., Sager, T. N. & Chopp, M. (2011) Effects of posttraumatic carbamylated erythropoietin therapy on reducing lesion volume and hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome in rats following traumatic brain injury, *Journal of neurosurgery.* **114**, 549-59.

160. Xiong, Y., Mahmood, A., Qu, C., Kazmi, H., Zhang, Z. G., Noguchi, C. T., Schallert, T. & Chopp, M. (2010) Erythropoietin improves histological and functional outcomes after traumatic brain injury in mice in the absence of the neural erythropoietin receptor, *Journal of neurotrauma.* **27**, 205-15.

161. Atif, F., Yousuf, S., Sayeed, I., Ishrat, T., Hua, F. & Stein, D. G. (2013) Combination treatment with progesterone and vitamin D hormone is more effective than monotherapy in ischemic stroke: the role of BDNF/TrkB/Erk1/2 signaling in neuroprotection, *Neuropharmacology.* **67**, 78-87.

162. Bian, X. X., Yuan, X. S. & Qi, C. P. (2010) Effect of recombinant human erythropoietin on serum S100B protein and interleukin-6 levels after traumatic brain injury in the rat, *Neurol Med Chir (Tokyo).* **50**, 361-6.

163. Cherian, L., Goodman, J. C. & Robertson, C. (2011) Improved cerebrovascular function and reduced histological damage with darbepoietin alfa administration after cortical impact injury in rats, *The Journal of pharmacology and experimental therapeutics.* **337**, 451-6.

164. Jin, W., Kong, J., Lu, T., Wang, H., Ni, H., Wu, J., Dai, Y., Jiang, J. & Liang, W. (2011) Erythropoietin prevents secondary brain injury induced by cortical lesion in mice: possible involvement of Nrf2 signaling pathway, *Ann Clin Lab Sci.* **41**, 25-32.

165. Valable, S., Francony, G., Bouzat, P., Fevre, M. C., Mahious, N., Bouet, V., Farion, R., Barbier, E., Lahrech, H., Remy, C., Petit, E., Segebarth, C., Bernaudin, M. & Payen, J. F. (2010) The impact of erythropoietin on short-term changes in phosphorylation of brain protein kinases in a rat model of traumatic brain injury, *J Cereb Blood Flow Metab.* **30**, 361-9.

166. Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Qu, C., Schallert, T. & Chopp, M. (2010) Delayed administration of erythropoietin reducing hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome following traumatic brain

injury in rats: comparison of treatment with single and triple dose, *Journal of neurosurgery.* **113**, 598-608.

167. Zhang, Y., Xiong, Y., Mahmood, A., Meng, Y., Liu, Z., Qu, C. & Chopp, M. (2010) Sprouting of corticospinal tract axons from the contralateral hemisphere into the denervated side of the spinal cord is associated with functional recovery in adult rat after traumatic brain injury and erythropoietin treatment, *Brain research.* **1353**, 249-57.

168. Miskowiak, K. W., Vinberg, M., Harmer, C. J., Ehrenreich, H., Knudsen, G. M., Macoveanu, J., Hansen, A. R., Paulson, O. B., Siebner, H. R. & Kessing, L. V. (2010) Effects of erythropoietin on depressive symptoms and neurocognitive deficits in depression and bipolar disorder, *Trials.* **11**, 97.

169. Wustenberg, T., Begemann, M., Bartels, C., Gefeller, O., Stawicki, S., Hinze-Selch, D., Mohr, A., Falkai, P., Aldenhoff, J. B., Knauth, M., Nave, K. A. & Ehrenreich, H. (2011) Recombinant human erythropoietin delays loss of gray matter in chronic schizophrenia, *Mol Psychiatry.* **16**, 26-36, 1.

170. Caltagirone, C. (2005) Neurodegenerazione, la morte dei neuroni, le malattie neurodegenerative.

171. Ehringer, H. & Hornykiewicz, O. (1960) [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system], *Klinische Wochenschrift.* **38**, 1236-9.

172. Chio, A., Magnani, C. & Schiffer, D. (1998) Prevalence of Parkinson's disease in Northwestern Italy: comparison of tracer methodology and clinical ascertainment of cases, *Movement disorders : official journal of the Movement Disorder Society.* **13**, 400-5.

173. Langston, J. W., Ballard, P., Tetrud, J. W. & Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis, *Science.* **219**, 979-80.

174. Fahn, S. & Cohen, G. (1992) The oxidant stress hypothesis in Parkinson's disease: evidence supporting it, *Annals of neurology.* **32**, 804-12.

175. Calne, D. B. (1992) The free radical hypothesis in idiopathic parkinsonism: evidence against it, *Annals of neurology.* **32**, 799-803.

176. Jenner, P., Schapira, A. H. & Marsden, C. D. (1992) New insights into the cause of Parkinson's disease, *Neurology.* **42**, 2241-50.

177. Duvoisin, R. C. (1993) The genetics of Parkinson's disease. A review, *Advances in neurology.* **60**, 306-15.

178. Morrish, P. K., Rakshi, J. S., Bailey, D. L., Sawle, G. V. & Brooks, D. J. (1998) Measuring the rate of progression and estimating the preclinical period of Parkinson's disease with [18F]dopa PET, *Journal of neurology, neurosurgery, and psychiatry.* **64**, 314-9.

179. Hornykiewicz, O., Kish, S. J., Becker, L. E., Farley, I. & Shannak, K. (1988) Biochemical evidence for brain neurotransmitter changes in idiopathic torsion dystonia (dystonia musculorum deformans), *Advances in neurology.* **50**, 157-65.

180. Jellinger, K. A. (2009) Absence of alpha-synuclein pathology in postencephalitic parkinsonism, *Acta neuropathologica.* **118**, 371-9.

181. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M. & Goedert, M. (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies, *Proceedings of the National Academy of Sciences of the United States of America.* **95**, 6469-73.

182. Forno, L. S. (1987) The Lewy body in Parkinson's disease, *Advances in neurology.* **45**, 35-43.

183. Trenkwalder, C. (1998) Sleep dysfunction in Parkinson's disease, *Clinical neuroscience.* **5**, 107-14.

184. Brown, R. G. & Marsden, C. D. (1990) Cognitive function in Parkinson's disease: from description to theory, *Trends in neurosciences.* **13**, 21-9.

185. Rascol, O., Brooks, D. J., Korczyn, A. D., De Deyn, P. P., Clarke, C. E. & Lang, A. E. (2000) A five-year study of the incidence of dyskinesia in patients with early Parkinson's disease who were treated with ropinirole or levodopa, *The New England journal of medicine.* **342**, 1484-91.

186. Metman, L. V., Del Dotto, P., LePoole, K., Konitsiotis, S., Fang, J. & Chase, T. N. (1999) Amantadine for levodopa-induced dyskinesias: a 1-year follow-up study, *Archives of neurology.* **56**, 1383-6.

187. Du, X., Wang, X. & Geng, M. (2018) Alzheimer's disease hypothesis and related therapies, *Transl Neurodegener.* **7**, 2.

188. Paul, L. (2011) Diet, nutrition and telomere length, *J Nutr Biochem.* **22**, 895-901.

189. Ostan, R., Monti, D., Gueresi, P., Bussolotto, M., Franceschi, C. & Baggio, G. (2016) Gender, aging and longevity in humans: an update of an intriguing/neglected scenario paving the way to a gender-specific medicine, *Clin Sci (Lond).* **130**, 1711-25.

190. Van Oyen, H., Nusselder, W., Jagger, C., Kolip, P., Cambois, E. & Robine, J. M. (2013) Gender differences in healthy life years within the EU: an exploration of the "health-survival" paradox, *Int J Public Health.* **58**, 143-55.

191. Arosio, B., Ostan, R., Mari, D., Damanti, S., Ronchetti, F., Arcudi, S., Scurti, M., Franceschi, C. & Monti, D. (2017) Cognitive status in the oldest old and centenarians: a condition crucial for quality of life methodologically difficult to assess, *Mech Ageing Dev.* **165**, 185-194.

192. Haroutunian, V., Purohit, D. P., Perl, D. P., Marin, D., Khan, K., Lantz, M., Davis, K. L. & Mohs, R. C. (1999) Neurofibrillary tangles in nondemented elderly subjects and mild Alzheimer disease, *Archives of neurology.* **56**, 713-8.

193. Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A., St George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M. & Neve, R. L. (1987) Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus, *Science.* **235**, 880-4.

194. Struble, R. G., Ala, T., Patrylo, P. R., Brewer, G. J. & Yan, X. X. (2010) Is brain amyloid production a cause or a result of dementia of the Alzheimer's type?, *J Alzheimers Dis.* **22**, 393-9.

195. van Helmond, Z., Miners, J. S., Kehoe, P. G. & Love, S. (2010) Higher soluble amyloid beta concentration in frontal cortex of young adults than in normal elderly or Alzheimer's disease, *Brain Pathol.* **20**, 787-93.

196. Morley, J. E., Farr, S. A., Banks, W. A., Johnson, S. N., Yamada, K. A. & Xu, L. (2010) A physiological role for amyloid-beta protein:enhancement of learning and memory, *J Alzheimers Dis.* **19**, 441-9.

197. Tampellini, D., Rahman, N., Gallo, E. F., Huang, Z., Dumont, M., Capetillo-Zarate, E., Ma, T., Zheng, R., Lu, B., Nanus, D. M., Lin, M. T. & Gouras, G. K. (2009) Synaptic activity reduces intraneuronal Abeta, promotes APP transport to synapses, and protects against Abeta-related synaptic alterations, *The Journal of neuroscience : the official journal of the Society for Neuroscience.* **29**, 9704-13.

198. Bennett, D. A., Schneider, J. A., Arvanitakis, Z., Kelly, J. F., Aggarwal, N. T., Shah, R. C. & Wilson, R. S. (2006) Neuropathology of older persons without cognitive impairment from two community-based studies, *Neurology.* **66**, 1837-44.

199. Aizenstein, H. J., Nebes, R. D., Saxton, J. A., Price, J. C., Mathis, C. A., Tsopelas, N. D., Ziolko, S. K., James, J. A., Snitz, B. E., Houck, P. R., Bi, W., Cohen, A. D., Lopresti, B. J., DeKosky,

S. T., Halligan, E. M. & Klunk, W. E. (2008) Frequent amyloid deposition without significant cognitive impairment among the elderly, *Archives of neurology.* **65**, 1509-17.

200. Holmes, C., Boche, D., Wilkinson, D., Yadegarfar, G., Hopkins, V., Bayer, A., Jones, R. W., Bullock, R., Love, S., Neal, J. W., Zotova, E. & Nicoll, J. A. (2008) Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial, *Lancet.* **372**, 216-23.

201. Salloway, S., Sperling, R., Gilman, S., Fox, N. C., Blennow, K., Raskind, M., Sabbagh, M., Honig, L. S., Doody, R., van Dyck, C. H., Mulnard, R., Barakos, J., Gregg, K. M., Liu, E., Lieberburg, I., Schenk, D., Black, R., Grundman, M. & Bapineuzumab 201 Clinical Trial, I. (2009) A phase 2 multiple ascending dose trial of bapineuzumab in mild to moderate Alzheimer disease, *Neurology.* **73**, 2061-70.

202. Bird, T. D. (2008) Genetic aspects of Alzheimer disease, *Genet Med.* **10**, 231-9.

203. Musiek, E. S. & Holtzman, D. M. (2015) Three dimensions of the amyloid hypothesis: time, space and 'wingmen', *Nat Neurosci.* **18**, 800-6.

204. Sala, G., Galimberti, G., Canevari, C., Raggi, M. E., Isella, V., Facheris, M., Appollonio, I. & Ferrarese, C. (2003) Peripheral cytokine release in Alzheimer patients: correlation with disease severity, *Neurobiol Aging.* **24**, 909-14.

205. Bauer, J., Strauss, S., Schreiter-Gasser, U., Ganter, U., Schlegel, P., Witt, I., Yolk, B. & Berger, M. (1991) Interleukin-6 and alpha-2-macroglobulin indicate an acute-phase state in Alzheimer's disease cortices, *FEBS Lett.* **285**, 111-4.

206. Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschy, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., McGeer, P. L., O'Banion, M. K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G. & Wyss-Coray, T. (2000) Inflammation and Alzheimer's disease, *Neurobiol Aging.* **21**, 383-421.

207. Song, W., Hooli, B., Mullin, K., Jin, S. C., Cella, M., Ulland, T. K., Wang, Y., Tanzi, R. E. & Colonna, M. (2017) Alzheimer's disease-associated TREM2 variants exhibit either decreased or increased ligand-dependent activation, *Alzheimers Dement.* **13**, 381-387.

208. Yuan, P., Condello, C., Keene, C. D., Wang, Y., Bird, T. D., Paul, S. M., Luo, W., Colonna, M., Baddeley, D. & Grutzendler, J. (2016) TREM2 Haplodeficiency in Mice and Humans Impairs

the Microglia Barrier Function Leading to Decreased Amyloid Compaction and Severe Axonal Dystrophy, *Neuron.* **92**, 252-264.

209. Bolos, M., Perea, J. R. & Avila, J. (2017) Alzheimer's disease as an inflammatory disease, *Biomol Concepts.* **8**, 37-43.

210. Hong, S., Dissing-Olesen, L. & Stevens, B. (2016) New insights on the role of microglia in synaptic pruning in health and disease, *Curr Opin Neurobiol.* **36**, 128-34.

211. Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Ragozzino, D. & Gross, C. T. (2011) Synaptic pruning by microglia is necessary for normal brain development, *Science.* **333**, 1456-8.

212. Casati, M., Ferri, E., Gussago, C., Mazzola, P., Abbate, C., Bellelli, G., Mari, D., Cesari, M. & Arosio, B. (2018) Increased expression of TREM2 in peripheral cells from mild cognitive impairment patients who progress into Alzheimer's disease, *Eur J Neurol.* **25**, 805-810.

213. Swomley, A. M. & Butterfield, D. A. (2015) Oxidative stress in Alzheimer disease and mild cognitive impairment: evidence from human data provided by redox proteomics, *Archives of toxicology.* **89**, 1669-80.

214. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. & Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease, *The international journal of biochemistry & cell biology.* **39**, 44-84.

215. Bhat, A. H., Dar, K. B., Anees, S., Zargar, M. A., Masood, A., Sofi, M. A. & Ganie, S. A. (2015) Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight, *Biomed Pharmacother.* **74**, 101-10.

216. Friedland-Leuner, K., Stockburger, C., Denzer, I., Eckert, G. P. & Muller, W. E. (2014) Mitochondrial dysfunction: cause and consequence of Alzheimer's disease, *Prog Mol Biol Transl Sci.* **127**, 183-210.

217. Cheignon, C., Tomas, M., Bonnefont-Rousselot, D., Faller, P., Hureau, C. & Collin, F. (2018) Oxidative stress and the amyloid beta peptide in Alzheimer's disease, *Redox Biol.* **14**, 450-464.

218. Sultana, R. & Butterfield, D. A. (2008) Redox proteomics studies of in vivo amyloid beta-peptide animal models of Alzheimer's disease: Insight into the role of oxidative stress, *Proteomics Clinical applications.* **2**, 685-96.

219. Nunomura, A., Tamaoki, T., Motohashi, N., Nakamura, M., McKeel, D. W., Jr., Tabaton, M., Lee, H. G., Smith, M. A., Perry, G. & Zhu, X. (2012) The earliest stage of cognitive

impairment in transition from normal aging to Alzheimer disease is marked by prominent RNA oxidation in vulnerable neurons, *Journal of neuropathology and experimental neurology.* **71**, 233-41.

220. Floyd, R. A. & Hensley, K. (2002) Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases, *Neurobiol Aging.* **23**, 795-807.

221. Caldwell, C. C., Yao, J. & Brinton, R. D. (2015) Targeting the prodromal stage of Alzheimer's disease: bioenergetic and mitochondrial opportunities, *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics.* **12**, 66-80.

222. Daulatzai, M. A. (2017) Cerebral hypoperfusion and glucose hypometabolism: Key pathophysiological modulators promote neurodegeneration, cognitive impairment, and Alzheimer's disease, *Journal of neuroscience research.* **95**, 943-972.

223. Blass, J. P., Gibson, G. E. & Hoyer, S. (2002) The role of the metabolic lesion in Alzheimer's disease, *J Alzheimers Dis.* **4**, 225-32.

224. Banks, W. A. (2015) The blood-brain barrier in neuroimmunology: Tales of separation and assimilation, *Brain Behav Immun.* **44**, 1-8.

225. Erickson, M. A., Dohi, K. & Banks, W. A. (2012) Neuroinflammation: a common pathway in CNS diseases as mediated at the blood-brain barrier, *Neuroimmunomodulation.* **19**, 121-30.

226. Collins, S. M., Surette, M. & Bercik, P. (2012) The interplay between the intestinal microbiota and the brain, *Nat Rev Microbiol.* **10**, 735-42.

227. Cryan, J. F. & Dinan, T. G. (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour, *Nature reviews Neuroscience.* **13**, 701-12.

228. Sampson, T. R. & Mazmanian, S. K. (2015) Control of brain development, function, and behavior by the microbiome, *Cell Host Microbe.* **17**, 565-76.

229. Campolo, M., Esposito, E., Ahmad, A., Di Paola, R., Paterniti, I., Cordaro, M., Bruschetta, G., Wallace, J. L. & Cuzzocrea, S. (2014) Hydrogen sulfide-releasing cyclooxygenase inhibitor ATB-346 enhances motor function and reduces cortical lesion volume following traumatic brain injury in mice, *Journal of neuroinflammation.* **11**, 196.

230. Impellizzeri, D., Cordaro, M., Bruschetta, G., Siracusa, R., Crupi, R., Esposito, E. & Cuzzocrea, S. (2017) N-Palmitoylethanolamine-Oxazoline as a New Therapeutic Strategy to Control Neuroinflammation: Neuroprotective Effects in Experimental Models of Spinal Cord and Brain Injury, *J Neurotrauma.* **34**, 2609-2623.

231. Pellow, S., Chopin, P., File, S. E. & Briley, M. (1985) Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, *Journal of neuroscience methods.* **14**, 149-67.

232. Ahmad, A., Crupi, R., Campolo, M., Genovese, T., Esposito, E. & Cuzzocrea, S. (2013) Absence of TLR4 reduces neurovascular unit and secondary inflammatory process after traumatic brain injury in mice, *PloS one.* **8**, e57208.

233. Prut, L. & Belzung, C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review, *European Journal of Pharmacology.* **463**, 3-33.

234. Impellizzeri, D., Campolo, M., Bruschetta, G., Crupi, R., Cordaro, M., Paterniti, I., Cuzzocrea, S. & Esposito, E. (2016) Traumatic Brain Injury Leads to Development of Parkinson's Disease Related Pathology in Mice, *Frontiers in neuroscience.* **10**.

235. Kawai, T. & Akira, S. (2007) Signaling to NF-kappaB by Toll-like receptors, *Trends Mol Med.* **13**, 460-9.

236. Gugliandolo, E., D'Amico, R., Cordaro, M., Fusco, R., Siracusa, R., Crupi, R., Impellizzeri, D., Cuzzocrea, S. & Di Paola, R. (2018) Neuroprotective Effect of Artesunate in Experimental Model of Traumatic Brain Injury, *Front Neurol.* **9**, 590.

237. Cordaro, M., Siracusa, R., Crupi, R., Impellizzeri, D., Peritore, A. F., D'Amico, R., Gugliandolo, E., Di Paola, R. & Cuzzocrea, S. (2018) 2-Pentadecyl-2-Oxazoline Reduces Neuroinflammatory Environment in the MPTP Model of Parkinson Disease, *Molecular neurobiology*, 1-16.

238. Cordaro, M., Siracusa, R., Crupi, R., Impellizzeri, D., Peritore, A. F., D'Amico, R., Gugliandolo, E., Di Paola, R. & Cuzzocrea, S. (2018) 2-Pentadecyl-2-Oxazoline Reduces Neuroinflammatory Environment in the MPTP Model of Parkinson Disease, *Mol Neurobiol*.

239. Mahmoodzadeh, T., Kashani, M. H. K., Ramshini, H., Moslem, A. & Mohammad-Zadeh, M. (2016) Effect of Camellia sinensis on Spatial Memory in a Rat Model of Alzheimer's Disease, *J Biomed.* **1**, e5340.

240. Botton, P. H., Costa, M. S., Ardais, A. P., Mioranzza, S., Souza, D. O., da Rocha, J. B. T. & Porciuncula, L. O. (2010) Caffeine prevents disruption of memory consolidation in the inhibitory avoidance and novel object recognition tasks by scopolamine in adult mice, *Behavioural Brain Research.* **214**, 254-259.

241. Mathiasen, J. R. & DiCamillo, A. (2010) Novel object recognition in the rat: a facile assay for cognitive function, *Curr Protoc Pharmacol.* **Chapter 5**, Unit 5 59.

242. Siracusa, R., Impellizzeri, D., Cordaro, M., Crupi, R., Esposito, E., Petrosino, S. & Cuzzocrea, S. (2017) Anti-Inflammatory and Neuroprotective Effects of Co-UltraPEALut in a Mouse Model of Vascular Dementia, *Front Neurol.* **8**, 233.

243. Sawant, S., Gokulan, R., Dongre, H., Vaidya, M., Chaukar, D., Prabhash, K., Ingle, A., Joshi, S., Dange, P., Joshi, S., Singh, A. K., Makani, V., Sharma, S., Jeyaram, A., Kane, S. & D'Cruz, A. (2016) Prognostic role of Oct4, CD44 and c-Myc in radio-chemo-resistant oral cancer patients and their tumourigenic potential in immunodeficient mice, *Clin Oral Investig.* **20**, 43-56.

244. Varghese, F., Bukhari, A. B., Malhotra, R. & De, A. (2014) IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples, *PLoS One.* **9**, e96801.

245. Ohkawa, H., Ohishi, N. & Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Analytical biochemistry.* **95**, 351-8.

246. Soni, M. G., Burdock, G. A., Christian, M. S., Bitler, C. M. & Crea, R. (2006) Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods, *Food Chem Toxicol.* **44**, 903-15.

247. Miraglia, N., Bianchi, D., Trentin, A., Volpi, N. & Soni, M. G. (2016) Safety assessment of non-animal chondroitin sulfate sodium: Subchronic study in rats, genotoxicity tests and human bioavailability, *Food Chem Toxicol.* **93**, 89-101.

248. Liu, Y., Sun, J. D., Song, L. K., Li, J., Chu, S. F., Yuan, Y. H. & Chen, N. H. (2015) Environment-contact administration of rotenone: A new rodent model of Parkinson's disease, *Behav Brain Res.* **294**, 149-61.

249. Zheng, A., Li, H., Cao, K., Xu, J., Zou, X., Li, Y., Chen, C., Liu, J. & Feng, Z. (2015) Maternal hydroxytyrosol administration improves neurogenesis and cognitive function in prenatally stressed offspring, *J Nutr Biochem.* **26**, 190-9.

250. Sedelis, M., Schwarting, R. K. & Huston, J. P. (2001) Behavioral phenotyping of the MPTP mouse model of Parkinson's disease, *Behavioural brain research.* **125**, 109-25.

251. Cordaro, M., Siracusa, R., Crupi, R., Impellizzeri, D., Peritore, A. F., D'Amico, R., Gugliandolo, E., Di Paola, R. & Cuzzocrea, S. (2018) 2-Pentadecyl-2-Oxazoline Reduces Neuroinflammatory Environment in the MPTP Model of Parkinson Disease, *Mol Neurobiol.* **55**, 9251-9266.

252. Fleming, S. M., Mulligan, C. K., Richter, F., Mortazavi, F., Lemesre, V., Frias, C., Zhu, C., Stewart, A., Gozes, I., Morimoto, B. & Chesselet, M. F. (2011) A pilot trial of the microtubule-

interacting peptide (NAP) in mice overexpressing alpha-synuclein shows improvement in motor function and reduction of alpha-synuclein inclusions, *Molecular and cellular neurosciences.* **46**, 597-606.

253. Siracusa, R., Paterniti, I., Cordaro, M., Crupi, R., Bruschetta, G., Campolo, M., Cuzzocrea, S. & Esposito, E. (2018) Neuroprotective Effects of Temsirolimus in Animal Models of Parkinson's Disease, *Mol Neurobiol.* **55**, 2403-2419.

254. Araki, T., Kumagai, T., Tanaka, K., Matsubara, M., Kato, H., Itoyama, Y. & Imai, Y. (2001) Neuroprotective effect of riluzole in MPTP-treated mice, *Brain Res.* **918**, 176-81.

255. Paterniti, I., Campolo, M., Siracusa, R., Cordaro, M., Di Paola, R., Calabrese, V., Navarra, M., Cuzzocrea, S. & Esposito, E. (2017) Liver X receptors activation, through TO901317 binding, reduces neuroinflammation in Parkinson's disease, *PloS one.* **12**, e0174470.

256. Lee, K. W., Zhao, X., Im, J. Y., Grosso, H., Jang, W. H., Chan, T. W., Sonsalla, P. K., German, D. C., Ichijo, H., Junn, E. & Mouradian, M. M. Apoptosis signal-regulating kinase 1 mediates MPTP toxicity and regulates glial activation, *PLoS One.* **7**, e29935.

257. Paterniti, I., Briguglio, E., Mazzon, E., Galuppo, M., Oteri, G., Cordasco, G. & Cuzzocrea, S. (2010) Effects of Hypericum Perforatum, in a rodent model of periodontitis, *BMC Complement Altern Med.* **10**, 73.

258. Cordaro, M., Impellizzeri, D., Gugliandolo, E., Siracusa, R., Crupi, R., Esposito, E. & Cuzzocrea, S. (2016) Adelmidrol, a Palmitoylethanolamide Analogue, as a New Pharmacological Treatment for the Management of Inflammatory Bowel Disease, *Molecular pharmacology.* **90**, 549-561.

259. Siracusa, R., Paterniti, I., Bruschetta, G., Cordaro, M., Impellizzeri, D., Crupi, R., Cuzzocrea, S. & Esposito, E. (2016) The Association of Palmitoylethanolamide with Luteolin Decreases Autophagy in Spinal Cord Injury, *Mol Neurobiol.* **53**, 3783-3792.

260. Petrosino, S., Cordaro, M., Verde, R., Schiano Moriello, A., Marcolongo, G., Schievano, C., Siracusa, R., Piscitelli, F., Peritore, A. F., Crupi, R., Impellizzeri, D., Esposito, E., Cuzzocrea, S. & Di Marzo, V. (2018) Oral Ultramicronized Palmitoylethanolamide: Plasma and Tissue Levels and Spinal Anti-hyperalgesic Effect, *Front Pharmacol.* **9**, 249.

261. Ogawa, N., Hirose, Y., Ohara, S., Ono, T. & Watanabe, Y. (1985) A simple quantitative bradykinesia test in MPTP-treated mice, *Res Commun Chem Pathol Pharmacol.* **50**, 435-41.

262. Weber, D., Davies, M. J. & Grune, T. (2015) Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions, *Redox Biol.* **5**, 367-380.

263. Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D. A. & Stella, A. M. (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity, *Nature reviews Neuroscience.* **8**, 766-75.

264. Hall, E. D., Sullivan, P. G., Gibson, T. R., Pavel, K. M., Thompson, B. M. & Scheff, S. W. (2005) Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury, *Journal of neurotrauma.* **22**, 252-65.

265. Cornelius, C., Crupi, R., Calabrese, V., Graziano, A., Milone, P., Pennisi, G., Radak, Z., Calabrese, E. J. & Cuzzocrea, S. (2013) Traumatic brain injury: oxidative stress and neuroprotection, *Antioxid Redox Signal.* **19**, 836-53.

266. Jenner, P. (1998) Oxidative mechanisms in nigral cell death in Parkinson's disease, *Mov Disord.* **13 Suppl 1**, 24-34.

267. Greenamyre, J. T., MacKenzie, G., Peng, T. I. & Stephans, S. E. (1999) Mitochondrial dysfunction in Parkinson's disease, *Biochem Soc Symp.* **66**, 85-97.

268. Huang, W. J., Zhang, X. & Chen, W. W. (2016) Role of oxidative stress in Alzheimer's disease, *Biomed Rep.* **4**, 519-522.

269. Tarozzi, A., Angeloni, C., Malaguti, M., Morroni, F., Hrelia, S. & Hrelia, P. (2013) Sulforaphane as a potential protective phytochemical against neurodegenerative diseases, *Oxid Med Cell Longev.* **2013**, 415078.

270. Vauzour, D., Ravaioli, G., Vafeiadou, K., Rodriguez-Mateos, A., Angeloni, C. & Spencer, J. P. (2008) Peroxynitrite induced formation of the neurotoxins 5-S-cysteinyl-dopamine and DHBT-1: implications for Parkinson's disease and protection by polyphenols, *Arch Biochem Biophys.* **476**, 145-51.

271. Aruoma, O. I., Bahorun, T. & Jen, L. S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts, *Mutation research.* **544**, 203-15.

272. Vauzour, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C. & Spencer, J. P. (2008) The neuroprotective potential of flavonoids: a multiplicity of effects, *Genes Nutr.* **3**, 115-26.

273. Kelsey, N. A., Wilkins, H. M. & Linseman, D. A. (2010) Nutraceutical antioxidants as novel neuroprotective agents, *Molecules.* **15**, 7792-814.

274. Singh, A., Tripathi, P., Yadawa, A. K. & Singh, S. (2020) Promising Polyphenols in Parkinson's Disease Therapeutics, *Neurochem Res.* **45**, 1731-1745.

275. Phan, C. W., David, P., Naidu, M., Wong, K. H. & Sabaratnam, V. (2015) Therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases: diversity, metabolite, and mechanism, *Crit Rev Biotechnol.* **35**, 355-68.

276. Pan, S. Y., Zhou, S. F., Gao, S. H., Yu, Z. L., Zhang, S. F., Tang, M. K., Sun, J. N., Ma, D. L., Han, Y. F., Fong, W. F. & Ko, K. M. (2013) New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics, *Evid Based Complement Alternat Med.* **2013**, 627375.

277. Thongbai, B., Rapior, S., Hyde, K. D., Wittstein, K. & Stadler, M. (2015) Hericium erinaceus, an amazing medicinal mushroom, *Mycological Progress.* **14**, 91.

278. Lu, Q. Q., Tian, J. M., Wei, J. & Gao, J. M. (2014) Bioactive metabolites from the mycelia of the basidiomycete Hericium erinaceum, *Nat Prod Res.* **28**, 1288-92.

279. Zhang, C. C., Yin, X., Cao, C. Y., Wei, J., Zhang, Q. & Gao, J. M. (2015) Chemical constituents from Hericium erinaceus and their ability to stimulate NGF-mediated neurite outgrowth on PC12 cells, *Bioorganic & medicinal chemistry letters.* **25**, 5078-82.

280. Yi, Z., Shao-long, Y., Ai-hong, W., Zhi-chun, S., Ya-fen, Z., Ye-ting, X. & Yu-ling, H. (2015) Protective effect of ethanol extracts of Hericium erinaceus on alloxan-induced diabetic neuropathic pain in rats, *Evidence-Based Complementary and Alternative Medicine.* **2015**.

281. Rahman, M. A., Abdullah, N. & Aminudin, N. (2014) Inhibitory effect on in vitro LDL oxidation and HMG Co-A reductase activity of the liquid-liquid partitioned fractions of Hericium erinaceus (Bull.) Persoon (lion’s mane mushroom), *BioMed research international.* **2014**.

282. Wang, J. C., Hu, S. H., Su, C. H. & Lee, T. M. (2001) Antitumor and immunoenhancing activities of polysaccharide from culture broth of Hericium spp, *Kaohsiung J Med Sci.* **17**, 461-7.

283. Zhang, Z., Liu, R.-N., Tang, Q.-J., Zhang, J.-S., Yang, Y. & Shang, X.-D. (2015) A new diterpene from the fungal mycelia of Hericium erinaceus, *Phytochemistry Letters.* **11**, 151-156.

284. Mori, K., Ouchi, K. & Hirasawa, N. (2015) The Anti-Inflammatory Effects of Lion's Mane Culinary-Medicinal Mushroom, Hericium erinaceus (Higher Basidiomycetes) in a Coculture System of 3T3-L1 Adipocytes and RAW264 Macrophages, *International journal of medicinal mushrooms.* **17**, 609-18.

285. Yang, B. K., Park, J. B. & Song, C. H. (2003) Hypolipidemic effect of an Exo-biopolymer produced from a submerged mycelial culture of Hericium erinaceus, *Biosci Biotechnol Biochem.* **67**, 1292-8.

286. Tsai-Teng, T., Chin-Chu, C., Li-Ya, L., Wan-Ping, C., Chung-Kuang, L., Chien-Chang, S., Chi-Ying, H. F., Chien-Chih, C. & Shiao, Y. J. (2016) Erinacine A-enriched Hericium erinaceus mycelium ameliorates Alzheimer's disease-related pathologies in APPswe/PS1dE9 transgenic mice, *J Biomed Sci.* **23**, 49.

287. Chan, Y.-C., Chen, C.-C., Lee, L.-Y. & Chen, W.-P. (2019) Active substances for preventing hearing deterioration, the composition containing the active substances, and the preparation method thereof in, Google Patents,

288. Lee, K. F., Chen, J. H., Teng, C. C., Shen, C. H., Hsieh, M. C., Lu, C. C., Lee, K. C., Lee, L. Y., Chen, W. P., Chen, C. C., Huang, W. S. & Kuo, H. C. (2014) Protective effects of Hericium erinaceus mycelium and its isolated erinacine A against ischemia-injury-induced neuronal cell death via the inhibition of iNOS/p38 MAPK and nitrotyrosine, *Int J Mol Sci.* **15**, 15073-89.

289. Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J. & Bertoli, S. (2015) Cultivation, Genetic, Ethnopharmacology, Phytochemistry and Pharmacology of Moringa oleifera Leaves: An Overview, *Int J Mol Sci.* **16**, 12791-835.

290. Rodríguez-Pérez, C., Quirantes-Piné, R., Fernández-Gutiérrez, A. & Segura-Carretero, A. (2015) Optimization of extraction method to obtain a phenolic compounds-rich extract from Moringa oleifera Lam leaves, *Industrial Crops and Products.* **66**, 246-254.

291. Pan, W., Cao, Z., Liu, D. & Jiao, Y. (2020) Protective Effect of Diphenhydramine against Traumatic Brain Injury in Rats via Modulation of Oxidative Stress and Inflammation, *Pharmacology.* **105**, 47-53.

292. Ahmed, A. U., Sarvestani, S. T., Gantier, M. P., Williams, B. R. & Hannigan, G. E. (2014) Integrin-linked kinase modulates lipopolysaccharide- and Helicobacter pylori-induced nuclear factor kappaB-activated tumor necrosis factor-alpha production via regulation of p65 serine 536 phosphorylation, *J Biol Chem.* **289**, 27776-93.

293. Russo, M. V. & McGavern, D. B. (2016) Inflammatory neuroprotection following traumatic brain injury, *Science.* **353**, 783-5.

294. Bains, M. & Hall, E. D. (2012) Antioxidant therapies in traumatic brain and spinal cord injury, *Biochim Biophys Acta.* **1822**, 675-84.

295. Kumar, A., Alvarez-Croda, D. M., Stoica, B. A., Faden, A. I. & Loane, D. J. (2016) Microglial/Macrophage Polarization Dynamics following Traumatic Brain Injury, *J Neurotrauma.* **33**, 1732-1750.

296. Donat, C. K., Scott, G., Gentleman, S. M. & Sastre, M. (2017) Microglial Activation in Traumatic Brain Injury, *Front Aging Neurosci.* **9**, 208.

297. Kernie, S. G., Erwin, T. M. & Parada, L. F. (2001) Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice, *J Neurosci Res.* **66**, 317-26.

298. Zhao, M., Liang, F., Xu, H., Yan, W. & Zhang, J. (2016) Methylene blue exerts a neuroprotective effect against traumatic brain injury by promoting autophagy and inhibiting microglial activation, *Mol Med Rep.* **13**, 13-20.

299. Raghupathi, R., Graham, D. I. & McIntosh, T. K. (2000) Apoptosis after traumatic brain injury, *J Neurotrauma.* **17**, 927-38.

300. Feart, C., Samieri, C., Alles, B. & Barberger-Gateau, P. (2013) Potential benefits of adherence to the Mediterranean diet on cognitive health, *Proc Nutr Soc.* **72**, 140-52.

301. Scarmeas, N., Luchsinger, J. A., Stern, Y., Gu, Y., He, J., DeCarli, C., Brown, T. & Brickman, A. M. (2011) Mediterranean diet and magnetic resonance imaging-assessed cerebrovascular disease, *Ann Neurol.* **69**, 257-68.

302. Stupans, I., Kirlich, A., Tuck, K. L. & Hayball, P. J. (2002) Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants, *J Agric Food Chem.* **50**, 2464-9.

303. Richard, N., Arnold, S., Hoeller, U., Kilpert, C., Wertz, K. & Schwager, J. (2011) Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages, *Planta medica.* **77**, 1890-7.

304. Lavelli, V. (2002) Comparison of the antioxidant activities of extra virgin olive oils, *J Agric Food Chem.* **50**, 7704-8.

305. Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B. & Bartsch, H. (2000) The antioxidant/anticancer potential of phenolic compounds isolated from olive oil, *Eur J Cancer.* **36**, 1235-47.

306. de la Puerta, R., Martinez Dominguez, M. E., Ruiz-Gutierrez, V., Flavill, J. A. & Hoult, J. R. (2001) Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission, *Life Sci.* **69**, 1213-22.

307. CREA, R., BITLER, C. M., BOLIN, L. M. & PONTONIERE, P. (2012) Anti-inflammatory activity of hydroxytyrosol, *European Journal of Nutraceuticals & Functional Foods*, 26.

308. Brunetti, G., Di Rosa, G., Scuto, M., Leri, M., Stefani, M., Schmitz-Linneweber, C., Calabrese, V. & Saul, N. (2020) Healthspan Maintenance and Prevention of Parkinson's-like Phenotypes with Hydroxytyrosol and Oleuropein Aglycone in C. elegans, *Int J Mol Sci.* **21**.

309. Miller, I. N. & Cronin-Golomb, A. (2010) Gender differences in Parkinson's disease: clinical characteristics and cognition, *Mov Disord.* **25**, 2695-703.

310. Bagli, E., Goussia, A., Moschos, M. M., Agnantis, N. & Kitsos, G. (2016) Natural Compounds and Neuroprotection: Mechanisms of Action and Novel Delivery Systems, *In vivo.* **30**, 535-47.

311. Jing, X., Wei, X., Ren, M., Wang, L., Zhang, X. & Lou, H. (2016) Neuroprotective Effects of Tanshinone I Against 6-OHDA-Induced Oxidative Stress in Cellular and Mouse Model of Parkinson's Disease Through Upregulating Nrf2, *Neurochem Res.* **41**, 779-86.

312. Zhao, F., Wu, T., Lau, A., Jiang, T., Huang, Z., Wang, X. J., Chen, W., Wong, P. K. & Zhang, D. D. (2009) Nrf2 promotes neuronal cell differentiation, *Free radical biology & medicine.* **47**, 867-79.

313. Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., Kraft, A. D., Lee, J. M., Li, J. & Johnson, J. A. (2009) The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease, *Antioxid Redox Signal.* **11**, 497-508.

314. Belcher, J. D., Chen, C., Nguyen, J., Zhang, P., Abdulla, F., Nguyen, P., Killeen, T., Xu, P., O'Sullivan, G., Nath, K. A. & Vercellotti, G. M. (2017) Control of Oxidative Stress and Inflammation in Sickle Cell Disease with the Nrf2 Activator Dimethyl Fumarate, *Antioxid Redox Signal.* **26**, 748-762.

315. Lastres-Becker, I., Garcia-Yague, A. J., Scannevin, R. H., Casarejos, M. J., Kugler, S., Rabano, A. & Cuadrado, A. (2016) Repurposing the NRF2 Activator Dimethyl Fumarate as Therapy Against Synucleinopathy in Parkinson's Disease, *Antioxid Redox Signal.* **25**, 61-77.

316. Mutter, F. E., Park, B. K. & Copple, I. M. (2015) Value of monitoring Nrf2 activity for the detection of chemical and oxidative stress, *Biochem Soc Trans.* **43**, 657-62.

317. Zagoura, D., Canovas-Jorda, D., Pistollato, F., Bremer-Hoffmann, S. & Bal-Price, A. (2017) Evaluation of the rotenone-induced activation of the Nrf2 pathway in a neuronal model derived from human induced pluripotent stem cells, *Neurochemistry international.* **106**, 62-73.

318. Scuto, M., Di Mauro, P., Ontario, M. L., Amato, C., Modafferi, S., Ciavardelli, D., Trovato Salinaro, A., Maiolino, L. & Calabrese, V. (2019) Nutritional Mushroom Treatment in Meniere's Disease with Coriolus versicolor: A Rationale for Therapeutic Intervention in Neuroinflammation and Antineurodegeneration, *Int J Mol Sci.* **21**.

319. Miquel, S., Champ, C., Day, J., Aarts, E., Bahr, B. A., Bakker, M., Banati, D., Calabrese, V., Cederholm, T., Cryan, J., Dye, L., Farrimond, J. A., Korosi, A., Laye, S., Maudsley, S., Milenkovic, D., Mohajeri, M. H., Sijben, J., Solomon, A., Spencer, J. P. E., Thuret, S., Vanden Berghe, W., Vauzour, D., Vellas, B., Wesnes, K., Willatts, P., Wittenberg, R. & Geurts, L. (2018) Poor cognitive ageing: Vulnerabilities, mechanisms and the impact of nutritional interventions, *Ageing Res Rev.* **42**, 40-55.

320. Bayram, B., Ozcelik, B., Grimm, S., Roeder, T., Schrader, C., Ernst, I. M., Wagner, A. E., Grune, T., Frank, J. & Rimbach, G. (2012) A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression, *Rejuvenation Res.* **15**, 71-81.

321. Rodriguez-Morato, J., Xicota, L., Fito, M., Farre, M., Dierssen, M. & de la Torre, R. (2015) Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases, *Molecules.* **20**, 4655-80.

322. Martinez-Huelamo, M., Rodriguez-Morato, J., Boronat, A. & de la Torre, R. (2017) Modulation of Nrf2 by Olive Oil and Wine Polyphenols and Neuroprotection, *Antioxidants (Basel).* **6**.